The P53 Independent Functions of Estrogen-Activated MDM2 in Cell Signaling and Mammary Architecture

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The p53 independent functions of estrogen-activated MDM2 in cell signaling and mammary architecture

By

Nandini Kundu

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

The p53 independent functions of estrogen-activated MDM2 in cell signaling and mammary architecture

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Nandini Kundu

Advisor: Dr. Jill Bargonetti

Estrogen receptor positive (ER+) breast cancers often have MDM2 overexpression indicating a critical role for MDM2 in breast cancer tumorigenesis. The cancer genome atlas (TCGA) found that increased MDM2 expression is one of the four pathways that correlate with all breast cancer subtypes. MDM2 is mainly known as the negative regulator of wild type p53. However, aggressive breast cancers often have MDM2 overexpression and mutant p53 (mtp53). We previously reported that MDM2 provides an estrogen-mediated proliferative advantage to MCF-7 breast cancer cells (ER+, MDM2 overexpression, wild type p53), independent of wild type p53 in both 2D and 3D culture conditions. This and other studies suggest that MDM2 has a p53-independent role in tumorigenesis. To examine the estrogen-induced p53-independent roles of MDM2, we generated T47D breast cancer cells (ER+, MDM2 overexpression, mtp53 L194F) with shRNA to MDM2. As seen previously, estrogen treatment increased MDM2, and MDM2 knockdown did not change p53 protein levels. MDM2 knockdown inhibited estrogen mediated cell proliferation in 2D and 3D anchorage independent soft agar and matrigel culture. MDM2 knockdown decreased mass size, induced lumen formation and significantly
reduced the number of phospho-histone H3 positive cells per mass indicating a decrease in mitotic rate. MDM2 knockdown also decreased Rb phosphorylation and E2F1 protein levels. Moreover, blocking estrogen signaling by estrogen antagonist Fulvestrant decreased MDM2 protein levels and phosphorylation of Rb. Our data place MDM2 as a central hub for estrogen-mediated p53-independent signal transduction. We demonstrate that MDM2 provides advantage to estrogen-induced breast cancer cell proliferation and disruption of mammary architecture through ER-MDM2-phosphoRb-E2F1 signaling pathway.
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CHAPTER 1:
Introduction
1.1. The *mdm2* gene and protein

1.1.1. MDM2 discovery as an oncogene

MDM2 (murine double minute 2) is overexpressed in approximately 10% of all human cancers and plays an important role in tumor development (Momand, Jung, Wilczynski, & Niland, 1998). The *mdm2* gene was first discovered as the transforming gene in the spontaneously transformed mouse cell line, 3T3-DM, where it is overexpressed 50 fold due to genomic amplification on double minute chromosomes (acentromeric extrachromosomal nuclear bodies). This overexpression of MDM2 promotes a growth advantage and spontaneous transformation to the 3T3-DM cells (Cahilly-Snyder, Yang-Feng, Francke, & George, 1987; Fakharzadeh, Trusko, & George, 1991). MDM2 overexpression can occur via different mechanisms including gene amplification (Meddeb et al., 1996; Oliner, Kinzler, Meltzer, George, & Vogelstein, 1992), increased transcription (Bueso-Ramos et al., 1993; Watanabe et al., 1994) and enhanced translation (Landers, Cassel, & George, 1997; Landers, Haines, Strauss, & George, 1994).

1.1.2. The *mdm2* gene and its regulation

The human *mdm2* gene is approximately 33 kilobases (kb) in size and located on chromosome 12 (q14.3-q15) (Oliner et al., 1992; Weaver et al., 2008). It consists of 12 exons with two promoters, P1 and P2, p53-independent and
p53-dependent respectively (Barak, Gottlieb, Juven-Gershon, & Oren, 1994; Zauberman, Flusberg, Haupt, Barak, & Oren, 1995). The transcription from P1 promoter represents the basal transcription of mdm2 gene. There are two p53 binding sites in the first intron upstream of P2 promoter. The transcription from the P2 promoter is induced by activated p53 (Zauberman et al., 1995). The P1 and the P2 derived mdm2 transcripts encode identical full length MDM2 protein, as the start site of translation of mdm2 is located within exon 3 (Barak et al., 1994; Perry, Piette, Zawadzki, Harvey, & Levine, 1993). A number of p53 independent transcription factor binding sites have been identified in the P2 promoter region, some of which are Ets/Ap-1, E boxes, Smad2/3, RXR and GC boxes (review (Manfredi, 2010)). Thus, several transcription factors regulate the transcription of mdm2 from the P2 promoter including p53 (Barak, Juven, Haffner, & Oren, 1993; Juven, Barak, Zauberman, George, & Oren, 1993), Ets/Ap-1 (Phelps, Darley, Primrose, & Blaydes, 2003), MYCN (Slack et al., 2005), Smad2/3 (Araki et al., 2010), RXRγ (Xu et al., 2009)(Xu2009) and Sp1 (Bond et al., 2004).

MDM2 is overexpressed at both the protein and mRNA levels in many different cancers, including breast cancers. Much of the overexpressed MDM2 is due to transcription from the P2 promoter as mdm2 transcript from this promoter is more efficiently translated (C. Y. Brown, Mize, Pineda, George, & Morris, 1999). Upstream of the P2 promoter, there is a binding site for the transcriptional activator, Sp1. A single nucleotide polymorphism (T to G) is found at position 309 in the mdm2 gene adjacent to the P2 promoter. This polymorphism increases the
transcription of \textit{mdm2} gene from P2 promoter in a Sp1 dependent manner and leads to high expression of \textit{mdm2} transcripts and protein, which is associated with accelerated tumor formation in humans (Bond et al., 2004).

\subsection*{1.1.3. MDM2 protein structure}

The full-length \textit{mdm2} transcript is translated into a protein of 491 amino acids with several functional domains (Marechal et al., 1997). MDM2 protein has three major regions: the N-terminus (aa1 – 113), the central region (aa114 – 300) and the C-terminus (aa301 – 491).

The amino terminal domain forms a deep hydrophobic cleft and binds to the N-terminal transactivation domain of p53, inhibiting p53 transactivation (J. Chen, Lin, & Levine, 1995). The N-terminus of MDM2 also interacts with C-terminus of p53 and is necessary for p53 degradation (Poyurovsky et al., 2010). Additionally, a novel algorithm showed that the N-terminus of MDM2 contains a SWIB domain, similar to the one contained in SWI/SNF chromatin remodeling complex that facilitates transcription (Bennett-Lovsey, Hart, Shirai, & Mizuguchi, 2002).

The nuclear localization and nuclear export signal allow MDM2 to shuttle back and forth between the cytoplasm and the nucleus (Freedman & Levine, 1998; Roth, Dobbelstein, Freedman, Shenk, & Levine, 1998). The central region of MDM2 has an acidic domain and a zinc finger domain. A number of proteins including L5, L11, L26 ribosomal proteins, p14ARF and Rb tumor suppressors and the acetyltransferase p300 have been shown to interact with this central
region of MDM2 (Bouska & Eischen, 2009). The acidic domain of MDM2 also interacts with the central region of p53, the DNA binding domain, and this interaction is essential for p53 ubiquitination by MDM2 (Ma et al., 2006; Wallace, Worrall, Pettersson, Hupp, & Ball, 2006).

The C-terminus of MDM2 consists of a RING finger domain, which is responsible for the E3 ubiquitin ligase activity (Fang, Jensen, Ludwig, Vousden, & Weissman, 2000; Honda, Tanaka, & Yasuda, 1997). Amino acids 464-471 in the RING finger domain function as the nucleolar localization signal (Lohrum, Ashcroft, Kubbat, & Vousden, 2000). Thus, the C-terminus promotes its E3 ubiquitin ligase activity, has nucleotide binding activity and encompasses the nucleolar localization signal. The RING domain also contains the oligomerization domain, regulated by the very last nine amino acids.

Figure 1: MDM2 protein structure: Schematic representation of MDM2 full-length protein showing its distinct domains. The N terminal domain consists of p53 binding domain and SWIB domain. The central region comprises of nuclear localization signal (NLS), nuclear export signal (NES), acidic domain and zinc finger domain. The C terminal domain includes ring finger domain and nucleolar localization domain (NoLS). The exons corresponding to each domain are marked above and putative post-translational modifications of proteins are shown below. (Adapted from Okoro, Rosso, & Bargonetti, 2012)

Interestingly, exon 12 of mdm2 gene encodes one-third of the protein coding sequence; comprises of zinc finger region, the nucleolar localization signal (NoLS) and the RING finger domain. MDM2 plays a role of hub protein
where it interacts with a plethora of other proteins and acts as a transducer of different signaling pathways (Fahraeus & Olivares-Illana, 2014).

Apart from full-length, there are over 40 transcripts of splice variants transcribed from *mdm2* gene by alternative and aberrant splicing (Bartel, Taubert, & Harris, 2002). Overexpression of splice variants is observed under stressful conditions and in late stage and aggressive tumors. The most well characterized splice variants studied (*mdm2-a, mdm2-b, mdm2-c*) lack the exons encoding the p53 binding domain, suggesting a p53-independent functions of these splice variants (reviewed in (Okoro et al., 2012)). It is unknown if all the splice variant transcripts of *mdm2* are translated into proteins *in vivo*. Studies have shown 5 splice variants, *mdm2 a-e* can be translated into proteins in *in vitro* systems (Sigalas, Calvert, Anderson, Neal, & Lunec, 1996). Recently, studies from our lab have developed an antibody against exon4-10 splice junctions and identified MDM2-C isoform as an endogenously expressed protein in cancer tissue and cells (Okoro et al., 2013). Understanding the different forms of splice variants and their role would help us to understand the oncogenic potential of MDM2 and its family of splice variants.

### 1.2 MDM2 – p53 Regulatory Feedback Loop

The *mdm2* gene is one of the many p53 target genes (Barak et al., 1993; Perry et al., 1993). The p53 protein binds near the *mdm2* P2 promoter and transcriptionally upregulates *mdm2* expression (Juven et al., 1993; Wu, Bayle, Olson, & Levine, 1993; Zauberman et al., 1995). MDM2 protein is a well-known
negative regulator of tumor suppressor p53 and targets p53 for degradation by
ubiquitination (Haupt, Maya, Kazaz, & Oren, 1997; Honda et al., 1997; Kubbutat,
Jones, & Vousden, 1997). However, MDM2 can also ubiquitinate itself and
induce its own degradation (Fang et al., 2000; Honda & Yasuda, 2000). MDM2
protein directly inhibits p53-mediated transactivation by binding to and blocking
the p53 transactivation domain, thereby hindering p53 interaction with
transcriptional co-activators (J. Chen et al., 1995; Momand, Zambetti, Olson,
George, & Levine, 1992; Oliner et al., 1993; Wu et al., 1993). During stressful
conditions as in DNA damage, the interaction of MDM2 and p53 is hindered due
to posttranslational modifications of both MDM2 and p53 (Kruse & Gu, 2009;
Marine & Lozano, 2010; Vousden & Prives, 2009). In response to stress signals,
MDM2 is phosphorylated by several kinases (Marine & Lozano, 2010), which
inhibit its E3 ubiquitin ligase activity. The p53 protein is also phosphorylated and
acetylated (Kruse & Gu, 2009). As degradation of p53 by MDM2 is blocked, this
results in increased p53 protein stability and activity as a transcription factor for
growth arrest, apoptosis and DNA repair (Meek & Knippschild, 2003; Moll &
Petrenko, 2003). It has been shown that acetylation of p53 is necessary for p53
activation (Tang, Zhao, Chen, Zhao, & Gu, 2008), as it hinders recruitment of
MDM2 to p53-responsive elements, thus inhibiting MDM2 mediated repression.
However, several studies showed that posttranslational modifications are not
required for p53 activation. It has been shown that disruption of MDM2–p53
complex alone by small molecule inhibitor (Nutlin-3) results in p53 activation
(Thompson et al., 2004; Vassilev et al., 2004). MDM2 depletion also increases

Figure 2: MDM2-p53 regulatory feedback loop: p53 binds to MDM2 promoter and increases MDM2 protein level, while MDM2 being an E3 ubiquitin ligase degrades p53, thereby maintaining the tumor suppressor p53 at a low level under normal conditions. Under stressful conditions, some of which are listed above, the feedback loop is interfered due to posttranslational modifications in both MDM2 and p53; MDM2 can no longer degrade p53. p53 is stabilized and leads to activation of its target genes. It is believed that p53 activation depends on the level of stress, as depicted above. Mild stress causes growth arrest whole severe stress results in apoptosis. (Adapted from (Levine & Oren, 2009))

This critical MDM2-p53 auto-regulatory feedback loop has been demonstrated very clearly by many in vivo experiments. Homozygous deletion of mdm2 in the mouse germline results in embryonic lethality. Interestingly, simultaneous deletion of both mdm2 and p53 rescues the lethal phenotype (Jones, Roe, Donehower, & Bradley, 1995; Montes de Oca Luna, Wagner, &
Mice that have a hypomorphic allele of *mdm2*, expressing 30% of the total MDM2 protein level compared to normal mice, have reduced body weight, show developmental defects and are more radiosensitive (Mendrysa et al., 2003). Furthermore, it had been shown that in adult mice, MDM2 is important for continuous inhibition of the lethal functions of p53 (Ringshausen, O'Shea, Finch, Swigart, & Evan, 2006).

There are several other ways by which p53 and MDM2 interacts with each other. MDM2 targets ribosomal protein RPL26, which is necessary for p53 translation, for ubiquitin dependent degradation; thus inhibiting p53 protein translation (Ofir-Rosenfeld, Boggs, Michael, Kastan, & Oren, 2008; Takagi, Absalon, McLure, & Kastan, 2005). Surprisingly, the RING finger domain of MDM2 binds with p53 mRNA in the region coding for MDM2 binding site, stimulating p53 translation (Candeias et al., 2008; Naski et al., 2009; Yin, Stephen, Luciani, & Fahraeus, 2002). These studies indicate that MDM2 has a dual function, synthesis and degradation of p53, and add complexity to the MDM2-p53 feedback loop.

### 1.3. Importance of MDM2 in ER+ breast cancers

#### 1.3.1 Clinical Breast Cancer Subtypes and Hormone Receptor Status

Breast cancer is one of the most common cancers and is the second leading cause of death among women, after lung cancer (American Cancer
Although breast cancer is a complex and heterogeneous disease, clinically a pathologist determines the hormone receptor status for determining the course of treatment. According to the American Cancer Society 2016, invasive breast cancers are categorized on the basis of hormone receptor and Her2 into following types:

- **Hormone receptor positive breast cancer**: These breast cancers are positive for either estrogen receptor or progesterone receptor or both. In this cancer, these hormones fuel the growth and proliferation of cancer cells. About two-thirds of breast cancers have at least one of these receptors.

- **Hormone receptor negative breast cancer**: These breast cancers are negative for estrogen and progesterone receptors.

- **HER2 positive breast cancer**: These breast cancers have elevated protein levels of the growth-promoting protein, HER2/neu (often called HER2).

- **HER2 negative breast cancer**: These breast cancers do not have increased levels of HER2 protein, hence are HER2 negative.

- **Triple negative breast cancer**: These breast cancers are not positive for estrogen or progesterone receptor and do not have increased levels of HER2 protein.

- **Triple positive breast cancer**: These breast cancers are positive for estrogen receptor and/or progesterone receptors and have elevated levels of HER2.
It is necessary to understand the molecular categories of breast cancer due to heterogeneity in breast cancer. Based on gene expression profiling, The Cancer Genome Atlas (TCGA) has classified breast cancer into 4 different subtypes - Luminal A, Luminal B, Basal-like and HER2-enriched (HER2E) ("Comprehensive molecular portraits of human breast tumours," 2012). The majority of the luminal breast cancers are estrogen receptor positive, most of the basal-like are triple negative and HER2E comprises those that not only have HER2 DNA amplification but also HER2-amplicon-associated genes. This study indicated that many of the clinically observed categories occur not only across but also within these molecular subtypes of breast cancer and understanding the heterogeneity might increase the efficiency of treatment.

1.3.2. MDM2 is overexpressed in ER+ breast cancer

One of the pathways that are identified to be impaired in breast cancer is the p53-MDM2 pathway. MDM2 overexpression occurs in all molecular subtypes of breast cancer. Patients with MDM2 overexpression in their cancers have a worse prognosis than patients with cancers that do not test positive for such overexpression. Therefore MDM2 expression is a negative prognostic marker for breast cancer (Turbin et al., 2006). Previous studies have shown that MDM2 overexpression occurs in many ER+ breast tumors (Hori, Shimazaki, Inagawa, Itabashi, & Hori, 2002; Marchetti et al., 1995). There is also a strong positive correlation between MDM2 overexpression and ERα expression in primary human breast tumors and human breast cancer cell lines (Baunoch et al., 1996; Brekman, Singh, Polotskaia, Kundu, & Bargonetti, 2011; Gudas et al., 1995; Hori
et al., 2002; Marchetti et al., 1995; Sheikh, Shao, Hussain, & Fontana, 1993; Swetzig, Wang, & Das, 2016). Importantly, MDM2 overexpression occurs in the ER+ category of the molecular subtypes (Swetzig et al., 2016). It has generally been noted that ER positive status correlated with MDM2 overexpression indicating that MDM2 plays an important role in estrogen receptor positive breast cancers.

In ER+ MCF7 and T47D breast cancer cells, \textit{mdm2} transcript from P2 promoter is activated by p53 dependent and p53 independent mechanism (Phelps et al., 2003). In MCF7 cells having wild type p53, activation of p53 induced \textit{mdm2}-P2 transcript. However, in T47D cells, the p53 being loss of function (Phelps et al., 2003; Polotskaia et al., 2015), was not able to induce \textit{mdm2}-P2 transcript (Phelps et al., 2003). We and others have shown that estrogen treatment increases MDM2 at both mRNA and protein levels (Baunoch et al., 1996; Brekman et al., 2011; Gudas et al., 1995; Marchetti et al., 1995; Sheikh et al., 1993). The increase in \textit{mdm2} mRNA is due to increased transcription with estrogen treatment (Kinyamu & Archer, 2003; Saji et al., 1999). ER\textsubscript{\(\alpha\)} enhances transcription via its ability to interact and activate the AP1 transcription factor (Paech et al., 1997). Mutation in AP1 site in T47D cells results in loss of 52.5\% P2 promoter activity (Phelps et al., 2003). This appears to be the mechanism of how ER alpha enhances MDM2 expression. Additionally, \textit{mdm2} transcription is increased due to a naturally occurring SNP at position 309 from T to G increasing the affinity for binding of the transcription factor Sp1 at P2 promoter of \textit{mdm2} gene (Bond et al., 2004). Elevated MDM2 accelerates tumor
formation in a gender specific and hormone dependent manner (Bond et al., 2006; Bond & Levine, 2007). Furthermore studies have shown that estrogen preferentially simulates transcription of MDM2 from the SNP309 G allele, resulting in heightened MDM2 protein levels in estrogen responsive cell lines in a genotype specific fashion. Thus, cells having homozygous G/G SNP have more MDM2 protein levels than cells having heterozygous T/G SNP or cells having normal genotype T/T in the presence of estrogen (Hu et al., 2007).

1.4. p53 independent activities of MDM2: Non-canonical pathways of MDM2

MDM2 has been shown to interact with several other proteins, in addition to p53. This gives a hint of MDM2 functions that are p53-independent, referred to here as “non-canonical functions of MDM2”. There are several examples of p53-independent functions of MDM2 in vivo. In vivo mouse model studies have shown that p53⁻/⁻ mice with an overexpression of mdm2 (transgenic) or mdm2⁺⁻ mice develop a different tumor spectrum than p53⁻/⁻ mice alone (Jones, Hancock, Vogel, Donehower, & Bradley, 1998). MDM2-transgenic mice homozygous for the transgene (MDM2⁰⁺/⁰⁺) develop tumors at a more rapid pace as compared to heterozygous (MDM2⁰⁺/⁻) mice (Jones et al., 1998). The targeted expression of MDM2 in mice mammary tissues uncouples the S phase and mitosis, leading to aneuploidy, independently of p53 and inhibits the development of mammary glands (Lundgren et al., 1997). Expression of MDM2 splice variants lacking the p53 binding domain increases tumorigenesis in mice (Ganguli & Wasylyk, 2003). Several studies have shown that MDM2 directly or by interacting with other
factors influences genome stability, cell cycle and DNA repair (Bouska & Eischen, 2009).

MDM2 interacts with Rb protein, a tumor suppressor and a negative regulator of cell cycle progression from G1 to S phases (Yamasaki, 2003). MDM2 binds to Rb via its central region (Sdekk et al., 2004) and degrades it by targeting it for ubiquitination (Uchida et al., 2005) and proteosomal degradation (by promoting its association with C8 proteosome subunit) (Sdekk et al., 2005). MDM2 via its N-terminal hydrophobic pocket interacts with E2F1, a transcriptional factor that positively regulates the G1-S phase transition (Mundle & Saberwal, 2003). MDM2 binds the catalytic subunit of DNA polymerase ε, which has role in DNA repair, recombination and replication and stimulates its activity (Asahara et al., 2003; Vlatkovic et al., 2000). MDM2 also binds with ribosomal proteins L5, L11 and L23, independent of 80S ribosome and polysome (Dai & Lu, 2004; Dai et al., 2004; Y. Zhang et al., 2003). This suggests that MDM2 interacts with these ribosomal proteins and alters ribosomal biogenesis. As alteration and disturbances in ribosomal biogenesis lead to tumorigenesis, this suggests MDM2 overexpression might alter ribosomal biogenesis, promoting tumorigenesis. Furthermore, MDM2 plays a major role in estrogen induced cell proliferation in MCF-7 breast cancer cells in a p53 independent manner (Brekman et al., 2011). Collectively these data indicate that MDM2 has tumorigenic potential independent of p53.
CHAPTER 2:
Materials and Methods
2.1. Cell culture

2D culture: Human breast cancer cells T47D (mdm2 SNP309 G/G, mutant p53) and MCF7 (mdm2 SNP309 T/G, wild type p53) cells were obtained from American Type Culture Collection (ATCC). They were grown in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (FBS, Gemini) and 50U/ml penicillin and 50µg/ml streptomycin (Mediatech) at 5% CO₂ 37°C humidified incubator. Cell lines with the inducible mdm2 knockdown were generated and described previously in (Brekman et al., 2011). T47D cells with constitutive knockdown to mdm2 were also generated. T47D cells were infected with MLP vector (a generous gift from Scott Lowe) containing mdm2 151656 shRNA or empty vector by the retroviral mediated gene transfer method. In short, empty or shmdm2 containing vector were transfected using the calcium phosphate method to phoenix packaging cells to make virus that was used to infect T47D cells and then they were selected with 5µg/ml puromycin.

2.2. Molecular cloning of shmdm2 cell lines

The pSM2c plasmids with shmdm2 151656 (position 1793….1811; shRNA sequence: CTGTCTATAAGAGATTAT) were cloned into doxycycline-inducible vector STGM PGK PURO (done by Angelika Brekman) and constitutive vector MLP.1224. Reverse tetracycline transcriptional activator (rtTA) plasmid was used to induce shRNA expression from STGM PGK PURO vector in tissue culture experiments. Empty STGM PGK PURO and MLP.1224 vectors were used as controls.
2.3. Molecular cloning of MDM2 overexpression cell lines

PCR cloning of the IRES pT7 vector plasmid pT7CF1-CGST-HA-His-GFP (Thermo Scientific) containing mdm2-FL (full-length) (cloning done by Danielle Okorro) was done with the following primers so the IRES sequence is retained along with full length mdm2.

mdm2 Cla1 Forward primer:
5'TAAGCAATCGATCGAATTAATTCCGGTTATTTTC3'

mdm2 Xba1 Reverse primer:
5'TGCTTATCTAGACTAGGGGAATAAGTTAGCACAAC3'

The PCR product and pLNCX vector (a gift from Dr. Carol Prives from Columbia University) were digested with Cla1 and Xba1 (New England Biolabs, Inc.). The gel purified PCR product-containing Cla1-IRES-mdm2FL-Xba1 was inserted into CMV pLNCX plasmid via ligation. The pLNCX-IRES-mdm2FL ligated DNA were transformed and plasmids were purified using Qiagen mini/Maxi prep kits.

2.4. Generation of cell lines containing stable constitutive shmdm2 and stable constitutive MDM2 overexpression.

For stable shRNA knockdown cell lines and stable MDM2 overexpression cell lines, MLP vector with shRNA or pLNCX vector with mdm2FL were introduced into MCF7 and T47D cells via retroviral gene transfer method and selected with Puromycin for MLP vector for 3 days and G418 for pLNCX vector for 7 days. Briefly, phoenix packaging cells were transfected by calcium
phosphate method using MLP or pLNCX vector. Media containing the generated viruses was harvested and MCF7 or T47D cells were infected with the plasmids. After selection with the appropriate drugs, the newly generated cell lines were grown in DMEM media supplemented with 10% FBS (Gemini) and 2500 units of penicillin-streptomycin.

2.5. Anchorage independent growth assay

Cells were grown in 2D culture for 3 days with or without doxycycline. They were trypsinized, washed with 1X clear HBBS three times and then counted. Cells with equal seeding densities were mixed with 0.3% Noble Agar (Sigma A5431) in phenol-red-free-DMEM media containing 10% charcoal stripped FBS (Gemini), antibiotics, 10nM estrogen and with or without doxycycline. They were seeded onto 35mm dishes coated with 0.5% Nobel gar in the same media. They were then topped with the same media. Estrogen-treated growth media with or without doxycycline were replaced every 3 days. Colonies were counted after 3-3.5 weeks.

2.6. Cell culture in matrigel

Cells were grown in 2D culture for 3 days with or without doxycycline. They were trypsinized, washed with 1X clear HBBS three times and then counted. They were seeded on top of 40µl solidified matrigel (Cultrex) in phenol-red-free-DMEM media containing 10% charcoal stripped FBS (Gemini), antibiotics, 10nM estrogen and with or without doxycycline. Media were changed
every 3 days. Masses were grown for 3-3.5 weeks, fixed with 4% paraformaldehyde, permeabilized, blocked and stained with propidium iodide or actin immunostaining by Rhodamine-Phalloidin (Cytoskeleton BK005) or primary antibody phosho histone H3 (Millipore) followed by Alexa Fluor conjugated secondary antibody (Invitrogen) and mounted with Vectashield mounting medium containing DAPI (Fisher Scientific). Imaging was performed using confocal microscope using laser scanning spectral confocal microscope TCS SP2.

2.7. Treatments

Inducible shmdm2 or vector control cell lines were grown in complete media described above, with or without doxycycline (4µg/ml for T47D and 2µg/ml for MCF7) for shRNA induction for 3 days. The growth media were then replaced with phenol-red-free DMEM media containing 10% charcoal stripped FBS (Gemini) and antibiotics and treated with 10nM estrogen (17β-estradiol, Sigma) and/or 7µM tamoxifen (Sigma-Aldrich) and/or 10µM Fulvestrant (Sigma-Aldrich) wherever applicable. Fresh media were supplemented every 72 hours.

2.8. Live cell imaging

Cells were seeded at 20x10^3 per well in a 12-well glass bottom plate (MatTek, Ashland, MA, USA). After treatment, cells were stained with 50 ul ReadyProbes Cell Viability Imaging Kit Blue/Red (Life Technologies Cat# R37610) for 15 min at room temp. z-stack image of stained cells were taken by confocal microscopy using a Nikon A1 confocal microscope with 20x objective.
Propidium iodide: red fluorescence; Nuclear DNA: blue fluorescence. Dr. Jill Bargonetti performed this protocol.

2.9. Fluorescence Activated Cell Sorting (FACS)

FACS was performed on FACScan device (BD Biosciences). After treatments, cells were harvested, washed, resuspended in PBS containing 2% bovine serum albumin, 0.1% sodium azide, fixed in 30% ethanol and stored overnight at 4°C. Before analysis, propidium iodide staining and RNase treatment were performed for 30 minutes at 37°C.

2.10. Whole cell protein extract

Cells were pelleted from 2D culture at 1100 rpm for 7 mins at 4°C and washed 3 times with 1X ice-cold PBS. The cells were then suspended in RIPA buffer (0.1% SDS, 1% IGEPAL NP-40, 0.5% Deoxycholate, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 50mM Tris-Cl pH8) with 1mM PMSF, 8.5µg/ml Aprotinin, 2µg/ml Leupeptin, sodium fluoride (5mM), sodium orthovanadate (1mM) and phosphatase cocktail inhibitor (Sigma) following standard protocol.

2.11. Purification of MDM2 expressed in bacteria

After IPTG induction for 4 hours, BL21DE3 (having plasmid pRSETA HDM2; generous gift from Lindsey Mayo lab) bacterial cell pellets were collected and lysed with lysis buffer (pH 8: 100mM Na₂H₃PO₄, 100mM Tris-Cl, 8M Urea, 1mM PMSF) by frequent vortexing. It was then centrifuged for 25 minutes in 4°C.
at 10,000g. The supernatant was collected, mixed with Ni-NTA agarose beads (Qiagen, #30210) in the ratio of 1:3 (bead to supernatant) and rocked for an hour at 4°C. After a quick spin as the beads settled, the supernatant was transferred and labeled as “unbound”. The pellet was washed with wash buffer (pH 6.3: 100mM Na$_2$H$_2$PO$_4$, 100mM Tris-Cl, 8M Urea, 1mM PMSF) in 1:2 (bead to buffer) 2 times and the supernatant was labeled as “wash”. The remaining pellet was mixed in 2.5 X the elution buffer (pH 5.9: 100mM Na$_2$H$_2$PO$_4$, 100mM Tris-Cl, 8M Urea, 100mM EDTA pH8, 1mM PMSF) and supernatant was collected after quick spun and labeled as “elute”. This was repeated 2 more times. Small aliquots taken at each elute and run on a gel to confirm purification of MDM2 compared to wash and unbound. The third elute of purified MDM2 was used in \textit{in vitro} kinase assay. Alla Polotskaia and Jun Yeob Kim did this protocol.

\textbf{2.12. Nuclear Extracts}

Pellets were collected of cells with and without overnight estrogen treatment. The pellets were suspended in 5X packed cell volumes with cytoplasmic extraction buffer (CEB: 10mM Hepes pH 7.9; 1.5mM MgCl$_2$; 10mM KCl; 0.5mM PMSF, 0.5 mM DTT; 8.5μg/ml Aprotinin, 2μg/ml Leupeptin; and phosphatase inhibitor mixture I (Sigma)). After washing, cells were resuspended with a 20-gauge needle in 2X packed cell volumes of CEB and incubated on ice for 10 min. After centrifugation (10 min at 12,000 rpm at 4 °C) the supernatant was removed to give the cytoplasmic fraction. The pellet was resuspended in kinase reaction buffer (20 mmol/L Tris pH 7.5; 7.5 mmol/L MgCl$_2$; 1 mmol/L DTT;
0.5 mmol/L ethyleneglycol-bis [β-aminoethyl]-N,N,N',N'-tetraacetic acid [EGTA]; 25 mmol/L β-glycerophosphate; 0.5 mmol/L sodium orthovanadate; 1 mmol/L PMS; 2 µg/ml leupeptin, 1.5 µg/ml aprotinin and phosphatase inhibitor mixture). Cells were resuspended with a 20-gauge needle and the cell suspension was rocked for 30 min at 4 °C and then centrifuged for 30 min at 13,000 rpm at 4 °C. The supernatant was the nuclear fraction.

2.13. In vitro kinase assay

The nuclear extract made in kinase buffer was incubated with 1µl purified MDM2 in elution buffer or elution buffer alone (control) and ATP regeneration system (1mM ATP, 1mM magnesium chloride, 10mM phosphocreatine, 50µg/ml creatine kinase) for 30 minutes at 30°C. The total reaction volume was 50µl. The reaction was terminated with 4X NuPAGE Lithium dodecyl Sulfate buffer (Life Technologies) and western blot assay was performed immediately.

2.14. Western blot assay

Protein extracts from 2D culture were prepared with 4X NuPAGE Lithium dodecyl Sulfate buffer (Life Technologies) and 20mM DTT. The samples were heated at 70°C for 10 min and then 100 mM Iodoacetamide (Sigma) was added after heating. Samples were separated by SDS-PAGE followed by electrotransfer onto a nitrocellulose membrane. The resulting membrane was blocked with 5% non-fat milk (Biorad) in 1X PBS-0.1% Tween-20 or 5% Bovine Serum Albumin (BSA) in 1X TBS-0.1% Tween-20 (for probing phospho Rb) and probed with
primary antibody overnight at 4°C. The membrane was washed with 1X PBS-0.1% Tween-20 or 1X TBS-0.1% Tween-20 followed by probing with secondary antibody. The protein signal was detected by chemiluminescence using the Super Signal Kit (Pierce) and autoradiography using Hyblot CL films (Denville Scientific).

2.15. Antibodies

AntiMDM2 antibody – 1:1:1 mix of mouse monoclonal antibodies 4B2, 2A9, 4B11; antip53 antibody - 1:1:1 mix of mouse monoclonal antibodies 240,421,1801; rabbit polyclonal antiActin (Sigma-Aldrich) or anti-Actin HRP (Santa Cruz); phospho Rb (Ser807/811: Cell Signaling); Total Rb (Cell Signaling); E2F1 (Cell Signaling) and secondary anti-mouse and anti-rabbit antibodies (Sigma-Aldrich) were used for western blot analysis.
CHAPTER 3:
Inducible \textit{mdm2} knockdown in 2D culture conditions inhibits estrogen-mediated cell proliferation in G/G SNP309 ER positive breast cancer cells in a p53 independent manner.
3.1. Introduction

About two-thirds of breast cancers are estrogen receptor positive (Gruvberger et al., 2001). Many of the estrogen receptor positive breast cancer cells have high levels of MDM2 (Baunoch et al., 1996; Bueso-Ramos et al., 1996; Gudas et al., 1995; Marchetti et al., 1995; Sheikh et al., 1993). MDM2 is upregulated in the presence of estrogen (Hori et al., 2002). Increased MDM2 protein levels occurs in ER + breast cancer independently of p53 using AP1 and ETS transcription factors (Phelps et al., 2003). A naturally occurring polymorphism (T to G) at position 309 in the mdm2 P2 promoter increases the DNA binding capacity of the transcription activator, Sp1, resulting in increased transcription of mdm2 (Bond et al., 2004). Sp1 is a well-characterized cotranscriptional activator of ER (Safe, 2001; Saville et al., 2000). This indicates that estrogen signaling enhances mdm2 transcription level especially in ER+ breast cancers harboring SNP 309 (T to G) at mdm2 P2 promoter (Bond et al., 2006). Additionally, estrogen receptor dependent increase of mdm2 transcription was shown to be mediated at least in part, by ER binding to MDM2 promoter that harbors the SNP309 locus (Kinyamu & Archer, 2003). Estrogen preferentially stimulates transcription from SNP309 G allele of mdm2 gene (Hu et al., 2007). Estrogen treatment increases MDM2 protein level much higher in SNP309 G/G ER+ cells than SNP 309 T/G ER+ cells, as compared to the normal T/T ER+ cells (Hu et al., 2007).

Studies from our lab have shown that there is an estrogen dependent increase of MDM2 protein (consistent with other studies) that positively
contributes to estrogen-mediated cell proliferation in a wild type p53 independent manner (Brekman et al., 2011). This was the time I joined the laboratory and we extended this study to investigate the p53 independent role of MDM2 in estrogen signaling in the ER+ breast cancer cells. We carried out our experiments using the T47D ER+ human breast cancer cell line, which has MDM2 overexpression due to SNP 309 G/G and L149F mutant p53. This mutant p53 is a loss of function since p53 knockdown in this cell line does not alter cell proliferation (Polotskaia et al., 2015) and DNA damage response cannot activate p53 (Phelps et al., 2003). Thus T47D cell lines serves as an ideal model system to study the p53-independent functions of MDM2.

3.2. Results

Using T47D cells, we generated cell lines containing inducible mdm2 shRNA knockdown. Doxycycline-inducible shRNA constructs targeting \textit{mdm2} were a generous gift from Scott Lowe at Cold Spring Harbor Laboratories. The \textit{sh}mdm2 constructs were introduced into T47D cells by retroviral gene transfer method to generate a pool of T47D.sh\textit{mdm2} cells (Brekman et al., 2011). By adding different concentration of doxycycline, the T47D.sh\textit{mdm2} pool was verified for MDM2 knockdown, while reduction of MDM2 was not observed in the vector control T47D.STGM cells (Figure 3 A). We carried out the experiments using 4\(\mu\text{g/ml}\) or lower dosage of doxycycline since higher dosage inhibited proliferation in vector T47D/STGM cells. From this pool of T47D.sh\textit{mdm2} cells, stable clones of T47D.sh\textit{mdm2} were selected by seeding the cells at limited
densities. Each of the clones was verified for efficiency of MDM2 knockdown, some of which were shown in Figure 3 B & C. When the stable cell lines carrying inducible shmdm2 were selected, we began to study the p53-independent role of MDM2 in estrogen-mediated cell proliferation in clonal lines as indicated.

**Figure 3: Characterization of shmdm2 pool and single cell clones of T47D cell lines for MDM2 knockdown.** T47D cells with control vector, mdm2.shRNA pool or mdm2.shRNA clones (3B6, 4C2, 4F1) were treated with various concentrations of doxycycline (Dox) for six days to induce shRNA expression. (A) Western blot analysis of MDM2 treated with 2,4,6 and 8 µg/ml doxycycline in T47D.STGM (vector control) and T47D.shmdm2 pool. +ve control (lane1) is from protein extract of MCF cells. (B&C) Western blot analysis of MDM2 treated with 2,3 and 4 µg/ml doxycycline in T47D.STGM (vector control) and T47D.shmdm2 clones 3B6, 4C2 and 4F1.

Using these established cell lines, we first assessed whether MDM2 protein levels increase with estrogen treatment and the efficiency of MDM2 knockdown upon shmdm2 induction. T47D cells were treated with 10nM estrogen for 5 days with and without shmdm2 induction and proteins were assessed by western blot analysis. The first step was to confirm that MDM2 proteins levels would increase with estrogen treatment in T47D cells. As expected, estrogen treatment led to an increase in MDM2 protein levels (Figure 4, compare lanes 1-3 and 5-7). We also observed that mdm2 shRNA induction...
reduced MDM2 protein levels both in the presence and absence of estrogen treatment (Figure 4, compare lanes 1-2, 3-4) while similar induction in the vector control had no effect on MDM2 (Figure 4, compare lanes 5-6, 7-8). Importantly, p53 protein levels are elevated with estrogen and do not decease with knockdown of MDM2 (Figure 4, compare lanes 1-2, 3-4).

To determine whether MDM2 knockdown had any effect on cell proliferation, we treated T47D cells with the above conditions and assessed cell proliferation using Guava viacount. Upon estrogen treatment, there was a robust increase in cell number (Figure 4). Importantly, with MDM2 knockdown there was a significant reduction in number (Figure 4), suggesting MDM2 was playing an important role in estrogen-driven cell proliferation. Thus, we concluded that there was a positive correlation with increased MDM2 protein levels and enhanced proliferation with estrogen treatment. This part of the study was included in the publication (Brekman et al., 2011). Since the p53 protein levels do not alter with MDM2 knockdown and MDM2 inhibition leads to a decrease in estrogen-mediated cell proliferation, this suggested a p53 independent role for MDM2 in the activation of estrogen-mediated cell proliferation (Brekman et al., 2011).
Figure 4: MDM2 knockdown in estrogen-treated T47D cells decreases cell proliferation. Western blot analysis of MDM2, p53 and actin protein levels from whole cell lysates and cell count of clonal T47D cells using Guava via count with mdm2 shRNA or control vector. T47D cells carrying mdm2.shRNA or control vector were treated with 2 µg/ml doxycycline (shRNA induction) for 3 days to induce shRNA expression, followed by 10nM estrogen for 5 days in presence or absence of 2 µg/ml of DOX. (Brekman et al., 2011)

3.3. Discussion

Estrogen is the driver of ER+ breast cancer as estrogen works through estrogen receptor and stimulates proliferation by activating a plethora of ER target genes that signals proliferation (Beckmann, Niederacher, Schnurch, Gusterson, & Bender, 1997). Importantly, a positive correlation exists between MDM2 overexpression and ER alpha expression (Hori et al., 2002; Marchetti et al., 1995).

Consistent with other studies, we have shown that in ER positive breast cancer cell line having mutant p53, T47D cells, estrogen treatment increased MDM2 protein levels. However, unlike the MDM2-p53 central dogma, p53 protein was not reduced with estrogen-mediated increase in MDM2. Importantly, MDM2 knockdown inhibited estrogen-mediated cell proliferation but did not increase p53
protein level. Interestingly, p53 levels remained unaltered with MDM2 knockdown in the presence of estrogen. In ER+ MCF7 breast cancer cell line having wild type p53, our laboratory reported similar observation (Brekman et al., 2011). Thus MDM2 knockdown resulted in inhibition of estrogen-mediated cell proliferation in ER positive breast cancer cells having wild type p53 and mutant p53, thus independent of p53 status.
CHAPTER 4:
Estrogen-mediated growth in 3D culture conditions requires MDM2 for oncogenic phenotypes.
4.1. Introduction

3D culture unlike the monolayer 2D culture conditions, mirrors the tissue microenvironment in terms of cellular communication and interaction with the extracellular matrix, and can better recapitulate the behavior of cancer cells in vivo (Lovitt, Shelper, & Avery, 2014). The anchorage independent colony formation assay is a form of 3D culture where no substrate, such as extracellular matrix (ECM) proteins are available for cell attachment (Thierbach & Steinberg, 2009). It is often associated with in vivo malignancies and is considered a hallmark of cellular transformation (Pavelic et al., 1980; Wada, Akiyoshi, Nakamura, & Tsuji, 1984).

Normal mammary epithelial cells have a unique organized architecture that gets disrupted in malignancy (Wiechmann & Kuerer, 2008). One of the hallmarks of breast carcinogenesis is the disruption of mammary tissue architecture. Within the mammary gland, there is a complex network of branching ducts. These ducts exit from sac-like structures called lobules. A cross section of duct reveals a hollow lumen, which is lined by an inner layer of glandular epithelial cells, having a polarized morphology, specialized cell-cell contact and attachment to an underlying basement membrane. These normal mammary epithelial cells rapidly lose many aspects of the differentiated state upon dissociation and culture on plastic substrata as they loose the signals from the extracellular matrix (Kenny et al., 2007). Growing cells in laminin rich matrigel, which consist of gelatinous protein mixture that resembles the complex
extracellular environment found in breast tissues, can largely restore the in vivo mammary architecture. Non-malignant mammary cells when grown in 3D laminin-rich matrix undergo small rounds of cell division after which they organize into polarized (with apical-basal polarity) growth arrested colonies having a hollow lumen. In contrast, malignant cells have disrupted architecture, with loss of tissue polarity and filled lumen (Kenny et al., 2007).

In order to understand the biological significance of MDM2 in estrogen signaling, we examined the function of MDM2 in breast cancer cell proliferation in 3D culture using the anchorage independent colony formation assay in soft agar and the growth of masses in matrigel. We also examined whether estrogen-activated MDM2 plays any role in disrupting the mammary architecture.

4.2. Results

It was previously reported that in ER+ breast cancer cell lines, MCF7 and T47D, estrogen treatment induces anchorage-independent cell growth as seen by the formation of large multicellular colonies in a soft agar assay (Y. Jiang et al., 2003; Stevens & Meech, 2006). Additionally, overexpression of MDM2 in MCF7 cells in the presence of estrogen provides a growth advantage (Saji et al., 1999). Previous unpublished work by Angelika Brekman from our laboratory showed a significant decrease in colony formation in soft agar for MCF7 cells in the presence of estrogen with MDM2 knockdown as compared to vector control cells, where shRNA induction had no effect on colony size (Figure 5 A, A. Brekman, unpublished data). To see if this was independent of an influence on
wild type p53 we examined effect of MDM2 knockdown on anchorage independent growth in the presence of estrogen in T47D cells.

Since MDM2 is required for the estrogen mediated cell proliferation in 2D culture and in 3D culture in MCF7 cells, we hypothesized that MDM2 will similarly be required for the cell proliferation in the 3D culture in T47D cells. To test this hypothesis, the cells were grown in the presence of estrogen in soft agar for 3-3.5 weeks with and without MDM2 knockdown. T47D cells formed large multicellular colonies in the presence of estrogen and with MDM2 knockdown colony size was significantly decreased (Figure 5 B). Representative images of the colonies formed with and without shRNA induction are shown in Figure 5 C. These experiments both in MCF7 and T47D cells showed that MDM2 knockdown inhibited large colony formation in soft agar indicating that MDM2 is required for estrogen-mediated anchorage independent growth. Furthermore because this was seen for T47D cells it indicated that the MDM2 mediated effect was p53 independent.

We also studied the function of MDM2 in 3D matrigel culture conditions, recapitulating the in vivo glandular architecture (Kenny et al., 2007). ER+ MCF7 and T47D cells when grown in matrigel form colonies with disorganized architecture called masses (Kenny et al., 2007). We previously published that MDM2 knockdown in MCF7 cells leads to a substantial decrease in the number of large masses (Brekman et al., 2011) and hypothesized that similar to the MCF7 cells, MDM2 will be required for estrogen-mediated cell growth in matrigel in T47D cells that do not have wild type p53. We observed the growth of masses
with or without MDM2 knockdown. We categorized the masses formed when T47D cells were grown in the presence of estrogen in matrigel into 5 different categories - very small (yellow), small (red), intermediate (green), large (purple) and very large (blue); as shown in Figure 5 D. With MDM2 knockdown we observed a robust decrease in large (purple) and very large (blue) masses and an increase in very small (yellow) and small (red) masses as compared to the vector control. We did not observe any masses of very small (yellow) and small (red) categories in the vector control. However, in clonal T47D.shmdm2 cell lines even without shRNA induction, we observed very small (yellow) and small (red) masses potentially due to a very low expression of shmdm2 without shRNA induction. In conclusion, this experiment showed that MDM2 protein was required for large and very large mass formation in matrigel, independently of wild type p53. Therefore, regardless of different p53 status, we observed a growth inhibitory effect that correlated with MDM2 knockdown. This demonstrates that estrogen-activated MDM2 in ER+ breast cancer cells promotes growth of colonies in 3D culture system.
Figure 5: MDM2 depletion of estrogen-treated breast cancer cell inhibits colony formation in 3D culture conditions.

A. Number of large colonies (50µm or larger) determined by counting the colonies of MCF7 cells when grown in soft agar in presence of estrogen and in presence and absence of shRNA induction, in bright field, in inverted fluorescence microscope. Average of three independent experiments are shown.

B. Number of large colonies (100µm or larger) determined by counting the colonies of T47D cells when grown in soft agar in presence of estrogen and in presence and absence of shRNA induction, in bright field, in inverted fluorescence microscope. Average of two independent experiments are shown.

C. A representative image of colony formed when T47D cells is grown in presence of estrogen in soft agar for 3 weeks.

D. T47D cells grown in matrigel for 3 weeks in presence of estrogen and in presence or absence of 4µg/ml doxycycline, were fixed and stained with propidium iodide. Masses were categorized in 5 different groups and the number of masses in each group were counted and presented as percent of mass in total population. * represents a p value ≤ 0.05, ** represents a p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents a p value ≤ 0.0001.
represents a p value ≤ 0.0001. The p value was determined by 2-tailed Student t-test.

Our data showed that MDM2 has a stimulatory role in colony formation in 3D culture that mirrors in vivo conditions. We were interested to investigate if MDM2 is responsible for disruption of mammary architecture. Consistent with previous findings (Kenny et al., 2007), we observed T47D cells grown in matrigel formed masses with disrupted architecture and filled lumen (Figure 6 A, bottom panel). Interestingly with MDM2 depletion, we observed that a significant number of masses had ductal lumen architecture, comprised of luminal clearance and less disrupted architecture (Figure 6 A, middle panel). Among the masses having ductal lumen architecture, there were a small number of masses having ordered acinus-like morphology and a perfect hollow lumen architecture (Figure 6 A, top panel). Overall, we observed that MDM2 knockdown was correlated with a statistically significant increase in the percent of masses with ductal lumen (Figure 6 B), restoring a nearly normal architecture. This indicated that cells with MDM2 knockdown had the potential to revert towards a normal mammary architecture.
Figure 6: MDM2 depletion in ER+ breast cancer cells with mutant p53 leads to formation of lumen and reverts mammary architecture towards a normal state.

A. A representative image from confocal immunofluorescence microscopy labeling DAPI, GFP and F-Actin of estrogen treated inducible clonal T47D.shmdm2 cells grown in 3D-matrigel in the presence and absence of 4 µg/ml doxycycline (dox) for 3 weeks is shown. The first and middle panel shows hollow lumen and ductal lumen respectively in the presence of shRNA expression to mdm2; the GFP (Kumar et al.) indicates shRNA expression to mdm2. The third panel shows mass structure (disruption of normal breast cancer architecture) in the absence of shRNA expression to mdm2.

B. T47D cells grown in matrigel for 3 weeks in presence of estrogen and in presence and absence of 4 µg/ml dox, were fixed, stained with F-Actin and mounted with DAPI. Confocal z-stack images were acquired. Masses with lumen were counted and presented as percent of total population of masses grown in 3Dmatrigel. An average of two independent experiments are shown. Confocal z-stack images of approximately 30 and 50 masses in each group were analyzed in two independent experiments respectively. * represents a p value ≤ 0.05, ** represents a p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents a p value ≤ 0.0001. The p value was determined by 2-tailed Student t-test.
We also studied the effect of MDM2 on mitosis by staining the masses growing in matrigel with phospho-histone H3 antibody. Our hypothesis was that MDM2 knockdown would reduce the number of mitotic cells in the masses. There is a positive correlation between phosphorylation of histone H3 and mitotic chromosome condensation starting to occur in the early prophase (Hendzel et al., 1998; Hendzel et al., 1997). Phospho-histone H3 has been used as a reliable and easy method to count mitosis in many tumors, including breast (Ribalta, McCutcheon, Aldape, Bruner, & Fuller, 2004; Skaland et al., 2007; Veras, Malpica, Deavers, & Silva, 2009). Using T47D cells grown in the presence of estrogen, we stained masses with antibodies for mitotic marker phospho-histone H3 to determine if the luminal clearance phenotype is associated with decreased mitosis. Representative images are shown in Figure 7 A, wherein MDM2 knockdown was associated with ductal lumen phenotype. Interestingly, accompanied with MDM2 knockdown there was a dramatic decrease in the average number of mitotic cells (cells stained positive for phosphor-histone H3 in masses) in both the inducible clones and constitutive MDM2 knockdown T47D cells when compared to vector control (Figure 7 B and 7 C). Several studies have shown that MDM2 overexpression drives cell cycle progression to S phase (Frum et al., 2014; Lundgren et al., 1997). To determine if in T47D cells MDM2 overexpression drives cells to S phase, we asked if MDM2 knockdown would affect cell cycle progression. In order to accomplish this, we grew T47D cells with inducible MDM2 knockdown in the presence of estrogen using standard 2D culture conditions and used Fluorescence-activated cell sorting (FACS) to
determine cell cycle progression. As expected, estrogen treatment increased the number of cells in S phase. With MDM2 knockdown in the presence of estrogen, there was a significant reduction from 13.16% to 7.79% in number of cells in S phase (Figure 7 D). This demonstrated that in ER+ breast cancer cells, MDM2 knockdown inhibited estrogen-mediated cell proliferation and negatively influenced S phase cell cycle progression.
Figure 7: MDM2 knockdown impedes mitosis and significantly decreases the number of cells in S phase in the presence of estrogen.

A. Representative confocal Z-stack image of DAPI, phospho-histone H3 and GFP in estrogen-treated inducible clonal T47D.shmdm2 cells in the presence and absence of 4µg/ml doxycycline. B. & C. T47D cells (B. inducible shmdm2 clonal and vector pool; C. constitutive shmdm2 and vector pool) grown in presence of estrogen in 3D matrigel for 3.5 weeks. The cells were fixed, permeabilized, blocked, stained with phospho-histoneH3 antibody and mounted with DAPI. Images were taken with confocal microscope. Quantitative analysis of phospho-histone H3 positive cells were performed by capturing optical Z-stack sections of masses and dividing the number of positive phospho-histone H3 cells by the total number of masses. Confocal z-stack images of approximately 50-60 masses (as indicated) were acquired in each group. Results of one of the two independent experiments are shown. D. Cell cycle analysis by FACS (Fluorescence Activated Cell Sorting). T47D cells were harvested, fixed and stained with propidium iodide and subjected to cell cycle analysis by FACS. Cell numbers were presented as percent of cells in S phase in a total population of 10,000 cells and analyzed by FACS in each group. Average of 4 independent experiments are shown. * represents a p value ≤ 0.05, ** represents a p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents a p value ≤ 0.0001. The p value was determined by 2-tailed Student t-test.
4.3. Discussion

In 2D culture system, we showed that estrogen treatment elevated MDM2 protein levels, and MDM2 inhibition led to reduced estrogen-mediated proliferation; indicating that MDM2 is required in estrogen-mediated cell proliferation. Here we observed that MDM2 knockdown in soft agar (3D culture) led to drastic reduction of colonies, indicating MDM2 is required for the anchorage independent growth in the presence of estrogen. This observation is consistent with other studies showing that overexpression of MDM2 in estrogen-treated MCF7 cells provides a growth advantage (Saji et al., 1999). Since the growth advantage was observed in both ER+ breast cancer cell lines, having wild type p53 (MCF7) and mutant p53 (T47D), this result suggests that MDM2 is required for anchorage independent growth, independent of p53 status.

To understand the biological significance, we continued our studies by growing ER+ breast cancer cells in matrigel, which recapitulates the *in vivo* glandular architecture. Previous studies from our lab have published that MDM2 knockdown in estrogen treated MCF7 cells inhibited the estrogen-mediated growth of masses (Brekman et al., 2011). To study the p53 independent effects of MDM2 in estrogen-mediated growth of masses, we carried out experiments in T47D cells that have a loss-of-function mutant p53. We categorized the size of masses in 5 different categories – very large, large, intermediate, small and very small. We observed that upon depletion of MDM2 in estrogen-treated T47D cells, the larger masses were replaced by smaller masses, indicating inhibition of
growth, independent of p53. However, further studies need to be done to understand how estrogen-activated MDM2 stimulates proliferation in 3D culture.

We also studied the physiological relevance of MDM2 in ER+ breast cancer by investigating if MDM2 has any role in disrupting the mammary architecture since distortion of architecture occurs in breast carcinogenesis. We observed that a significant number of the masses with MDM2 knockdown had partial luminal clearance. This phenotype resembled a near normal architecture or an intermediate phase between disrupted architecture and normal mammary architecture. This suggests that MDM2 is important and central in estrogen signaling as it is involved, at least by part, in disrupting mammary architecture. More experiments need to be done to assess if the apical-basal polarity is restored upon depletion of MDM2 and to understand if the ductal lumen phenotype was observed due to inhibition of cell proliferation or initiation of cell death.

We also observed that MDM2 knockdown in the presence of estrogen reduced the number of cells in the masses undergoing mitosis by detecting phospho-histone H3 (mitotic marker) positive cells. A recent study showed that MDM2 drives mitotic catastrophe in podocytes of glomerulus in kidney, resulting in loss of podocyte (Mulay et al., 2013). However, a previous study has shown that targeted overexpression of MDM2 in mouse mammary glands caused an increase in DNA synthesis with a reduction in mitosis, resulting in multinucleated cells associated with hyperplasia. MDM2 has been shown to suppress mitosis in a p53 independent manner (Lundgren et al., 1997). These data are not
consistent with our finding. This could be due to the fact that the condition of both the experiments are different. In our study, estrogen-activated MDM2 was in a physiological level while in their study (Lundgren et al., 1997) MDM2 protein was overexpressed in mammary gland. The level of MDM2 protein is an important factor in the context of tumor formation.

Although we observed that MDM2 depletion in the presence of estrogen inhibited mitosis, we still need to dissect whether MDM2 regulates DNA synthesis or mitosis. Our observation could be due to the fact that MDM2 depletion might inhibit DNA synthesis, thus resulting in reduced mitosis. Another possibility is that estrogen activated MDM2 might increase both DNA synthesis and mitosis. Further experiments need to be done to resolve these issues. Whether MDM2 increases cells in S phase can be resolved by staining with antibodies against PCNA to detect cells undergoing DNA synthesis in S phase.

In conclusion, we observed that estrogen-activated MDM2 is required for colony formation in a p53 independent manner in 3D culture conditions. MDM2 knockdown in the presence of estrogen resulted in partial luminal clearance and led to an intermediate stage, ushering towards a near normal mammary architecture, suggesting that estrogen-activated MDM2 plays a role, at least in part, to disrupt normal mammary architecture. MDM2 depletion resulted in inhibition of mitosis as observed in immunofluorescence staining with phospho-histone H3 in 3D culture and a reduction in the number of cells in S phase in 2D culture. Thus, our data demonstrated that MDM2 is important in estrogen signaling.
CHAPTER 5:
Estrogen-activated MDM2 provide proliferative advantage through the Rb-E2F1 pathway.
5.1. Introduction

The growth proliferative effect of estrogen is due to the ability of estrogen to induce cell cycle progression from G1 to S phase (Lewis-Wambi & Jordan, 2009). Two of the proteins that accelerate the transition of cell cycle from G1 to S phase are phosphorylated Rb and E2F1. In the early G1 phase of cell cycle, hypophosphorylated Rb binds with the transcription factor E2F1 and inhibits E2F1 activation. In the mid G1 phase, several cyclin dependent kinases (CDK2, CDK4, and CDK6) phosphorylate Rb (Harbour & Dean, 2000). This in turn sets E2F1 free and thus E2F1 is released, binds to DNA with its binding partner DP, and induces transcription of E2F1 target genes ribonucleotide reductase (RR), thymidylate synthase (TS), thymidine kinase (TK) and dihydrofolate reductase (DHFR) thereby, resulting in increased proliferation (DeGregori, Kowalik, & Nevins, 1995; Wang, Dong, Saville, & Safe, 1999). Estrogen treatment induces cell cycle progression through G1 to S phase transition (Prall, Sarcevic, Musgrove, Watts, & Sutherland, 1997) and promotes Rb phosphorylation (Altucci et al., 1996). MDM2 stimulates E2F1 transcriptional activity (Martin et al., 1995), increases protein stability of E2F1 (Zhang et al., 2005), disrupts Rb-E2F1 complex and inhibits tumor suppressive functions of Rb by degrading it (Sdek et al., 2005; Uchida et al., 2005). MDM2 depletion results in the reduction of phosphorylated Rb and E2F1 in prostate cancer cells (Zhang, Li, Wang, Agrawal, & Zhang, 2003). We and others have shown estrogen increases MDM2
both in message and protein levels (Brekman et al., 2011; Hu et al., 2007). Additionally, E2F1 gets upregulated by estrogen (Wang et al., 1999) and estrogen treatment preferentially stimulates loading of RNA polymerase II to SNP 309 G allele of mdm2 gene and increasing MDM2 protein level (Hu et al., 2007). Since we observed that MDM2 knockdown in the presence of estrogen decreased the number of cells in S phase (Figure 7 D), and E2F1 and hyperphosphorylated Rb play a central role in driving the cells to S phase; we hypothesized that MDM2 imparts the estrogen-mediated proliferative advantage in a p53 independent manner through Rb-E2F1 pathway. We next investigated the downstream targets of estrogen-activated MDM2 signaling.

5.2. Results

In order to determine the p53-independent downstream targets of MDM2 signaling in the presence of estrogen, we asked how Rb phosphorylation and E2F1 protein levels were altered with MDM2 knockdown. We observed that estrogen treatment increased MDM2 and shRNA induction caused a robust decrease of MDM2 protein in T47D shmdm2 cell line. Consistent with our previous wild type p53 studies (Brekman et al., 2011), p53 protein levels in T47D cells were not altered with MDM2 knockdown (Figure 8 A compare lanes 7 to 8). We saw an increase of E2F1 and phospho Rb protein with estrogen treatment, consistent with other studies (Altucci et al., 1996; Stender et al., 2007). Interestingly, accompanied with MDM2 knockdown in the presence of estrogen, a robust decrease in E2F1 and phospho Rb protein was observed in the inducible shRNA clones from T47D cell lines (Figure 8 A, compare lanes 8 to 7, Figure 8
B). However, the decrease in phospho Rb protein was not statistically significant (Figure 8 B) potentially due to the toxicity of induction by doxycycline. To eliminate the effects caused by doxycycline, we performed similar western blot experiments in the T47D cells with constitutive MDM2 knockdown. We observed a similar result as in inducible shmdm2 knockdown cell line, where estrogen treatment increased MDM2, phospho Rb, E2F1 protein level and MDM2 knockdown in the presence of estrogen correlated with reduced E2F1 and phospho Rb protein levels (Figure 8 C & 8 D). The reduction of phospho Rb protein levels with MDM2 knockdown was statistically significant. However, the reduction of E2F1 protein level with MDM2 knockdown showed a trend but the data tested by student t-test was not significant. This is possibly because of the large error bar of standard deviation as in each of the biological replicate experiments, a reduction of E2F1 with MDM2 depletion was observed.
Figure 8: MDM2 influences estrogen mediated cell proliferation through the Rb-E2F1 pathway in G/G mdm2 SNP 309 ER+ T47D breast cancer cells.

A. Inducible clonal T47D cells with mdm2 shRNA or control vector were treated with and without 4µg/ml doxycycline (dox) for 3 days to induce shRNA expression, followed by 10nM estrogen for 5 days in the presence and absence of dox. A representative image of western blot analysis of MDM2, phospho Rb, E2F1, p53 and Actin protein levels from 50µg whole cell protein extract is shown. B. ImageJ analysis was performed for MDM2, phosphor Rb and E2F1 protein levels normalized to Actin. The graph represents average of three independent experiments with standard deviation in inducible clonal T47D cells with mdm2 shRNA or control vector. C. A constitutive pool of T47D cells with mdm2 shRNA or control vector were treated with 10nM estrogen for 5 days. A representative image of western blot analysis of MDM2, phospho Rb, E2F1, total Rb and Actin protein levels from 50µg whole cell protein extract is shown. D. ImageJ analysis was performed for MDM2, phosphor Rb and E2F1 protein levels normalized to Actin. Graph represents average of three independent experiments with standard deviation in constitutive pool of T47D cells with mdm2 shRNA or control vector. * represents a p value ≤ 0.05, ** represents a p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents a p value ≤ 0.0001. The p value was determined by 2-tailed Student t-test.

In summary, in ER+ T47D cells, estrogen treatment led to an increase in MDM2, phospho Rb and E2F1 protein level. MDM2 depletion was correlated with decrease in phospho Rb and E2F1 protein level in the presence of estrogen.

To further test the role of MDM2 in estrogen signaling, we examined the protein levels of these prospective downstream targets in MCF7 cells having wild type p53. Estrogen treatment increased MDM2 protein levels (Figure 9 A, compare lane 3 and 7 to 1 and 5) and MDM2 knockdown by mdm2 shRNA showed reduced MDM2 protein levels as compared to the vector control (Figure 9 A, compare lanes 6 and 8 to lanes 2 and 4). Phospho Rb protein levels also increased in the presence of estrogen (Figure 9 A, compare lanes 3, 4 and 7 to lanes 1 and 5) and upon MDM2 knockdown in presence of estrogen phospho Rb
protein levels were decreased (Figure 9 A, compare lane 8 to 7). The total Rb protein increased in the presence of estrogen and did not change with MDM2 knockdown. E2F1 protein levels increased with estrogen treatment (Figure 9 A, compare lanes 3, 4 and 7 to lanes 1 and 5) and with MDM2 knockdown E2F1 protein levels were reduced (Figure 9 A, compare lane 8 to 7). When three independent experiments were analyzed by imageJ analysis and averaged we calculated an 85% decrease of MDM2 protein levels correlated with a 45% decrease of E2F1 and 81% decrease of phospho Rb protein levels (Figure 9 B).
Figure 9: MDM2 influences estrogen mediated cell proliferation through the Rb-E2F1 pathway in T/G mdm2 SNP 309 ER+ MCF7 breast cancer cells. 

A. Inducible clonal MCF7 cells with mdm2 shRNA or control vector were treated with and without 2µg/ml doxycycline (dox) for 3 days to induce shRNA expression, followed by 10nM estrogen for 5 days in the presence and absence of dox. A representative image of western blot analysis of phospho Rb, Total Rb, E2F1, MDM2 and Actin protein levels from 50µg whole cell protein extract is shown. 

B. Quantitative analysis of percentage change in signal intensity of western blot protein levels by ImageJ in lane 8 compared to lane 7. Relative reduction of MDM2, E2F1 and phospho Rb with shRNA induction to mdm2 in estrogen-treated inducible clonal MCF7.shmdm2 cells was quantified by ImageJ analysis. The graph represents average of three independent experiments with standard deviation.
In conclusion, MDM2 inhibition in the presence of estrogen led to a reduction of phospho Rb and E2F1, in T47D cells with mutant p53 and in MCF7 cells with wild type p53. This demonstrates that regardless of the p53 status, estrogen mediated cell proliferation is through MDM2-mediated increases in phospho Rb and the stimulation of E2F1.

We observed that estrogen stimulated MDM2 in ER+ breast cancer cells increased Rb phosphorylation as MDM2 knockdown in the presence of estrogen reduced phospho Rb protein level. We questioned if MDM2 alone or in coordination with estrogen treatment is responsible for increased phospho Rb protein level. If MDM2 alone were responsible, then MDM2 overexpression would result in increased phospho Rb protein level in the absence of a stimulus for proliferation. Since overexpression of MDM2 causes forced cell cycle progression to S phase and we observed that MDM2 depletion correlated with decrease of phospho Rb protein level in the presence of estrogen and Rb phosphorylation occurs when cell cycle progresses to S phase, we hypothesized that MDM2 protein might drive Rb phosphorylation. To test if MDM2 alone increased phosphorylation of Rb, we performed an *in vitro* kinase assay (described in Materials and Methods) with purified MDM2 added to nuclear protein extract from cells grown with and without overnight estrogen treatment in ER+ breast cancer cells. Interestingly, in nuclear protein extract from MCF7 or T47D cells grown in the absence of estrogen treatment, we saw that addition of purified MDM2 increased phosphorylation of Rb protein (Figure 10). This provides further evidence that MDM2 alone drives phosphorylation of Rb in ER+
breast cancer cells as even in the absence of estrogen (a driver for estrogen-mediated cell proliferation), Rb phosphorylation was observed when MDM2 protein was added in an *in vitro* kinase assay. Other studies have shown that MDM2 depletion is shown to reduce Rb phosphorylation (Z. Zhang et al., 2003). Extract from estrogen treated cells also showed that the increase in phospho Rb occurred upon addition of 1µl of purified MDM2 (Figure 10). Our data suggests that MDM2 can promote Rb phosphorylation both *in vivo* and *in vitro*. Taken together, our results indicate that estrogen provokes signals to increase MDM2 protein and this estrogen-stimulated MDM2 promotes signal transduction for increasing phosphorylation of Rb.
Figure 10: MDM2 drives phosphorylation of Rb in ER+ breast cancer cells. In vitro kinase assay was performed to detect phosphorylation of Rb with and without overnight estrogen treatment in presence and absence of bacterially purified MDM2. A representative image of Western blot analysis of MDM2, phospho Rb and total Rb protein level from nuclear extract of MCF7 and T47D cells are shown. The numbers below phospho Rb blot represent the ImageJ values of two independent experiments normalized to lamin A that was used as a loading control of nuclear extract.
5.3. Discussion

Estrogen increases the number of cells in S phase as a number of proteins that are affected by estrogen play a role in increasing the rate of progression from G1 to S phase, including the induction of early response ER target genes (c-myc and c-fos) (Lamb, Ladha, McMahon, Sutherland, & Ewen, 2000; Prall, Rogan, Musgrove, Watts, & Sutherland, 1998; Prall, Rogan, & Sutherland, 1998). Here we show MDM2 as one of the proteins that is elevated by estrogen and estrogen-activated MDM2, at least in part, plays a role in G1-S phase transition. Estrogen has been shown to increase phosphorylation of Rb (Foster, Henley, Bukovsky, Seth, & Wimalasena, 2001) and Rb phosphorylation stimulates proliferation (Fribourg, Knudsen, Strobek, Lindhorst, & Knudsen, 2000).

Phosphorylation of Rb is important in estrogen signaling since phosphorylation-defective mutant of pRb in mammary gland results in inhibition of ductal growth and proliferation (Z. Jiang & Zacksenhaus, 2002). Additionally, an elevation of hyperphosphorylation of Rb is observed in ER+ breast cancer cells that are hypersensitized to estrogen signaling. The persistent hyperphosphorylation of Rb in hyper-sensitized ER+ breast cancer cells led to increase in cells in S phase (Balasenthil & Vadlamudi, 2003; Knudsen, Buckmaster, Chen, Feramisco, & Wang, 1998). Also, a recent study showed that Rb phosphorylation at sites S807/S811 protects cells from apoptosis (Antonucci, Egger, & Krucher, 2014). This study and others (Adegbola & Pasternack, 2005)
further supports the fact that hyperphosphorylation of Rb stimulated proliferation. However, the mechanism is still unknown.

Here we showed that estrogen stimulated MDM2 increased phospho Rb and E2F1 protein levels in both ER+ cell lines, having wild type p53 (MCF7) and mutant p53 (T47D) as MDM2 knockdown reduced phospho Rb and E2F1 protein levels in the presence of estrogen. Our data is consistent with studies done in prostate cancer cells with different p53 status (wild type, mutant and null), where knockdown of MDM2 led to reduction of phospho Rb and E2F1 (Z. Zhang et al., 2003). Moreover, other studies in prostate cancer cell lines have shown that MDM2 increases protein stability of E2F1 by displacing SCF$^{Skp2}$, an E3 ligase of E2F1, from E2F1 in a p53 and Rb independent manner (Zhang et al., 2005). It is quite possible that estrogen-stimulated MDM2 stabilizes E2F1 protein level by displacing the E3 ubiquitin ligase for E2F1, SCF$^{Skp2}$. Further experiments needs to be done to test this possibility.

We have shown that by biochemical in vitro kinase assay that MDM2 overexpression led to an increase in phosphorylated Rb protein levels. Interestingly, the increase in phosphorylated form of Rb was observed in absence of estrogen. This data confirmed the fact that MDM2 can drive phosphorylation of Rb, even in the absence of proliferative stimuli, like, estrogen. Our data is consistent with other study that shows MDM2 knockdown is associated with decrease in phosphorylated Rb (Z. Zhang et al., 2003). Thus, we demonstrated that MDM2 is a central hub in estrogen-mediated p53 independent cell proliferation.
CHAPTER 6:
Combinations of MDM2 knockdown and estrogen antagonist provide better inhibition of E2-ERα-MDM2-phosphoRb axis.
6.1. Estrogen plays an important role in breast cancer.

Estrogen plays an important role in the development of female breast tissue (Gruber, Tschugguel, Schneeberger, & Huber, 2002). Female aromatase deficient patients who are incapable of converting \(C_{19}\) steroids (eg, testosterone) to estrogen, have impaired breast development at the onset of puberty (MacGillivray, Morishima, Conte, Grumbach, & Smith, 1998). Estrogen also plays an important role in the onset and progression of breast cancer (Pike, Spicer, Dahmoush, & Press, 1993; Platet, Cathiard, Gleizes, & Garcia, 2004) as more than 70% of primary breast cancers in women are estrogen receptor positive (ER+) and depend on estrogen for growth (Beckmann et al., 1997; Clark, Osborne, & McGuire, 1984; Masood, 1992).

6.2. Estrogen signaling, a target for ER+ breast cancer treatment.

Estrogen works through estrogen receptors (ER), which are members of ligand-modulated nuclear transcription factors. There are two types of ER: ER alpha (ER\(\alpha\)) and ER beta (ER\(\beta\)) (Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996). ER\(\alpha\) is believed to be the predominant target of estrogen in breast tissue (Anderson, Chatterjee, Ershler, & Brawley, 2002) as 2/3\(rd\) of breast cancer are ER+ (Clark et al., 1984).

The ligand estrogen binds to ER with high affinity and specificity, followed by release of heat shock protein (HSP)-90 from ER, receptor dimerization, and nuclear localization (Beato, 1989; MacGregor & Jordan, 1998). The estrogen-ER complex through the DNA binding domain of ER binds to specific DNA
sequences, the Estrogen Response elements (ERE), and increases the transcription of estrogen-regulated genes which involves a wide range of genes, directly or indirectly associated with proliferation, invasion, survival or angiogenesis in breast cancer. The estrogen receptor has 2 regions, termed activation functions (AF): AF1 and AF2, which recruit transcriptional co-activators and co-repressors to the transcriptional complex, depending on the type of ligand (estrogen agonist or antagonist) (Beato, 1989). When ER binds to estrogen, coactivator molecules are recruited and transcription is promoted (Figure 11). On the other hand, if ER binds to estrogen antagonist, it inhibits interaction of the ER with other transcription machinery and thus inhibits transcription of estrogen responsible genes (Beato, 1989).

6.3. Tamoxifen, a drug for endocrine therapy to breast cancer

Tamoxifen is a competitive inhibitor of estrogen binding to ER, thus blocking the proliferation-signaling pathway of estrogen. Tamoxifen has mixed agonist and antagonist effects, depending on target tissues as tamoxifen acts like an anti-estrogen in breast tissue and estrogen-like in other tissues like bone. Hence, it is also known as SERM, or selective estrogen receptor modulator (Osborne, 1998). Early stage ER-positive breast cancer patients are often treated with tamoxifen as adjuvant therapy (Davies et al., 2011).

Mechanism of action:
Tamoxifen binds with estrogen receptor in the ligand-binding domain, ER gets released from heat shock protein (HSP)-90 and receptor dimerization occurs. This
is followed by sequence specific DNA binding to an ERE. As tamoxifen inhibits AF2 function in breast cancer cells, tamoxifen-ER complex inhibits transcription of ER-responsive genes. However, a partial transcription of ER target genes occurs through AF1 (Figure 11) (MacGregor & Jordan, 1998).

6.4. Fulvestrant, an ER antagonist with no agonist activity.

Fulvestrant (‘Faslodex’) is an ER antagonist that binds to ER, accelerates degradation of ER and thereby blocks estrogen signaling (Osborne, Wakeling, & Nicholson, 2004). As it acts as an anti-estrogen throughout the body, it is not a SERM. It is used in clinic to treat ER+, advanced and metastatic breast cancer patients.

Mechanism of action:

Chemically, Fulvestrant is drastically different from tamoxifen. It is a 7α-alkylsulphinyl analogue of 17β-estradiol, having a binding affinity with ER of 89% to that of estradiol, compared to tamoxifen having a binding affinity with ER of 2.5% to that of estradiol (Wakeling & Bowler, 1987). Fulvestrant competitively inhibits binding of estradiol to ER. Fulvestrant-ER complex impairs receptor dimerization and nuclear-cytoplasmic shuttling, thus inhibiting nuclear localization of the receptor. Any Fulvestrant-ER complex that enters the ER is transcriptionally inactive as both AF1 and AF2 subunits are disabled (Figure 11). Fulvestrant treatment causes accelerated degradation of the ER protein, thus making the Fulvesrant-ER complex very unstable. As Fulvestrant treatment
reduces ERα protein levels, this result in reduction in estrogen regulated gene expression (Osborne et al., 2004).

Figure 11: Schematic representation of mechanism of action of Estradiol, Tamoxifen and Fulvestrant. Taken from: Fulvestrant: A new type of estrogen receptor antagonist for the treatment of advanced breast cancer Buzdar, A.U. Drugs Today 2004, 40(9): 751 ISSN 1699-3993.

6.5. Results

The therapy of choice for ER+ breast cancer patients is anti-estrogen receptor targeted. Tamoxifen is the first drug approved as chemopreventive agent for women with high risk for developing ER+ breast cancer. Currently, tamoxifen is administered by all stages of ERα+ breast cancer patients (Fisher et al., 1998). Using T47D cells, we analyzed cell cycle progression using FACS analysis. As expected, estrogen treatment significantly increased the percentage
of cells in S phase and the additional treatment of tamoxifen caused a significant reduction (Figure 12 A). MDM2 knockdown alone significantly reduced the percentage of cells in S phase in estrogen treated T47D cells. The reduction with MDM2 knockdown is similar in quantification to tamoxifen treatment. Interestingly, MDM2 knockdown combined with tamoxifen treatment in the presence of estrogen further significantly reduced the number of cells in S phase (Figure 12 A). It is possible that we may have further reduced number of cells in S phase upon combination treatment of both tamoxifen and MDM2 knockdown, due to a better reduction of MDM2 protein level with combination treatment as both MDM2 knockdown by inducible shRNA to \textit{mdm2} and tamoxifen treatment alone causes partial decrease of MDM2 protein. 4-hydroxytamoxifen (the active metabolite of tamoxifen) is shown to downregulate MDM2 protein level significantly (Swetzig et al., 2016). Another study has shown that MDM2 depletion in p53-deficient MCF7 cells sensitizes cells to tamoxifen suggesting MDM2 might play a positive role in developing tamoxifen resistance in breast cancer cells (Shostak et al., 2014). Furthermore it is known that tamoxifen has partial estrogen-agonist activity with some estrogen responsive genes remaining transcriptionally active (Inoue et al., 2002).

In order to eliminate the partial agonist effect of estrogen by tamoxifen, we used Fulvestrant. Fulvestrant has been used with success for targeting MDM2 pathway (Dolfi et al., 2014; Swetzig et al., 2016). We first tested if simultaneously knocking down MDM2 and estrogen antagonist might further reduce MDM2 protein level. To investigate if that’s the case, we looked into the protein levels of
MDM2 in estrogen treated inducible T47D.shmdm2 cell lines with and without shmdm2 induction in the presence and absence of Fulvestrant. There was a better reduction of MDM2 protein levels with the combination of MDM2 depletion by inducible shRNA to mdm2 and Fulvestrant in the presence of estrogen (Figure 12 B). This observation is consistent with other reports that showed that MDM2 is reduced by Fulvestrant (Dolfi et al., 2014; Swetzig et al., 2016) and tamoxifen (Swetzig et al., 2016). Interestingly, we observed that correlated with additive reduction of MDM2 protein, this combination treatment also led to increased inhibition phospho Rb (Figure 12 B). Increase in phospho Rb protein level is an indication of increased number of cells in the S phase of cell cycle and hence is considered to be a marker of cell proliferation (Hallstrom & Nevins, 2009). We were interested to examine whether the combination of Fulvestrant and MDM2 knockdown caused a further reduction of cell viability compared to both of them alone. We saw that in the presence of estrogen, simultaneous treatment of Fulvestrant with MDM2 knockdown caused a further reduction in cell viability, measured as percentage of mitochondrial activity by MTT assay (Figure 12 C).
Figure 12: Combination knockdown of MDM2 and anti-estrogen therapy enhances growth inhibition of MDM2 overexpressing ER+ breast cancer cells.

A. Cell cycle analysis by FACS (Fluorescence Activated Cell Sorting). 4µg/ml doxycycline treated T47D cells grown in presence and absence of 10nM estrogen and 7µM tamoxifen for 5 days were harvested, fixed and stained with propidium iodide and subjected to cell cycle analysis by FACS. Cells numbers were presented as percent of cells in S phase in a total population of 10,000 cells analyzed by FACS in each group. Average of 4 independent experiments are shown.

B. T47D.shmdm2 cells were treated with 10nM estrogen and 10µM Fulvestrant for 5 days. A representative western blot analysis of MDM2, phosphoRb and Actin protein levels from 50µg whole cell protein extract is shown. The numbers below each blot represent imageJ values of the representative blot normalized to Actin.

C. The MTT assay was performed in T47D.vector and inducible clonal T47D.shmdm2 clonal cell lines after treatments. The results were shown as percentage mitochondrial activity, represents an average of 2 independent experiments. Graph represents average of three independent experiments (A.) and average of two independent experiments (C.) with error bars presenting ± mean standard deviation.
The representative images of the cells by light microscopy (Figure 13 A) and by confocal microscopy (Figure 13 B) demonstrated more reduction in the number of cells with simultaneous treatment of Fulvestrant and MDM2 knockdown, compared to each of them alone. Importantly, while cell proliferation was reduced by combination treatments, we did not observe an increase in cell death by confocal microscopy. These data indicate that Fulvestrant is not strong enough to block the MDM2 pathway. This suggests that pharmacological inhibition of estrogen signaling along with MDM2 inhibition though not additive but is better way to inhibit estrogen signaling.

Figure 13: Representative images showing combination knockdown of MDM2 and anti-estrogen therapy enhances growth inhibition of MDM2 overexpressing ER+ breast cancer cells. 

A. Inducible clonal T47D cells with mdm2 shRNA or control vector were treated with and without 4µg/mL doxycycline for 3 days to induce shRNA expression, followed by 10nM estrogen and 10µM Fulvestrant for 5 days. Representative images are shown in bright field and in GFP fluorescence. B. A representative live cell image by confocal microscopy with 20X objective of T47D.vector and inducible clonal T47D.shmdm2 clonal cell lines after treatments. Red fluorescence represents staining with propidium iodide. Blue fluorescence represents staining of nuclear DNA.
6.6. Discussion

We hypothesized that MDM2 was important in estrogen signaling and tested if knockdown of MDM2 would have the same effects as inhibition of estrogen signaling. Importantly, in support of our hypothesis, we observed similar suppressive effects with MDM2 knockdown or inhibition of estrogen signaling alone, and slightly improved inhibition upon both conditions. This improved suppressive effect in decrease of cell viability (as measured by MTT assay) and reduction in number of cells in S phase of cell cycle upon knocking down MDM2 in the presence of estrogen antagonist (Fulvestrant or Tamoxifen) was correlated with additive reduction of MDM2, when compared with each of the treatments alone. Importantly, along with the growth suppressive effect with simultaneous knockdown of MDM2 and treatment with estrogen antagonists that is correlated with the decrease of MDM2 protein levels, an additive reduction in phospho Rb protein levels were also observed. Hyper phosphorylation of Rb is correlated with increased number of cells in the S phase of cell cycle (Balasenthil & Vadlamudi, 2003; Knudsen et al., 1998). This demonstrated that MDM2 is a central hub in estrogen signaling as growth suppressive effects of ER+ breast cancer cells were correlated with decrease in MDM2 protein levels.
CHAPTER 7:
Conclusion, Significance and Future directions
7.1. Conclusion

MDM2 overexpression occurs in many human cancers (Momand et al., 1998). There are an increasing number of reports that suggest MDM2 overexpression is correlated with tumor development, invasion and metastasis in a p53 dependent and p53 independent manner (Brekman et al., 2011; X. Chen et al., 2013; Gu, Findley, & Zhou, 2002; Jung et al., 2013; Ladanyi et al., 1993; Vaughan et al., 2011; Yang et al., 2006). According to the cancer genome atlas (TCGA), MDM2 overexpression occurs in all sub-categories of breast cancers ("Comprehensive molecular portraits of human breast tumours," 2012). MDM2 is also considered as a negative prognostic marker (Onel & Cordon-Cardo, 2004; Turbin et al., 2006). About two-third of the ER+ breast cancers overexpress MDM2. We have published that MDM2 provokes estrogen-mediated cell proliferation in a wild type p53 independent manner (Brekman et al., 2011).

In this thesis we show that MDM2 is central in estrogen signaling and propose a central signaling pathway (Figure 14). In ER positive breast cancer cells, estrogen treatment increased MDM2 protein levels and MDM2 depletion inhibited estrogen-mediated cell proliferation in a p53 independent manner in both 2D and 3D culture conditions. Interestingly our data suggest that estrogen-activated MDM2 in ER+ breast cancer cells having mutant p53, at least in some part, disrupted normal mammary architecture since MDM2 knockdown caused more normal mammary architecture of masses grown in matrigel. Additionally, MDM2 knockdown decreased the number of phospho histone positive cells per
mass in 3D matrigel culture, indicating reduction of mitotically active cells. FACS analysis of MDM2 knockdown in the presence of estrogen in 2D culture conditions revealed that there is a decrease in the percent of cells in S phase of cell cycle. We showed that estrogen-activated MDM2 provided an estrogen-mediated proliferative advantage through the Rb-E2F1 pathway. Estrogen treatment increased MDM2, phospho Rb and E2F1 protein levels while MDM2 depletion in the presence of estrogen decreased phospho Rb, and E2F1 protein. Recent studies show blocking estrogen signaling reduces MDM2 protein levels (Dolfi et al., 2014; Swetzig et al., 2016). We have shown that blocking estrogen signaling by the addition of Fulvestrant reduced MDM2 protein levels and phospho Rb protein levels, thus demonstrating the ER-MDM2-Phospho Rb-E2F1 signaling pathway.
Figure 14. Pathway showing p53-independent function of MDM2 is required for estrogen-mediated breast cancer proliferation and disruption in 3D mammary architecture: In ER+ breast cancer cells, estrogen is the driver of mammary epithelial tumorigenesis. MDM2 plays an important role in driving tumorigenesis. We have shown that estrogen-activated MDM2 led to increased levels of hyperphosphorylated Rb. Once Rb is hyperphosphorylated, E2F1 is released. This ER-MDM2-Phospho Rb-E2F1 axis promotes estrogen-mediated proliferation leading to mammary epithelial tumorigenesis and to a large extent is responsible for disrupting the mammary architecture. Inhibiting MDM2 alone or by blocking estrogen signaling or combination of both is correlated with decreased mammary epithelial tumorigenesis.

7.2. Significance

Estrogen receptor positive breast cancers are often treated with estrogen antagonists that block estrogen signaling. Fulvestrant, an estrogen antagonist is widely used to treat late stage ER positive breast cancers (Osborne et al., 2004). Our data demonstrates the oncogenic functions of MDM2 in a p53 independent manner. The positive functions of oncogenic MDM2 in pathways that are not dependent on p53 are not well studied. Our data emphasizes the importance of
MDM2 in estrogen signaling and indicates estrogen-activated MDM2 drives phosphorylation of Rb. ER+ breast cancer patients, who are treated with estrogen receptor antagonists often develop resistance to the drugs and MDM2 is high in those situations (Shostak et al., 2014). Our work suggests fulvestrant works, at least partly, by inhibiting MDM2 and phospho Rb proteins. MDM2 knockdown in masses grown in 3D matrigel inhibited mitosis and leads to a more normal mammary architecture. Our results indicate that MDM2 is a good therapeutic target for advanced ER positive breast cancers and especially to those that are resistant to estrogen antagonist therapy.

7.3. Future directions

To summarize our results, MDM2 is a hub and is central to estrogen signaling. Our results demonstrated an ER-MDM2-phosphoRb-E2F1 signal transduction pathway in ER positive breast cancers by using inducible knockdown as a variable. One of the important findings put forward in this thesis is that estrogen-activated MDM2 plays a role in disrupting mammary architecture and positively influences mitosis, suggesting that MDM2 could be a potential therapeutic target for ER positive breast cancers, especially those that are resistant to estrogen antagonist therapy.

To clarify our findings and expand on the molecular pathways of MDM2 in ER positive breast cancer, this project could be expanded in the following ways:

1. Demonstrate the ER-MDM2-phosphoRb-E2F1 signal transduction pathway in 2D culture with overexpression of MDM2 in ER+ breast cancer cell lines. 2.
Determine the downstream pathway targeted by MDM2 in estrogen-mediated cell proliferation with upregulation and downregulation of MDM2 for a broad comparison in 3D culture system. 3. Determine if MDM2 depletion in 3D matrigel culture restores apical-basal polarity and if increased expression of MDM2 provokes increased disorder. 4. Determine if MDM2 depletion in a wild type p53 background is able to restore normal or near normal architecture. 5. Determine if MDM2 overexpression in normal mammary epithelial cells results in disruption of mammary architecture. 6. Determine if ductal lumen phenotype correlated with MDM2 knockdown is associated with inhibition of cell proliferation and/or cell death. 7. Resolving the potential drawbacks of shRNA based knockdown strategy. 8. Examine if MDM2 increases global phosphorylation of proteins and understand the mechanism of MDM2 activating Rb phosphorylation.

7.3.1. Demonstrate the ER-MDM2-phosphoRb-E2F1 signal transduction pathway in 2D culture with overexpression of MDM2 in ER+ breast cancer cell lines.

To investigate the downstream target of MDM2 signaling in estrogen-mediated cell proliferation we overexpressed MDM2 in ER+ breast cancer cell lines, MCF7 and T47D. Since we observed a strong correlation between increased MDM2 protein levels in the presence of estrogen with increased E2F1 and phospho Rb protein levels, we thought that the increased protein levels of phospho Rb and E2F1 is due to elevated MDM2 protein levels that occurred in
the presence of estrogen. If this were true, then overexpression of MDM2 even in the absence of estrogen would increase E2F1 and phospho Rb protein level.

To determine the role of MDM2, we generated stable cell lines that constitutively express MDM2 from its cDNA. The MDM2 constructs used were cloned from pT7 vector containing IRES expressing MDM2 to pLNCX vector, a gift from Carol Prives laboratory from Columbia University. The IRES-MDM2-containing pLNCX constructs were then infected into T47D and MCF7 cells by retroviral gene transfer method (described in Materials and Methods). We experienced difficulties in establishing the MDM2 overexpressing cell lines, as after some passages MDM2 overexpression was not found. The fact that MDM2 overexpression was lost has been reported previously (see review (Deb, 2003)).

To observe the effects of MDM2 overexpression, we extracted proteins at very early time points, 24 hours post infection and after 3 passages post selection with 1.5 mg/ml G418. 24 hours post infection of MDM2 overexpressing pLNCX constructs in T47D (Figure 15 A) and MCF7 (Figure 15 B) cell lines, we observed an increase in MDM2 protein level, indicating that there was MDM2 overexpression. Along with an increase in MDM2, there was an increase in E2F1 protein level (third lane in Figure 15 A & B). An increase in phospho Rb protein levels were not seen in either of the cell lines with MDM2 overexpression but a shift in the migration of phospho Rb protein bands was observed. This could possibly be due to increased phosphorylation of Rb. We were also interested to see if MDM2 could be stably overexpressed in these newly constructed cell lines. MCF7 cells 24 hours post infection with pLNCX vector containing MDM2 cDNA
were grown under selection with 1.5 mg/ml G418 for 7 days and then passaged. At passage 3, MDM2 overexpression was observed (Figure 15 C). Upon comparing the parental cell line and pLNCX overexpressing MDM2, we observe an increase in phospho Rb and E2F1 protein levels. Phospho Rb and E2F1 protein levels were not increased upon comparing it with vector alone. We think that the increase in phospho Rb and E2F1 protein levels in the vector control was due to interfering effects of G418. p53 protein levels remain unchanged with MDM2 overexpression compared to vector alone and parental in MCF7 cells (Figure 15 C).
Figure 15: MDM2 overexpression increases E2F1 and phospho Rb protein level. T47D (A) and MCF7 (B&C) cells were transfected with pLNCX vector containing MDM2. A representative image of western blot analysis of MDM2, E2F1, phospho Rb, Total Rb and actin protein levels from 50µg whole cell extract after 24 hours post transfection (A and B) and 3 passages after selection with 1.5mg/ml G418 (C) is shown.

Discussion

Several attempts were made to overexpress endogeneous MDM2 in breast cancer cells in order to compare knockdown and overexpression experiments. Unfortunately, it was challenging to obtain a stable ER+ cell line having MDM2 overexpression. After 24 hours post retroviral infection, we saw
MDM2 overexpression in both ER+ cell lines, MCF7 and T47D, in correlation with increase in E2F1 and phospho Rb protein. This indicated that MDM2 overexpression led to elevation in E2F1 and phospho Rb protein levels, thereby signaling to increased proliferation. We also observed a large number of floaters in pool of MCF and T47D cells overexpressing MDM2. However, we lost stable MDM2 overexpression at later passages of MDM2 overexpressing cell lines. Our results are consistent with the results reported by the Deb laboratory in the review (Deb, 2003) wherein it is reported that engineered stably overexpressing MDM2 cell lines can express MDM2 only in early passages (D. R. Brown, Thomas, & Deb, 1998; Deb, 2003). It is possible that high MDM2 overexpression is lethal to the cells and as a result they die and cells that do not have MDM2 overexpression outnumber the population. That MDM2 acts as both an oncogene and as a tumor suppressor in different experimental conditions has been reported in the review (Manfredi, 2010).

MDM2 overexpression in the compound eyes of Drosophila resulted in small or rough eye phenotype with disorganized bristle (Folberg-Blum, Sapir, Shilo, & Oren, 2002). In this case no apoptosis or proliferation is observed. MDM2 overexpression in wing imaginal disc in Drosophila activates caspase, degrades MDM2 and induces apoptosis with a gnarled phenotype of the wings. In these cells proliferative activity by MDM2 was not observed. It was hypothesized that ectopically expressed MDM2 in the wing cells might be degraded and wing cells having MDM2 overexpression were discarded upon induction of apoptosis (Folberg-Blum et al., 2002). Additionally, the growth
inhibitory effects of MDM2 have been documented in cell lines. MDM2 overexpression from cDNA in normal fibroblasts led to growth arrest (D. R. Brown et al., 1998), supporting the growth inhibitory role of overexpressed MDM2. It is possible that inhibitory role of MDM2 gets promoted when MDM2 is expressed beyond a threshold level. Since somehow the MDM2 overexpressing cells undergoes a growth arrest, the cells in the pool that did not get infected (i.e. the cells not having MDM2 overexpression) had a selective growth advantage. This possibly could be an explanation as to why MDM2 overexpression is lost in pool of MDM2 overexpression cells lines in later passages.

Future experiments need to be done to improve the passage numbers having MDM2 overexpression. The G418 concentration needs to be optimized so that MDM2 overexpression occurs without its toxic effects. One approach could be to reduce its concentration and do a continuous selection so that the cells in the pool that did not get infected do not take over the population. This continuous selection with low dose G418 might help us to obtain stable MDM2 overexpression cell lines and provide an important tool for proof-of-principle experiments.

7.3.2. Determine the downstream pathway targeted by MDM2 in estrogen-mediated cell proliferation in 3D culture system.

Previous work (Brekman et al., 2011) and current data in our laboratory indicate that MDM2 is central in estrogen signaling and MDM2 plays a very important role in estrogen-mediated cell proliferation. We have shown that
estrogen-activated MDM2 is required for estrogen-mediated cell proliferation in ER positive breast cancer cells having mutant p53 in both 2D and 3D culture conditions, demonstrating the oncogenic functions of MDM2 that are p53 independent. Since 3D culture is more physiologically relevant system, as it resembles the in vivo conditions, it is important to determine the downstream pathways targeted by estrogen-activated MDM2 in this system. Estrogen treatment increases cell proliferation by increasing the number of cells in the S phase of the cell cycle (Lewis-Wambi & Jordan, 2009). MDM2 overexpression also forces cells to enter S phase (Lundgren et al., 1997). Our data showed that estrogen-activated MDM2 increased the number of cells in S phase. We have also shown with 2D culture in estrogen-treated T47D and MCF7 cells that along with elevated MDM2 protein levels, there was an increase in the phospho Rb and E2F1 protein levels. We have also shown that with knockdown of MDM2 in the presence of estrogen, there was a decrease in E2F1 and phospho Rb protein levels. We thus showed that MDM2 provides an estrogen-mediated proliferative advantage through an ER-MDM2-PhosphoRb-E2F1 signaling pathway. Therefore we need to examine this pathway in the 3D matrigel culture system by performing western blot from protein extracts from masses growing in the matrigel in the presence of estrogen to see if phospho Rb, E2F1 protein levels decrease with MDM2 knockdown. To further demonstrate the signaling pathway, similar western blot experiments are required to be performed to see if phospho Rb, E2F1 protein levels increase with MDM2 overexpression in MDM2-overexpression lines. In addition to studying this pathway in ER positive breast
cancer cells, similar studies can also be made in normal mammary epithelial cells MCF10A overexpressing MDM2 to further verify our results. Additionally, a global MDM2-driven proteome study should be made to understand what proteins or pathways MDM2 is targeting to provide a proliferative advantage in the presence of estrogen.

7.3.3. Determine if MDM2 depletion in 3D matrigel culture restores apical-basal polarity.

Normal mammary epithelial cells in vivo have an organized architecture of hollow lumen surrounded by single layer of epithelial cells having apical basal polarity (Kenny et al., 2007). Mammary epithelial cells lose this organization when grown in 2D culture but recapitulate in vivo glandular architecture when grown in 3D matrigel conditions. Breast cancer cells have disrupted morphology. The role of MDM2 in the mammary architecture is a very under studied area. We have shown for the first time that MDM2 depletion in estrogen-treated ER positive breast cancer cells was correlated with appearance of ductal lumen, a phenotype of near normal mammary architecture, suggesting that estrogen-activated MDM2 played a role in disrupting mammary architecture. Thus it is important to see if MDM2 knockdown restores the apical basal polarity.

Apical basal polarity is often identified by highly conserved proteins whose localization provide ‘identity’ to apical and basolateral domains. The Par protein (consists of Par3, Par6 and atypical protein kinase (aPKC)) and Crump protein (consists of Crumbs, Pals1/Stardust and Patj/Disc lost) complex establishes
apical domain and tight junction (TJ) assembly, while Scribble protein complex (consists of Scribble, lethal giant larvae (Lgl) and Disc large (Dlg)) determines the basolateral domain (Tanoss & Rodriguez-Boulan, 2008). The TJ formation is a marker for establishment of apical-basal polarity in the epithelial architecture. Proteins that are responsible for formation of TJs are Zona-occludin-1 (ZO-1) and occludins (Shin, Fogg, & Margolis, 2006). Immunostaining of masses growing in matrigel culture with markers for apical-basal polarity and TJs would be important to understand if polarity is resumed after MDM2 knockdown in the presence of estrogen. Preliminary data showed that with MDM2 knockdown in the presence of estrogen, there was an increase in tight junction marker ZO-1 protein levels (Figure 16). It is important to determine if there is increased localization of ZO-1 protein at the cell membrane and at cell-cell junctions, thereby restoring epithelial integrity. An increase of ZO-1 protein levels was observed with estrogen treatment. This is consistent with a report (Jimenez-Salazar et al., 2014) showing that estrogen treatment increases ZO-1 mRNA and protein levels and stimulates TJ disruption as ZO-1 protein translocate from cytoplasm to the nucleus in the presence of estrogen, while treatment with anti-estrogen ICI 182, 780 inhibits the estrogen-induced nuclear translocation. Therefore, it is necessary to determine if MDM2 knockdown in the presence of estrogen restores the TJ formation. Additionally, localization of proteins responsible for apical basal polarity should also be examined using immunofluorescence of masses growing in matrigel with proteins that resides in the apical and basal domains. To further demonstrate the role of MDM2 in establishing apical-basal polarity, these experiments are
required to be performed in MDM2-overexpressing ER positive breast cancer cell lines and examine if MDM2 overexpression disrupts apical basal polarity.

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Figure 16: MDM2 knockdown in the presence of estrogen increased tight junction protein marker ZO-1. T47D.MLP (vector) and T47D.MLPshmdm2 (constitutive mdm2 knockdown cell line) were grown in the presence and absence of 10nM estrogen for 5 days. Proteins were extracted from whole cell extract and run in SDS-PAGE. Western blot analysis was performed using MDM2, ZO-1 and Actin antibodies.

7.3.4. Determine if MDM2 depletion in a wild type p53 background is able to restore normal or near normal architecture.

One of the hallmarks of breast carcinogenesis is the disruption of mammary architecture. We have shown that MDM2 knockdown in T47D cells caused partial lumen clearance (ductal lumen phenotype) in breast cancer cells having mutant p53. This showed that MDM2, at least in part, was responsible for disrupting mammary architecture (Figure 6). We predict that we will observe luminal clearance with MDM2 knockdown in ER positive breast cancer cells.
having wild type p53 (like, MCF7 cells) because the MDM2 should keep the p53-independent pathway intact. In addition, to completely rule out the possible role of p53 in influencing the formation of lumen, experiments need to be carried out by simultaneously knocking down MDM2 and wild type p53 in ER positive breast cancer cell lines.

7.3.5. **Determine if MDM2 overexpression in normal mammary epithelial cells result in disruption of mammary architecture.**

Breast cancer cell lines when grown in matrigel are often associated with filled lumen. We have shown that estrogen-activated MDM2 contributed to filled lumen phenotype. To further confirm this oncogenic role of MDM2, experiments need to be done in normal mammary epithelial cells MCF10A overexpressing MDM2. MCF10A when grown in matrigel form organized mammary architecture with hollow lumen surrounded by single layer of growth-arrested and polarized epithelial cells (Kenny et al., 2007). It would be important to see if MDM2 overexpression in MCF10A cells grown in matrigel causes the colonies to have disrupted mammary architecture.

7.3.6. **Determine if ductal lumen phenotype correlated with MDM2 knockdown is associated with inhibition of cell proliferation and/or cell death.**

In our studies, we have observed that MDM2 depletion was correlated with ductal lumen phenotype when T47D cells were grown in 3D-matrigel. In 2D
culture conditions, we have demonstrated that MDM2 depletion inhibited cell proliferation, thereby dampening estrogen-mediated cell proliferation. However, in 2D culture, we did not observe any cell death with MDM2 knockdown. It is very well established that proliferation of cells in physiologically relevant 3D culture conditions is very different than 2D culture system, as in the former, there are signals from the extracellular matrix proteins in which the cells grow and form a characteristic architecture that resemble the *in vivo* conditions. Thus, it is important to examine whether cell death is associated with MDM2 knockdown in 3D culture condition.

Cell death is necessary for luminal clearance and maintenance of hollow lumen (Debnath et al., 2002); which are the characteristics of normal mammary architecture. Our hypothesis is partial luminal clearance would be accompanied by cell death. Cell death in 3D matrigel culture could be tested by *in situ* live/dead cell analysis after staining cells with ethidium bromide and calcein. Ethidium bromide selectively stains dead cells while calcein marks viable cells (Debnath et al., 2002). We expect to see that dead cells in the middle of the lumen and viable cells surrounding the lumen in most of the masses with MDM2 knockdown. Alternatively, we might not see any dead cells in the luminal space. This could possibly happen if all the dead cells are cleared at the time we perform staining. It would be necessary to monitor the growth of masses at regular intervals as this event might occur at early time points.

Previous studies have shown that overcome apoptosis is crucial for tumor cells to have filled lumen. Combination of increased proliferation and inhibition of
apoptosis have been shown to accelerate lumen filling, thereby disrupting mammary architecture (Debnath et al., 2002). Thus it is important to understand how MDM2 depletion inhibits apoptosis in 3D culture. In our studies, we have demonstrated the ER-MDM2-phosphoRb-E2F1 axis. We have shown that estrogen activated MDM2 increased E2F1 and phospho Rb protein levels, thereby inhibiting the tumor suppressive properties of Rb and activating E2F1, leading to increased cell proliferation. We have seen that in the presence of estrogen along with increase of MDM2, anti-apoptotic BCI2 protein is increased in both ER positive breast cancer cells MCF7 (Brekan et al., 2011) and T47D (Figure 17). Importantly with MDM2 knockdown, in the presence of estrogen, BCI2 protein levels were decreased in both these cell lines (Figure 17). It is possible that estrogen-activated MDM2 might increase proliferation by blocking tumor suppressive properties of Rb and activating E2F1, and inhibit apoptosis by activating BCI2, thereby poising as one of the factors responsible for disrupting mammary architecture, resulting in filled lumen. In order to test this possibility, western blot experiments of protein extracts from cells grown in 3D-matrigel culture conditions could be performed. We expect to see that with MDM2 knockdown in the presence of estrogen, BCI2 protein levels were decreased, along with reduction of phospho Rb and E2F1.
Figure 17: MDM2 knockdown in the presence of estrogen reduced anti-apoptotic protein BCL2. A representative image of western blot analysis of MDM2, BCL2 and Actin protein levels from 50µg whole cell protein extract in constitutive pool of T47D cells with mdm2 shRNA or control vector after 48 hours of estrogen treatment is shown.

7.3.7. Resolving the potential drawbacks of shRNA based knockdown strategy.

In order to study the role of MDM2 in breast cancer cell proliferation, we employed the use of RNAi technology to knockdown MDM2. We studied the effects of MDM2 knockdown in reducing the protein levels of its downstream targets and altering architecture towards a normal state. RNAi gene silencing can occur either by introduction of a synthetic small interfering RNA (siRNA) or a vector-based short hairpin RNA (shRNA) (Rao, Senzer, Cleary, & Nemunaitis, 2009). siRNAs comprise of 21-23 nucleotides (nt) double-stranded RNAs with 2
nucleotide overhangs on the 3’ ends. These siRNAs are then processed by a RNA-induced silencing complex (RISC). On the other hand, shRNAs are folded into stem-loop structures that are then processed by endonuclease Dicer and then incorporated into RISC. Both of these RNAi based gene silencing techniques are very efficient and hence are extensively used for gene silencing. Recent studies have shown that RNAi based gene silencing have some off-target effects that affect transcript expression and translation of unintended mRNA (Echeverri et al., 2006; Jackson et al., 2003).

siRNA based knockdown strategies often have more number of off-target effects compared to shRNA based knockdown strategies. The reduced off-target effects in shRNA based knockdown are due to differences in RNAi pathways in both these strategies. Processing an shRNA resembles the endogenous mechanism and the quantity of shRNA produced in the cells is in physiological limits. On the other hand, siRNAs are usually used in much higher quantity. This leads to an increase in lethality due to excess and toxic concentration that competes with endogenous miRNA over RNAi machinery like Argonaute-2, a component of the RNA-induced silencing complex (RISC). This accounts for its saturation of RNAi machinery leading to off target effects and lethality (Singh, Narang, & Mahato, 2011). To reduce the off-target effects, we have used shRNA strategy of gene silencing. However, lethal effect are also seen to some extent in shRNA expression driven by highly active RNA polymerase III type promoters (H1 or U6) (Grimm & Kay, 2007; Grimm et al., 2006). Later studies have shown that this lethal effects are significantly reduced upon using mir-30 based shRNA
which is under the control of a RNA polymerase II promoter, as it does not saturate the RNAi machinery (Giering, Grimm, Storm, & Kay, 2008). We used mir-30 based shmdm2 constructs to alleviate the toxicity without reducing the efficiency of knockdown.

mir30 based shRNA knockdown of MDM2 used in this study, is an efficient strategy over siRNA based gene silencing. However, the seed region of shRNA might have short mismatches with regions of non-targeted mRNA, resulting in knockdown of off-target genes (Jackson et al., 2006). That the shRNA was targeted and the effects observed was due to knockdown of target gene could be strengthened by using different shRNA constructs against the target mRNA. If the results using different constructs targeting different regions of the same mRNA were repetitive, then the probability that this effect was due to interference of off-target would be dampened. It would establish the fact that the observed effects were due to target gene knockdown. In our study, we have used a number of clones having shmdm2 construct in the same region to validate our findings. For further validation, we have to repeat the experiments using different shRNA constructs targeting different regions of mdm2 gene. We have several shmdm2 constructs targeting different regions in exon 12 and constructs targeting different regions of other exons of mdm2 gene. As a proof of principle, rescue experiments could also be performed. We would overexpress MDM2 having non-sense mutation in the inducible mdm2 knockdown cell lines such that the endogenous MDM2 is knocked down and MDM2 having non-sense mutation is overexpressed as it is unperturbed by the shRNA construct. In this experiment if
we observed reverted results, then we would be able to validate our results one more time. For example, with MDM2 knockdown in human breast cancer cells with mutant p53, we observed a ductal luminal phenotype leading towards a normal mammary architecture. If the mutant MDM2 having nonsense mutation is expressed, such that shRNA induction lead to reduction of endogenous MDM2 and with MDM2 overexpression mass architecture accompanied by a dramatic reduction of ductal luminal phenotype compared to MDM2 knockdown alone is observed, we would be able to demonstrate that MDM2 was responsible for disrupting mammary architecture. Similarly, to further verify that MDM2 signals to the Rb-E2F1 pathway, we by the same strategy overexpress MDM2 having nonsense mutations in the inducible shmdm2 human breast cancer cell lines such that although endogenous MDM2 protein level decrease but the functionally active nonsense mutant MDM2 was expressed, and we saw no reduction in downstream target proteins. In this way, we would further validate our findings that MDM2 is required for estrogen to signal Rb-E2F1 pathway for proliferation.

7.3.8. To examine if MDM2 increases global phosphorylation of proteins and to understand the mechanism of MDM2 activating Rb phosphorylation.

Data from the in vitro kinase assay shows that MDM2 can work in some way to increase the phosphorylation of Rb. It is unclear now as to how MDM2 promoted signal transduction for increasing phosphorylation of Rb. It is also possible that MDM2 promoted signal transduction that increases phosphorylation of proteins other than Rb. Our hypothesis is MDM2 promotes signal transduction
to phosphorylate a large number of proteins. Thus, to test if increased MDM2 signals a global phosphorylation of proteins, we would perform a radioactive in vitro kinase assay using nuclear extracts from MCF7 and T47D cell lines, purified MDM2 and $^{32}$P-labeled ATP. The radiolabelled ATP would be separated from the radiolabelled substrate by SDS-PAGE electrophoresis and transferred to nitrocellulose membrane. The amount of phosphorylated substrate could be quantified by standard autoradiography. To further strengthen the idea that this increased phosphorylation was driven by MDM2 and was independent of p53, we would perform the radioactive in vitro kinase assay in MEFs (mouse embryonic fibroblast) that are null for MDM2 and p53 because they are derived from a knockout mouse. We would also incubate the nuclear extracts in MEFs with purified MDM2 and $^{32}$P-labeled ATP. We expected to see a lot of bands (from the radioactive labeled phosphorylated proteins) with the addition of purified MDM2 when compared to extracts that were not incubated with purified MDM2 protein, indicating that MDM2 phosphorylated a plethora of proteins in all the three above mentioned cell lines. This will demonstrate that MDM2 in ways independent of p53, would drive global phosphorylation of proteins.

Although the above experiments would help us to demonstrate that MDM2 played a role in driving phosphorylation of plethora of proteins, including Rb, it still remained unclear how MDM2 was influencing the phosphorylation event. One possibility was MDM2 influencing the phosphorylation by its E3 ubiquitin ligase activity. Our hypothesis was MDM2 through its E3 ubiquitin ligase activity inhibited inhibitor of cyclin or cyclin dependent kinase (CDK). We can test this
hypothesis by knocking down endogenous MDM2 by using our stable inducible or constitutive MDM2 knockdown engineered cell lines, and extracting nuclear fraction, followed by in vitro kinase assay using the nuclear extracts and incubating with purified mutant MDM2 that do not have the E3 ligase activity. We expect to detect no phosphorylation of Rb and/or other proteins. This would demonstrate the E3 ligase activity is needed for the phosphorylation event.

Another question that still remained unclear was what kinase was downstream of MDM2 pathway that activated the phosphorylation of Rb. Its been well studied that Cyclin D along with CDK (Cyclin dependent kinases)4/6 plays an important role in phosphorylating Rb and cell cycle progression to S phase (Harbour & Dean, 2000). This suggests that CDK4 might be the kinase used by MDM2 to activate Rb phosphorylation. CDK4 activity is checked by phosphorylation of histone H1 (Godden-Kent et al., 1997). Thus, in order to test if CDK4 was the kinase that was activated MDM2 to increase Rb phosphorylation, we would have to test if MDM2 increased phosphorylation of histone H1. Our hypothesis is MDM2 would activate CDK4 to increase RN phosphorylation. In order to test the hypothesis we would perform the in vitro kinase assay with purified MDM2 added to nuclear extracts. We would expect to see that addition of purified MDM2 increased phosphorylation of histone H1, suggesting that MDM2 promoted signal transduction for increasing phosphorylation of Rb by activating CDK4.

It is possible that MDM2 is inhibiting a CDK inhibitor to activate phosphorylation event. One of the CDK inhibitors is p21 (Harper, Adami, Wei,
Keyomarsi, & Elledge, 1993). In MCF7 cells with MDM2 knockdown the p21 protein level increases (Brekman et al., 2011) as MDM2 degrades p21 protein (Jin, Lee, Zeng, Dai, & Lu, 2003). Moreover, in the in vitro kinase assay we see that with the addition of MDM2 there is an increase in the phosphorylation of Rb. It is possible that high MDM2 protein lead to degradation of p21. p21 is a CDK inhibitor and inhibits the phosphorylation of Rb. Our hypothesis is MDM2 would decrease p21 protein levels resulting in increased Rb phosphorylation. In order to test this, we would perform the in vitro kinase assay with purified MDM2 added to nuclear extracts. We would expect to see that addition of purified MDM2 in MCF7 cells; p21 protein levels would decrease along with the corresponding increase in histone H1 phosphorylation, indicating that CDK4 was activated. However, in T47D cells with MDM2 knockdown in the presence of estrogen the p21 protein levels do not change. It is not clear whether MDM2 is degrading p21 in this cell line (Brekman et al., 2011). Since we expect that p21 protein levels not to change with MDM2 knockdown, we observed a moderate increase in phosphorylation of Rb with the addition of MDM2 (Figure 10). Here, some other protein that is inhibitor of CDK4 might plays a role, like p27 (Toyoshima & Hunter, 1994). It would be important to test if with the addition of MDM2, p27 protein levels were reduced and the activity of CDK4 was increased. In order to test this, we would perform the in vitro kinase assay with purified MDM2 added to nuclear extracts. We would expect to see that addition of purified MDM2 in T47D cells, p27 would decrease along with the corresponding increase in histone H1 phosphorylation, indicating that CDK4 was activated. To further enquire if p21 or p27 was
degraded by MDM2, we can perform the in vitro kinase assay in the presence of proteasome inhibitor. We expect to see an increased p21 or p27 protein levels in the presence of proteasome inhibitor in the presence of purified MDM2. We expect that along with the increased p21 protein level there would be a corresponding reduction in phosphorylation of Rb and histone H1. These experiments would help us to understand the mechanism of how MDM2 drives the phosphorylation of Rb.
CHAPTER 8: Perspective
In this thesis, we have shown the p53-independent oncogenic function of estrogen-activated MDM2 in providing an estrogen-mediated proliferative advantage and disruption of mammary architecture through an ER-MDM2-phosphoRb-E2F1 pathway. The p53 independent oncogenic pathway of MDM2 is less studied than the p53-dependent pathway of MDM2. In this study, we investigated the requirement of MDM2 in estrogen-mediated tumorigenic phenotypes. We have shown in more physiologically relevant 3D anchorage-independent soft agar and matrigel culture that growth of colonies or masses in the presence of estrogen required estrogen-activated MDM2. We studied the p53-independent oncogenic effects of MDM2 on tumor phenotypes in the presence of estrogen in more depth by exploring mammary architecture in laminin rich matrigel microenvironment. We observed that MDM2 knockdown in the presence of estrogen resulted in luminal clearance. This suggests that estrogen works through MDM2 in disrupting mammary architecture.

Estrogen treatment increases phosphorylation of Rb (Altucci et al., 1996; Foster et al., 2001) and we have shown estrogen-activated MDM2 contributed to the increase in phosphorylation, thus indicating ER-MDM2-phosphoRb signaling axis. Fulvestrant, a novel ERα antagonist, is highly effective in treating late stage metastatic breast cancer, inhibits estrogen-activated MDM2 (Dolfi et al., 2014; Swetzig et al., 2016). We have shown that in addition to blocking the estrogen-mediated upregulation of MDM2, Fulvestrant also inhibited estrogen-mediated upregulation of Rb phosphorylation. This finding further supported our data indicating the ER-MDM2-phospho-Rb axis. We also showed that combinations of
Fulvestrant treatment and MDM2 knockdown further reduced estrogen-mediated cell proliferation and Rb phosphorylation suggesting that MDM2 is a central hub in estrogen signaling pathway. This also indicated that targeting MDM2 might be an alternative treatment approach for ER positive breast cancers, especially those that are resistant to anti-estrogen therapy. However, further experiments need to be performed to understand the MDM2-driven proteome and demonstrate that targeting MDM2 restores apical-basal polarity of mammary architecture.


Swetzig, W. M., Wang, J., & Das, G. M. (2016). Estrogen receptor alpha (ERalpha/ESR1) mediates the p53-independent overexpression of


