The role of Mdm2 in estrogen-mediated breast cancer cell proliferation

by

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Abstract

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Adviser: Dr. Jill Bargonetti

Estrogen signaling is important in breast cancer development and progression. Mdm2, a negative regulator of the p53 tumor suppressor, is often over-expressed in estrogen receptor positive breast cancers. To study the role of Mdm2 in the estrogen-mediated breast cancer cell proliferation, we examined the effect of estrogen on the p53-Mdm2 pathway in estrogen receptor positive and p53 wild-type MCF-7 breast cancer cells. Estrogen-mediated increase in cell proliferation correlated with increased Mdm2, but no concomitant decrease in the p53 protein level. Blocking Mdm2 expression with inducible shRNA inhibited estrogen-mediated cell proliferation and colony formation in soft agar. Mdm2 knockdown in the presence of estrogen increased p21 and the percent of cells in the G1 phase. Interestingly, knockdown of p53 had no effect on the estrogen-mediated cell proliferation. Estrogen also up-regulated the Mdm2 protein levels in cells exposed to the DNA damaging agent, etoposide, and the Mdm2 inhibitor, Nutlin-3. In turn, estrogen inhibited etoposide- and Nutlin-3-induced transcription of *puma*, a pro-apoptotic p53 target gene, without changing the p53 protein levels or p53 recruitment to the chromatin. The decrease in *puma* gene transcription correlated with a decrease in Puma protein and an increase in Bcl-2 protein, an anti-apoptotic estrogen receptor target. Overall, our findings suggest that estrogen signals to an Mdm2-mediated pathway to provoke cell proliferation and that this pathway is associated with inhibition of the G1 checkpoint.

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CHAPTER 1:

INTRODUCTION

1.1 Discovery and functions of Mdm2

The murine double minute 2 (*mdm2*) gene was discovered on double minute chromosomes (acentromeric extrachromosomal nuclear bodies) in a spontaneously transformed mouse BALB/c cell line (3T3-DM), a derivative of the NIH-3T3 cell line (Cahilly-Snyder et al. 1987). The *mdm2* gene was found to be over-expressed by amplification greater than 50-fold, to provide growth advantage and to promote spontaneous transformation (Fakharzadeh et al. 1991).

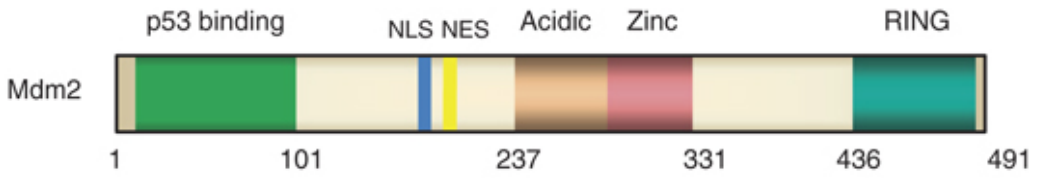

Figure 1: Domain structures of Mdm2. *p53 binding domain, nuclear localization signal (NLS), nuclear export signal (NES), acidic domain, zinc finger domain and RING finger domain (Lee and Gu 2010).*

Biochemically, the Mdm2 protein functions as an E3 ubiquitin ligase (Honda et al. 1997). The RING motif is common in E3 ligases and is responsible for the E3 ligase activity of Mdm2 (Figure 1). The main p53 interaction domain is encoded by the N terminus 100 amino acids of Mdm2, which binds the N terminal transactivation domain of p53. More recently, the N terminus of Mdm2 has also been shown to interact with the C terminus of p53 (Poyurovsky et al. 2010). The nuclear localization and nuclear export signals shuttle Mdm2 back and forth between the cytoplasm and the nucleus. The central region of Mdm2 contains an acidic domain and a zinc finger domain. A number of proteins have been shown to interact with this region of Mdm2, including L5, L11 and L26 ribosomal proteins, p14ARF and Rb tumor suppressors, and the acetyltransferase p300 (Bouska and Eischen 2009). The acidic domain of Mdm2 has also been shown to interact with the central region of p53, the DNA binding domain, and this interaction was shown to be essential for p53 ubiquitination by Mdm2 (Ma et al. 2006, Wallace et al. 2006).

The C terminus of Mdm2 contains the RING finger domain, which is responsible for the ubiquitin ligase function of Mdm2 and also serves as a binding site for the MdmX protein, an Mdm2 homolog (Tanimura et al. 1999).

1.2 The role of Mdm2 in the p53 pathway

1.2.1 p53 functions as a tumor suppressor

The p53 tumor suppressor primarily acts as a transcription factor (Riley et al. 2008) inducing over 200 known target genes (Wei et al. 2006, Zhao et al. 2000). Through its DNA binding domain, p53 associates with consensus response elements, located in promoters and intronic sequences of numerous genes, and regulates their expression (Laptenko and Prives 2006). Many of these genes encode proteins that control cell cycle progression, senescence, DNA repair and apoptosis (Vousden and Prives 2009) (Figure 2). Stressful stimuli, such as DNA damage and oncogene activation, lead to p53 activation and subsequent up-regulation of its target genes expression (Levine et al. 2006). Additionally, non-transcriptional activities of p53 have been implicated in its apoptotic response where p53 has a direct function in the intrinsic mitochondrial cell death pathway (Vaseva and Moll 2009).

p53 is considered to be an important barrier to tumor development because it prevents proliferation of cells that have sustained DNA damage and/or have abnormally active oncogenes (Meek 2009). Probably for this reason, in over 50% of all human cancers the *p53* gene is found to be mutated or completely deleted (Hollstein et al. 1991, Vogelstein et al. 2000). However, in addition to mutations in the *p53* gene, p53 may be compromised through improper activity of the p53 negative regulators.

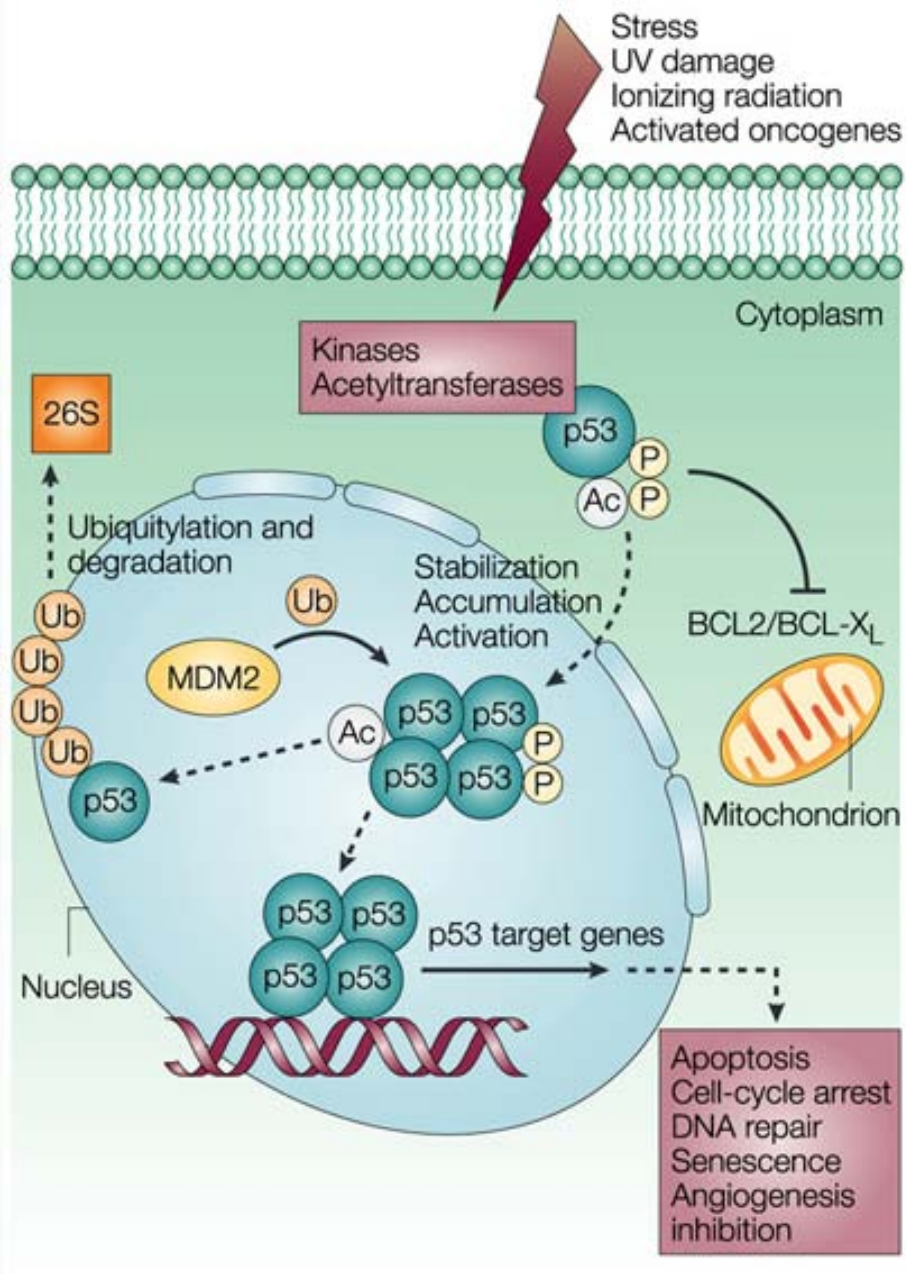

Figure 2: Activation of p53 and cellular responses. *Stress signals activate various kinases and acetyltransferases, which post-translationally modify p53. This stabilizes p53 and activates p53 transcriptional activity, where p53 interacts with sequence specific DNA binding sites of its target genes. The transcriptional activation leads to diverse cellular responses such as apoptosis, cell-cycle arrest and DNA repair. When p53 is no longer needed, it is targeted for ubiquitination by Mdm2 and is degraded by the proteasome. p53 can also act outside of the nucleus to induce apoptosis by binding with anti-apoptotic proteins such as Bcl-2. (From (Bode and Dong 2004)).*

1.2.2 The p53-Mdm2 auto-regulatory feedback loop

Under normal conditions, the p53 tumor suppressor activity is inhibited. Mdm2 is a major negative regulator of p53. Soon after its discovery, Mdm2 was shown to form a complex with the tumor suppressor p53 concealing its transactivation domain and inhibiting p53 transcriptional activity (Barak and Oren 1992, Momand et al. 1992, Oliner et al. 1993). Later on, Mdm2 was shown to function as an E3 ubiquitin ligase that mediates p53 ubiquitination and degradation (Kubbutat et al. 1997).

The critical role of Mdm2 in the negative regulation of p53 has been shown *in vivo.* Homozygous deletion of the *mdm2* gene in mice results in embryonic lethality at the blastocyst stage due to inappropriate apoptosis. Importantly, this phenotype can be rescued by simultaneous deletion of the *p53* gene (Jones et al. 1995, Montes de Oca Luna et al. 1995). Evidently, loss of MdmX in mice, an Mdm2 homolog, also leads to p53-dependent embryonic lethality, though at a later stage of development and due to inappropriate proliferation (Finch et al. 2002, Parant et al. 2001). Thus, the balance between p53, Mdm2 and MdmX protein levels is critical in development and cell survival. Mice with a hypomorphic allele of *mdm2*, expressing about 30% of the total Mdm2 level as compared to normal mice, and mice haploinsufficient for *mdm2* and *mdmx* have decreased body weight, exhibit defects in development and are more radiosensitive than normal mice (Mendrysa et al. 2003, Terzian et al. 2007). All these phenotypes are p53-dependent. Furthermore, in adult mice, Mdm2 is also critical for continuous suppression of the lethal activity of p53 (Ringshausen et al. 2006).

Regulation of p53 by Mdm2 occurs via a negative feedback loop (Figure 3). The p53 protein binds the *mdm2* P2 promoter and transcriptionally up-regulates *mdm2* expression (Barak et al. 1993, Juven et al. 1993, Perry et al. 1993). In turn, the Mdm2 protein, via its E3 ubiquitin

ligase activity, targets p53 to the proteasome for degradation (Haupt et al. 1997, Honda et al. 1997, Kubbutat et al. 1997). A chain of at least four ubiquitin molecules is believed to be required for efficient proteasomal degradation (Thrower et al. 2000). Transfection studies show that Mdm2 promotes the addition of polyubiquitin chains and degradation of p53 when expressed at high levels and monoubiquitination and nuclear export of p53 when expressed at low levels (Li et al. 2003). Interestingly, a few groups have shown that Mdm2 mediates a monomeric ubiquitination of p53 on multiple lysine residues instead of polymeric ubiquitination (Lai et al. 2001) and the p300 protein, through its E4-like ubiquitin ligase activity, cooperates with Mdm2 in polyubiquitination of p53 (Grossman et al. 2003, Grossman et al. 1998, Zhu et al. 2001). Mdm2 can also ubiquitinate itself and induce its own degradation (Fang et al. 2000, Honda and Yasuda 2000).

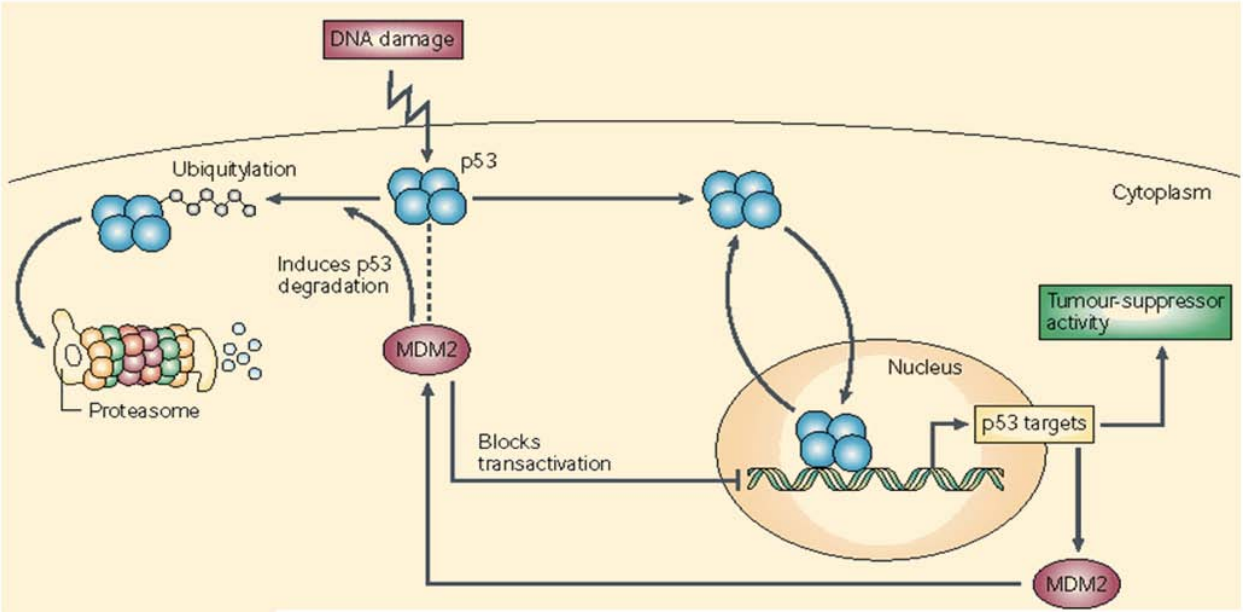

Figure 3: The p53-Mdm2 auto-regulatory feedback loop. *p53 stimulates the expression of Mdm2. Mdm2 inhibits p53 by blocking its transcriptional activity and also by targeting p53 for proteasomal degradation. (Adapted from (Chene 2003)).*

Interestingly, there is evidence that the p53 protein can be degraded independently of Mdm2 (Lee and Gu 2010). Following induction of the p53 protein expression in otherwise p53- and Mdm2-null mice the p53 protein levels decrease (Ringshausen et al. 2006), suggesting that a functional negative feedback loop exists even in the absence of Mdm2. Additional candidates for regulation of p53 ubiquitination and degradation are Cop1, Pirh2 and ARF-BP1 (Brooks and Gu 2006), Topors (Rajendra et al. 2004), CARPs (Yang et al. 2007) and Synoviolin (Yamasaki et al. 2007). However, the role of these proteins in the regulation of p53 levels has not been confirmed *in vivo*. Furthermore, the delay in p53 degradation in the absence of Mdm2 and the subsequent lethal pathologies indicate that the Mdm2 protein is the key regulator of p53 degradation *in vivo* (Marine and Lozano 2010).

In addition to regulating p53 stability, Mdm2 directly inhibits p53-mediated transactivation by binding to and blocking the p53 transactivation domain (Figure 3) (Momand et al. 1992, Oliner et al. 1993, Thut et al. 1997, Wu et al. 1993), thus hindering p53 interaction with transcriptional co-activators (Lin et al. 1994, Thut et al. 1997). In mice with a hypomorphic allele of *mdm2,* both transcriptional activation and apoptotic functions of p53 are increased, but the level of p53 protein does not coordinately increase (Mendrysa et al. 2003). This suggests that Mdm2 can inhibit the transcriptional activation and apoptotic functions of p53 in a manner independent of degradation. A chromatin-associated and transcriptionally incompetent p53-Mdm2 complex has been detected in cancer cell lines, where Mdm2 localizes to p53-responsive elements in a p53-dependent manner (Arva et al. 2005, White et al. 2006). These studies show that when the p53-induced transcription is activated by DNA damage, Mdm2 association with p53-responsive elements is reduced, suggesting that Mdm2 transiently localizes to p53 target genes and is released during transactivation. In fact, during p53 activation, acetylation of p53,

which was shown to be indispensible for p53 activation, blocks the recruitment of Mdm2 to the p53-responsive promoters thus alleviating Mdm2-mediated transcriptional repression of p53 (Tang et al. 2008).

Interestingly, a recent *in vivo* study has shown that the Mdm2-p53 interaction, without Mdm2-mediated p53 ubiquitination, cannot control p53 activity sufficiently. Specifically, mice with abrogated Mdm2 E3 ubiquitin ligase activity, but retained p53 binding ability, exhibited p53-dependent embryonic lethality, suggesting that the Mdm2 E3 ubiquitin ligase activity is required for early mouse embryonic development (Itahana et al. 2007). In fact this particular study strongly argues that the primary physiological function of Mdm2 is to promote p53 degradation (Clegg et al. 2008). However, a possibility exists that the Mdm2's E3 ubiquitin ligase activity is required for ubiquitination of other proteins at the p53-responsive promoters (Minsky and Oren 2004) or that monoubiquitination rather then polyubiquitination of p53 and or other proteins by Mdm2 is required for transcriptional repression. In addition, it is also possible that this particular mutation in the RING domain of Mdm2 (C462A) affects the conformation of the Mdm2 protein, and therefore, this mutant Mdm2 loses its inhibitory activity towards p53 not because it has abrogated E3 ubiquitin activity, but because it is conformationally different from the wild-type Mdm2.

More recently, additional components in the p53-Mdm2 auto-regulatory feedback loop have been discovered. Mdm2 was shown to inhibit *p53* mRNA translation by targeting the L26 ribosomal protein for degradation (Ofir-Rosenfeld et al. 2008, Takagi et al. 2005). Surprisingly, Mdm2 has also been shown to stimulate *p53* mRNA translation by binding directly to the *p53* mRNA (Candeias et al. 2008, Naski et al. 2009, Yin et al. 2002). Furthermore, such interaction of Mdm2 with *p53* mRNA suppressed Mdm2's capacity to promote p53 ubiquitination, leading

to p53 protein accumulation. These new findings add to the complexity of the p53-Mdm2 auto-regulatory feedback loop and suggest that Mdm2 may harbor a dual function towards p53.

Different stress signals lead to disruption of the p53-Mdm2 interaction which allows for p53 activation and consequent cellular responses. The protein levels of p53 and its negative regulator Mdm2 normally oscillate in response to a stress signal, which in turn may allow cells to repair their DNA without risking the irreversible consequences of continuous p53 activation (Lahav 2008, Lev Bar-Or et al. 2000). DNA damage induces phosphorylation and acetylation of p53, thus disrupting p53 interaction with Mdm2 and activating p53 transcriptional activity (Kruse and Gu 2009, Vousden and Prives 2009). Recently, it has been shown that acetylation is indispensible for p53 activation, as it abrogates Mdm2-mediated repression of p53 by blocking Mdm2 recruitment to p53-responsive promoters, which in turn leads to p53 activation independent of its phosphorylation status (Tang et al. 2008). Mdm2 has also been shown to be phosphorylated after DNA damage and thus lose its E3 ubiquitin activity towards p53 (Cheng and Chen 2010, Cheng et al. 2009). Interestingly, several studies imply that the p53 post-translational modifications are not required for p53 function. Disruption of the p53-Mdm2 complex by small molecule inhibitors (e.g. Nutlin-3) is sufficient for p53 pathway activation (Thompson et al. 2004, Vassilev et al. 2004). Mdm2 knockdown also activates the p53 pathway without inducing p53 phosphorylation or acetylation of key p53 residues (Giono and Manfredi 2007). In addition, a recent study has shown that while the p53 protein levels transiently increase due to benign breaks in DNA that occur during normal cell division, p53 activity during these bursts is kept in check by methylation of several lysine residues at the p53 C terminus (Berger 2010, Loewer et al. 2010). However, the role of Mdm2 in endogenous regulation of p53 under these conditions has not been examined.

1.2.3 Mdm2 over-expression inhibits p53 activity

The involvement of Mdm2 in the p53 pathway alludes to the reason for Mdm2's transformation potential. Consistent with the *in vivo* evidence that Mdm2 is indispensable for p53 activity inhibition (Jones et al. 1995, Montes de Oca Luna et al. 1995), Mdm2 over-expression often occurs in human tumors that retain a wild-type *p53* genotype (Landers et al. 1994, Momand et al. 1998). Mdm2 over-expression can occur due to gene amplification (Oliner et al. 1992, Reifenberger et al. 1993), gene rearrangements (Leach et al. 1993), enhanced translation (He et al. 1994, Landers et al. 1994), and increased transcription due to a naturally occurring single nucleotide polymorphism at position 309 (SNP309) in the *mdm2* gene P2 promoter (Bond JL et al. 2004, Sheikh et al. 1993).

1.3 p53-independent function of Mdm2 in cell cycle regulation and cell proliferation

Mdm2 over-expression has been shown to be associated with altered cell cycle regulation (Bouska and Eischen 2009). Mdm2 over-expression drives cell cycle progression and increases the percentage of cells in the S phase of the cell cycle (Lundgren et al. 1997). This suggests that Mdm2 over-expression can induce cell cycle progression and thereby increase the likelihood of passing on mutations that were not corrected during the G1 checkpoint. In part, this increased proliferation is due to p53 inhibition by Mdm2. However, Mdm2 has also been shown to interact, independently of p53, with p21, Rb and E2F proteins, which are involved in the G1 to S transition (Figure 4).

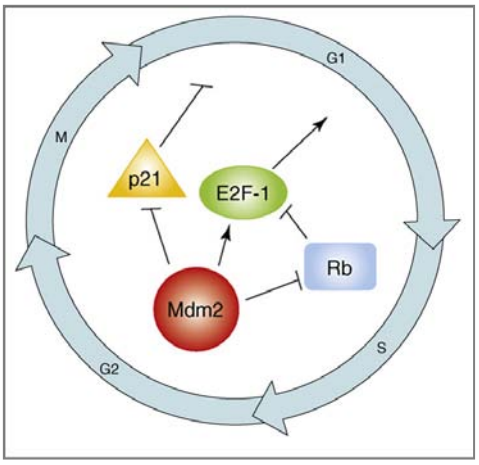

Figure 4: Mdm2 regulates cell cycle transition through the G1 checkpoint. *Mdm2 forces cells into the S phase by activating E2F and by facilitating Rb and p21 proteasomal degradation. Adapted from (Bouska and Eischen 2009).*

The p21 cyclin-dependent kinase inhibitor is a cell-cycle regulator that inhibits the G1 to S phase transition. Since p21 is a transcriptional target of p53 (el-Deiry et al. 1993), Mdm2 can inhibit p21 expression through its negative regulation of p53. In addition, Mdm2 has been shown to regulate p21 independently of p53. In cells lacking p53, Mdm2 over-expression results in decreased p21 protein level, whereas Mdm2 knockdown increases p21 and decreases proliferation. This occurs by Mdm2 binding to p21, inducing a conformational change in p21 and thus increasing p21's interaction with the C8 proteasome subunit (Jin et al. 2003, Xu et al. 2010, Zhang Z. et al. 2004b).

Mdm2 has been shown to promote cell cycle progression by simultaneously inducing E2F activity and inhibiting Rb (Figure 4). Hypo-phosphorylated Rb binds and inhibits E2F, a transcription factor that controls genes necessary for G1 to S cell cycle progression, thus inducing a G1 arrest (Mundle and Saberwal 2003). Rb phosphorylation by cyclin-dependent kinases promotes Rb-E2F dissociation, thereby promoting the transcriptional activation of E2F target genes (Mundle and Saberwal 2003). Mdm2 induces E2F transcriptional activation and thus cell cycle progression (Martin et al. 1995). Mdm2 has also been shown to regulate the Rb protein stability through two mechanisms: Mdm2 ubiquitinates Rb (Uchida et al. 2005), and

independently of its ubiquitin ligase activity, Mdm2 directly promotes Rb association with the C8 proteasome subunit (Sdek et al. 2005), thus targeting Rb for proteasomal degradation.

1.4 The role of Mdm2 in breast cancer

1.4.1 Estrogen plays a major role in breast cancers

Estrogen is important in growth and differentiation of normal mammary gland (Gruber et al. 2002), and also plays a major role in the onset and progression of breast cancers (Pike et al. 1993, Platet et al. 2004). Anti-estrogen endocrine therapy is the therapy of choice for the majority of women with breast cancers (Jordan and Brodie 2007). Estrogen acts via its receptors (estrogen receptors: ERα and ERβ), which belong to the nuclear receptor superfamily of ligand-activated transcription factors and control physiological and pathological processes largely by regulating gene transcription (McDonnell and Norris 2002, Pearce and Jordan 2004). ERα is believed to be the predominant target of estrogen in breast tissue (Anderson et al. 2002, Fuqua et al. 2003). About 2/3 of human breast tumors have high ERα levels (Clark et al. 1984) and depend on estrogen for growth (Beckmann et al. 1997). The mitogenic effects of estrogen are largely attributed to its ability to increase the expression of key cell-cycle and survival regulatory genes in hormone responsive tissues, eventually leading to tumor progression (Prall et al. 1997, Sommer and Fuqua 2001). For example, estrogen induces expression of the anti-apoptotic gene *bcl-2* thus inhibiting apoptosis (Perillo et al. 2000) and also stimulates Myc expression to aid in cell survival (Rodrik et al. 2006).

1.4.2 Mdm2 over-expression contributes to tumorigenesis in breast tissue

Mdm2 mRNA and protein over-expression has been observed in breast cancers (Bueso-Ramos et al. 1996, Meek and Knippschild 2003). Transgenic mice with *mdm2* gene expression specifically in the mammary gland develop mammary tumors, suggesting that over-production of Mdm2 contributes to tumorigenesis in breast tissue (Lundgren et al. 1997). Evaluation of the relationship between Mdm2 protein expression and survival in patients with breast carcinoma shows that patients with Mdm2-positive tumors have worse survival than patients with Mdm2-negative tumors (Turbin et al. 2006).

1.4.3 Mdm2 is over-expressed in estrogen receptor α positive breast cancers

Mdm2 appears to be over-expressed in ERα positive ($ER\alpha^+$) human breast cancers (Hori et al. 2002, Marchetti et al. 1995). $ER\alpha^+$ breast cancer cell lines also exhibit higher expression of Mdm2 mRNA and protein levels as compared to ERα negative cell lines (Gudas et al. 1995, Sheikh et al. 1993).

In addition to containing high basal levels of Mdm2 mRNA and protein, $ER\alpha^+$ breast cancer cell lines show increased Mdm2 expression in the presence of estrogen (Saji et al. 1999). Estrogen promotes recruitment of ERα to the *mdm2* P2 promoter, suggesting a role for ERα in the regulation of *mdm2* gene expression (Kinyamu and Archer 2003). High Mdm2 protein expression correlates with an increased level of transcription from the *mdm2* gene P2 promoter through binding sites of AP1-ETS family transcription factors and upstream nGGGGC boxes, but independently of p53 (Okumura et al. 2002b, Phelps et al. 2003).

A single nucleotide polymorphism at position 309 in the *mdm2* gene P2 promoter region (SNP309 T to G) leads to Mdm2 over-expression (Bond JL et al. 2004) and correlates with

accelerated tumor formation in a gender-specific and estrogen-dependent manner (Bond G. L. and Levine 2007, Bond G. L. et al. 2006). Because the SNP309 G nucleotide increases SP1 transcriptional co-activator binding affinity and SP1 is a co-activator of ERα, it could potentially augment the transcriptional regulation of Mdm2 by estrogen. Evidently, estrogen treatment preferentially stimulates RNA polymerase II loading to the SNP309 G allele of the *mdm2* gene, and preferentially increases Mdm2 protein levels in SNP309 G/G cells (T47-D), intermediately in SNP309 T/G cells (MCF7), and only marginally in SNP309 T/T cells (ZR75-1) (Hu et al. 2007).

1.4.4 Mdm2 modulates estrogen receptor α function

Mdm2 has been shown to regulate ERα. In the presence of estrogen, Mdm2 over-expressing MCF-7 cells show increased proliferation and increased *in vitro* transcriptional activity of ERα (Saji et al. 2001). Furthermore, Mdm2 has been shown to interact directly with the ERα in a ternary complex with p53 and to be involved in ERα degradation (Duong et al. 2007).

1.5 The role of p53 in breast cancer

The *p53* gene is the most commonly mutated gene in human cancers (Vogelstein et al. 2000). Resistance to chemotherapy in breast cancers correlates with the presence of inactivating mutations in the *p53* gene (Berns et al. 2000, Geisler et al. 2001). However, *p53* mutations in breast cancers occur in only 20-30% of the cases (Caleffi M 1994, Coles et al. 1992, Hartmann et al. 1997, Pharoah et al. 1999). And in fact, the occurrence of p53 mutations in breast cancers is a late event and is found primarily in ERα negative (ERα-) tumors (Caleffi M 1994, Hartmann et

al. 1997). This suggests that in breast cancers p53 activity is suppressed primarily through the estrogen signaling pathway and/or through the negative regulators of p53.

1.5.1 Estrogen receptor α inhibits p53 activity

ERα can inhibit p53 transcriptional activity by directly interacting with the p53 protein on the chromatin (Konduri et al. 2010, Liu G. et al. 2000, Sayeed et al. 2007). In MCF-7 breast cancer xenografts, radiation disrupts the p53-ERα interaction and increases p53-mediated transcriptional activity (Liu W. et al. 2009). Estrogen has also been shown to affect the post-translational modifications of p53. In MCF-7 breast cancer cells, estrogen inhibits resveratrol-induced phosphorylation and acetylation of p53, decreases p53-DNA binding and inhibits apoptosis (Zhang S. et al. 2004a).

CHAPTER 2:

MATERIALS AND METHODS

Cell culture

MCF-7, ZR75-1 and MCF10A normal immortalized mammary epithelial cell lines were obtained from the American Type Culture Collection (ATCC). MCF-7 and ZR75-1 cells were grown in RPMI 1640 medium (Mediatech), 10% FBS (Gemini) and 2,500 units of penicillin-streptomycin (Mediatech) at 5% CO_2 37^0C humidified incubator. MCF10A cells were grown in a 1:1 mixture of DMEM and Ham's F-12 medium (Mediatech) containing 0.1 μg/ml cholera enterotoxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisol, 20 ng/ml epidermal growth factor, 5% horse serum and 2,500 units of penicillin-streptomycin at 5% CO_2 37^0C humidified incubator.

Cloning of *mdm2* and *p53* shRNA constructs

pSM2c vectors with six different shRNAs for *mdm2*, four different shRNAs for *mdmx*, and three different shRNAs for *p53* (see Table 1 below) were cloned into a doxycycline-inducible vector, STGM PGK PURO (see Figure 5). An empty STGM PGK PURO vector was used as a control. Reverse tetracycline transcriptional activator (rtTA) expressing plasmid was used to induce shRNA expression in the presence of doxycycline from the STGM PGK PURO vector in tissue culture experiments. Vectors were a generous gift from Scott Lowe and Agustin Chicas.

Figure 5: Map of doxycycline-inducible construct for shRNA expression. *shRNA oligos for mdm2, mdmx or p53 were inserted between XhoI and EcoRI restriction sites. GFP expression was used to assess shRNA expression.*

Table 1: *mdm2, mdmx* and *p53* shRNA sequences.

Clone ID	Position	shRNA sequence
mdm2		
254930	802…820	CAATTAGTGAGACAGAAGA
151656	1793…1811	CTGTCTATAAGAGAATTAT
254556	573…591	GTGCCAAGCTTCTCTGTGA
262120	1924...1942	CCTACTTTGGTAGTGGAAT
151657	1836...1854	GAATTTAGACAACCTGAAA
254553	484...502	GCCAGTATATTATGACTAA
mdmx		
11941	278…296	CTATTTAGGTCAGTACATA
13023	246…264	GGTGAAATGTTCACTGTTA
196120	247…265	GTGAAATGTTCACTGTTAA
151660	1097...1115	GAAGGATTGGTATTCAGAT
p53		
825	825…844	GCATCTTATCCGAGTGGAA
1018	1018…1039	CCGGCGCACAGAGGAAGAGAA
2120	2120…2139	GAGGATTTCATCTCTTGTA

Generation of MCF-7 clonal cell lines containing inducible shRNAs

rtTA plasmid and STGM PGK PURO plasmid (containing shRNA for *mdm2, mdmx,* or *p53*) were introduced into the MCF-7 cells by retrovirus-mediated gene transfer method. Briefly, Phoenix packaging cells were transfected by calcium phosphate method with either the rtTA plasmid or with the STGM PGK PURO plasmid. Medium containing the generated viruses was harvested and MCF-7 cells were co-infected with the two plasmids. After selection with puromycin (STGM PGK PURO) and hygromycin (rtTA), clonal MCF-7 cell lines were generated by limited dilution method. During and after infections, MCF-7 cells were grown in DMEM medium supplemented with 10% FBS (Gemini) and 2,500 units of penicillin-streptomycin. Clonal cell lines were selected based on the level of protein knockdown and GFP

fluorescence. To induce shRNA expression, cells were treated with 2 μg/ml doxycycline for six days or as indicated in the figures.

Treatments

Estrogen (17β-estradiol, E2), Etoposide (ETOP), Nutlin-3 (NUT) and DMSO were purchased from Sigma. 24 hours prior to treatments, growth medium was changed to phenol-red-free RPMI 1640 (Invitrogen) containing 10% charcoal-stripped FBS (Gemini) and antibiotics. Fresh medium was supplemented every 72 hours. All estrogen treatments were carried out in RPMI medium.

Quantitative reverse transcription-PCR (qRT-PCR)

RNA was isolated using QIAshredder columns and RNeasy Mini Kit (Qiagen). 5 μg of RNA was used for cDNA synthesis using High Capacity cDNA Archive Kit reagents (Applied Biosystems). 150 ng of cDNA was combined with Taqman Universal Master Mix and Applied Biosystems Assays on Demand primers/probes for *puma* (Hs00248075_m1), *mdm2* (Hs00242813_m1), *p21* (Hs00355782_m1) and *actin* (4352935E). PCR reaction was carried out in 7500 Sequence Detection System (Applied Biosystems). P-values were calculated by student t-test.

Whole cell protein extract

Cells were harvested and washed in 1xPBS. Cells were lysed in RIPA buffer (0.1% SDS, 1% NP-40, 0.5% Deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-Cl pH8, 1 mM PMSF, 8.5 μg/ml Aprotinin and 2 μg/ml Leupeptin) following standard protocol.

Histone protein extract

Cells were harvested and washed in 1xPBS. Cells were resuspended in Triton Extraction Buffer (TEB) at cell density of 10^7 cells/ml (4 ml for 150 mm plate of cells). TEB recipe: in 50 ml PBS: 0.5 % v/v Triton X 100, 2 mM PMSF and 0.02% w/v NaN_3. Cells were lysed on ice for 10 min with gentle stirring, followed by centrifugation for 10 min at 1200 rpm 4^0C. The pellets were washed in half the volume of TEB and centrifuge for 10 min at 1200 rpm 4^0C. Pellets were resuspended in 0.2 N HCl at a cells density of $4x10^7$ cells/ml (~100-200 µl). Acid extraction was done by rocking the samples 4^0C overnight, followed by centrifugation for 10 min at 2000 rpm 4^0C to rid of cell debry.

Western blot

50 µg of protein extract were separated by 10% SDS-PAGE and electro-transferred to nitrocellulose membrane. Immunoblotting was done with p53 antibodies (pAb421, pAb240 and pAb1801); Mdm2 (SMP-14 from Santa Cruz sc-965 or from Sigma M4308); ERα (Santa Cruz HC-20); MdmX (Bethyl laboratories BL1258); Bcl-2 (100 Santa Cruz sc-509); Puma (Cell Signaling 4976); p21 (Ab-1 Oncogene Research Science OP64); H2AX (Millipore MAB3406); γH2AX Ser139 (Millipore, 05-636); Actin (Sigma A2066).

Immunofluorescence

Cells, grown and treated on coverslips, were fixed with 4% Formaldehyde and permeabilized with 0.5% Triton-X-100. Immunohistochemistry was done with p53 (FL-393 Santa Cruz sc-6243), Mdm2 (SMP-14 Santa Cruz sc-965) or γH2AX Ser139 (Millipore, 05-636) antibodies followed by incubation with FITC-conjugated anti-mouse (Jackson ImmunoResearch 715-095-

150) or Alexa-conjugated anti-rabbit (Invitrogen A11037). Coverslips were mounted onto slides using Vectashield mounting medium with DAPI (Fisher Scientific NC9524612). Images were collected by PerkinElmer UltraVIEW ERS Spinning Disc Microscope.

Chromatin immunoprecipitation (ChIP)

Cells were incubated with 1% Formaldehyde for 30 min at 5% CO_2 37^0C humidified incubator, followed by 0.125 M Glycine treatment for 5 min. Cells were lysed in RIPA buffer (0.1% SDS, 1% NP-40, 0.5% Deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-Cl pH8, 1 mM PMSF, 8.5 µg/ml Aprotinin, 2 µg/ml Leupeptin and Phosphatase Inhibitor Cocktail 1 (Sigma)). Lysates were sonicated 10 times (1 min pulse and 1 min rest) in a Branson Digital Sonifier and spun down for 30 min 13,000 rpm at 4^0C. 400 µg of cell lysates were subjected to overnight incubation at 4^0C with 2 µg of p53 (Ab-6 Calbiochem OP43); Mdm2 (N-20 Santa Cruz sc-813); RNA polymerase II (RNAPII H-224 Santa Cruz sc-9001) or non-specific IgG (Santa Cruz, IgG mouse sc-2025, IgG rabbit sc-2027). 50 µl of 25% beads slurry of protein A/G Plus Agarose beads (Santa Cruz sc-2003), pre-blocked with 0.3 mg/ml sheared herring sperm DNA (Invitrogen, 15634-017), were added to immunoprecipitation samples for 2 hours at 4^0C, followed by washes: *(1)* 0.1% SDS, 1% Triton-X-100, 20 mM Tris pH8.1, 150 mM NaCl; *(2)* 0.1% SDS, 1% Triton-X-100, 20 mM Tris pH8.1, 500 mM NaCl; *(3)* 0.25 M LiCl, 1% NP-40, 1% Deoxycholate, 1 mM EDTA, 10 mM Tris pH8; and *(4)* twice with TE pH8. Immunoprecipitated chromatin was de-crosslinked overnight at 65^0C with 1 mg/ml ProteinaseK, 1% SDS and 0.1M $NaHCO_3$. For total DNA input, 40 µg were similarly de-crosslinked. DNA fragments were purified using Qiagen QiaQuick kit (Qiagen) and amplified by real-time quantitative PCR in 7500 Sequence Detection System (Applied Biosystems). The primers and

probes sequences for p53 responsive elements (p53-REs) in *puma*, *mdm2* and *p21* were based on (Kaeser and Iggo 2002). Same primers were used for TATA box regions in *puma* and *mdm2* genes because of the close proximity. *p21* TATA box primers were based on (Gomes et al. 2006). See table 2 below. P-values were calculated by student t-test.

Table 2: Primers used in chromatin immunoprecipitation.

puma p53-REs and TATA box	forward primer: GCGAGACTGTGGCCTTGTGT reverse primer: CGTTCCAGGGTCCACAAAGT probe: TGTGAGTACATCCTCTGGGCTCTGCCTG
mdm2 p53-REs and TATA box	forward primer: GGTTGACTCAGCTTTTCCTCTTG reverse primer: GGAAAATGCATGGTTTAAATAGCC probe: GCTGGTCAAGTTCAGACACGTTCCGAA
p21 p53-Res	forward primer: GTGGCTCTGATTGGCTTTCTG reverse primer: CTGAAAACAGGCAGCCCAA probe: TGGCATAGAAGAGGCTGGTGGCTATTTTG
p21 TATA box	forward primer: TATATCAGGGCCGCGCTG reverse primer: GGCTCCACAAGGAACTGACTTC

***mdm2* siRNA transfection**

Cells were seeded in media with no antibiotics. After 24 hours, 10 µl Lipofectamine2000 (Invitrogen) was incubated for 5 min with 240 µl Optimem (Invitrogen). 0.2 nmol (100 nM) of non-specific or *mdm2* siRNA (Dharmacon) were resuspended in 250 µl Optimem and combined with Lipofectamine2000. After 20 min, 500 µl siRNA-Lipofectamine2000 mix was added to cells with 1.5 ml Optimem. Six hours later, 1.5 ml of RPMI growth medium with 20% FBS was supplemented, to have 10% final FBS concentration. The next day, treatments were carried out as described in the figures.

Viability and cell count assay

Viability (cell permeability) and number of cells were determined by the Guava Viacount assay according to manufacturer's protocol (Millipore). Graphs show means and standard errors of three independent experiments. P-values were calculated by student t-test.

MTT assay

Percent of cell proliferation was determined by the MTT assay. After treatments, cells were incubated for 1 hour with the MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole). MTT was reduced to formazan by the mitochondrial reductase enzymes in the cells. Formazan crystals were dissolved in MTT solubility solution, containing Triton-X-100 and HCl in PBS. Graphs show means and standard errors of three independent experiments. P-values were calculated by student t-test.

Fluorescence activated cell sorting (FACS)

FACS was performed on a FACScan (BD Biosciences). After treatments, cells were harvested, washed, resuspended in PBS containing 2% bovine serum albumin and 0.1% sodium azide, fixed in 30% ethanol, and stored overnight at 4^0C. Before sorting, propidium iodide staining and RNase treatment were performed for 30 minutes at 37^0C. P-values were calculated by student t-test.

Cell culture in matrigel

MCF-7 cells were seeded at a density of $5x10^3$ cells per cm^2 on top of 50 µl solidified matrigel (BD Biosciences) in MEBM basal medium without phenol red (Lonza CC-3153) supplemented

with bullet kit components except for BPE (Lonza CC-4156), 10% charcoal FBS and 2% matrigel, in the presence of 10 nM estrogen and in the absence or presence of 2 μg/ml doxycycline. Medium was changed every three days. Brightfield pictures show mass structures that MCF-7 cells form in matrigel after 3 weeks. MCF-7 cells were also fixed directly in culture with 4% Formaldehyde and stained with propidium iodide. Confocal analysis was performed using Laser scanning spectral confocal microscope TCS SP2. Large, intermediate and small mass structures were counted and presented as percent of the total population. P-values were calculated by student t-test.

Colony formation in soft agar

5,000 MCF-7 cells were mixed with 0.3% Nobel agar (Sigma A5431) in growth medium, 10 nM estrogen, and with or without 2 μg/ml doxycycline. Cells were seeded onto 35 mm dishes coated with 0.5% Nobel agar in growth medium. Cells were then fed with the growth medium, estrogen, and with or without doxycycline. Plates were incubated at 5% CO_2 37^0C humidified incubator for 14 days. Culture medium was replaced every three days. Colonies were fixed in 4% formaldehyde, stained with 0.005% crystal violet and counted under dissecting microscope. Images were collected by inverted phase and fluorescent microscope. P-values were calculated by student t-test.

CHAPTER 3:

RESULTS

3.1 Examining the effects of estrogen-mediated Mdm2 over-expression on the p53 pathway.

3.1.1 Introduction

A majority of estrogen receptor α positive ($ERα^+$) breast cancers carries a wild-type *p53* gene (Hartmann et al. 1997, Pharoah et al. 1999). Therefore, understanding how p53 is inhibited and restoring its function are relevant issues in the breast cancer therapy field. p53 plays a central role in cell cycle arrest, DNA repair and apoptosis pathways (Vogelstein et al. 2000). In contrast, ERα positively regulates growth and development of various tissues, and promotes increased proliferation of breast cancer cells in the presence of estrogen (Pearce and Jordan 2004). ERα over-expression in breast cancers has been shown to correlate with high expression of the Mdm2 oncogene (Gudas et al. 1995, Hori et al. 2002, Sheikh et al. 1993). Since Mdm2 is a major negative regulator of p53, it is possible that in the $ERα^+$ breast cancers that over-express Mdm2, the delicate balance between the opposing functions of the p53-mediated tumor suppression and the ERα-mediated cell proliferation is disrupted.

In this study we examined the role of Mdm2 in the estrogen-mediated breast cancer cells proliferation. While Mdm2 has been implicated in estrogen's mechanism of action, the role that Mdm2 plays in this process has not been clearly defined. Our goal was to determine if in the $ERα^+$ breast cancer cells, estrogen-mediated cell proliferation depended on Mdm2 and whether Mdm2 was inhibiting the p53 pathway in this process (see Figure 6).

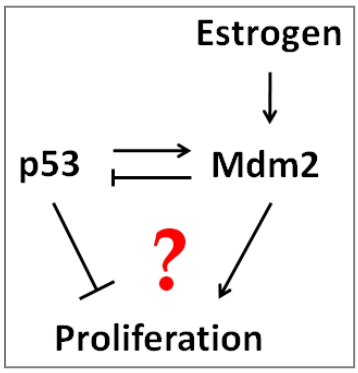

Figure 6: A model illustrating the fundamental hypothesis of this study. *Since estrogen is known to promote Mdm2 over-expression in breast cancer cells, we hypothesized that the estrogen-mediated cell proliferation is dependent on Mdm2. Since Mdm2 is a major negative regulator of p53, we proposed that the increased cell proliferation in the presence of estrogen occurs through inhibition of the p53 pathway via Mdm2.*

The experiments were carried out in the MCF-7 and ZR75-1 breast cancer cell lines, two commonly used breast carcinoma cell lines. Both cell lines have ERα and are considered ERα+ cells and also carry a wild-type *p53* (see Table 3). In one allele of the *mdm2* gene P2 promoter, the MCF-7 cells carry a G nucleotide instead of the commonly found T nucleotide (a single nucleotide polymorphism at position 309 (SNP309 T→G)), while the ZR75-1 cells carry two T alleles. Importantly, the G allele in the *mdm2* gene promoter has been shown to lead to Mdm2 over-expression (Bond JL et al. 2004) and to correlate with accelerated tumor formation in a gender-specific and estrogen-dependent manner (Bond G. L. and Levine 2007, Bond G. L. et al. 2006). Furthermore, estrogen treatment has been shown to up-regulate the Mdm2 protein level more robustly in the MCF-7 cells (SNP309 T/G) than in the ZR75-1 cells (SNP309 T/T) (Hu et al. 2007).

Table 3: Breast cancer cell lines used in this study.

Cell lines	**ERα**	**p53**	**Mdm2 SNP309**
MCF-7	+	wild-type	T/G
ZR75-1	+	wild-type	T/T

3.1.2 Results

A major proliferation and survival advantage is seen in MCF-7 cells treated with estrogen for five days (Rodrik et al. 2006, Shanmugam et al. 1999). When we treated MCF-7 cells with 10 nM estrogen for five days in otherwise steroids-depleted growth medium, we observed that the cell proliferation increased by 2.8 fold (Figure 7A). Importantly, the increase in MCF-7 cells proliferation in the presence of estrogen correlated with a dramatic increase in the Mdm2 protein level (Figure 7B). This observation served as a ground for our hypothesis that Mdm2 over-expression plays an important role in the estrogen-mediated cell proliferation. Interestingly, we observed that there were different forms of the Mdm2 protein present in both the untreated and estrogen treated cells, as the Mdm2 Western blot showed several bands for the Mdm2 protein (Figure 7B). The different forms of Mdm2 detected in the untreated cells were all up-regulated in the presence of estrogen (Figure 7B).

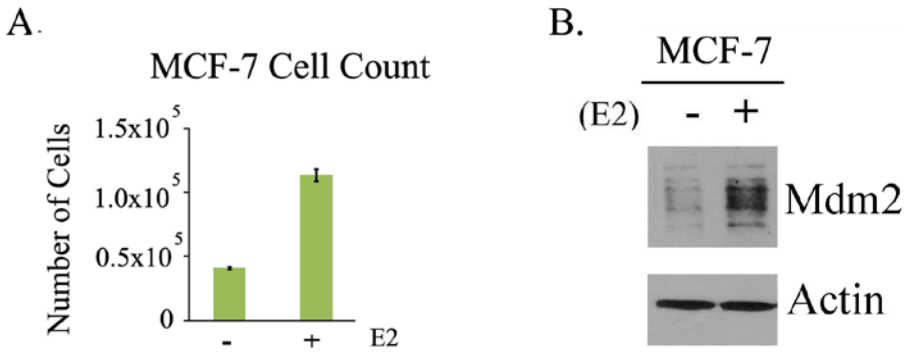

Figure 7: Prolonged estrogen treatment increased cell proliferation and the Mdm2 protein level in the MCF-7 cells. *MCF-7 cells were grown in the presence of 10 nM estrogen (E2) for five days. (A) 10,000 cells were seeded at the beginning of estrogen treatment. Number of cells after five days was determined by Guava Viacount assay. (B) Mdm2 and Actin protein levels in whole cell lysates were analyzed by Western blot.*

In the previous experiment (see Figure 7 above), we observed that prolonged estrogen treatment substantially increased the Mdm2 protein level which correlated with increased MCF-7 cells proliferation. Therefore, we hypothesized that estrogen may also promote an increase in the Mdm2 protein level in the presence of a stress signal, which in turn would block the p53-mediated response to the stess. As a stress signal we used two different drugs: a DNA damaging agent, etoposide, and also a small molecule Mdm2 inhibitor, Nutlin-3. Etoposide is a cytotoxic chemotherapeutic agent that causes DNA double strand breaks by interfering with the scission-reunion reaction of the topoisomerase II (van Maanen et al. 1988). Nutlin-3 is a small molecule cis-imidazoline analog, which inhibits the interaction between the N termini of Mdm2 and p53 by binding to the hydrophobic pocket in the N terminus of Mdm2 (Vassilev et al. 2004).

Initally, we examined the effects of etoposide and Nutlin-3 treatments on MCF-7 cell proliferation in the presence of a prolonged estrogen treatment. We observed that etoposide treatment for 48 hours in the presence of estrogen increased the percent of cells in the G2/M phase of the cell cycle and concomitantly decreased the percent of cells in the G1 phase (Figure 8A, etoposide). In turn, Nutlin-3 treatment for 24 hours in the presence of estrogen increased the percent of cells in the G1 phase and decreased the percent of cells in the S phase (Figure 8A, Nutlin-3). This suggests that etoposide treatment caused the cells to slow down at the G2/M checkpoint transition, while Nutlin-3 treatment caused a G1 arrest-like state. The 24 hours of Nutlin-3 treatment inhibited cell proliferation to a lesser extent than the 48 hours of etoposide treatment (Figure 8B), probably because of the difference in the duration of the treatments. However, further studies are needed to determine that for sure. In future studies it will be important to determine the IC_{50} (Inhibitory Concentration) for etoposide and Nutlin-3 drugs (time curves and drug concentration curves). Also, a BrdU incorporation assay (BrdU is a

synthetic thymidine analog) is needed to determine if the cells are completely arrested after the treatments or just slow down in their transition through the cell cycle checkpoints. Lack of BrdU incorporation after etoposide or Nutlin-3 treatments would indicate that the drugs induced an arrest at the corresponding phases of the cell cycle.

In conclusion, we observed that in the MCF-7 cells, etoposide and Nutlin-3 treatments in the presence of estrogen increased the percent of cell populations in the G2/M and G1 cell cycle phases respectively (Figure 8A) and moderately decreased cell proliferation (Figure 8B) without affecting the cellular membrane integrity, as measured by the cell viability assay (Figure 8C). This suggests that the drugs, at the conditions used, inhibited cell proliferation and did not induce cell death. In all the subsequent experiments, unless otherwise indicated, the above conditions were used for etoposide and Nutlin-3 treatments.

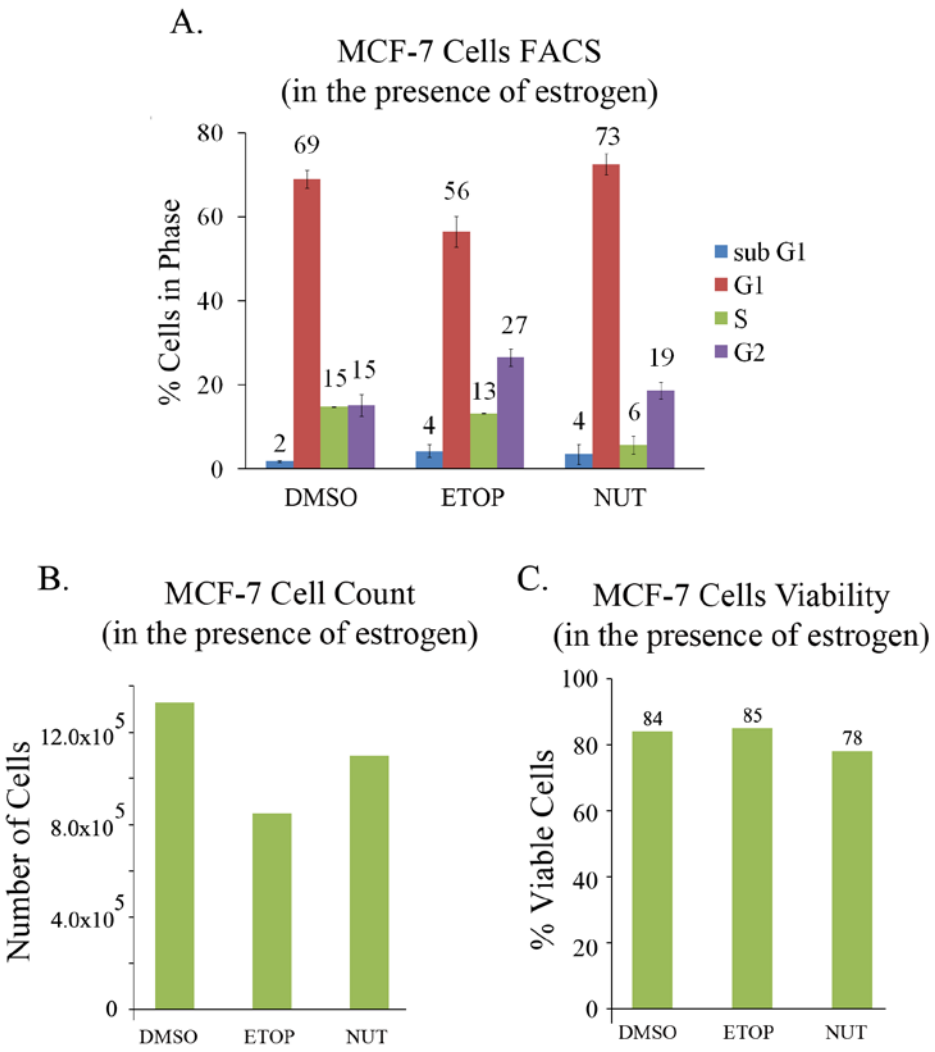

Figure 8: In MCF-7 cells, etoposide and Nutlin-3 treatments in the presence of estrogen increased the percentage of cells in the G2/M and G1 cell cycle phases respectively and moderately decreased cell proliferation. *MCF-7 cells were treated with 10 nM estrogen (E2) for five days, 50 µM etoposide (ETOP) for 48 hours and 10 µM Nutlin-3 (NUT) for 24 hours. (A) Cell cycle phases were determined by fluorescence activated cell sorting (FACS). (B) Number of cells and (C) cell viability based on membrane permeability were determined by Guava Viacount assay at the end of the treatments.*

Because etoposide and Nutlin-3 treatments in the presence of estrogen inhibited MCF-7 cells proliferation only moderately, we wanted to determine if such an ineffective response to the drugs correlated with increased Mdm2 protein expression. Drug treatments alone (etoposide or Nutlin-3) increased the protein level of Mdm2 (Figure 9, compare lanes 1-3-5). Mdm2 is a p53 target gene. Therefore, the drug-induced increase in the Mdm2 protein level was probably due to activation of the p53 transcriptional activity by these drugs (see more on this in Figure 11 below). Importantly, we observed that similarly to increasing the basal protein level of Mdm2, prolonged estrogen treatment further up-regulated the Mdm2 protein level after both etoposide and Nutlin-3 treatments (Figure 9, compare lanes 1-2, 3-4, 5-6).

In addition, we also examined if estrogen had any effect on the p53 protein expression. Since one of the major functions of Mdm2 is to promote p53 ubiquitination and subsequent proteasomal degradation, we hypothesized that in the presence of estrogen the increased Mdm2 protein level would lead to a decrease in the p53 protein level. However, it was surprising to see that while estrogen increased the Mdm2 protein level, the p53 protein level did not decrease, but in fact increased as well (Figure 9, compare lanes 1-2).

To determine if estrogen influenced the p53 protein level after a drug treatment, we treated the cells with etoposide or Nutlin-3 in the absence and in the presence of estrogen. DNA damage induced by etoposide treatment increased the p53 protein level (Figure 9, compare lanes 1-3), while estrogen treatment caused no significant change (Figure 9, compare lanes 3-4). Similarly, the p53 protein level increased dramatically after Nutlin-3 treatment (Figure 9, compare lanes 1-5) while estrogen had no effect (Figure 9, compare lanes 5-6).

In conclusion, we observed that in the MCF-7 breast cancer cells estrogen-mediated up-regulation of basal and drug-induced Mdm2 protein levels did not lead to p53 protein down-regulation.

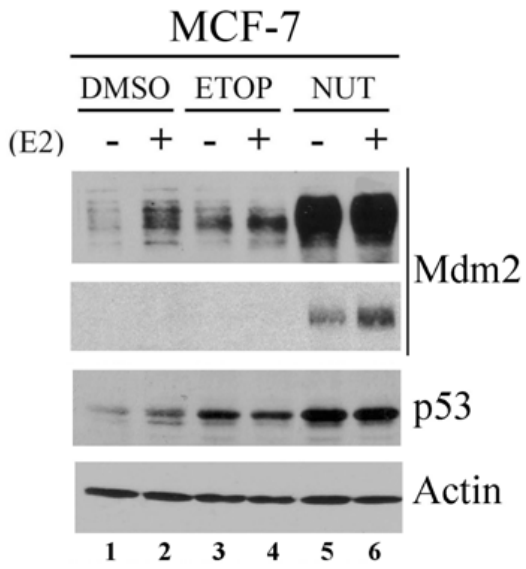

Figure 9: In MCF-7 cells, prolonged estrogen treatment increased the basal and the drug-induced Mdm2 protein levels, but did not down-regulate the p53 protein levels. *MCF-7 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. Mdm2, p53 and Actin protein levels of whole cell lysates from MCF-7 cells were analyzed by Western blot. Lower strip of Mdm2 shows a lighter exposure.*

Importantly, in a recently published study a comparison of the Mdm2 protein expression in the MCF-7 and the ZR75-1 breast cancer cells in the presence of estrogen showed that estrogen treatment robustly increased the Mdm2 protein level in the MCF-7 cells, but not in the ZR75-1 cells (Hu et al. 2007). This work showed that the increase in the Mdm2 protein expression was mediated by the SNP309 G allele in the *mdm2* gene promoter. As mentioned earlier, the MCF-7 cells are SNP309 heterozygous T/G, while the ZR75-1 cells are SNP309 homozygous T/T. To follow up on our observations in the MCF-7 cells (see Figure 9 above), we also examined the effect of prolonged estrogen treatment on the Mdm2 protein expression in the ZR75-1 cells. Importantly, similarly to Hu et al. findings, we observed that estrogen treatment did not increase the Mdm2 protein level in the ZR75-1 cells (Figure 10, compare lanes 1-2) as it did in the MCF-7 cells (see Figure 9 above, compare lanes 1-2).

In addition to examining the effect of prolonged estrogen treatment on the basal Mdm2 protein expression in the ZR75-1 cells, we also carried out experiments to determine if estrogen had any effect on the Mdm2 protein expression in the presence of a drug treatment. Interestingly, in contrast to the MCF-7 cells, where etoposide treatment increased the Mdm2 protein level (see Figure 9 above, compare lanes 1-3), in ZR75-1 cells etoposide treatment decreased the Mdm2 protein level (Figure 10, compare lanes 1-3). Importantly, similar to the fact that estrogen did not affect the basal level of the Mdm2 protein in the ZR75-1 cells (Figure 10, compare lanes 1-2), estrogen addition to the etoposide treatment also had no effect on the Mdm2 protein level (Figure 10, compare lanes 3-4). Likewise, while Nutlin-3 treatment dramatically increased the Mdm2 protein level in the ZR75-1 cells (Figure 10, compare lanes 1-5), estrogen addition to the Nutlin-3 treatment had no effect on the Mdm2 protein level (Figure 10, compare lanes 5-6).

It is not clear why in contrast to the MCF-7 cells, where etoposide treatment increased the Mdm2 protein level, etoposide treatment decreased the Mdm2 protein level in the ZR75-1 cells. Activation of kinases (e.g. ATM and ATR) by DNA damage is known to induce Mdm2 auto-degradation (Stommel and Wahl 2004) and this is probably what we observed in the ZR75-1 cells. In turn, it is possible that in the MCF-7 cells, this pathway is compromised and therefore the Mdm2 protein was not degraded after DNA damage in these cells. In addition, a major difference between the MCF-7 and ZR75-1 cell lines with respect to the Mdm2 protein regulation is that the MCF-7 cells carry a homozygous deletion for the *ink4a/arf* gene (Ikediobi et al. 2006, Musgrove et al. 1995). The Ink4a/ARF protein binds to Mdm2, sequesters it to the nucleolus (Weber et al. 1999) and also promotes Mdm2 degradation (Zhang Y. et al. 1998). Therefore, it is possible that DNA damage (induced by etoposide) activated the Ink4a/ARF pathway in the ZR75-1 cells but not in the MCF-7 cells, which, in turn, led to Mdm2 degradation in the ZR75-1 cells but not in the MCF-7 cells. It is not known, however, if the Ink4a/ARF expression is regulated by DNA damage.

We also examined if estrogen had any effect on the p53 protein expression in the absence and in the presence of a drug treatment. Prolonged estrogen treatment appeared to have no effect on the basal p53 protein level in the ZR75-1 cells (Figure 10, compare lanes 1-2). As expected, DNA damage induced by etoposide treatment led to an increase in the p53 protein level (Figure 10, compare lanes 1-3), while estrogen had no effect (Figure 10, compare lanes 3-4). And likewise, Nutlin-3 treatment increased the p53 protein level dramatically (Figure 10, compare lanes 1-5), while estrogen had no effect (Figure 10, compare lanes 5-6).

In conclusion, we observed that similarly to the previously published findings (Hu et al. 2007), in the ZR75-1 cells estrogen did not up-regulate the Mdm2 protein expression as it robustly did in the MCF-7 cells. And in addition, we found that in the presence of a stress signal, such as DNA damage or p53 activation by a small molecule inhibitor, estrogen also had no effect on the Mdm2 protein levels in the ZR75-1 cells while it up-regulated the Mdm2 protein expression in the MCF-7 cells.

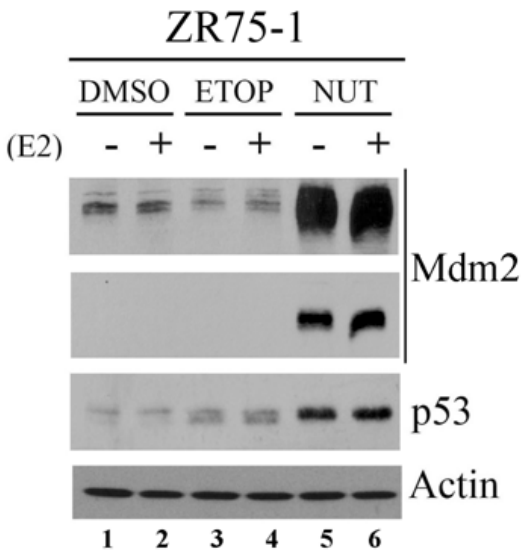

Figure 10: In ZR75-1 cells, prolonged estrogen treatment did not affect the Mdm2 and p53 protein levels. *ZR75-1 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. Mdm2, p53 and Actin protein levels of whole cell lysates from ZR75-1 cells were analyzed by Western blot. Lower strip of Mdm2 shows a lighter exposure.*

Mdm2 is a major negative regulator of p53. We observed that in the MCF-7 breast cancer cells prolonged estrogen treatment increased both the basal and the drug-induced Mdm2 protein levels. However, though the Mdm2 expression was up-regulated in the presence of estrogen, the p53 protein stability was sustained. Therefore, we hypothesized that perhaps the increased Mdm2 protein expression affected the p53 protein transcriptional activity without affecting the p53 protein stability.

To examine the effect of prolonged estrogen treatment on the p53 transcriptional activity in the MCF-7 cells, we looked at the effect of estrogen treatment on etoposide- and Nutlin-3-mediated fold activation of three different p53 target genes: *puma* (involved in apoptosis), *mdm2* (involved in negative regulation of p53) and *p21* (involved in cell cycle arrest). We observed that the basal expression of the p53 target genes *puma* and *p21* was significantly down-regulated after prolonged estrogen treatment (Figure 11, compare black and gray bars in DMSO samples). Furthermore, while, as expected, etoposide and Nutlin-3 treatments up-regulated the expression of all three p53 target genes that we examined (Figure 11, compare black bars between DMSO, etoposide and Nutlin-3 samples), p53 target genes expression was differently affected when estrogen was added to the treatments (Figure 11, compare black and gray bars). Specifically, estrogen inhibited etoposide- and Nutlin-3-induced *puma* transactivation, but not that of the *mdm2* and *p21* genes (Figure 11, compare black and gray bars).

In conclusion, we observed that in the MCF-7 cells prolonged estrogen treatment robustly inhibited basal and drug-induced expressions of the pro-apoptotic p53 target gene *puma*. Additionally, estrogen robustly inhibited the basal expression of a growth-arrest p53 target gene *p21*, but had no effect on its expression in the presence of the stress-inducing stimuli.

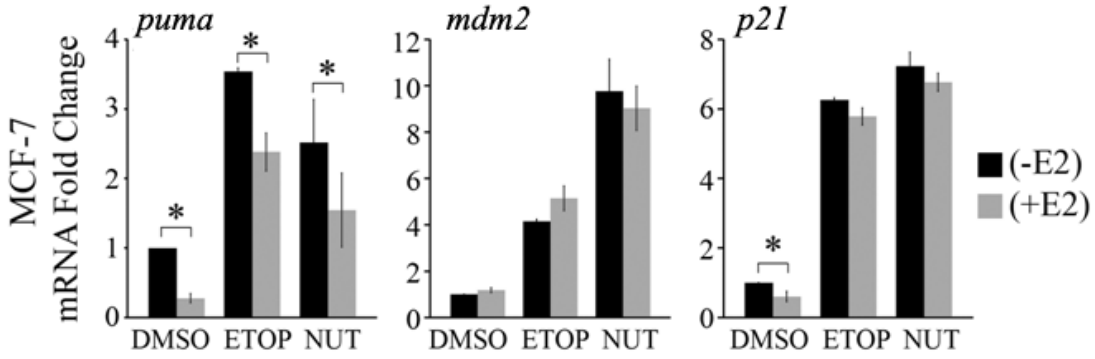

Figure 11: In MCF-7 cells, prolonged estrogen treatment inhibited basal expressions of puma and p21 genes and also inhibited etoposide- and Nutlin-3-mediated expression of puma. *MCF-7 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. Relative levels of puma, mdm2 and p21 mRNA transcripts were determined by quantitative real-time RT-PCR. Values were normalized to DMSO samples and actin levels. Graphs show means and standard errors of two independent experiments. * p < 0.05 (determined by student t-test).*

Because we observed that in the MCF-7 cells estrogen robustly inhibited the basal and the drug-induced expressions of the pro-apoptotic p53 target gene *puma*, we wanted to determine if this inhibition in transcription was functionally important. Therefore, in addition to examining the mRNA levels of *puma*, we also carried out Western blot experiments to determine if estrogen affected the protein levels of Puma as well. In direct correspondence with the transcription data, where estrogen decreased both the basal and the drug-induced *puma* mRNA expression, we observed that prolonged estrogen treatment in the absence and in the presence of etoposide and Nutlin-3 treatments decreased the protein levels of Puma (Figure 12, compare lanes 1-2, 3-4, 5-6).

Importantly, the pro-apoptotic protein Puma plays a major role in apoptosis induction by negatively regulating the anti-apoptotic protein Bcl-2 (Yu and Zhang 2009). In turn, the *bcl-2* gene is an ERα target gene and estrogen has been shown to inhibit apoptosis by inducing the *bcl-2* gene expression in the MCF-7 cells (Perillo et al. 2000). Therefore, in addition to examining the effect of prolonged estrogen treatment on the Puma protein expression we also examined how estrogen affected the Bcl-2 protein expression in the MCF-7 cells. We observed that prolonged estrogen treatment both in the absence and in the presence of etoposide and Nutlin-3 treatments increased the protein levels of Bcl-2 (Figure 12, compare lanes 1-2, 3-4, 5-6).

In conclusion, we observed that in the MCF-7 breast cancer cells, prolonged estrogen treatment, both in the absence and in the presence of etoposide and Nutlin-3 drugs, coordinately decreased the pro-apoptotic Puma protein levels while increasing the anti-apoptotic Bcl-2 protein levels. This observation suggests that in the MCF-7 breast cancer cells estrogen signals towards the anti-apoptotic pathway by decreasing Puma and by simultaneously increasing Bcl-2, and thus increases cell survival during induced proliferation.

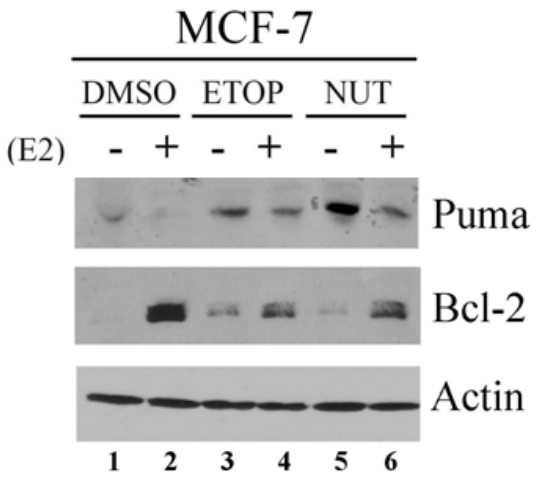

Figure 12: In MCF-7 cells, prolonged estrogen treatment in the absence and in the presence of etoposide and Nutlin-3 drugs decreased Puma and increased Bcl-2 protein levels. *MCF-7 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. Protein levels of Puma, Bcl-2 and Actin from whole cell lysates were analyzed by Western blot.*

Because in the ZR75-1 cells prolonged estrogen treatment did not up-regulate the Mdm2 protein expression (basal and drug-induced) as it robustly did in the MCF-7 cells, we hypothesized that in the ZR75-1 cells the p53 transcriptional activity would not be affected by the estrogen treatment. Similar to the MCF-7 cells, to examine the effect of prolonged estrogen treatment on the p53 transcriptional activity in the ZR75-1 cells, we looked at the effect of estrogen treatment on etoposide- and Nutlin-3-mediated fold activation of three different p53 target genes: *puma*, *mdm2* and *p21*.

We observed that in the ZR75-1 cells, prolonged estrogen treatment did not decrease the basal and the etoposide-mediated activation of the three p53 target genes that we examined (Figure 13, compare black and gray bars in DMSO and etoposide samples). Importantly, this observation supported our hypothesis. But surprisingly, prolonged estrogen treatment significantly reduced the Nutlin-3-mediated activation of all three p53 target genes (Figure 13, compare black and gray bars in Nutlin-3 treatment).

Interestingly, though both cell lines, MCF-7 and ZR75-1, are ERα positive and carry a wild type p53, estrogen affected differently the p53 transcriptional activity in the two cell lines. It is possible that since the MCF-7 cells carry the G allele for the SNP309 (T→G) and over-express the Mdm2 protein in the presence of estrogen, the basal expression of *puma* and *p21* mRNA transcripts was inhibited in the MCF-7 cells, but not in the ZR75-1 cells. And similarly, etoposide-mediated *puma* induction was inhibited in the MCF-7 cells, but not in the ZR75-1 cells. However, it is not clear why in the ZR75-1 cells estrogen inhibited the Nutlin-3-mediated expression of all three genes that we examined, while in the MCF-7 cells only *puma* was inhibited. Since in the ZR75-1 cells, estrogen treatment did not up-regulate the Mdm2 protein level, the inhibition of Nutlin-3-induced gene expression in these cells was probably independent

of Mdm2. It possible, for example, that a wide range of genes (including the p53 target genes) is affected in the ZR75-1 cells when the Nultin-3 treatment is combined with estrogen.

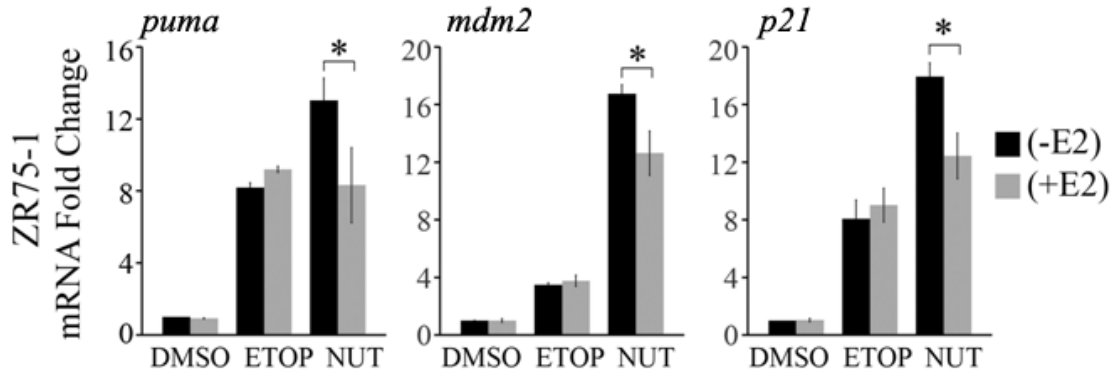

Figure 13: In ZR75-1 cells, prolonged estrogen treatment did not affect the basal and the etoposide-mediated puma, mdm2 and p21 genes expression, but inhibited the Nutlin-3-mediated expression of all three genes. *ZR75-1 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. Relative levels of puma, mdm2 and p21 mRNA transcripts were determined by quantitative real-time RT-PCR. Values were normalized to DMSO samples and actin levels. Graphs show means and standard errors of two independent experiments. * $p < 0.05$ (determined by student t-test).*

Estrogen signaling is mediated primarily through the ERα protein, a nuclear transcription factor (McDonnell and Norris 2002, Pearce and Jordan 2004). Therefore, in addition to examining the effect of prolonged estrogen treatment on the p53-Mdm2 pathway in the MCF-7 and ZR75-1 breast cancer cells, we also examined how estrogen affected the ERα protein levels in these cells. We observed that in both MCF-7 and ZR75-1 cells prolonged estrogen treatment decreased the ERα protein levels (Figure 14A and 14B, compare lanes 1-2). In the ZR75-1 cells, however, the estrogen-mediated decrease in the ERα protein level was more robust than in the MCF-7 cells (Figure 14A and 14B, compare lanes 1-2).

Interestingly, estrogen has been shown to negatively regulate the ERα protein level via the proteasomal degradation in an Mdm2-dependent manner, where Mdm2 acts as an E3 ubiquitin ligase towards ERα (Duong et al. 2007). We observed that the Mdm2 protein level increased after estrogen treatment in the MCF-7 cells but not in the ZR75-1 cells. Therefore, if the estrogen-mediated degradation of the ERα protein was Mdm2-dependent, then one would expect to see a greater degradation of the ERα protein in the cells that had more Mdm2 (the MCF-7 cells). But in turn, we observed the opposite. The ZR75-1 cells, that showed no increase in the Mdm2 protein after estrogen treatment, had lower ERα protein level after estrogen treatment than the MCF-7 cells (Figure 14A and 14B, compare lanes 1-2). This suggests that the estrogen-mediated down-regulation of the ERα protein in the MCF-7 and ZR75-1 cells depends not only on the Mdm2 protein.

In addition, we also examined the ERα protein levels after etoposide and Nutlin-3 treatments in the absence and in the presence of a prolonged estrogen treatment. Similar to the estrogen treatment, etoposide also decreased the ERα protein levels in both cell lines (Figure 14A and 14B, compare lanes 1-3), and the effect was additive when estrogen and etoposide

treatments were combined (Figure 14A and 14B, compare lanes 3-4). Nutlin-3 treatment also slightly reduced the ERα protein levels in both cell lines (Figure 14A and 14B, compare lanes 1-5), while the addition of estrogen reduced the ERα protein level in the ZR75-1 cells but not in the MCF-7 cells (Figure 14A and 14B, compare lanes 5-6). Interestingly, it was recently shown that other agents, such as UV and RITA (a small molecule that inhibits the interaction between p53 and Mdm2 by binding to the p53 protein (Sun et al. 1998)) also affect the estrogen-dependent ERα turnover (Duong et al. 2007).

In conclusion, our observations suggest that agents that signal to the p53 pathway (e.g. etoposide and Nutlin-3) also affect the ERα signaling pathway, implicating the existence of a cross-talk between the two pathways.

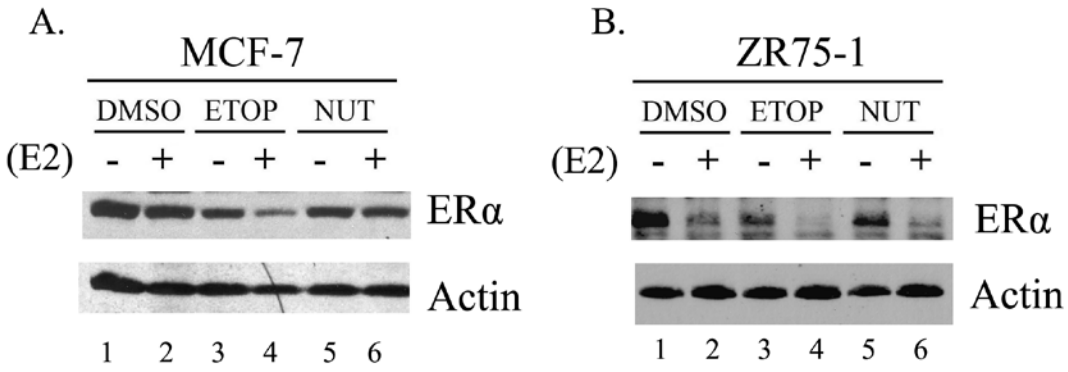

Figure 14: Estrogen, etoposide and Nutlin-3 treatments decreased the estrogen receptor α (ERα) protein levels in both MCF-7 and ZR75-1 cells. *MCF-7 and ZR75-1 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. Protein levels of ERα and Actin from whole cell lysates of MCF-7 (A) and ZR75-1 (B) cells were analyzed by Western blot.*

We observed that in the MCF-7 breast cancer cells prolonged estrogen treatment decreased both the basal and the drug-induced expression of the p53 target gene *puma* while the p53 protein levels did not decrease. Therefore, we hypothesized that the estrogen-mediated inhibition of *puma* expression might have been due to nuclear exclusion of the p53 protein after estrogen treatment. In fact, nuclear exclusion of p53 has been shown to occur following estrogen treatment in the breast cancer cells (Molinari et al. 2000, Moll et al. 1992). We, therefore, evaluated by immunofluorescent staining if the nuclear exclusion of p53 could explain the estrogen-mediated inhibition of *puma* mRNA expression in the MCF-7 cells.

When we examined how prolonged estrogen treatment affected the cellular localization of the p53 protein in the MCF-7 cells, we observed that estrogen treatment did not lead to a nuclear exclusion of the p53 protein (Figure 15A, DMSO treatment). And in fact, it appeared that estrogen treatment moderately increased the nuclear localization of the p53 protein (Figure 15A, DMSO treatment). This increase in the p53 protein staining in the presence of estrogen, correlated with the estrogen-mediated increase in the p53 protein level we described earlier in the Western blot analysis. When the cells were treated with etoposide, the p53 nuclear staining increased (Figure 15A, etoposide treatment). Interestingly, some p53 remained localized to the cytoplasm. Importantly, prolonged estrogen treatment did not block the etoposide-mediated p53 protein nuclear localization, but in fact moderately increased it (Figure 15A, etoposide treatment). When the cells were treated with Nutlin-3, the p53 protein staining increased dramatically in the nuclei while estrogen did not appear to have any significant effect (Figure 15A, Nutlin-3 treatment). The size of the nuclei, however, appeared to be smaller when estrogen was added to the Nutlin-3 treatment (Figure 15A, Nutlin-3 treatment, DAPI staining).

In addition to examining the effect of prolonged estrogen treatment on the p53 protein cellular localization in the MCF-7 cells, we also examined how estrogen treatment affected the cellular localization of the Mdm2 protein in these cells. Importantly, earlier we observed by Western blot analysis that prolonged estrogen treatment increased both the basal and the drug-induced Mdm2 protein levels. Concomitantly with the Western blot data, it seemed that estrogen treatment moderately increased the nuclear localization of the basal and the etoposide-induced Mdm2 protein (Figure 15A, DMSO and etoposide treatments). Interestingly, both the Mdm2 and the p53 proteins were evident in punctate nuclear foci before and after estrogen treatment. When the cells were treated with Nutlin-3, the Mdm2 protein staining increased dramatically in the nuclei of the cells, whereas estrogen addition to the treatment did not appear to have any significant effect (Figure 15A, Nutlin-3 treatment).

In the ZR75-1 cells, in parallel to what we observed by the Western blot analysis of the p53 and Mdm2 protein levels, estrogen did not appear to have any effect on neither the p53 nor the Mdm2 cellular localization in the absence and in the presence of the drug treatments (Figure 15B).

In conclusion, we observed that in MCF-7 breast cancer cells prolonged estrogen treatment did not block the basal and the drug-induced p53 protein nuclear localization. This suggests that the estrogen-mediated inhibition of the p53 target gene *puma* expression was not due to a decrease in the nuclear localization of the p53 protein in the presence of estrogen.

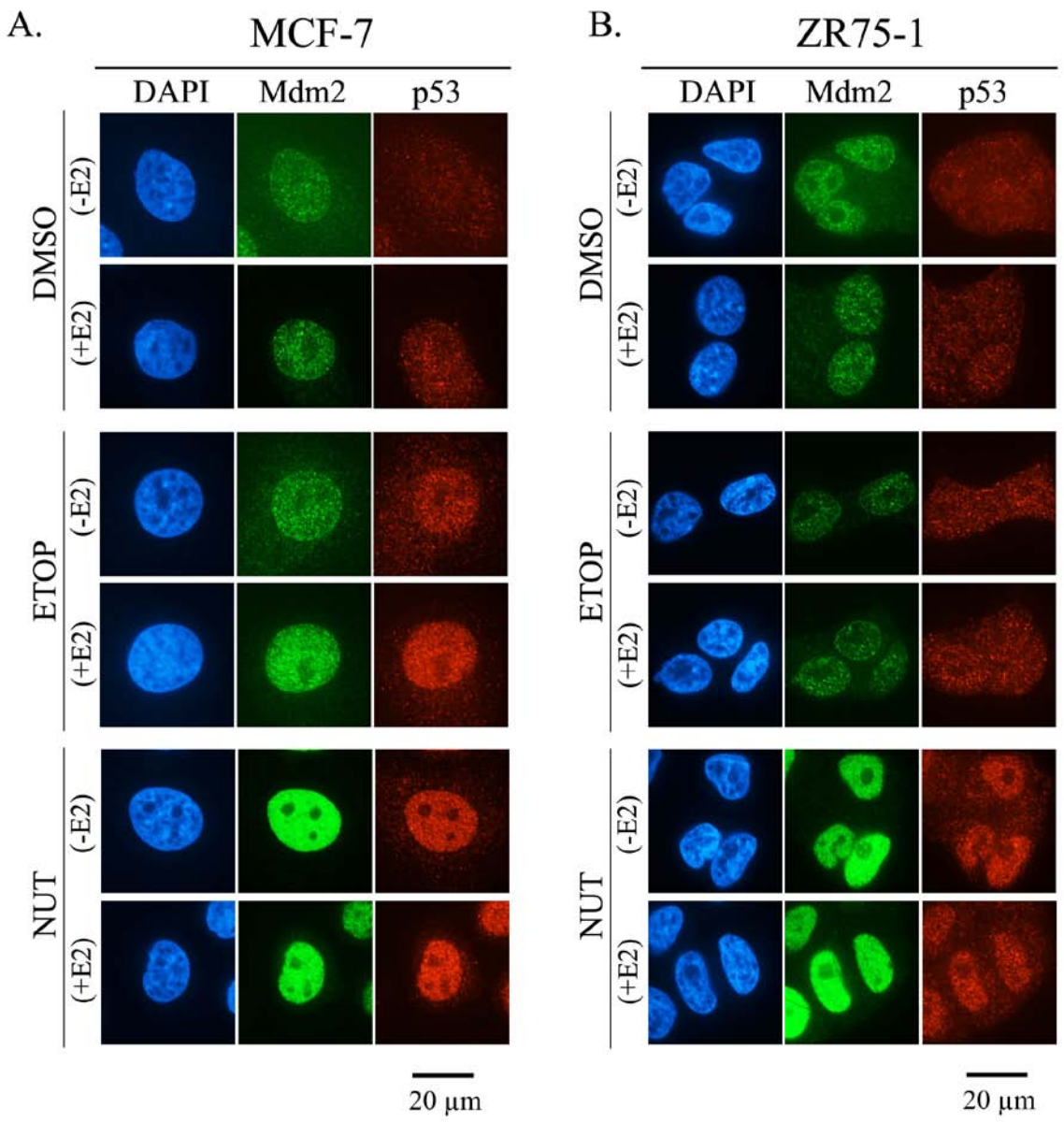

Figure 15: Prolonged estrogen treatment increased the nuclear localization of p53 and Mdm2 in untreated and etoposide treated MCF-7 cells, while the cellular localization of p53 and Mdm2 did not change after estrogen treatment in the ZR75-1 cells. *MCF-7 (A) and ZR75-1 (B) cells were treated with 10 nM estrogen (E2) for five days, 50 µM etoposide (ETOP) for 48 hours and 10 µM Nutlin-3 (NUT) for 24 hours. Immunofluorescence was carried out for p53 and Mdm2 proteins. Nuclear DNA was stained with DAPI. Confocal images were collected by PerkinElmer UltraVIEW ERS Spinning Disc Microscope. Representative images of cells from three independent experiments are shown.*

In the MCF-7 breast cancer cells, estrogen treatment alone or in combination with etoposide and Nutlin-3 treatments, increased the protein levels of Mdm2 without decreasing the protein levels of p53. In parallel, we observed that both the basal and the drug-induced expression of the p53 target gene *puma* was inhibited in the presence of estrogen. In the following experiment we hypothesized that though estrogen treatment did not reduce the p53 protein levels, the estrogen-mediated inhibition of the p53 target gene *puma* mRNA expression was due to reduced p53 protein recruitment to the p53-resposive elements (p53-REs) in the *puma* gene.

To determine if prolonged estrogen treatment decreased the ability of the nuclear p53 protein to interact with the p53-REs in the *puma* gene, we carried out quantitative chromatin immunoprecipitation (ChIP) experiments. Additionally, by this method we also examined the p53 protein recruitment to the p53-REs in the *mdm2* and *p21* genes. As expected, we observed that both etoposide and Nutlin-3 treatments increased the p53 protein recruitment to the p53-REs in all three genes that we examined (Figure 16A, compare black bars only). Evidently, the p53 protein recruitment was higher in the Nutlin-3 treatment than in the etoposide treatment, which correlated with higher p53 protein level in the Nutlin-3 treatment when analyzed by a Western blot. In contrast to our hypothesis, however, estrogen treatment did not significantly affect the p53 protein recruitment to the *puma* gene (Figure16A, compare black and gray bars, $p>0.05$). And similarly, estrogen treatment did not affect the p53 protein recruitment to the *mdm2* and *p21* genes (Figure 16A, compare black and gray bars, $p>0.05$).

The Mdm2 protein is known to be recruited to the chromatin in a p53-dependent manner (White et al. 2006). And in fact, in addition to regulating the p53 protein stability, Mdm2 directly inhibits the p53 protein transcriptional activity by co-localizing with p53 on the chromatin

(Momand et al. 1992, Oliner et al. 1993, Thut et al. 1997, Wu et al. 1993). Importantly, a chromatin-associated and transcriptionally incompetent p53-Mdm2 complex has been detected in various cancer cell lines that over-express Mdm2, where the Mdm2 protein localizes to the p53-REs in the p53 target genes (Arva et al. 2005). Because of our observation of increased Mdm2 following estrogen treatment and the existing evidence in the literature, we hypothesized that the estrogen-mediated decrease in the *puma* mRNA expression was due to increased Mdm2 protein recruitment to the p53-REs in the *puma* gene.

Similar to the p53 protein, to determine if prolonged estrogen treatment affected the ability of the Mdm2 protein to interact with the p53-REs in the *puma* gene, we carried out quantitative ChIP experiments. As a control, we also examined the effect of estrogen treatment on the Mdm2 protein recruitment to the p53-REs in the *mdm2* and *p21* genes. First we examined how the drug treatment alone (etoposide and Nutlin-3) affected the Mdm2 protein recruitment to the chromatin. We observed that etoposide treatment did not affect the relative binding of the Mdm2 protein to the *puma* p53-REs, while Nutlin-3 treatment increased Mdm2 recruitment to the *puma* p53-REs by 1.5 folds (Figure 16B, compare black bars in *puma*). Similarly, Mdm2 protein recruitment to the p53-REs of the *mdm2* and *p21* genes also increased only in the Nutlin-3 treated cells (Figure 16B, compare black bars in *mdm2* and *p21*). When we examined how estrogen affected the Mdm2 protein recruitment to the chromatin, we observed that in the presence of estrogen, there was a trend of a slightly higher levels of Mdm2 protein recruitment in etoposide and Nutlin-3 treatments (Figure 16B, compare black and gray bars), but the increase was not significant ($p>0.05$).

In conclusion, our observations suggest that the estrogen-mediated inhibition of the basal and the drug-induced *puma* mRNA expression in the MCF-7 breast cancer cells was not due to a

decrease in the p53 protein interaction with the p53-REs in the *puma* gene. Furthermore, we determined that in the presence of a prolonged estrogen treatment, though the cellular Mdm2 protein level increased, estrogen did not significantly increase the Mdm2 protein recruitment to the p53-REs in the *puma* gene.

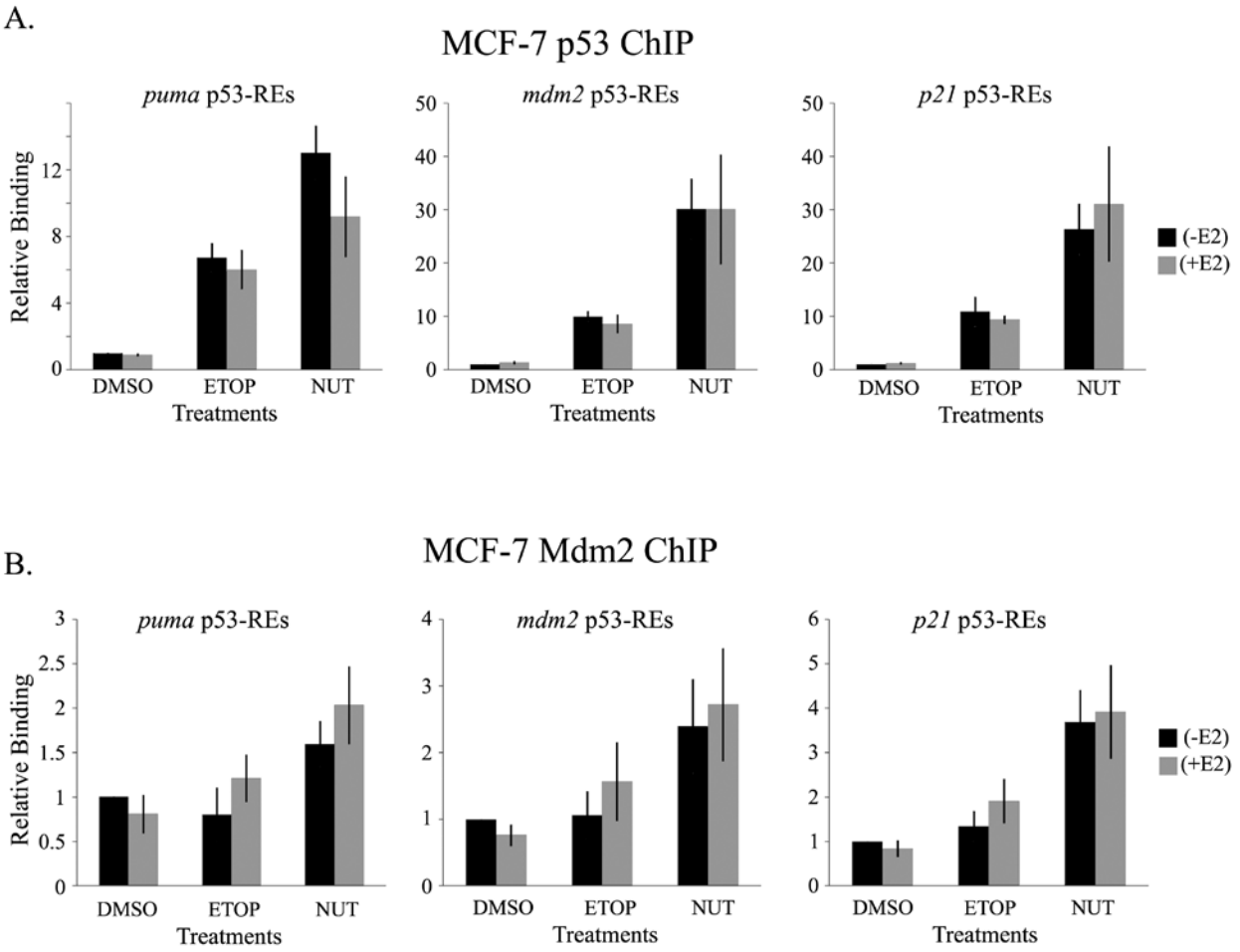

Figure 16: Prolonged estrogen treatment did not significantly affect the recruitment of the p53 and Mdm2 proteins to the p53-REs of puma, mdm2 and p21 genes in the MCF-7 cells. *MCF-7 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. 400 μg of cross-linked and sonicated whole cell lysates were subjected to chromatin immunoprecipitation using antibodies against p53 (A) and Mdm2 (B). ChIP with non-specific IgG was done to subtract the background. Immunoprecipitated DNA was amplified by real-time quantitative PCR with primers and FAM-labeled probes for p53-REs in puma, mdm2 and p21 genes (See table 2 on page 22). Values were normalized to IgG and inputs, followed by normalization to the DSMO samples. Graphs show means and standard errors of three independent experiments. The p-values comparing p53 and Mdm2 recruitments to the chromatin in the absence and in the presence of estrogen were greater than 0.05 (calculated by student t-test), suggesting that the changes in the relative binding of p53 and Mdm2 to the p53-resposive elements under the different treatments were not significant.*

So far in this study, we observed that in the MCF-7 breast cancer cells both the basal and the etoposide-induced expressions of the p53 target gene *puma* were strongly inhibited by prolonged estrogen treatment. In contrast, in the ZR75-1 cells the basal and the etoposide-induced *puma* mRNA expressions were not affected by prolonged estrogen treatment. Because estrogen treatment did not reduce the p53 protein level in the MCF-7 cells, we concluded that the decrease in the *puma* expression was not due to increased p53 degradation. Furthermore, by carrying out ChIP experiments we demonstrated that prolonged estrogen treatment did not reduce the p53 protein recruitment to the chromatin of *puma*. Importantly, since estrogen treatment robustly up-regulated the levels of the Mdm2 protein in the MCF-7 but not in the ZR75-1 cells, we hypothesized that the increased Mdm2 protein recruitment to the p53-REs in the *puma* gene was the reason for the estrogen-mediated inhibition of *puma* expression. However, though prolonged estrogen treatment increased the total cellular Mdm2 protein level in the MCF-7 cells, ChIP experiments revealed that prolonged estrogen treatment did not increase the recruitment of the Mdm2 protein to the chromatin in these cells.

Nonetheless, a possibility exists that the ChIP experiments performed after prolonged estrogen treatment (five days) do not represent the p53 and Mdm2 dynamic mode of interaction with the chromatin (White et al. 2006). Therefore, in the following experiments we asked similar questions with regards to how estrogen affects the p53-Mdm2 pathway as we did in the earlier experiments (by assessment of p53 target genes expression, p53 and Mdm2 protein levels, and p53 and Mdm2 recruitment to the chromatin), but the estrogen treatment duration was much shorter.

Initially, we carried out quantitative real-time RT-PCR experiments to determine if the short-term estrogen treatment (24 hours) of MCF-7 cells inhibited the basal expression of *puma*

and *p21* mRNA as the prolonged estrogen treatment did. Additionally, to determine if the effect of estrogen on the p53 target genes transcription was dose-dependent, the 24 hour estrogen treatments were done at increasing concentration (5, 10 and 100 nM). We hypothesized that since the five day estrogen treatment robustly inhibited the basal expression of *puma* and *p21* mRNA, then the shorter duration of estrogen treatment would have a similar effect. And indeed, we observed that in the MCF-7 cells estrogen treatment for 24 hours significantly reduced the basal expressions of the p53 target genes *puma* and *p21* (Figure 17A, compare black bars), while the basal expression of *mdm2* was not affected by estrogen (Figure 17A, compare black bars).

In addition, we also examined how the short-term estrogen treatment (24 hours) affected the etoposide-mediated up-regulation of the p53 target genes expression. Evidently, earlier in this study we showed that after a prolonged estrogen treatment (5 days), the etoposide-mediated expression of *puma* mRNA was inhibited in the MCF-7 cells. We observed that etoposide treatment alone increased the transcription from the *puma*, *mdm2* and *p21* genes in the MCF-7 cells (Figure 17A, compare black and gray bars in no estrogen samples). In parallel to the long-term estrogen treatment, we observed that etoposide-mediated expression of *puma* was inhibited in the presence of a short-term estrogen treatment (Figure 17A, compare gray bars). Interestingly, the effect of estrogen was not dose-dependent, as the differences between the increasing concentrations of estrogen were not significant (Figure 17A, compare gray bars, $p>0.05$). Additionally, in contrast to the long-term estrogen treatment, the short term estrogen treatment also inhibited the etoposide-mediated *mdm2* mRNA expression (Figure 17A, compare gray bars). Although the increasing concentrations of estrogen appeared to inhibit the etoposide-mediated expression of the *mdm2* mRNA in a dose-dependent manner, the changes were not significant (Figure 17A, compare gray bars, $p>0.05$).

Because in the ZR75-1 cells prolonged estrogen treatment did not inhibit the basal and the etoposide-mediated expressions of the p53 target genes (*puma*, *mdm2* and *p21*), we hypothesized that a short-term estrogen treatment would have a similar effect on the p53 target genes expression in these cells. And indeed, we observed that the short-term estrogen treatment had no effect on the basal and the etoposide-induced expressions of the *puma*, *mdm2* and *p21* genes (Figure 17B).

In conclusion, we observed that estrogen treatment for 24 hours, similar to the prolonged estrogen treatment, affected both the basal and the DNA damage-induced expressions of the p53 target genes in the MCF-7, but not in the ZR75-1 cells. Importantly, we observed that the basal expressions of *puma* and *p21* genes were inhibited by both prolonged and short-term estrogen treatments, and the etoposide-induced *puma* expression was also inhibited by both estrogen treatment conditions. But interestingly, the effect of short-term estrogen treatment differed from the prolonged estrogen treatment with regards to the etoposide-mediated induction of the *mdm2* mRNA expression. Specifically, while the etoposide-induced *mdm2* mRNA expression was not affected by the prolonged estrogen treatment, it was inhibited by the short-term estrogen treatment.

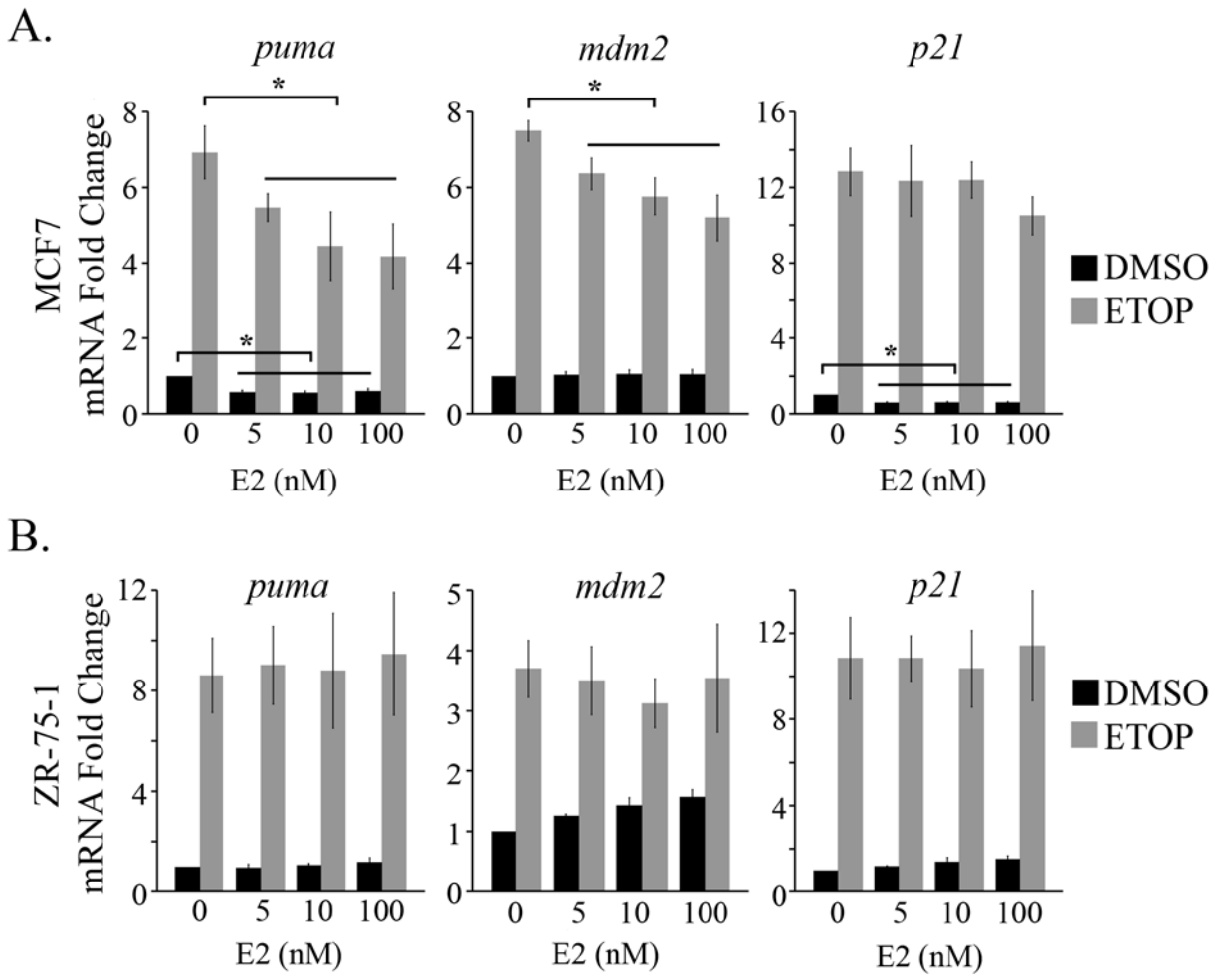

Figure 17: In MCF-7 cells, short-term estrogen treatment inhibited basal expression of puma and p21 genes and also inhibited etoposide-mediated expression of puma and mdm2 genes, while in ZR75-1 cells estrogen did not significantly affect puma, mdm2 and p21 genes expression. *MCF-7 and ZR75-1 cells were treated with 5, 10 and 100 nM estrogen (E2) for 24 hours and with 50 µM etoposide (ETOP) for 3 hours. Relative mRNA levels of puma, mdm2 and p21 transcripts in MCF-7 (A) and ZR75-1 (B) cells were determined by quantitative real-time RT-PCR. Values were normalized to DMSO samples and actin levels. Graphs show means and standard errors of three (MCF-7) and two (ZR75-1) independent experiments. * $p < 0.05$ (determined by student t-test).*

In the above RT-PCR experiment, we observed that the short-term estrogen treatment inhibited the basal expression of *puma* mRNA and also inhibited the etoposide-mediated inductions of *puma* and *mdm2* mRNA transcripts in the MCF-7 cells but not in the ZR75-1 cells. Therefore, we wanted to determine the corresponding protein levels of Mdm2 and p53 in the two cell lines. Additionally, to determine if the effect of estrogen on the Mdm2 and p53 proteins was dose-dependent, the 24 hour estrogen treatment was done at increasing concentration of 5, 10 and 100 nM. We hypothesized that similar to the prolonged estrogen treatment, the short-term estrogen treatment would also increase the protein level of Mdm2. Interestingly, we observed that in MCF-7 cells only the 10 nM estrogen treatment increased the protein level of Mdm2, while the 5 and the 100 nM estrogen treatments had no effect on the Mdm2 protein level (Figure 18A, compare lanes 1-4). Surprisingly, in the ZR75-1 cells, in contrast to the prolonged estrogen treatment where estrogen did not affect the Mdm2 protein level, the 5 and the 10 nM short-term estrogen treatments increased Mdm2 (Figure 18B, compare lanes 1-4).

In addition to examining the effect of the short-term estrogen treatment on the basal protein level of Mdm2, we also studied how estrogen affects the Mdm2 protein level in the presence of etoposide. The etoposide treatment was done for three hours in the absence or in the presence of a 24 hour estrogen treatment. In contrast to the 48 hour etoposide treatment (that was done in conjunction with the prolonged estrogen treatment protocol) where etoposide up-regulated the Mdm2 protein level, the three hour etoposide treatment did not affect the Mdm2 protein level in the MCF-7 cells (Figure 18A, compare lanes 1-5). Similarly, the three hour etoposide treatment did not affect the protein level of Mdm2 in the ZR75-1 cells (Figure 18B, compare lanes 1-5). In MCF-7 cells, when etoposide treatment was combined with 5 or 10 nM estrogen the Mdm2 protein levels increased, while the 100 nM estrogen treatment had no effect

(Figure 18A, compare lanes 5-8). In the ZR75-1 cells, when etoposide treatment was combined with 10 nM estrogen the Mdm2 protein level decreased, while the 5 and 100 nM estrogen had no effect (Figure 18B, compare lanes 5-8). In conclusion, it appeared that the short-term estrogen treatment affected the Mdm2 protein levels in both MCF-7 and ZR75-1 cells, but the effect was not dose-dependent. Furthermore, the effect of the short-term estrogen treatment on the Mdm2 protein level appeared to be sporadic as independent repeat experiments for the Mdm2 protein Western blot showed different changes in the Mdm2 protein level in response to the estrogen treatment (data not shown).

We also examined if the short-term estrogen treatment had any effect on the basal and the etoposide-induced p53 protein expression. We observed that in both MCF-7 and ZR75-1 cells, etoposide treatment for three hours increased the p53 protein level (Figures 18A and 18B, compare lanes 1-5). In the MCF-7 cells, the basal p53 protein level increased after 5 and 10 nM of short-term estrogen treatment, but not after 100 nM of estrogen (Figure 18A, compare lanes 1-4). The increase in the p53 protein level correlated with the earlier experiments, where prolonged estrogen treatments were carried out. In the ZR75-1 cells, short-term estrogen treatment had no effect on the p53 protein level (Figure 18B, compare lanes 1-4), and this again correlated with the effect of the prolonged estrogen treatment that we examined earlier. Interestingly, while estrogen treatment did not affect the drug-induced p53 protein level in the ZR75-1 cells (Figure 18B, compare lanes 5-8), estrogen further up-regulated the etoposide-induced p53 protein level in the MCF-7 cells (Figure 18A, compare lanes 5-8).

In addition, we also examined if the short-term estrogen treatment affected the ERα protein levels. We observed that similarly to the five day estrogen treatment, estrogen treatment for 24 hours decreased the ERα protein levels in both MCF-7 and ZR75-1 cells in the absence

and in the presence of etoposide (Figures 18A and 18B, compare lanes 1-4, 5-8). This result, similar to the prolonged estrogen treatment, supported what other investigators have reported in the literature with regards to how estrogen affects the ERα protein stability (Callige and Richard-Foy 2006).

In conclusion, in both MCF-7 and ZR75-1 cells short-term estrogen treatment did not up-regulate the Mdm2 protein level in a concentration-dependent manner and in fact there was no consistency between the replicate experiments (data not shown). Below only one of four different experiments for Mdm2 Western blots is shown (Figure 18). Since the estrogen treatment was relatively short, this observation may suggest that the Mdm2 protein level changes wildly and/or oscillates in both MCF-7 and ZR75-1 cells, but after a five day estrogen treatment the Mdm2 protein level becomes stably elevated in the MCF-7 cells but not in the ZR75-1 cells. The short-term estrogen treatment, however, did not reveal conclusively what effect estrogen had on the Mdm2 protein expression. Importantly, the effect of the 24 hour estrogen treatment on the p53 protein level was consistent between the independent replicate experiments, where each time estrogen treatment up-regulated both the basal and the etoposide-induced p53 protein levels in the MCF-7 cells but not in the ZR75-1 cells. This observation, however, was quite surprising as estrogen treatment decreased the expression of some of the p53 target genes in the MCF-7 cells. Therefore, to determine if estrogen affected the p53 protein recruitment to the chromatin and whether that was the reason for the reduced p53 target genes expression, we carried out the following ChIP experiments.

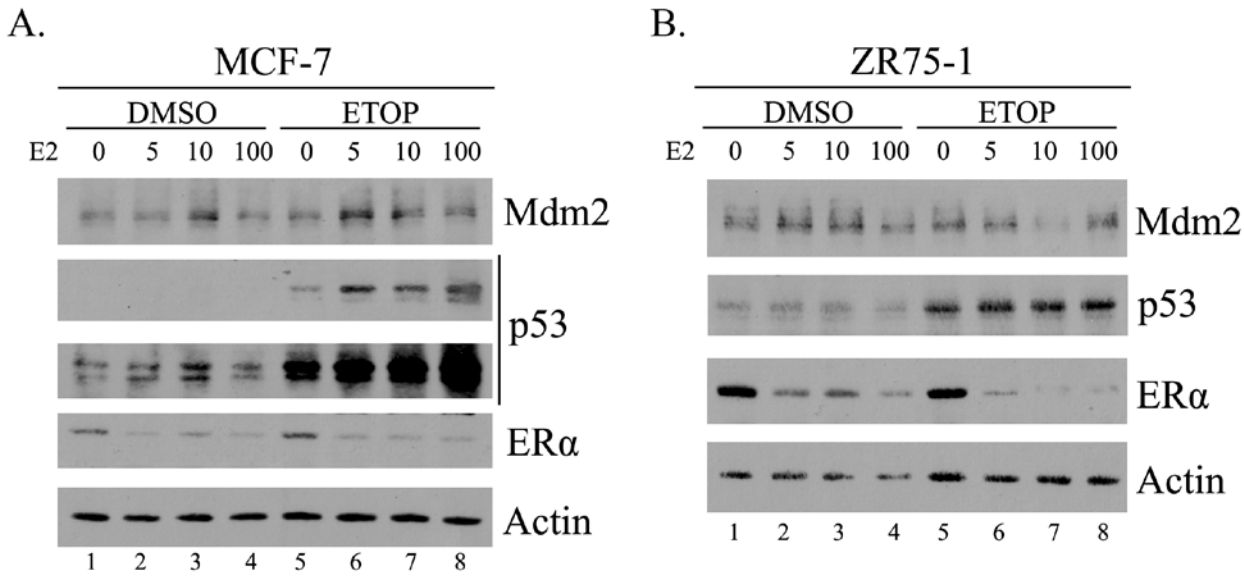

Figure 18: Assessment of Mdm2, p53 and ERα protein levels after a short-term estrogen treatment in the absence and in the presence of etoposide. *MCF-7 and ZR75-1 cells were treated with 5, 10 and 100 nM estrogen (E2) for 24 hours and with 50 µM etoposide (ETOP) for 3 hours. Mdm2, p53, ERα and Actin protein levels of whole cell lysates from MCF-7 (A) and ZR75-1 (B) cells were analyzed by Western blot.*

To determine if the short-term estrogen treatment affected the ability of the p53 protein to interact with the p53-REs in *puma* and *mdm2* genes in the MCF-7 cells, we carried out quantitative ChIP experiments. Earlier, we observed that in MCF-7 cells estrogen treatment decreased the basal mRNA level of *puma* and the etoposide-induced mRNA levels of *puma* and *mdm2*. We hypothesized that this occurred due to reduced p53 recruitment to the chromatin. However, p53 ChIP experiments revealed that estrogen did not reduce the p53 protein recruitment to the p53-REs in the *puma* and *mdm2* genes (Figure 19A).

Though the short-term estrogen treatment did not robustly increase the Mdm2 protein level as the prolonged estrogen treatment did, we hypothesized that the inhibition in the *puma* and the *mdm2* mRNA expression in the presence of estrogen could be due to increased Mdm2 protein recruitment to the chromatin. We observed, however, that neither etoposide nor estrogen treatments affected the levels of the Mdm2 protein recruitment to the p53-REs in *puma* and *mdm2* genes (Figure 19B). Interestingly, the basal level of the Mdm2 protein on the p53-REs in the *puma* gene was almost twice as high as the one on the p53-REs of the *mdm2* gene, but again it did not change in the presence of the treatments (Figure 19B).

Since the etoposide-induced mRNA expression from the *puma* and the *mdm2* genes was inhibited in the presence of estrogen, we examined if estrogen affected the RNA polymerase II (RNAP II) recruitment to the chromatin. We hypothesized that since estrogen inhibited the expression of these genes, it was possible for estrogen to affect the RNAP II recruitment to these genes. We observed, however, that estrogen did not significantly affect the RNAP II recruitment to the transcription start sites (TATA boxes) of the *puma* and the *mdm2* genes (Figure 19C). The transcription start sites in the two genes are located in close proximity to the p53-REs (Gomes

and Espinosa 2010, Xiao et al. 1998), and therefore the same primers that were used for the p53 and Mdm2 ChIP experiments were also used for the RNAP II ChIP experiments.

In conclusion, the results from the p53 and Mdm2 ChIP experiments after the short-term estrogen treatment correlated with the results from the ChIP experiments after a prolonged estrogen treatment. Estrogen treatment inhibited the expression of certain p53 target genes, but did not affect the recruitment of the p53 or the Mdm2 proteins to the chromatin of these genes. Interestingly, estrogen also had no effect on the RNAP II recruitment to the chromatin of the inhibited genes. It is possible, however, that estrogen decreased the recruitment of the phosphorylated form of RNAP II that is involved in the elongation of RNAP II along the gene during the transcription process (RNAP II phosphorylated at Ser2 in the C-terminal domain (Komarnitsky et al. 2000)).

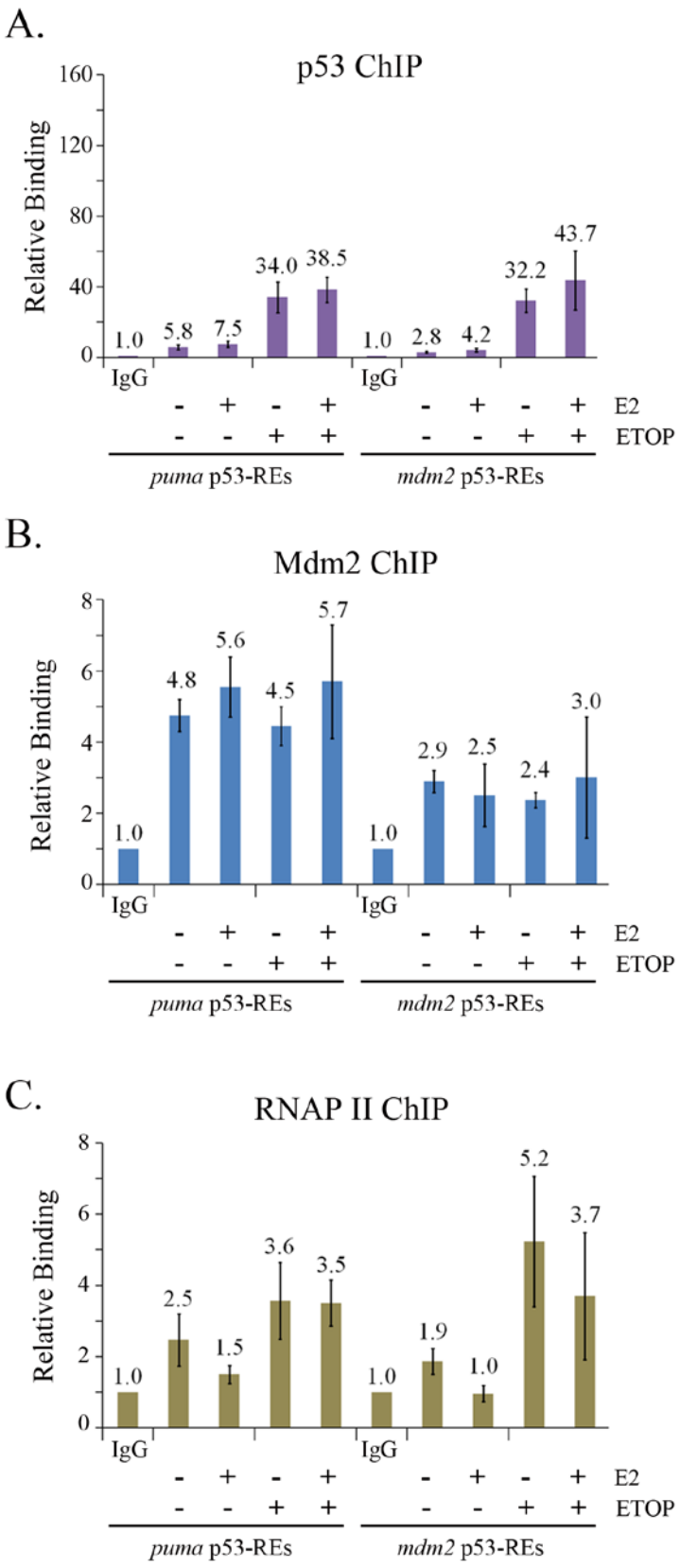

Figure 19: Short-term estrogen treatment had no significant effect on the p53, Mdm2 and RNAP II proteins recruitment to the puma and mdm2 genes in the MCF-7 cells. *MCF-7 cells were treated with 10 nM estrogen (E2) for 24 hours and 50 μM etoposide (ETOP) for three hours. 400 μg of cross-linked and sonicated whole cell lysates were subjected to chromatin immunoprecipitation using antibodies against p53 (A), Mdm2 (B) or RNAP II (C). ChIP with non-specific IgG was done to subtract the background. Immunoprecipitated DNA was amplified by real-time quantitative PCR with primers and FAM-labeled probes for p53-REs in puma and mdm2 genes. Values were normalized to IgG and inputs.*

3.1.3 Discussion

In this study we began to examine the role of the Mdm2 oncoprotein in the estrogen-mediated proliferation of ERα$^+$ breast cancer cells. Since estrogen is known to promote Mdm2 over-expression in breast cancer cells, we hypothesized that the estrogen-mediated cell proliferation is dependent on Mdm2. Importantly, we observed that there was a direct correlation between the estrogen-mediated proliferation of the MCF-7 breast cancer cells and the increased Mdm2 protein expression in the presence of estrogen in these cells. Exogenous Mdm2 over-expression in the MCF-7 cells has been shown to promote growth advantage (Saji et al. 1999), but the mechanism for this is not known. Furthermore, since Mdm2 is important in regulating the p53 tumor suppressor, and in fact is considered to be a major negative regulator of p53, we proposed that the increased cell proliferation in the presence of estrogen occurred through inhibition of the p53 pathway by Mdm2.

Analysis of estrogen effects on the expression and function of the p53 protein

Since one of the major functions of Mdm2 is to negatively regulate p53 by promoting its degradation, we hypothesized that in the presence of estrogen the induced Mdm2 over-expression would lead to p53 degradation. However, it was surprising to see that in the MCF-7 breast cancer cells, while estrogen increased the Mdm2 protein level, the p53 protein level did not decrease, but in fact increased as well. Interestingly, two independent studies have reported that estrogen can stabilize the p53 protein level in the MCF-7 cells (Okumura et al. 2002a) and that the estrogen receptor α (ERα) protects p53 from deactivation by Mdm2 by forming a complex with the p53 protein (Liu G. et al. 2000). Taken together, the evidence in the literature

and our data suggest that the p53 protein stability can be sustained in the presence of estrogen even when the Mdm2 protein level increases.

We hypothesized that since estrogen increased the expression of the Mdm2 protein, but the p53 protein stability was sustained, then perhaps the p53 transcriptional activity on the chromatin was affected. Importantly, in addition to regulating the p53 protein stability, Mdm2 is known to directly inhibit the p53 protein transcriptional activity on the chromatin by co-localizing with p53 at the p53-RE sites (Momand et al. 1992, Oliner et al. 1993, Thut et al. 1997, Wu et al. 1993). By co-localizing with p53 on the chromatin, Mdm2 is believed to hinder the interaction of the p53 protein with the transcriptional co-activators, which in turn can block transcription initiation (Lin et al. 1994, Thut et al. 1997). To determine if the p53 transcriptional activity is affected by estrogen, we looked at the effect of estrogen on the p53 target genes expression that are involved in three different pathways: *puma* (apoptosis), *mdm2* (p53 negative regulation), and *p21* (growth arrest). We observed that in the MCF-7 cells prolonged estrogen treatment robustly inhibited both the basal and the drug-induced (etoposide and Nutlin-3) expression of the pro-apoptotic p53 target gene *puma*. Additionally, we also observed that estrogen robustly inhibited the basal expression of the growth-arrest p53 target gene *p21*, but had no effect on its expression in the presence of the stress-inducing stimuli. This inhibition, however, occurred while estrogen in fact increased the basal p53 protein level and did not significantly change the drug-induced p53 protein level.

Since estrogen reduced the expression of the *puma* gene, but did not affect the p53 protein level, we conducted quantitative chromatin immunoprecipitation (ChIP) assays to see if the decrease in the *puma* transcription was due to reduced p53 protein recruitment to the chromatin. We saw, however, that the p53 protein recruitment to the *puma* p53-REs did not

change in the presence of estrogen. This suggests that estrogen may have affected the p53 protein activity at specific p53 target genes without substantially blocking the p53 protein DNA binding ability.

Since estrogen increased the basal- and the drug-induced Mdm2 protein level, we also examined if the estrogen-mediated inhibition of *puma* transcription was due to increased Mdm2 protein recruitment to the p53-REs in the *puma* gene. We observed, however, that estrogen did not lead to a significant increase in the Mdm2 protein recruited to the chromatin. These results suggest that the increased Mdm2 protein expression in the presence of estrogen is not sufficient to promote p53 degradation or to increase Mdm2 protein recruitment to the chromatin, but a possibility exists that the p53 transcriptional output is controlled by Mdm2 at an epigenetic level and that the changes in the Mdm2 protein level in the presence of estrogen are sufficient to affect the p53 protein activity on the chromatin. Evidently, Mdm2 has been shown to repress the p53-mediated transcription by promoting ubiquitination of histone H2 (Minsky and Oren 2004) and by recruiting methyltransferases to methylate p53 and histone H3 at target promoters (Chen L. et al. 2010). Importantly, the binding of the p53 protein to the *puma* promoter facilitates acetylation of the core histones (H3 and H4), which, in turn, leads to opening of the chromatin structure and transcriptional activation (Kaeser and Iggo 2004, Wang P. et al. 2007). Additionally, the estrogen-mediated increase in the Mdm2 protein may lead to the p300 transcriptional co-activator ubiquitination and subsequent degradation that could, in turn, result in reduced acetylation of p53 (Jin et al. 2004, Jin et al. 2002) and subsequently reduce p53 transcriptional activity. The role of the p300 acetyltransferase may be important in the estrogen-mediated inhibition of *puma*, since the expression of the *puma* gene is regulated by p300 (Iyer et al. 2004).

In addition, although estrogen treatment decreased the etoposide-induced p53 target gene *puma* transcription without reducing the p53 protein level, it is possible that estrogen affected the post-translational modifications on the p53 protein. For example, estrogen was shown to inhibit the resveratrol-activated p53 in the MCF-7 cells in part by interfering with the post-translational modifications of p53, which have been shown to be essential for p53-dependent DNA binding and consequent stimulation of downstream pathways (Zhang S. et al. 2004a). We observed that in the MCF-7 cells, p53 phosphorylation at Ser15 after etoposide treatment was very marginal and was not affected by estrogen (data not shown). For further investigation, it will be important to check if estrogen affects the post-translational modifications of the other key p53 residues that have been shown to be required for the activation of *puma* transcription (e.g. acetylation at Lys120, Lys164, Lys373, Lys382, and phosphorylation at Ser46) (Vousden and Prives 2009). Perhaps changes in the recruitment of the modified form of the p53 protein to the chromatin could explain the reduced *puma* transcription in the presence of estrogen.

It is highly likely that estrogen acts in a number of coordinated ways to block *puma* transcription. For example, estrogen can induce transient cyclical DNA methylation of active promoters that leads to transcription inhibition (Kangaspeska et al. 2008, Metivier et al. 2008). In turn, the *puma* gene is prone to be highly methylated, which results in reduced *puma* expression (Garrison et al. 2008). Therefore, it is possible that estrogen may promote *puma* gene methylation and thus inhibit its expression. Furthermore, estrogen has also been shown to up-regulate Myc expression (Rodrik et al. 2006). Importantly, adjacent to the location of the p53 binding site, the *puma* gene promoter contains E boxes which serve as an inhibitory binding site for Myc (Garrison et al. 2008). Therefore, it is possible that by inducing Myc expression estrogen can inhibit the expression of *puma*. In addition, the ERα protein can also bind to the p53

protein directly and thus potentially repress the p53 transcriptional activity on the chromatin as well (Konduri et al. 2010, Liu W. et al. 2006, Sayeed et al. 2007).

Because we did not obtain a direct evidence that estrogen affected the p53 protein activity when the *puma* expression was inhibited, a possibility exists that estrogen inhibited *puma* expression in a p53-independent manner. Therefore, it is important to determine if other transcription factor of the p53 family genes (p73 and p63) are affected by estrogen. For example, in response to a variety of stimuli p73 has been shown to activate *puma* gene expression by binding to the same p53-REs in the *puma* promoter as the ones that the p53 protein binds to (Matallanas et al. 2007, Melino et al. 2004, Ming et al. 2008). Therefore, it is possible that estrogen inhibited the p73-mediated transactivation of *puma*, and not that of the p53 protein. In support of this hypothesis, our preliminary experiments indicate that when p53 was knocked down by shRNA, *puma* transcription was still induced by etoposide treatment (data not shown).

Analysis of estrogen effects on the Mdm2 expression

Interestingly, when we examined the mechanism of how estrogen regulates the expression of Mdm2 we notice that while estrogen treatment increased the Mdm2 protein level, analysis of the *mdm2* mRNA level revealed that its expression was not significantly affected by the estrogen treatment. This observation may suggest that the estrogen-mediated Mdm2 over-expression in the MCF-7 cells was due to increased *mdm2* mRNA translation and/or due to inhibition of the Mdm2 protein degradation. Importantly, this observation does not rule out the possibility that estrogen regulates Mdm2 expression at the transcriptional level as well. In fact, an earlier study clearly showed by Northern blot analysis that estrogen treatment does increase the expression of the *mdm2* mRNA transcripts in the MCF-7 cells (Gudas et al. 1995). And

additionally, Arnold Levine's group also showed that in the presence of estrogen there is an increased RNAP II (RNA Polymerase II) recruitment to the *mdm2* gene promoter in the MCF-7 cells (Hu et al. 2007).

To analyze the *mdm2* mRNA expression in the absence and in the presence of estrogen treatment, in the quantitative real-time RT-PCR experiment we used primers that target an internal region of the *mdm2* mRNA transcript (exons 6 through 7). This region is known to be often spliced out during the *mdm2* mRNA processing (Bartel et al. 2002, Bartel et al. 2004). Therefore, a possibility exists that not all the *mdm2* transcripts (full length and spliced variants) were detected by this method. Hence, it will be important to systematically evaluate the expression of all the *mdm2* mRNA isoforms in the absence and in the presence of estrogen. This should be done by Northern blot analysis (using random labeling in order to detect all the possible isoforms of *mdm2*) as well as by quantitative real-time RT-PCR (using primers designed to target a specific region in the *mdm2* mRNA transcript that is common for all *mdm2* isoforms, and also primers that can detect and distinguish *mdm2* mRNA expression specifically from the P1 and the P2 promoters of the *mdm2* gene). These methods will allow us to see the estrogen-mediated changes in the different isoforms of the *mdm2* mRNA transcripts and to correlate them with the changes in the Mdm2 protein expression.

Interestingly, we observed that both the untreated and the estrogen-treated MCF-7 cells appeared to have multiple forms of the Mdm2 protein, as the Mdm2 Western blot showed several bands for Mdm2. The *mdm2* gene consists of 12 exons. The mRNA transcripts of the *mdm2* gene often lack several exons, as they are spliced out during mRNA processing (Bartel et al. 2002). In fact, alternative splicing of the *mdm2* gene has been shown to result in over 40 different *mdm2* splice variants that have been identified in both normal and tumorous tissues (Bartel et al. 2004,

Harris 2005). Importantly, the occurrence of high instances of the *mdm2* mRNA splicing has been associated with malignant cancer phenotypes (Bartel et al. 2001). In addition, the function of the Mdm2 protein is known to be modulated by post-translational modifications, such as ubiquitination, phosphorylation and sumoylation (Meek and Knippschild 2003). Therefore, the increase in the level of the different forms of the Mdm2 protein in the presence of estrogen suggest that estrogen may play an important role in the regulation of the *mdm2* transcript splicing and/or the post-translational modifications of the Mdm2 protein. Interestingly, a clinical study examining the correlation between the over-expression frequency of the *mdm2* splice variants and the clinicopathological features of breast cancers showed that there was no significant correlation between the two. However, cases with spliced *mdm2* transcripts tended to be of a more aggressive type of breast tumors (Hori et al. 2000).

Furthermore, to determine if estrogen regulates Mdm2 expression at the transcriptional or the translational levels, it will be important to examine the changes in the Mdm2 expression after estrogen treatment in the presence of a transcription inhibitor (α-amanitin) and a protein biosynthesis inhibitor (cyclohexamide). If estrogen regulates *mdm2* transcription, then in the presence of α-amanitin estrogen will not be able to up-regulate *mdm2* expression. And in turn, if estrogen regulates Mdm2 translation, then in the presence of cyclohexamide estrogen will not be able to up-regulate Mdm2 protein expression. It is important to keep in mind, however, that both mechanisms can occur simultaneously. In addition, it is also possible that estrogen regulates Mdm2 protein stability. Therefore, a pulse-chase cell labeling method (examining the level of [^{35}S]methionine-labeled Mdm2) will determine if the half-life of the Mdm2 protein increases in the presence of estrogen. If estrogen affects the stability of the Mdm2 protein and thus increases the half-life of Mdm2, than [^{35}S]methionine-labeled cells that were grown in the presence of

estrogen will retain more of the [^{35}S]methionine-labeled Mdm2 over-time than the [^{35}S]methionine-labeled cells that were grown in the absence of estrogen.

Estrogen signals towards the anti-apoptotic pathway by oppositely regulating the expression of Puma and Bcl-2

It has been shown earlier that the pro-apoptotic gene *puma* is among the genes that are expressed at reduced levels after estrogen treatment (Tozlu et al. 2006). We observed that though both MCF-7 and ZR75-1 cell lines are ERα^{+}, only in the MCF-7 cells the basal and the etoposide-induced *puma* mRNA expression was inhibited after estrogen treatment. This suggests that the ERα status in the breast cancer cells is not sufficient for the estrogen-mediated inhibition of *puma* gene expression. Evidently, in the MCF-7 cells, estrogen inhibited the basal and the drug-induced *puma* mRNA expression and in parallel up-regulated the Mdm2 protein level under the same conditions. Therefore, it is possible that estrogen requires Mdm2 to inhibit the *puma* mRNA expression. To further investigate this observation, it will be important to test if estrogen will still inhibit *puma* expression when the Mdm2 protein over-expression in blocked.

Estrogen has also been shown to inhibit apoptosis in the MCF-7 cells by inducing the expression of the anti-apoptotic gene, *bcl-2* (Perillo et al. 2000). Interestingly, we saw a coordinated up-regulation of Bcl-2 and a down-regulation of Puma in the MCF-7 cells. This suggests that estrogen may be signaling towards the anti-apoptotic pathway by decreasing Puma and by simultaneously increasing Bcl-2, and thus estrogen increases cell survival during the induced cell proliferation. Evidently, estrogen-derived oxidants have been shown to result in oxidative stress and DNA adducts (Chen Y. et al. 2000, Mobley and Brueggemeier 2004, Yared et al. 2002). Therefore, it is possible that the DNA-damaging effects of estrogen in combination

with suppression of the intrinsic apoptotic pathway could set the stage for the cancer cells to emerge from cell populations sustaining the DNA damage.

Summary of conclusions

1) Estrogen robustly up-regulated the expression of the Mdm2 protein in breast cancer cells that carry the SNP309 (T→G) in the *mdm2* gene (MCF-7 cells), but not in the cells that don't carry the SNP (ZR75-1 cells).

2) Estrogen-mediated Mdm2 over-expression correlated with increased cell proliferation.

3) Estrogen robustly inhibited both the basal and the drug-induced (etoposide and Nutlin-3) *puma* mRNA expression and in parallel decreased the Puma protein level.

4) Estrogen appeared to signal towards the anti-apoptotic pathway by decreasing Puma and by simultaneously increasing Bcl-2, and thus increasing cell survival during induced proliferation.

5) While estrogen decreased the p53 target gene *puma* expression, estrogen did not lead to p53 degradation, p53 nuclear exclusion or decreased p53 interaction with the chromatin.

6) Though estrogen increased the Mdm2 protein level and slightly increased the nuclear localization of Mdm2, estrogen did not increase the Mdm2 protein co-localization with the p53 protein on the chromatin.

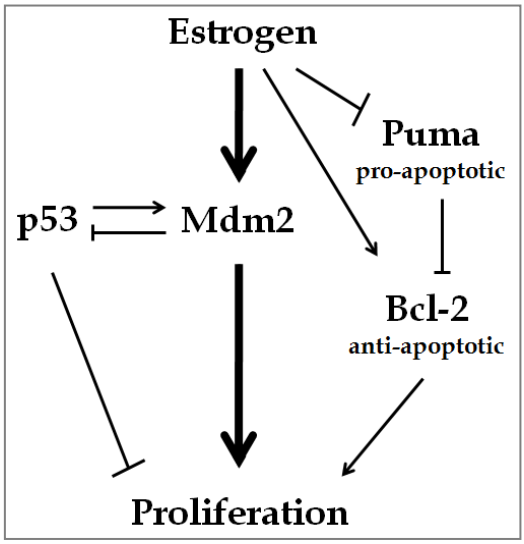

Figure 20: Mdm2 plays a central role in the estrogen-mediated cell proliferation. *The observed estrogen-mediated cell proliferation correlated with the Mdm2 protein over-expression in the MCF-7 cells. Our initial hypothesis was that the increased cell proliferation in the presence of estrogen occurred through inhibition of the p53 pathway by Mdm2. However, the data suggest that during this process Mdm2 targets other pathways as well. Specifically, we saw that the estrogen-mediated increase in Mdm2 did not lead to p53 protein degradation or increased Mdm2 co-localization with p53 on the chromatin. The pro-apoptotic p53 target Puma was robustly inhibited in the presence of estrogen, and since estrogen simultaleously up-regulated Mdm2 this suggests that Mdm2 may play a role in this process. And similarly, a posibility exists that Mdm2 may play a role in the estrogen-mediated up-regulation of the anti-apoptotic protein Bcl-2. Overall, the data suggest that during the estrogen-mediated cell proliferation several pathways may be targeted by estrogen via Mdm2.*

3.2 Determination of the roles of Mdm2 and p53 in the estrogen-mediated breast cancer cell proliferation.

3.2.1 Introduction

To investigate what role the Mdm2 protein plays during estrogen's signaling in the breast cancer cells, in the following part of this study we examined how the down-regulation of the Mdm2 expression affects the estrogen-mediated cell proliferation. Because we observed a strong correlation between the increased Mdm2 protein expression and cell proliferation in the presence of estrogen, we predicted that decreasing Mdm2 expression in the presence of estrogen would block cell proliferation. In addition, since Mdm2 is a major negative regulator of p53, we also investigated if the estrogen-mediated cell proliferation occurred due to the inhibition of the p53 pathway by Mdm2 (see Figure 21 below).

To determine the contribution of Mdm2 and p53 to the estrogen-induced cell proliferation, we transiently knocked down Mdm2 by siRNA and also generated stable cell lines containing shRNAs for inducible knockdown of Mdm2 and p53. The doxycycline-inducible shRNAs constructs targeting *mdm2* and *p53* using the mir-30 design (see Figure 5 on page 17 above) were a generous gift from Scott Lowe and Agustin Chicas form the Cold Spring Harbor Laboratory. The shRNA-containing constructs (see Table 1 on page 18 above) were introduced into the MCF-7 cells by retroviral gene transfer method. MCF-7 cells carrying various levels of the *mdm2* or the *p53* shRNAs were generated (pools), and subsequently, stable clones were selected by seeding the cells at limited densities (clones). Once the stable clonal cell lines carrying inducible shRNAs for *mdm2* and *p53* were generated, we began to examine the roles that the Mdm2 and p53 play during estrogen-mediated breast cancer cell proliferation.

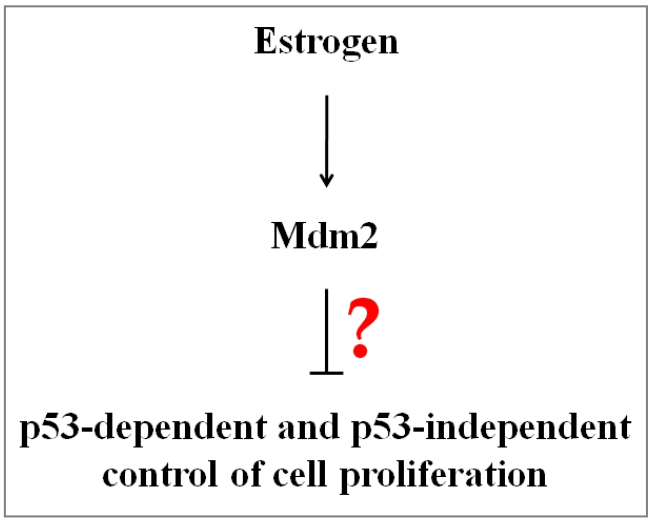

Figure 21: Examining the roles that Mdm2 and p53 play during the estrogen-mediated breast cancer cell proliferation. *Because there was a strong correlation between the increased Mdm2 protein expression and cell proliferation in the presence of estrogen, we investigated the role that Mdm2 and its major negative target, p53, play during this process. We predicted that decreasing Mdm2 expression in the presence of estrogen would block the estrogen-mediated cell proliferation. And in turn, blocking the p53 pathway, by knocking down p53, would further augment the estrogen-mediated cell proliferation.*

3.2.2 Results

In the previous section of this study, we observed that in the MCF-7 breast cancer cells transcription of certain p53 target genes was inhibited in the presence of estrogen. Since estrogen treatment increased the Mdm2 protein level in these cells, we hypothesized that Mdm2, a major p53 inhibitor, played an important role in the estrogen-mediated inhibition of the p53 target genes expression. To test this hypothesis, in the following experiment we transiently knocked down Mdm2 by siRNA and examined if the estrogen-mediated inhibition of the p53 target genes expression was relieved. Furthermore, since estrogen treatment did not increase the Mdm2 protein level in the ZR75-1 breast cancer cells and concomitantly did not robustly inhibit the expression of the p53 target genes in this cell line, we also compared the effect of the Mdm2 knockdown in the presence of estrogen between the two cell lines.

We observed that in both MCF-7 and ZR75-1 cell lines, *mdm2* siRNA reduced the Mdm2 protein level both in the absence and in the presence of the estrogen treatment (Figure 22A and 22C, compare lanes 1 and 5, and lanes 2 and 6, Mdm2) and also in the presence of an etoposide treatment (Figure 22A and 22C, compare lanes 3 and 7, and lanes 4 and 8, Mdm2). Similarly, the *mdm2* mRNA expression was reduced in both cell lines after the Mdm2 knockdown by siRNA in the presence of estrogen and etoposide treatments (Figure 22B and 22D, *mdm2*). Interestingly, knockdown of Mdm2 did not influence the p53 protein levels in the absence or in the presence of etoposide (Figure 22A and 22C, compare lanes 1-2 and 5-6, and lanes 3-4 and 7-8, p53). Furthermore, we observed that in both cell lines Mdm2 knockdown dramatically increased the p21 protein level, both in the presence and in the absence of estrogen and etoposide treatments (Figure 22A and 22C, compare lanes 1-2 and 5-6, and lanes 3-4 and 7-8, p21). In correlation, Mdm2 knockdown in the presence of estrogen resulted in increased expression of the *p21* mRNA

transcript, as determined by the quantitative real-time RT-PCR (Figure 22B and 22D, *p21*). And only the basal level of the *puma* mRNA transcripts increased after the Mdm2 knockdown, while the etoposide-mediated *puma* mRNA induction was not significantly affected (Figure 22B and 22D, *puma*).

In conclusion, in this experiment we observed that in both MCF-7 and ZR75-1 cell lines Mdm2 knockdown in the presence of estrogen similarly increased the basal and the etoposide-induced *p21* transcription and also increased the basal transcription of *puma*. Importantly, this experiment shows that in both cell lines the p53 target genes expression was similarly affected by the Mdm2 knockdown in the presence of estrogen. In addition (and surprisingly), we did not observe an increase in the p53 protein level when the Mdm2 expression was blocked. Following Mdm2 knockdown, however, the p53 target genes expression was up-regulated. This result suggests that while the p53 protein level did not increase following Mdm2 knockdown, the transcriptional activity of the p53 protein increased.

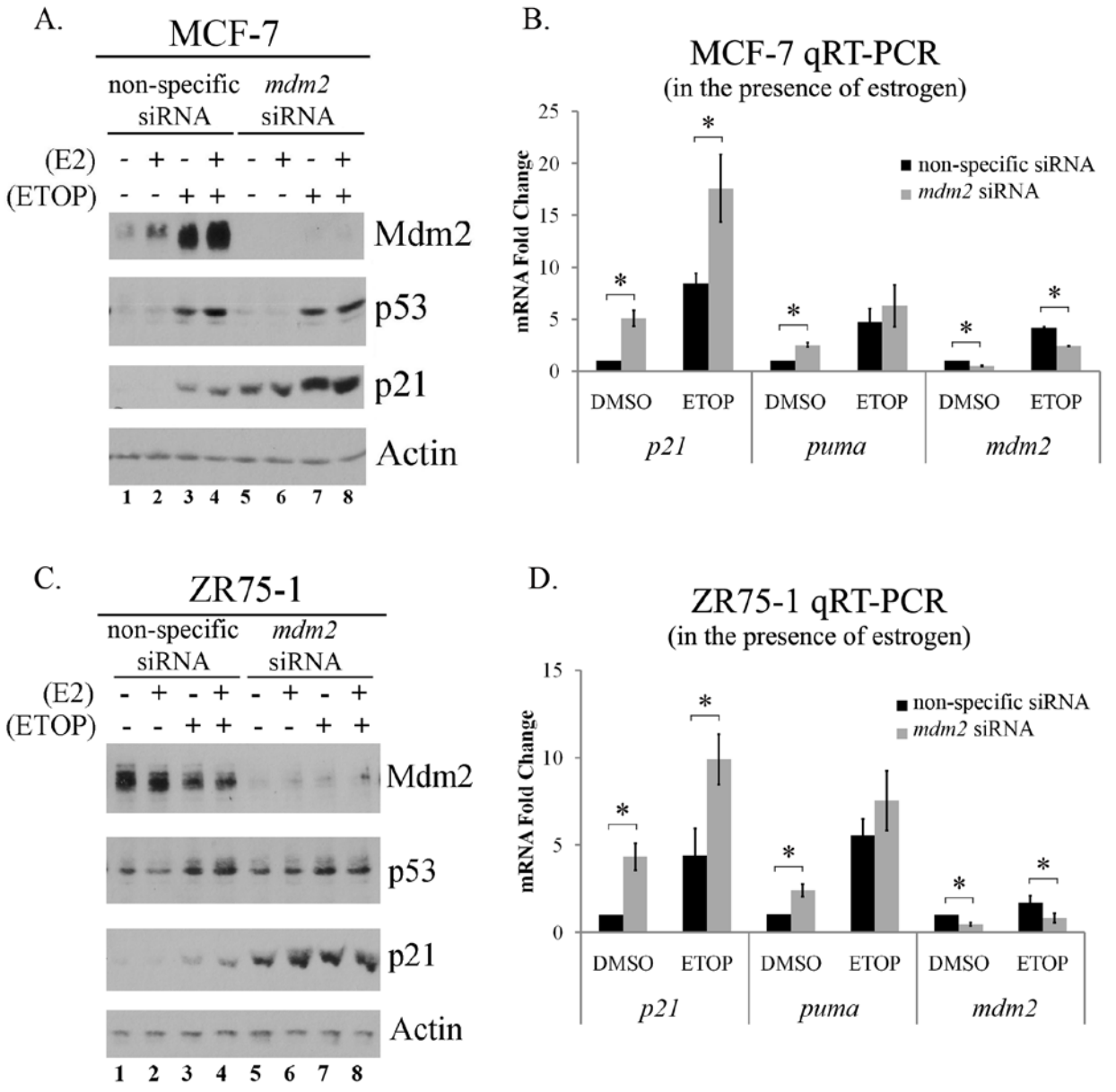

Figure 22: Mdm2 knockdown by siRNA potentiated the p53 transcriptional activity in the presence of estrogen in both MCF-7 and ZR75-1 cell lines. *MCF-7 and ZR75-1 cells were transfected with 100 nM of non-specific or mdm2 siRNA. 24 hours following transfection, cells were treated with 10 nM estrogen (E2) for 24 hours and 50 µM etoposide (ETOP) for 3 hours. Mdm2, p53, p21 and Actin protein levels from whole cell lysates of MCF-7 (A) and ZR75-1 (C) cells were analyzed by Western blot. Relative mRNA levels of puma, p21 and mdm2 genes in MCF-7 (B) and ZR75-1 (D) cells were determined by quantitative real-time RT-PCR. Results were normalized to control samples and actin values. Graphs show means and standard errors of two independent experiments. * $p<0.05$ (determined by student t-test).*

In the following experiment, we wanted to determine if the Mdm2 protein over-expression in the presence of estrogen contributed to the estrogen-mediated breast cancer cell proliferation. To achieve that, we examined the effect of the Mdm2 knockdown on the MCF-7 and ZR75-1 breast cancer cells proliferation in the presence of estrogen. Earlier in this study, we observed that during cell proliferation in the presence of estrogen, estrogen increased the Mdm2 protein level in the MCF-7 cells but not in the ZR75-1 cells. Therefore, in the following experiment we hypothesized that the Mdm2 knockdown in the presence of estrogen will inhibit the MCF-7 cells proliferation to a greater extent than the ZR75-1 cells proliferation.

In the MCF-7 cells, we observed that Mdm2 knockdown by siRNA in the presence of estrogen increased the percent of cells in the G1 phase and subsequently decreased the percent of cells in the S phase (Figure 23A). Cell proliferation was measured by the MTT colorimetric assay. In the MCF-7 cells, 18% inhibition of estrogen-mediated cell proliferation was observed after the Mdm2 knockdown (Figure 23B). Etoposide treatment inhibited the estrogen-mediated cell proliferation to a similar degree as the Mdm2 knockdown, while combination of etoposide treatment and Mdm2 knockdown did not lead to any further inhibition of cell proliferation (Figure 23B). In turn, the ZR75-1 cells appeared to be less sensitive to the Mdm2 knockdown than the MCF-7 cells, since Mdm2 depletion inhibited their proliferation only by 11% (Figure 23C). The ZR75-1 cells, however, were more sensitive to the etoposide treatment than the Mdm2 knockdown, and combination of the Mdm2 knockdown and etoposide treatment further inhibited cell proliferation (Figure 23C).

Importantly, in agreement with our hypothesis we observed that Mdm2 knockdown inhibited the estrogen-mediated MCF-7 cells proliferation more robustly than the estrogen-

mediated ZR75-1 cells proliferation. This suggests that the estrogen-mediated Mdm2 over-expression plays an important role during the estrogen-mediated breast cancer cell proliferation.

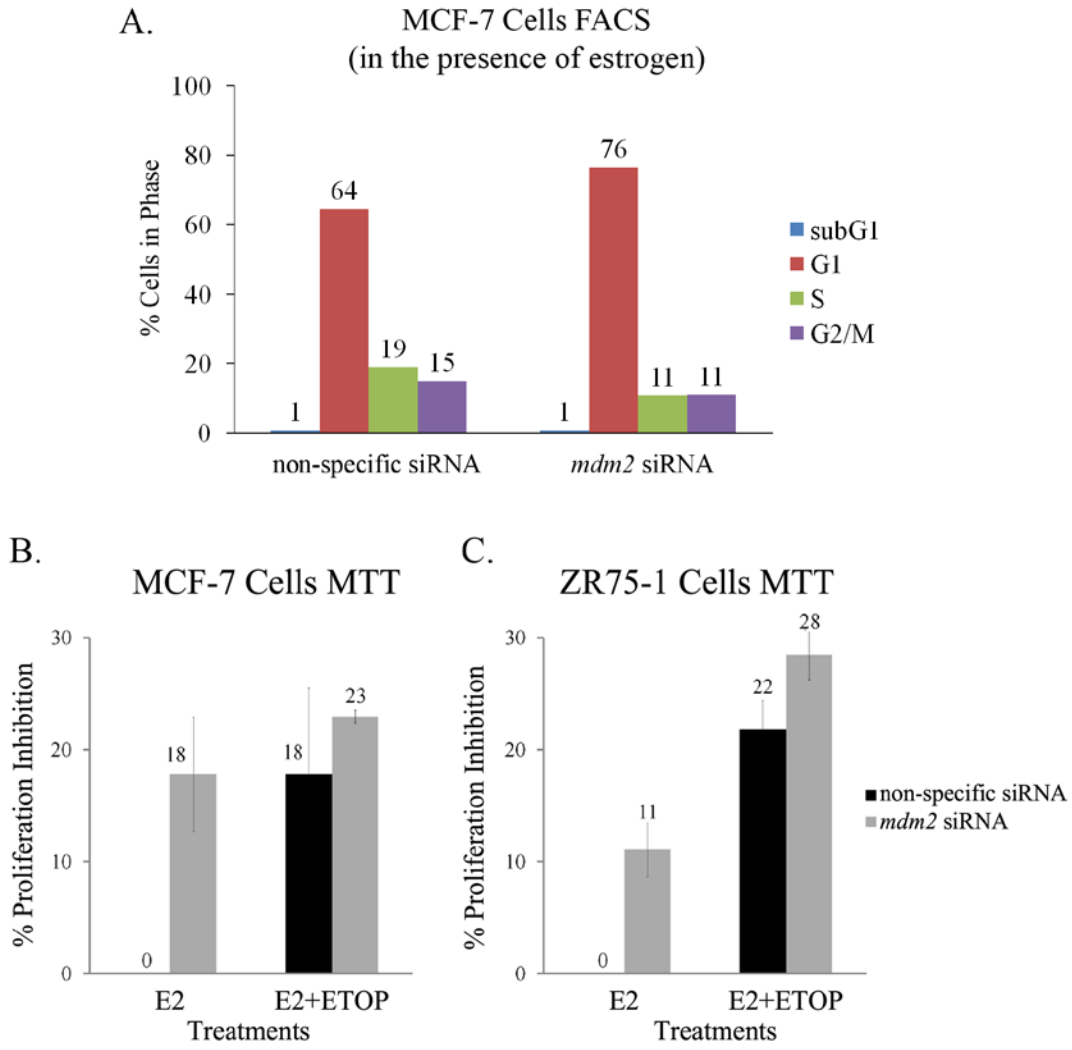

Figure 23: Mdm2 knockdown by siRNA inhibited the estrogen-mediated MCF-7 cells proliferation more robustly than the estrogen-mediated ZR75-1 cells proliferation. *(A) MCF-7 cells were transfected with 100 nM of non-specific or mdm2 siRNA. 24 hours following transfection, cells were treated with 10 nM estrogen (E2) for 48 hours. At the end of the treatments fluorescence activated cell sorting (FACS) was carried out. (B and C) Percent of cell proliferation inhibition was determined by the MTT assay. MCF-7 and ZR75-1 cells were transfected with 100 nM of non-specific or mdm2 siRNA. 24 hours following transfection, cells were treated with 10 nM estrogen (E2) for 48 hours and following additional 24 hours cell were treated with 50 μM etoposide (ETOP). Graphs show means and standard errors of three independent experiments.*

During the transient transfections with the siRNA, we observed that the cells were sensitive to the medium conditions, which made it difficult to conduct long term estrogen treatments and to study cell proliferation. Therefore, we generated stable cell lines containing shRNAs for inducible RNA interference. The doxycycline-inducible construct, carrying the shRNA, contains a GFP expressing marker (see Figure 5 on page 17 for the construct map details). When the MCF-7 cells carrying the shRNA for *mdm2* were treated with 2 µg/ml doxycycline for six days, we observed GFP expression as a sign for shRNA expression (Figure 24). Evidently, some GFP expression was visible in the absence of the doxycycline treatments, suggesting that the construct was slightly leaky. When clonal cell lines were selected for the use in the future experiments, we made sure to take this into consideration and selected only those clones that did not express any GFP in the absence of the doxycycline treatment.

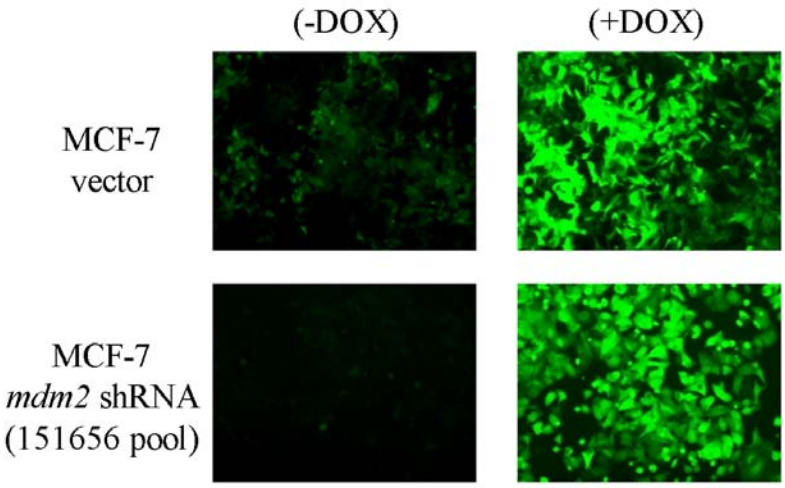

Figure 24: Assessment of the doxycycline-inducible construct expression in the MCF-7 cells. *MCF-7 cells with mdm2 shRNA (151656 pool) or control vector were treated with 2 µg/ml doxycycline for six days to induce shRNA expression. Images show GFP fluorescence.*

The following experiment illustrates that generating clonal cell lines containing the shRNA construct, increases the efficiency of the protein knockdown and the observed phenotype. The clonal MCF-7 cell line carrying the shRNA for *mdm2* (*mdm2* shRNA 151656, clone C4) was generated by limited dilution method from the MCF-7 *mdm2* shRNA-containing pool line (*mdm2* shRNA 151656, pool). Importantly, the Mdm2 protein knockdown appeared to be greater in the clonal line (Figure 25A, compare lanes 3-4 for the pool, and 5-6 for the clone, Mdm2). Furthermore, the increase in the p21 protein was greater in the MCF-7 clonal cell line as well (Figure 25A, compare lanes 3-4 for the pool, and 5-6 for the clone, p21). Interestinly, the p53 protein level was not affected by the Mdm2 protein knockdown (Figure 25A, compare lanes 3-4 for the pool, and 5-6 for the clone, p53). This observation is in agreement with what we observed earlier after Mdm2 knockdown with an siRNA. Importantly, the inhibition in cell proliferation after the Mdm2 knockdown was more effective in the clonal cell line than the one observed in the pool (Figure 25B, the MCF-7 cells proliferation is represented by the percent of the mitochondrial activity of the cells that could be considered to be proportional to the number of cells).

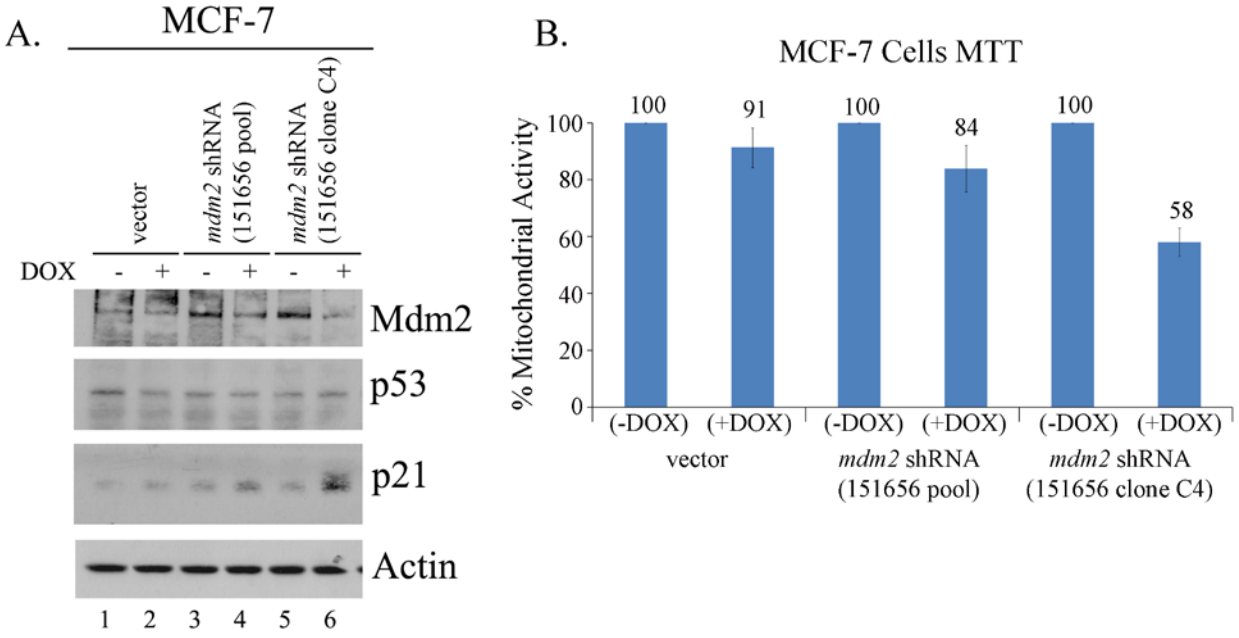

Figure 25: Characterization of the clonal MCF-7 cell line containing the shRNA for mdm2. *MCF-7 cells with control vector, mdm2 shRNA (151656 pool) or mdm2 shRNA (151656 clone C4) were treated with 2 µg/ml doxycycline for six days to induce shRNA expression. (A) Western blot analysis of Mdm2, p53, p21 and Actin protein levels in whole cell extracts. (B) Cell proliferation was measured by the MTT assay.*

Once we generated a clonal MCF-7 cell line with a good inducible Mdm2 knockdown, we began to examine the efficiency of the Mdm2 knockdown and its effect on the p53 and the p21 protein levels in the absence and in the presence of estrogen. Induction of the *mdm*2 shRNA by doxycycline treatment reduced the Mdm2 protein level and dramatically increased the p21 protein level in the MCF-7 cells (Figure 26A, compare lanes 3 and 4, Mdm2). The p53 protein level, however, was not affected by the Mdm2 knockdown (Figure 26A, compare lanes 3 and 4, p53). Interestingly, previously we observed that a transient Mdm2 knockdown by siRNA also did not affect the p53 protein level while the p53 target p21 increased dramatically.

The expression of the Mdm2 protein was also effectively blocked by the shRNA induction in the presence of estrogen (Figure 26B, compare lanes 1-3-4, Mdm2), while in the vector control cell line, doxycycline treatment had no effect on the Mdm2 protein level in the absence and in the presence of estrogen (Figure 26B, compare lanes 5-8, Mdm2). Interestingly, Mdm2 knockdown in the presence of estrogen promoted a greater up-regulation in the p21 protein level than in the absence of estrogen (Figure 26B, compare lanes 2 and 4, p21). We noticed that estrogen treatment alone increased the p21 protein level in the *mdm2* shRNA-containing cell line, but not in the vector control cell line (Figure 26B, compare lanes 1 and 3, and lanes 5 and 7, p21). In fact, in the vector control cell line, estrogen treatment decreased the protein level of p21 (Figure 26B, compare lanes 5 and 7, p21). Due to the inconsistency in the estrogen's effect on the p21 protein level in the *mdm2* shRNA-containing cell line and in the vector control cell line (when the *mdm2* shRNA was not expression, Figure 26B, compare lanes 1 and 3 and lanes 5 and 7, p21), we examined an additional MCF-7 clone (*mdm2* shRNA 151656, clone A4). Interestingly, in that clone we observed a similar increase in the p21 protein

level after estrogen treatment and an additive increase in the p21 protein level after the Mdm2 knockdown in combination with the estrogen treatment (data not shown).

Upon Mdm2 knockdown by shRNA, we also examined the changes in the ERα protein level. The Mdm2 E3 ubiquitin ligase has been shown to regulate the ERα protein level via the proteasomal degradation (Duong et al. 2007). We therefore, hypothesized that down-regulation of the Mdm2 protein by shRNA will increase the protein level of the ERα. Surprisingly, however, we observed that Mdm2 knockdown decreased the ERα protein level (Figure 26B, compare lanes 1 and 2). This result suggests that in addition to regulating ERα degradation, Mdm2 promotes ERα stability. Perhaps, in certain situations the ERα protein mono-ubiquitination by Mdm2 or simply the Mdm2-ERα complex formation is required for the protein stability, and when the Mdm2 expression is down-regulated, the ERα protein level cannot be sustained. In addition, similar to our earlier observations, estrogen treatment decreased the ERα protein level (Figure 26B, compare lanes 1 and 3). Whereas, combination of Mdm2 knockdown and estrogen treatment additively decreased the ERα protein level (Figure 26B, compare lanes 3 and 4). Interestingly, in the vector control cell line the basal level of the ERα protein was much lower than in the *mdm2* shRNA-containing cell line (Figure 26B, compare lanes 1 and 5). But regardless of that, doxycycline treatment in the vector control cell line did not affect the ERα protein level as it did in the mdm2 shRNA-containing cell line (Figure 26B, compare lanes 1 and 2, and lanes 5 and 6).

In conclusion, we observed that induction of the *mdm2* shRNA expression effectively reduced the Mdm2 protein level in the MCF-7 cells both in the absence and in the presence of the estrogen treatment. Furthermore, the decrease in the Mdm2 protein resulted in a robust increase in the p21 protein level. However, since the p53 protein level did not change after the

Mdm2 knockdown, it is possible that the increase in the p21 protein was partially p53-independent. In fact, the Mdm2 protein is known to regulate p21 degradation in a p53-independent manner by directly targeting p21 to the proteasome (Jin et al. 2003, Xu et al. 2010, Zhang Z. et al. 2004b). Importantly, these results suggest that the estrogen-mediated cell proliferation occurred though p21 inhibition by Mdm2.

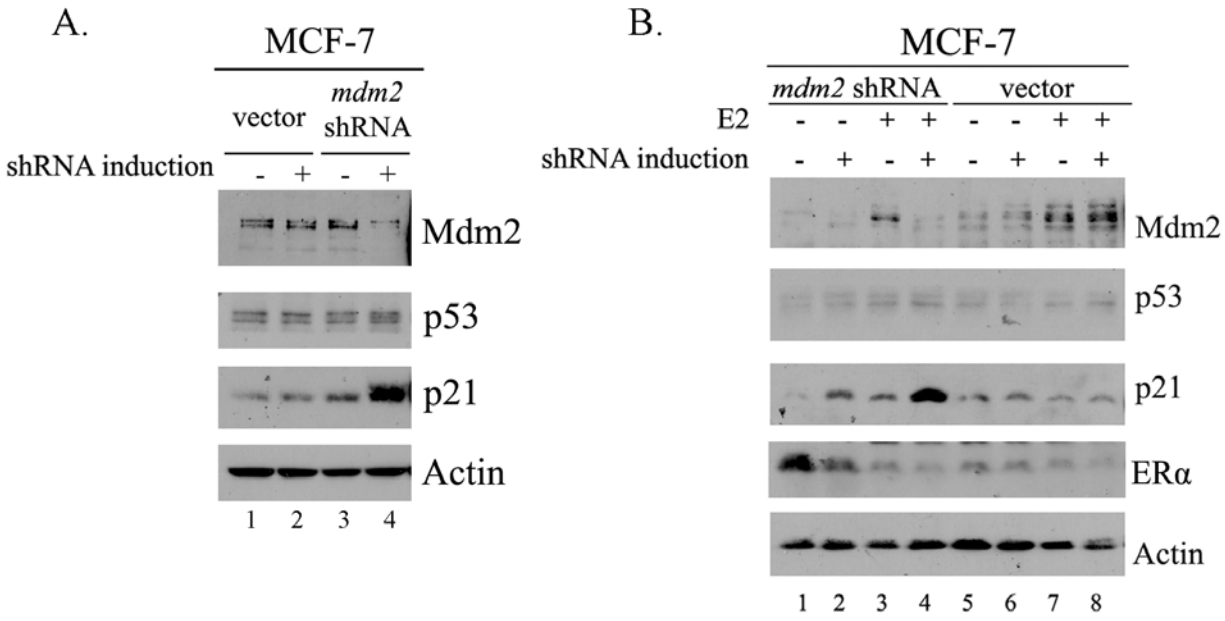

Figure 26: Assessment of the Mdm2 protein knockdown by shRNA in the absence and in the presence of an estrogen treatment in the MCF-7 cells. *Clonal MCF-7 cell lines with mdm2 shRNA (151656 clone C4) or control vector were treated with (A) 2 µg/ml doxycycline for six days or (B) 2 µg/ml doxycycline for three days followed by 10 nM estrogen (E2) for five days in the presence of doxycycline. Mdm2, p53, p21, ERα and Actin protein levels from whole cell lysates were analyzed by Western blot.*

Because we observed that the Mdm2 knockdown in the presence of estrogen robustly increased the p21 protein level, we hypothesized that after the Mdm2 knockdown the cells would lose their proliferative activity in the presence of estrogen. Therefore, we examined if the Mdm2 knockdown had any effect on the breast cancer cell proliferation in the presence of estrogen. We observed that Mdm2 down-regulation repressed cell proliferation both in the absence and in the presence of the estrogen treatment (Figure 27A, *mdm2* shRNA). While in the vector control cell line, doxycycline treatment did not significantly affect the cell proliferation in the absence or in the presence of estrogen (Figure 27A, vector). Importantly, by determining the fold change in the cell number under the different conditions, we observed that while the estrogen treatment increased the cell proliferation by 2.8 fold, when Mdm2 was knocked down the increase in the estrogen-mediated cell proliferation was only by 1.9 fold. Therefore, these results suggest that Mdm2 was required not only for the endogenous cell proliferation (in the absence of estrogen), but specifically for the estrogen-stimulated cell proliferation. In addition, since the knockdown of Mdm2 decreased the number of cells, it was important to determine if the decrease in the cell number was due to cell death or due to cell proliferation inhibition. Cell viability assay, determined by the assessment of the cell membrane integrity, showed that the cells remained viable after the Mdm2 knockdown both in the absence and in the presence of estrogen (Figure 27B). Therefore, we concluded that the decrease in the cell number after the Mdm2 knockdown was due to inhibition of cell proliferation and not due to an increase in cell death.

In conclusion, in agreement with our hypothesis these results suggest that the estrogen-mediated cell proliferation requires Mdm2 and, in turn, Mdm2 over-expression in the presence of estrogen inhibits the cell cycle regulator p21. And consecutively, this process can culminate in uncontrolled cell proliferation in the presence of estrogen.

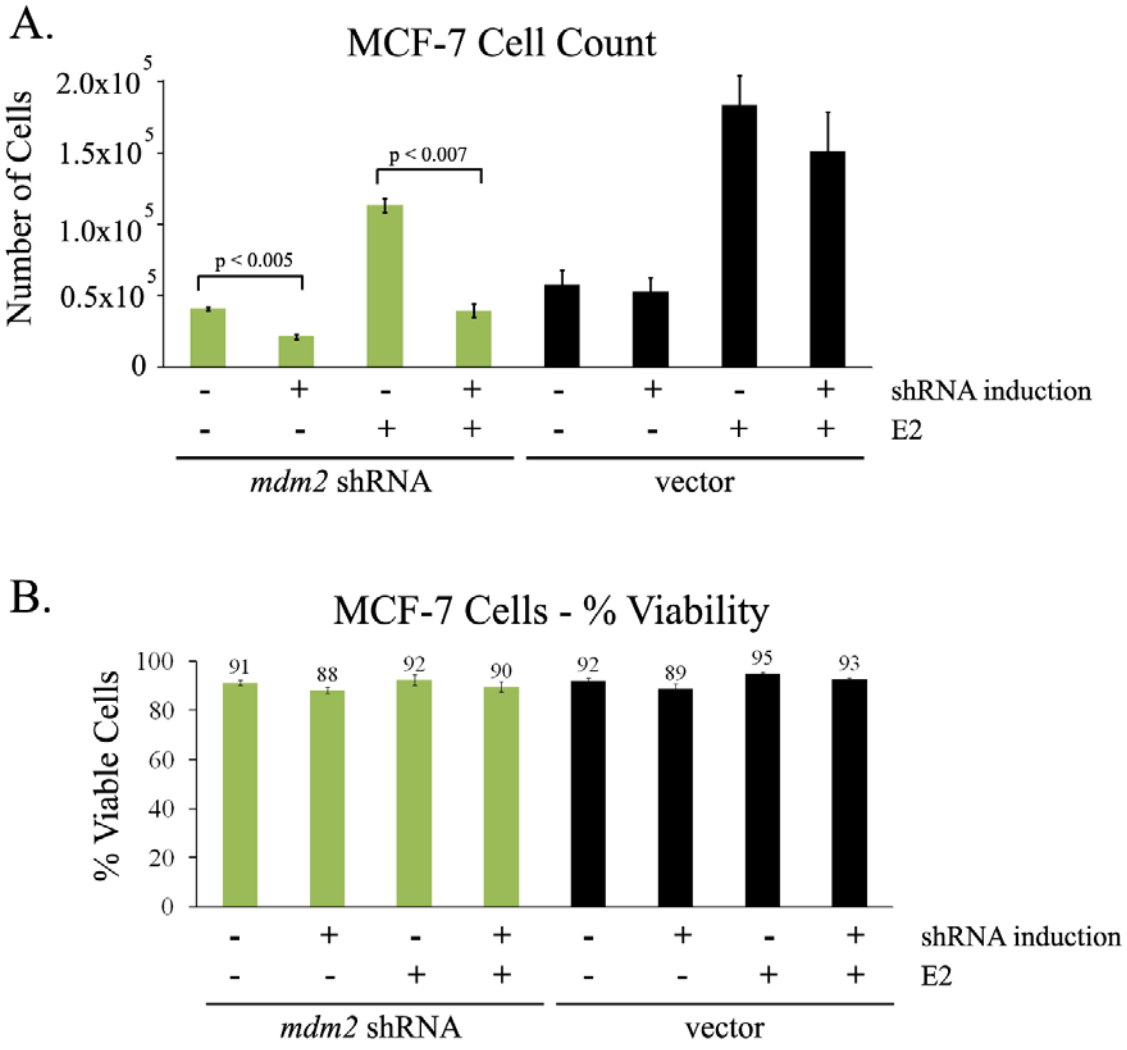

Figure 27: The estrogen-mediated MCF-7 cells proliferation was inhibited by the Mdm2 knockdown. *MCF-7 cells carrying control vector and clonal MCF-7 cell line with mdm2 shRNA (151656 clone C4) were treated with 2 µg/ml doxycycline for three days to induce shRNA expression, followed by 10 nM estrogen (E2) for five days in the presence of doxycycline. 10,000 cells were seeded at beginning of treatments. Number of cells (A) and cell viability based on membrane permeability (B) were determined by Guava Viacount assay at the end of the treatments.*

Since the MCF-7 cells did not die after the Mdm2 knockdown while the p21 protein level increased dramatically, we hypothesized that the decrease in the cell number was due to a change in the cell cycling during the estrogen-mediated cell proliferation. To examine the effect of estrogen, throughout the study estrogen treatments were carried out on cells that were grown in otherwise steroids-depleted medium. Therefore, when estrogen was not added to the medium the cells proliferated very slowly. And in fact, by FACS analysis we observed that the cell cycle distribution included a high percent of cells in the G1 phase and a low percent of cells in the S phase (data not shown). In the presence of estrogen, however, the cell proliferation rate increased dramatically and the percent of cells in the S phase increased (data not shown).

When the cell cycle profile was examined after the Mdm2 knockdown in the presence of estrogen, we saw that there was a significant increase in the percent of cells in the G1 phase and a significant decrease in the percent of cells in the S phase (Figure 28, *mdm2* shRNA). While in the vector control cell line, the percent distribution did not change after the doxycycline treatment (Figure 28, vector).

In conclusion, this result suggests that Mdm2 knockdown inhibited the estrogen-mediated MCF-7 breast cancer cells proliferation by slowing the cells down in the G1 to S transition. Importantly, this result implies that in the presence of estrogen Mdm2 over-expression inhibits the p21 protein and thus allows the proliferating cells to bypass the G1 checkpoint.

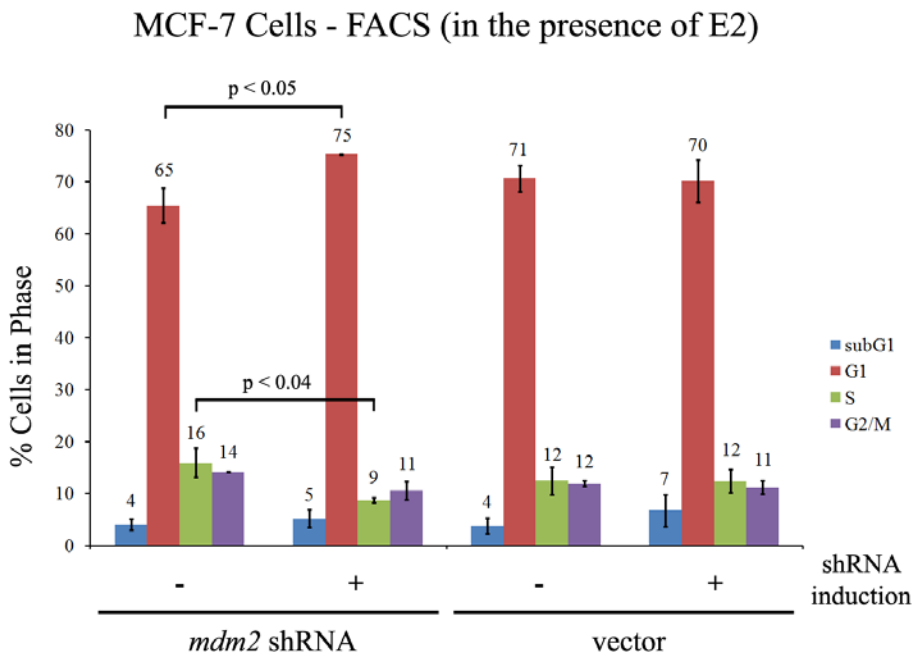

Figure 28: In MCF-7 cells, Mdm2 knockdown in the presence of estrogen inhibited the cell cycle transition at the G1 to S phases of the cell cycle. *MCF-7 cells carrying control vector and clonal MCF-7 cell line carrying the mdm2 shRNA (151656 clone C4) were treated with 2 µg/ml doxycycline for three days to induce shRNA expression, followed by 10 nM estrogen (E2) for five days in the presence of doxycycline. The percent of cells in the different cell cycle phases was determined by fluorescence activated cell sorting (FACS).*

Estrogen is known to inhibit apoptosis in the MCF-7 breast cancer cells by up-regulating the expression of the anti-apoptotic protein Bcl-2 (Perillo et al. 2000). Though we did not observe any increase in cell death after the Mdm2 knockdown in the presence of estrogen, we wanted to determine if decreasing the levels of the Mdm2 protein will affect the estrogen-mediated Bcl-2 expression. We hypothesized that Mdm2 over-expression in the presence of estrogen may be important for the estrogen-mediated signaling toward Bcl-2 over-expression.

Interestingly, we observed that following Mdm2 knockdown in the presence of estrogen, estrogen could no longer induce Bcl-2 expression to the same extent as it did in the presence of Mdm2 (Figure 29, compare lanes 1-3-4). The Bcl-2 protein level also somewhat decreased after doxycycline treatment in the vector control cell line (Figure 29, compare lanes 7 and 8), but the decrease was not as striking as it was in the *mdm2* shRNA-containing cell line (determined by relative quantification of bands intensities with the ImageJ software, data not shown). Doxycycline belongs to a well-known and widely used type of antibiotics, the tetracyclines (Smilack 1999). The bacteriostatic activity of tetracyclines lies in their capacity to inhibit protein synthesis. Because of the similarity between the prokaryotic protein synthesis machinery and that of eukaryotic mitochondria, tetracyclines are also able to interfere with the mitochondrial protein synthesis in the mammalian cells. Therefore, doxycycline, when used at high concentrations, can induce growth arrest and/or cell death (Saikali and Singh 2003). In fact, we observed that using higher concentrations of doxycycline appeared to be cytostatic to the MCF-7 cells (data not shown). And perhaps even at the lower concentrations of doxycycline, where the cell proliferation was not significantly inhibited, the expression of the mitochondrial proteins was affected. And therefore, doxycycline treatment reduced the Bcl-2 protein level in the vector control cell line.

In conclusion, this result suggests that since the estrogen-mediated up-regulation of the anti-apoptotic Bcl-2 protein expression was blocked by the Mdm2 knockdown, the over-expression of Mdm2 in the presence of estrogen is important for the estrogen-mediated induction of Bcl-2 expression and thus inhibition of apoptosis.

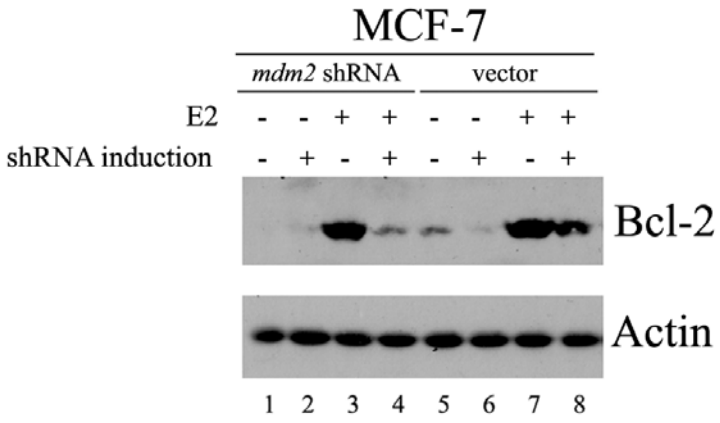

Figure 29: In MCF-7 cells, the estrogen-mediated up-regulation of the anti-apoptotic Bcl-2 protein was inhibited by the Mdm2 knockdown. *MCF-7 cells carrying control vector and clonal MCF-7 cell line with mdm2 shRNA (151656 clone C4) were treated with 2 µg/ml doxycycline for three days to induce shRNA expression, followed by 10 nM estrogen (E2) for five days in the presence of doxycycline. Western blot analysis of Bcl-2 and Actin protein levels from whole cell lysates.*

In the following experiment we compared the Mdm2 knockdown and the DNA damaging drug etoposide with respect to their effects on the MCF-7 cells proliferation in the presence of estrogen. Earlier in this study we observed that DNA damage induced by etoposide treatment inhibited MCF-7 cells proliferation by inducing cell cycle inhibition in the G2/M checkpoint. In turn, we observed that the Mdm2 knockdown in the presence of estrogen repressed MCF-7 cells proliferation by inhibiting the cell cycle transition at the G1 checkpoint. Therefore, we hypothesized that combination of Mdm2 knockdown and etoposide treatment would additively inhibit MCF-7 cells proliferation in the presence of estrogen.

By Western blot analysis of the cellular protein levels, we saw that etoposide slightly increased the Mdm2 protein level and robustly increased the p53 and the p21 protein levels (Figure 30A, compare lanes 1 and 2). *mdm2* shRNA induction decreased the Mdm2 protein level, while the level of the p53 protein did not change (Figure 30A, compare lanes 1 and 3). In agreement with earlier results, we observe that though the p53 protein expression did not increase upon Mdm2 knockdown, the p21 protein level increased (Figure 30A, compare lanes 1 and 3). Importantly, when etoposide treatment and Mdm2 knockdown were combined, the p21 protein level became even higher than in the etoposide treatment alone samples, while there was no further increase in the p53 protein level (Figure 30A, compare lanes 2 and 4). In contrast, in the vector control cell line when etoposide treatment and Mdm2 knockdown were combined, the p21 protein level was not different than when only the etoposide treatment was carried out (Figure 30A, compare lanes 6 and 8).

In agreement with our hypothesis, we observed that the estrogen-mediated MCF-7 cells proliferation was similarly inhibited by both etoposide treatment and Mdm2 knockdown (Figure 30B). Etoposide treatment caused a G2/M cell cycle arrest-like state and the Mdm2 knockdown

induced a G1 arrest-like state (Figure 30C). Furthermore, combination of the Mdm2 knockdown and etoposide treatment additively inhibited cell proliferation (Figure 30B). Interestingly, when Mdm2 was knocked down, the G2/M population in the etoposide treated cells decreased and the cells appeared to remain arrested in the G1 phase (Figure 30C).

In conclusion, we observed that Mdm2 knockdown in combination with etoposide treatment additively inhibited MCF-7 cells proliferation in the presence of estrogen. And importantly, we concluded that this inhibition in cell proliferation was mediated by the p21 protein since the additive inhibition of cell proliferation correlated with an additive increase in the p21 protein level.

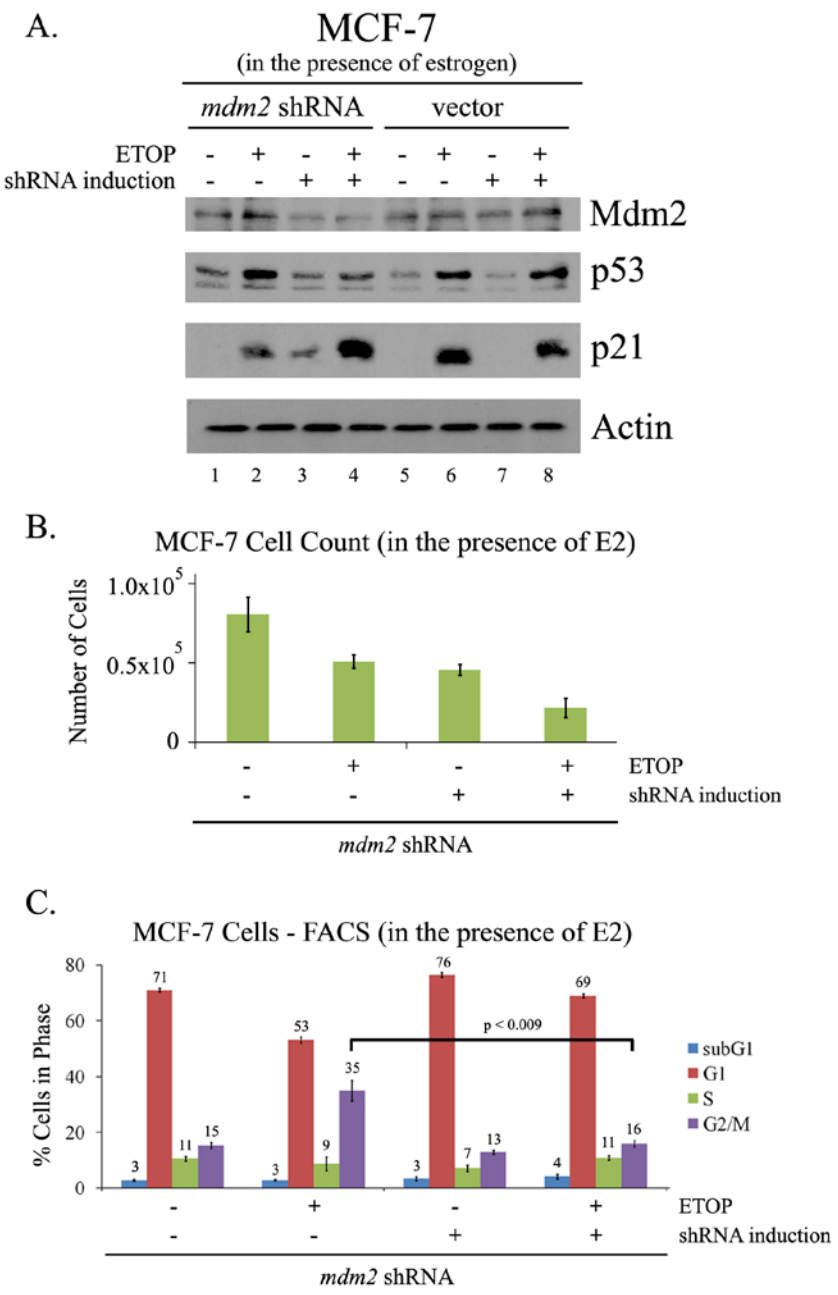

Figure 30: Mdm2 knockdown in combination with etoposide treatment additively inhibited MCF-7 cells proliferation in the presence of estrogen. *Clonal MCF-7 cells with mdm2 shRNA (151656 clone C4) or vector control were treated with 2 µg/ml doxycycline for three days, followed by 10 nM estrogen (E2) for five days and 50 µM etoposide (ETOP) for 48 hours in the presence of doxycycline. (A) Western blot analysis of Mdm2, p53, p21 and Actin protein levels from whole cell lysates. (B) 10,000 cells were seeded. The number of cells at the end of the treatment was determined by the Guava Viacount assay. (C) The percent of cells in the different cell cycle phases was determined by fluorescence activated cell sorting (FACS). (In B and C, the vector control cell line was omitted for clarity. No change was observed after Mdm2 knockdown).*

In the experiment described above, we observed that etoposide treatment and Mdm2 knockdown additively inhibited MCF-7 cells proliferation. Interestingly, combination of Mdm2 knockdown with chemotherapeutic agents or radiation therapy has been described to exhibit an additive effect on tumor growth both *in vitro* and *in vivo* (Liu T. G. et al. 2004, Wang H. et al. 2001, Zhang Z. et al. 2004c). Therefore, we wanted to determine if the additive inhibition of MCF-7 cells proliferation occurred because the etoposide-mediated inhibition of cell proliferation was improved by the Mdm2 knockdown, and thus the two mechanisms are part of the same pathway. We hypothesized, however, that since Mdm2 knockdown induced a G1 arrest-like state while etoposide treatment induced a G2/M arrest-like state, then the two events are involved in two independent pathways that regulate cell proliferation. And therefore, the etoposide-mediated inhibition of MCF-7 cells proliferation will not be augmented by the Mdm2 knockdown.

To test this hypothesis, in the following experiment first we treated the MCF-7 cells with doxycycline for six days to induce Mdm2 knockdown and then we exposed the cells to increasing concentrations of etoposide for an additional 48 hours while continuing the doxycycline treatment. Importantly, after the six days of Mdm2 knockdown the cells were counted and equal numbers of cells were seeded before the etoposide treatment. And therefore, the difference in the cell number that occurred because of the Mdm2 knockdown was not visible in this experiment. In support of our hypothesis, we observed that the etoposide-mediated inhibition of cell proliferation was not augmented by the Mdm2 knockdown (Figure 31), suggesting that cells with the Mdm2 protein down-regulated did not become more sensitive to the etoposide treatment.

Importantly, the result of this experiment suggests that Mdm2 knockdown and DNA damage induced by etoposide target two independent but parallel pathways that control cell proliferation. Furthermore, this result also suggests that Mdm2 does not play a role in the etoposide-mediated inhibition of cell proliferation and vice versa.

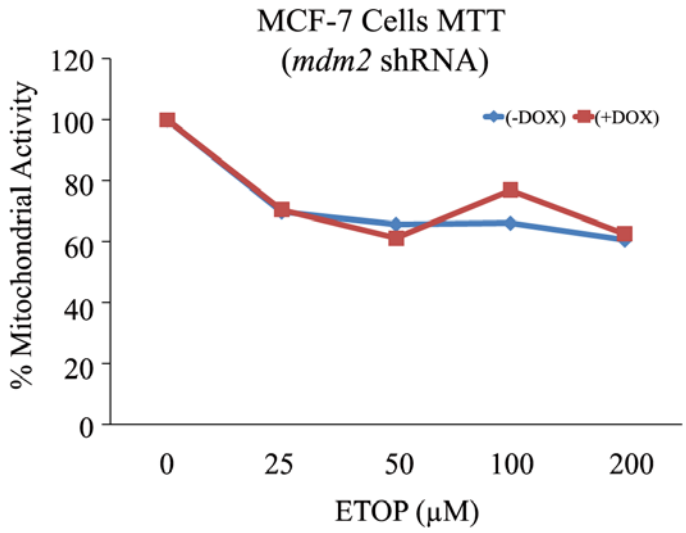

Figure 31: In MCF-7 cells, etoposide-mediated inhibition of cell proliferation was not augmented by the Mdm2 knockdown. *Clonal MCF-7 cells with mdm2 shRNA (151656 clone C4) were treated with 2 µg/ml doxycycline for six days. Then equal number of cells were seeded and treated with increasing concentrations of etoposide (25, 50, 100, 200 µM) for 48 hours in the absence or in the presence of 2 µg/ml doxycycline. Cell proliferation was determined by the MTT colorimetric assay, which measures the mitochondrial activity of the cells.*

We also investigated if the estrogen-mediated cell proliferation occurred due to inhibition of the p53 pathway by Mdm2. We hypothesized that if the p53 pathway was inhibited by estrogen treatment during the estrogen-mediated cell proliferation then blocking the p53 pathway would further augment the estrogen-mediated effect. Therefore, in the following experiment we examined how the p53 knockdown affected cell proliferation in the presence of estrogen. We reasoned that if the p53 pathway was indeed inhibited during the estrogen-mediated cell proliferation, than p53 knockdown would release this inhibition and that, in turn, will allow for even greater increase in the cell proliferation in the presence of estrogen.

Following expression of the *p53* shRNA in the MCF-7 cells, we observed a modest reduction in both p53 and p21 protein levels (Figure 32A, compare lanes 1 and 2). Interestingly, similar to our earlier observations estrogen treatment increased the level of the p53 protein while the level of the p21 protein decreased (Figure 32A, compare lanes 1 and 3). By Western blot analysis it appeared that expression of the *p53* shRNA in the presence of estrogen decreased the p53 protein level only slightly (Figure 32A, compare lanes 3 and 4), but quantification of the bands intensities by the ImageJ software showed that the p53 protein level decreased by 50% after the *p53* shRNA induction in the presence of estrogen (data not shown). In the vector control cell line we observe that there was no change in the p53 and p21 protein levels after doxycycline treatment in the absence or in the presence of estrogen (Figure 32A, compare lanes 5-8). While similar to the *p53* shRNA cell line, in the vector control cell line estrogen treatment decreased the protein level of p21 (Figure 32A, compare lanes 5-8). Interestingly, we observed that the estrogen-mediated increase in the Mdm2 protein level was not inhibited when the p53 protein was knocked down (Figure 32A, compare lanes 1-3-4). This suggests that the estrogen-mediated increase in the Mdm2 protein was not dependent on p53. In support of this observation, it has

been described earlier that estrogen can induce Mdm2 expression in the T-47D cell line, a breast cancer cell line that carries a mutant p53 (Hu et al. 2007).

With regards to the MCF-7 cells proliferation, after p53 knockdown we observed that there was a slight increase in the cell proliferation, but the increase was not significant, as the p value was greater than 0.05 (Figure 32B). Similarly, p53 knockdown in the presence of estrogen did not induce any further increase in the estrogen-mediated cell proliferation (Figure 32B). This result suggests that inhibition of the basal activity of the p53 protein did not affect the estrogen-mediated cell proliferation.

In conclusion, in this experiment we observed that estrogen treatment robustly increased MCF-7 cells proliferation, while no further increase in cell proliferation was achieved when the p53 protein was knocked down. This observation suggests that estrogen signaling promoted cell proliferation independently of p53 and that other pathways were affected during this process. Importantly, since the estrogen's impact on cell proliferation strongly depended on Mdm2, this result also suggests that a portion of the Mdm2 influence was p53-independent.

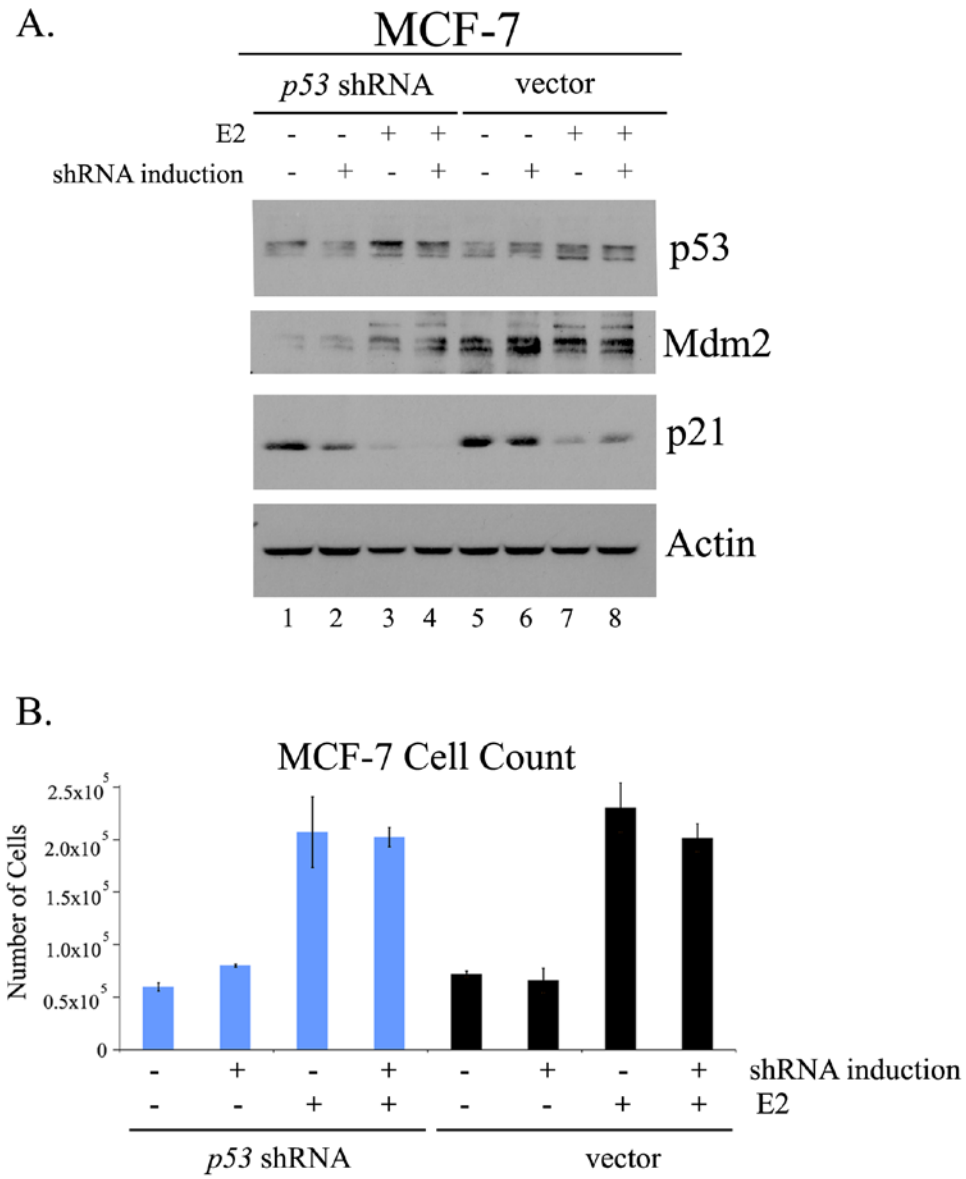

Figure 32: In MCF-7 cells, p53 knockdown did not affect the estrogen-mediated up-regulation of cell proliferation. *MCF-7 cells carrying control vector or clonal MCF-7 cell line with p53 shRNA (p53 2120 clone D11) were treated with 2 µg/ml doxycycline for three days to induce shRNA expression, followed by 10 nM estrogen (E2) for five days in the presence of doxycycline. 10,000 cells were seeded at beginning of treatments. (A) Western blot analysis of p53, Mdm2, p21 and Actin protein levels from whole cell lysates. (B) Number of cells was determined by Guava Viacount assay at the end of the treatments.*

3.2.3 Discussion

In response to estrogen, ERα induces transcription of target genes (e.g. *myc*, *cyclin-D1*, and *bcl-2*) to regulate cell growth and survival (Pearce and Jordan 2004). The mitogenic effects of estrogen are largely attributed to its ability to increase the expression of key cell cycle regulatory genes, which allow for G1 to S progression during the cell cycle (Prall et al. 1997). Importantly, we concluded that since the estrogen-mediated MCF-7 cells proliferation was inhibited by Mdm2 knockdown, the Mdm2 protein over-expression was required for the estrogen's signaling during this process. Specifically, our data show that Mdm2 knockdown led to an increase in the protein level of the cell cycle inhibitor, p21. The p21 cyclin-dependent kinase inhibitor is a cell-cycle regulator that inhibits the G1 to S phase transition. This suggests that Mdm2 inhibits p21 during cell cycle progression in the presence of estrogen and that estrogen robustly blocks this proliferative checkpoint pathway through the up-regulation of Mdm2.

To confirm this hypothesis it will be important to carefully examine the roles that Mdm2 and p21 play during the estrogen-mediated cell proliferation. To achieve that, one option would be to knockdown Mdm2 and p21 simultaneously. Since the Mdm2 knockdown alone leads to p21 protein increase, which, in turn, blocks the estrogen-mediated cell proliferation, then simultaneously knocking down p21 will allow the cells to continue to proliferate in the presence of estrogen even when the Mdm2 is knocked down.

Since p21 is a transcriptional target of p53, initially we hypothesized that Mdm2 was inhibiting the p21 protein by negatively regulating p53. In both MCF-7 and ZR75-1 breast cancer cell lines, the Mdm2 knockdown did not increase the p53 protein level, while the transcription of the p53 target gene *p21* increased. Therefore, it is important to note that down-

regulating Mdm2 in ERα^{+} breast cancer cells can functionally activate their wild-type p53 protein. These results suggest that estrogen uses an Mdm2-mediated pathway to provoke cell proliferation and that this pathway requires both p53-dependent and -independent signal transduction pathways.

To directly test if p53 is targeted during the estrogen-mediated Mdm2 over-expression and MCF-7 cells proliferation, we knocked down p53 by shRNA in the presence of estrogen. We observed, however, that while estrogen treatment robustly increased the MCF-7 cells proliferation, there was no further increase in cell proliferation when the p53 protein was knocked down. This observation suggests that the influence of Mdm2 during the estrogen-mediated cell proliferation was p53-independent. Importantly, Mdm2 has been shown to regulate p21 independently of p53. This occurs by Mdm2 binding to p21, inducing a conformational change in p21 and thus increasing p21's interaction with the C8 proteasome subunit (Jin et al. 2003, Xu et al. 2010, Zhang Z. et al. 2004b). Therefore, it is possible that estrogen regulates cell cycle progression by inducing Mdm2 over-expression, which, in turn lead to p21 inhibition, but all this occurs in a p53-independent manner.

Interestingly, we observed that the inhibition in the estrogen-mediated cell proliferation after the Mdm2 knockdown was comparable to that achieved by the DNA damaging agent etoposide. However, it appeared that the inhibition was achieved through two different pathways, where Mdm2 regulated the G1 checkpoint, while etoposide regulated the G2/M checkpoint. Furthermore, combination of etoposide treatment and Mdm2 knockdown additively inhibited cell proliferation. Taken together, these data suggest that it is important to consider Mdm2 as a target in the development of future breast cancer therapies.

In addition, we also observed that Mdm2 knockdown decreased the estrogen-mediated anti-apoptotic Bcl-2 protein expression. We also observed that the up-regulation in the Bcl-2 expression in the presence of estrogen correlated with inhibition of the pro-apoptotic Puma protein expression. Taken together, these results suggest that Mdm2 may play a role in the estrogen-mediated cell survival, which may be required during the high rate of cell proliferation that is induced by estrogen.

Summary of conclusions

1) Since the Mdm2 knockdown blocked the estrogen-mediated MCF-7 cells proliferation, the Mdm2 over-expression was required for the estrogen-mediated effect.

2) During the estrogen-induced cell proliferation Mdm2 over-expression in the presence of estrogen blocked p21 and allowed the cells to bypass the G1 checkpoint.

3) The p53 knockdown did not potentiate the estrogen-mediated MCF-7 cells proliferation, suggesting that other pathways were targeted by Mdm2 during the estrogen-mediated signaling.

4) The estrogen-mediated up-regulation of the anti-apoptotic Bcl-2 protein was blocked by the Mdm2 knockdown, suggesting that the estrogen-mediated Mdm2 over-expression may be important for the estrogen-mediated inhibition of apoptosis.

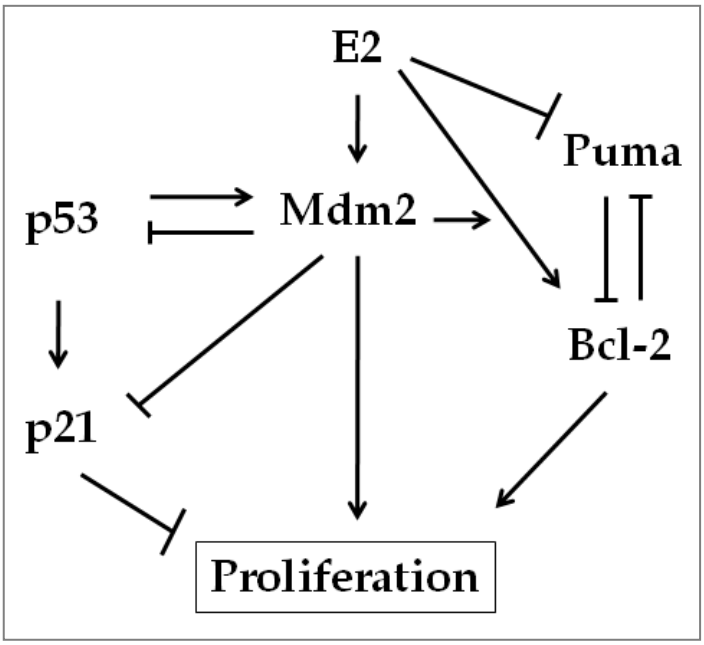

Figure 33: A model illustrating that Mdm2 plays a central role in the estrogen-mediated breast cancer cell proliferation. *The data suggest that Mdm2 inhibited p21 expression both via and independently of p53 and thus promoted the estrogen-mediated cell proliferation. In turn, this suggests that the inhibition of the p21 protein allowed the cells to bypass the G1 checkpoint during the estrogen-mediated cell proliferation. In addition, we observed that Mdm2 was required for the estrogen-mediated Bcl-2 up-regulation which, in turn, correlated with Puma down-regulation. The data suggest that Mdm2 affected several pathways during the estrogen-induced breast cancer cell proliferation, which are associated with the regulation of the G1 checkpoint and cell survival.*

3.3 Studying the role of Mdm2 in cell proliferation in 3D culture (soft agar and matrigel).

3.3.1 Introduction

Conducting experiments in 3D cultures, that closely resemble the environmental conditions found in tissues, can better recapitulate the behavior of cancer cells *in vivo*. In addition to examining the role of the Mdm2 protein in cell proliferation in the 2D cultures, we also studied the function of the Mdm2 protein in breast cancer cell proliferation in soft agar (3D cultures). Anchorage-independent colony formation of cultured cells in soft agar is often associated with *in vivo* malignancies and has been used as a marker for cellular transformation (Pavelic et al. 1980, Wada et al. 1984). MCF-7 cells have been shown to depend on estrogen for colony formation in soft agar (Stevens and Meech 2006). Furthermore, over-expression of Mdm2 in MCF-7 cells affords a growth advantage in soft agar in the presence of estrogen (Saji et al. 1999). Therefore, in the following experiments, we examined the effect of Mdm2 knockdown on anchorage-independent MCF-7 cell growth in soft agar in the presence of estrogen.

In addition to studying the role of Mdm2 in the anchorage-independent cell growth, we also examined the role of Mdm2 in cell growth in matrigel (3D cultures). The female breast is composed of several major duct systems, each comprising of numerous lobules. A lobule consists of a terminal branch-like system of lobular cells, which empty into the ducts. A cross section of the duct reveals that it has a hollow lumen, which is lined by an inner layer of epithelial cells. In ductal carcinoma *in situ* (DCIS), this inner layer of epithelial cells continues to proliferate and eventually fills in the lumen (Wiechmann and Kuerer 2008). The matrigel consists of a gelatinous protein mixture that resembles the complex extracellular environment found in many tissues. The growth of normal mammary epithelial cells in matrigel results in formation of polarized, growth-arrested acini-like spheroid structures that recapitulate several

aspects of glandular architecture *in vivo*. Mammary tumor cells, however, continue to proliferate into disorganized masses (Cordon-Cardo et al. 1994). Since MCF10A cells are considered to be normal immortalized mammary epithelial cells, we compared MCF-7 breast cancer cells to the MCF10A cells. Once we confirmed the different morphologies that MCF10A and MCF-7 cell lines form in matrigel, we began to determine if the Mdm2 knockdown affected the MCF-7 cells growth in matrigel.

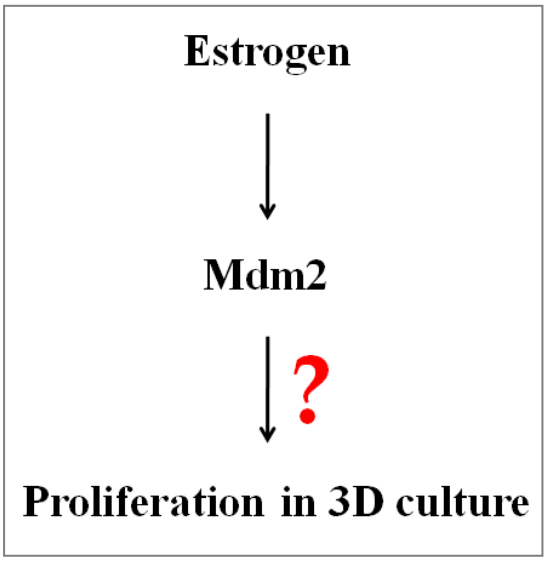

Figure 34: Examining whether the Mdm2 protein controls the estrogen-mediated cell proliferation in 3D culture. *In addition to examining the role of the Mdm2 protein in the 2D cultures, we also studied the function of the Mdm2 protein in the estrogen-mediated breast cancer cell proliferation in soft agar (anchorage-independent growth) and in matrigel (mass formation).*

3.3.2 Results

To study the role of the Mdm2 protein in the MCF-7 cells grown in soft agar in the presence of estrogen, in the preliminary experiment that is described below, we examine how the MCF-7 cells behaved in soft agar in the absence and in the presence of estrogen. We observed that MCF-7 cells, when grown for two weeks in growth medium containing complete FBS, formed large colonies in soft agar (Figure 35A, complete FBS). Interestingly, the MCF-7 cells did not form colonies in medium that was depleted of steroids (Figure 35A, charcoal-stripped FBS). In fact, higher magnification revealed that the cells did not grow in soft agar in the absence of steroids (Figure 35B, (-E2)). In the presence of estrogen, the MCF-7 cells proliferated and formed large and multicellular colonies in soft agar, though the colonies were not as big as the ones formed in growth medium with complete FBS (Figure 35A, compare complete FBS and charcoal-stripped FBS (+E2)).

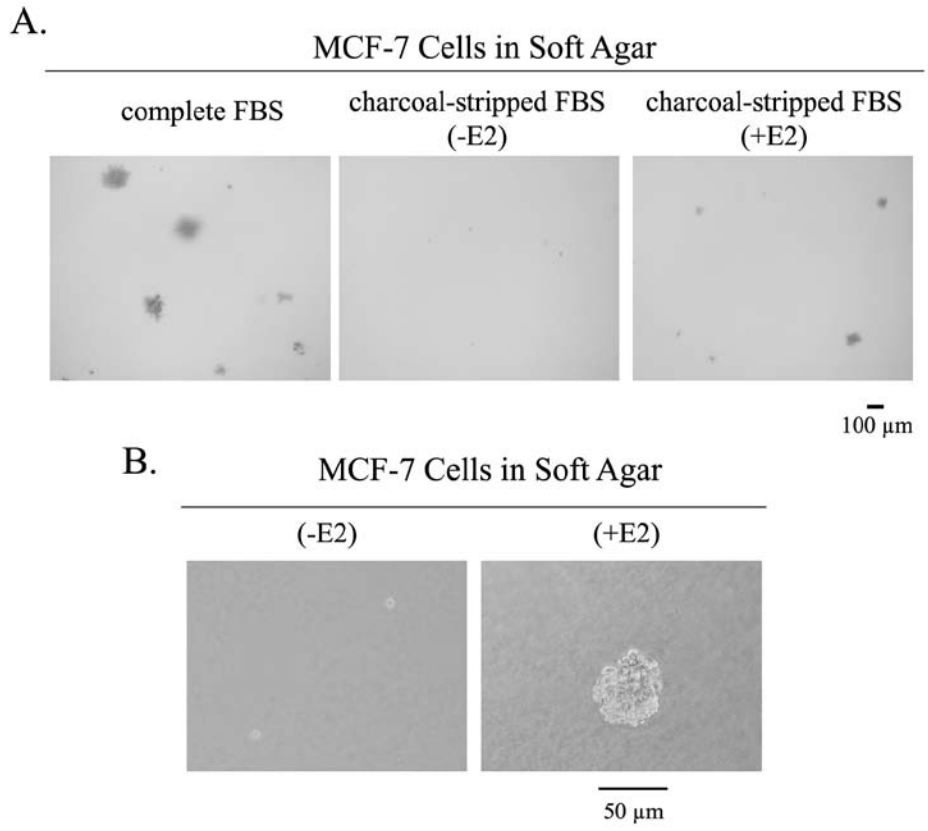

Figure 35: Estrogen was required for the MCF-7 colony formation in soft agar. *MCF-7 cells were grown for two weeks in soft agar. The agar was mixed and covered with different growth media: growth medium with complete FBS, growth medium with charcoal-stripped FBS and growth medium with charcoal-stripped FBS and 10 nM estrogen. Representative images of colonies formed by the MCF-7 cells in soft agar under the different growth media conditions are shown. 40x magnification (A) and 100x magnification (B).*

In the following experiment, we wanted to determine if the Mdm2 protein was required for the estrogen-mediated colony formation by the MCF-7 cells in soft agar. We hypothesized that since the Mdm2 protein was required for the estrogen-mediated cell proliferation in the 2D culture, then, similarly, the Mdm2 protein will be required for the cell growth in the 3D culture. To carry out this experiment, initially, we grew the cells in the 2D culture in the presence of complete growth medium and doxycycline to knockdown the expression of Mdm2. After six days of doxycycline treatment, we seeded the cells in soft agar, which contained growth medium supplemented with charcoal-stripped FBS, estrogen and doxycycline (for continuous Mdm2 knockdown). After growing the cells in soft agar for two weeks, we counted the large colonies that formed in the absence and in the presence of the Mdm2 knockdown. Importantly, in order to determine the effect of the *mdm2* shRNA expression on the MCF-7 cells growth in soft agar, in the Mdm2 knockdown samples only the GFP expressing colonies were counted.

We observed that upon the Mdm2 knockdown, the number of the large and multicellular colonies decreased dramatically (Figure 36A and Figure 36B, *mdm2* shRNA). Whereas, in the vector control cell line, doxycycline treatment had no effect on the colony formation by the MCF-7 cells in soft agar in the presence of estrogen (Figure 36A and Figure 36B, vector). Evidently, in the previous experiment, we observed that in the presence of estrogen the MCF-7 cells form colonies of an average size of 50 μm (Figure 35B). Importantly, the Mdm2 knockdown dramatically decreased the number of colonies of 50 μm in size or larger that formed in the presence of estrogen in soft agar (Figure 36B). This experiment shows that Mdm2 knockdown inhibited large colony formation of MCF-7 cells in soft agar in the presence of estrogen, and that Mdm2 is required for the estrogen-mediated anchorage-independent MCF-7 cell growth.

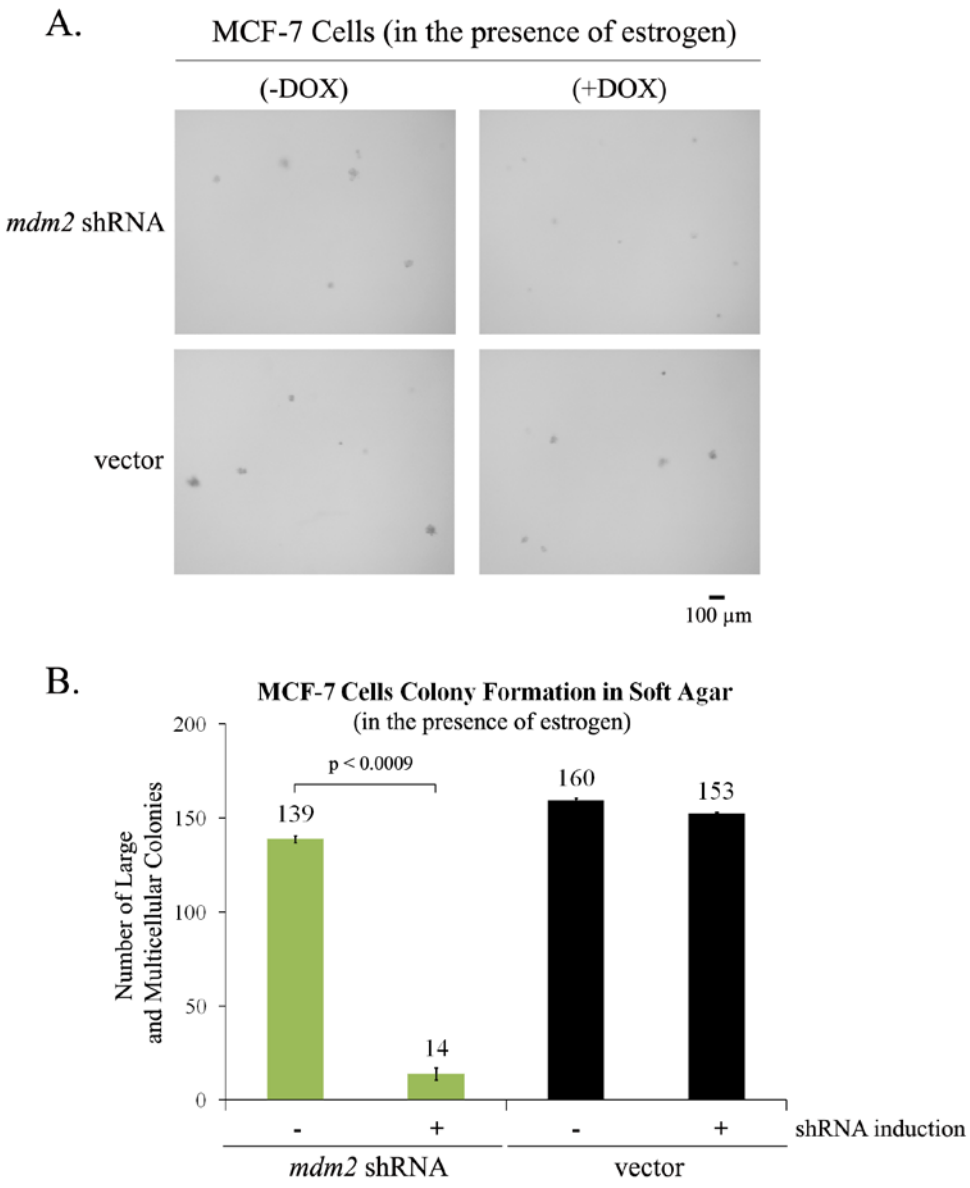

Figure 36: Mdm2 knockdown inhibited large (multicellular) colony formation when MCF-7 cells were grown in soft agar in the presence of estrogen. *MCF-7 cells were grown on plates (2D) for six days in the presence of 2 µg/ml doxycycline in growth medium with complete FBS. The cells were then washed, trypsinized and mixed with agar in growth medium containing charcoal-stripped FBS, 10 nM estrogen and 2 µg/ml doxycycline. Cells were grown in the agar for two weeks. (A) Representative images of colonies that MCF-7 cells formed in soft agar in the absence and in the presence of mdm2 shRNA induction (40x magnification). (B) The number of colonies (50 µm in size or larger) was determined by counting the colonies on the dissecting fluorescent microscope. In the mdm2 shRNA samples only the GFP expressing colonies were counted. Averages of two independent experiments are shown. Each experiment was done in three replicates. The p-value was determined by the Student t-test.*

To study the role of the Mdm2 protein in the MCF-7 cells grown in matrigel, in the preliminary experiment that is described below, we examine how the MCF-7 cells behaved in the 3D matrigel culture. In line with the published work from the Bissell laboratory (Kenny et al. 2007, Lu et al. 2002), we observed that the MCF-7 breast cancer cells form disorganized masses of different sizes in the matrigel (Figures 37A and 37B, MCF-7). Whereas the MCF10A cells, which are considered to be normal breast epithelial cells, formed organized and defined acini (Figures 37A and 37B, MCF10A). The intermediate masses that were formed by the MCF-7 cells somewhat resembled the MCF10A acini structures (Figures 37A, MCF-7 and MCF10A). However, a cross section of the intermediate MCF-7 mass structure showed that upon Mdm2 knockdown the lumen remain filled with cells (Figure 37C, PI staining).

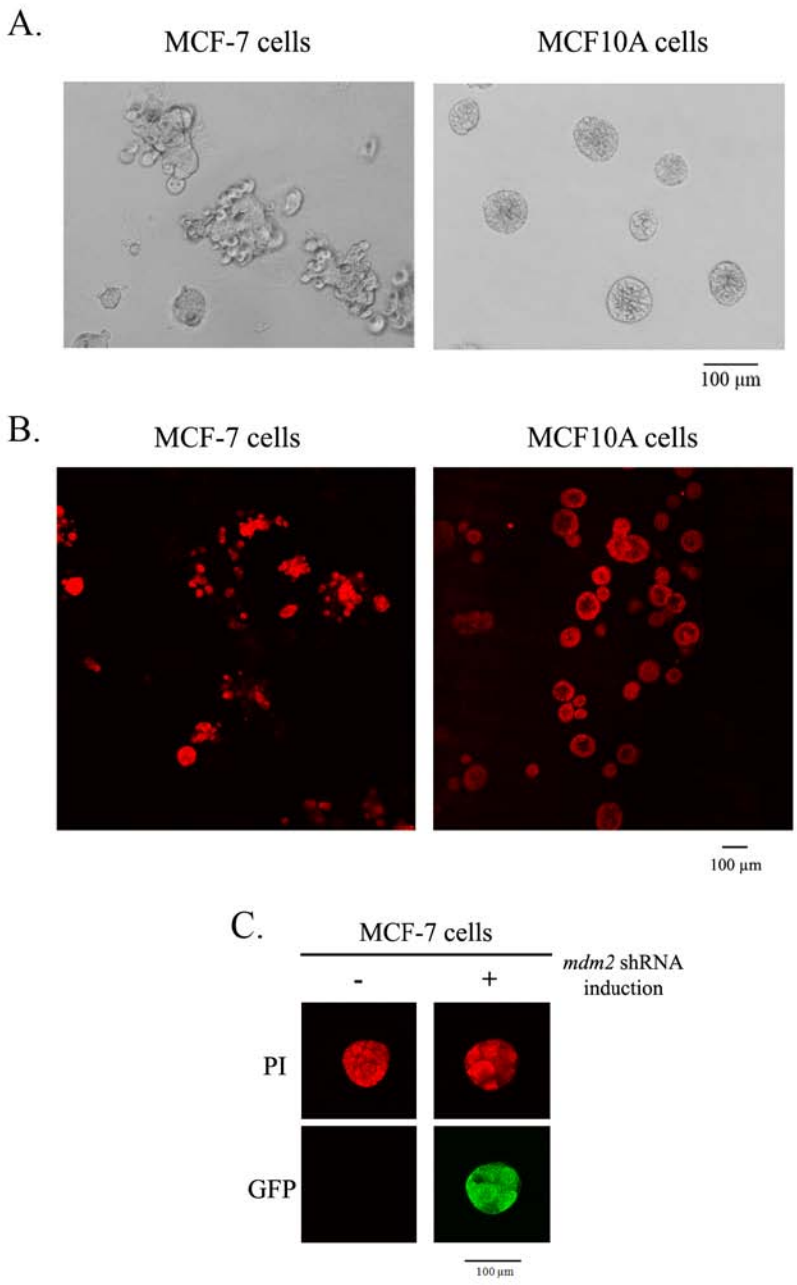

Figure 37: Assessment of the MCF-7 and MCF10A cells growth and morphology in matrigel. *MCF-7 and MCF10A cells were grown in matrigel for three weeks, followed by fixation in formaldehyde and propidium iodide staining. (A) MCF-7 and MCF10A breast cells morphologies in matrigel (3D culture). (B) Confocal analysis of MCF-7 and MCF10A cells morphologies. Propidium iodide staining is shown. (C) Confocal analysis of MCF-7 cells grown in matrigel for three weeks in the absence or presence of 2 µg/ml doxycycline. Propidium iodide staining and GFP expression are shown. Representative images of cells from two independent experiments are shown.*

In the following experiment, we wanted to determine what role the Mdm2 protein plays during the MCF-7 cells growth in matrigel. We hypothesized that since the Mdm2 protein was required for the estrogen-mediated cell proliferation in the 2D culture, then, similarly, the Mdm2 protein will be required for the MCF-7 cells growth in the matrigel. In the experiment described below, the cells were grown in the presence of estrogen in otherwise steroid-depleted growth medium. We observed that the MCF-7 cells grown in matrigel formed masses of three different sizes: large, intermediate and small (Figure 38A). Only a small percent of the structures (about 13%) had an intermediate mass size, which somewhat resembled the MCF10A acini structures (Figure 38A and 38B, mdm2 and vector control, no shRNA induction). While about 44-46% of the structures were either large or small (Figure 38B, mdm2 and vector control, no shRNA induction). Importantly, the Mdm2 knockdown decreased the number of the large structures and increased the number of the small structures that MCF-7 cells formed in the matrigel (Figure 38B, mdm2). While in the vector control cell line doxycycline treatment did not affect the distribution of the different mass morphologies (Figure 38B, vector).

In conclusion, this experiment showed that the Mdm2 protein was required for the large mass formation by the MCF-7 cells in matrigel, suggesting that Mdm2 over-expression may play a role in disorganized and invasive-like phenotypes of breast cancer cells.

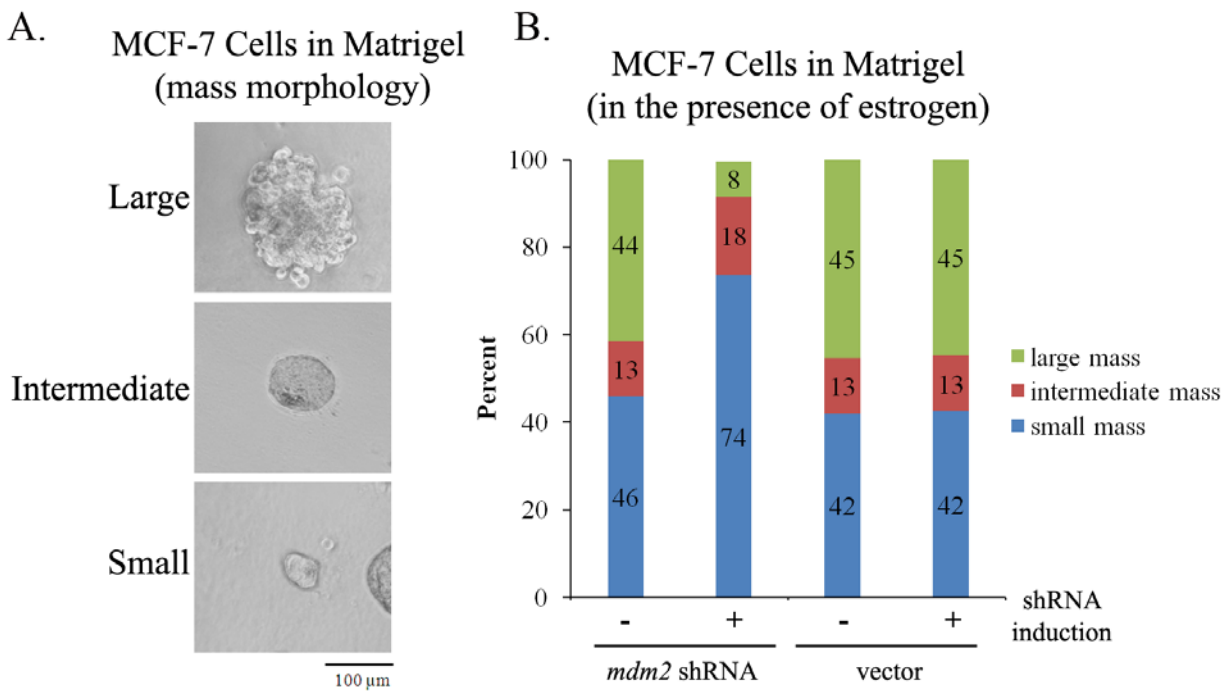

Figure 38: Mdm2 knockdown decreased the ability of the MCF-7 cells to form large masses in the matrigel in the presence of estrogen. *(A) MCF-7 cells, grown in matrigel for three weeks, formed mass structures of three different sizes: large, intermediate and small. (B) MCF-7 cells, grown in matrigel for three weeks in the presence of estrogen and in the absence or presence of 2 µg/ml doxycycline, were fixed and stained with propidium iodide. Masses of different sizes (large, intermediate and small) were counted and presented as percent of the total population. A total of about 300 structures were counted. In the mdm2 shRNA samples only the GFP expressing masses were counted. Averages of three independent experiments are shown. The significance in the percent change of large and small structures was determined by the student t-test ($p < 0.05$).*

3.3.3 Discussion

In the 2D culture, we have observed that Mdm2 protein levels become elevated in the presence of estrogen and that Mdm2 is required for the estrogen-mediated MCF-7 breast cancer cells proliferation. In soft agar (3D culture), our experimental observations confirmed that the MCF-7 cells acquired anchorage-independent growth advantage when grown in the presence of estrogen (Stevens and Meech 2006) and that this growth was dependent on Mdm2 (Saji et al. 1999).

When grown in matrigel (3D culture), the MCF-7 cells do not form acini, but exhibit a mass-like morphology (Kenny et al. 2007). This phenotype recapitulates the ductal carcinoma *in situ* (DCIS) breast cancer type that is characteristic of a filled in lumen appearance. Interestingly, we observed that the cell masses appeared to be of three different sizes: small, intermediate and large. The large masses slightly resembled the grape-like morphology, which is characteristic of the invasive and aggressive breast cancer cells (Kenny et al. 2007). This suggests that the MCF-7 cells exhibit a somewhat intermediate aggressive breast cancer cell phenotype in matrigel. The intermediate size masses were similar in size and shape to the acini that the normal mammary epithelial MCF10A cells form in matrigel. But the masses formed by the MCF-7 cells had a filled lumen as opposed to the MCF10A acini that had a hollow lumen. When we knocked down Mdm2 in the MCF-7 cells grown in matrigel, we observed that the large mass-like structures were replaced with smaller structures, suggesting that Mdm2 is important for the MCF-7 cells proliferation in matrigel. Furthermore, this observation also suggests that Mdm2 may be important for the invasive behavior of breast cancer cells. Therefore, additional studies on the role of the Mdm2 protein in the aggressive metastatic cells in the presence of estrogen are needed.

Summary of conclusions

1) Mdm2 is required for large colony formation in soft agar in the presence of estrogen, suggesting that Mdm2 is important for the estrogen-mediated anchorage-independent cell growth.

2) Mdm2 is required for the large mass formation in matrigel, suggesting that Mdm2 over-expression promotes disorganized and invasive-like phenotypes in breast cancer cells.

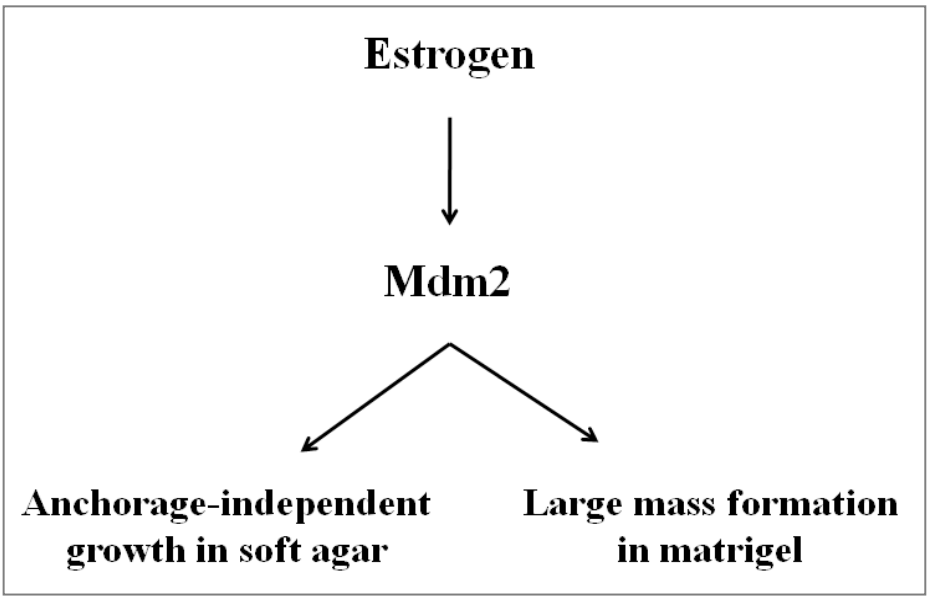

Figure 39: Mdm2 plays an important role in the estrogen-mediated MCF-7 cells proliferation in 3D culture. *In addition to playing a pivotal role during the estrogen-mediated cell proliferation in 2D culture, the Mdm2 protein is required for large colony formation in soft agar and large mass formation in matrigel.*

CHAPTER 4:

FUTURE DIRECTIONS

AND

PRELIMINARY DATA

4.1 Introduction

Our major conclusion from this study is that the estrogen-mediated MCF-7 breast cancer cell proliferation requires Mdm2. This was illustrated in a few key experiments. We observed that the increase in cell proliferation in the presence of estrogen correlated with an increase in the Mdm2 protein level. In turn, Mdm2 knockdown inhibited cell proliferation in the presence of estrogen. Importantly, this inhibition in cell proliferation was associated with inhibited transition through the G1 checkpoint.

To clarify our findings, this project could be expanded by addressing the following questions:

1. Determine if a p53-independent role of Mdm2 is required for the stimulation of the estrogen-mediated breast cancer cell proliferation.
2. Determine if MdmX plays a role in the estrogen-mediated breast cancer cell proliferation.
3. Determine the roles of Mdm2 and MdmX in drug-sensitivity of breast cancer cells in the presence of estrogen.
4. Determine the downstream pathway(s) targeted by Mdm2 in the presence of estrogen.

4.2 Determine if a p53-independent role of Mdm2 is required for the stimulation of the estrogen-mediated breast cancer cell proliferation.

Mdm2 is a major known inhibitor of the p53 tumor suppressor. Mdm2 over-expression often occurs in human tumors that retain a wild-type *p53* genotype (Landers et al. 1994, Momand et al. 1998). The Mdm2 protein has been shown to be over-expressed in the ERα$^+$ breast cancers (Bueso-Ramos et al. 1996, Hori et al. 2002, Marchetti et al. 1995) and to be associated with worse survival of patients (Turbin et al. 2006). In turn, though the *p53* gene is the most commonly mutated gene in human cancers (Vogelstein et al. 2000), mutations in the *p53* gene occur rarely in breast cancers (20-30%) (Caleffi M 1994, Coles et al. 1992, Hartmann et al. 1997, Pharoah et al. 1999). Considering these correlations, we hypothesized at the beginning of this study that in the ERα$^+$ breast cancers, the p53 activity may be suppressed through the estrogen-mediated over-expression of Mdm2.

Therefore, a great majority of our initial experiments focused on examining the effects of estrogen-mediated Mdm2 over-expression on the p53 pathway. We observed that estrogen robustly increased the Mdm2 protein levels in the MCF-7 breast cancer cells. Although estrogen treatment inhibited the transcription of several p53 target genes, the p53 protein level, nuclear localization and p53 interaction with the chromatin were not affected by estrogen. Interestingly, other groups have shown that ERα inhibits the p53 transcriptional activity by directly interacting with the p53 protein on the chromatin (Liu G. et al. 2000, Sayeed et al. 2007). And in fact, a sequential ChIP assay has demonstrated that ERα represses p53-mediated transcriptional activation in human breast cancer cells by recruiting nuclear receptor co-repressors and histone deacetylases (Konduri et al. 2010). These studies, however, did not address the role of Mdm2 in this process.

Overall, our finding and other published studies suggest that estrogen may be using an Mdm2-mediated pathway to provoke cell proliferation and this pathway may require the inhibition of both p53-dependent and p53-independent signal transduction pathways. In the future, we will carry out experiments to determine if a p53-independent role of Mdm2 is required for the stimulation of estrogen-mediated breast cancer cell proliferation.

Importantly, we observed that the estrogen-mediated MCF-7 cell proliferation was inhibited after Mdm2 knockdown, but was not affected by the p53 knockdown. This result suggests that the inhibition of the p53 protein basal activity does not affect the estrogen-mediated cell proliferation. Mdm2 knockdown by antisense oligonucleotides has been shown to inhibit cell proliferation, increase p21 protein, and increase sensitivity to chemotherapeutic agents and radiation in both MCF-7 (*p53* wild-type) and MDA-MB-468 (*p53* mutant, R273H) breast cancer cells and xenografts (Wang H. et al. 2001, Zhang Z. et al. 2004c). This supports our hypothesis that Mdm2 may promote cell proliferation and survival in a p53-independent manner. To determine if Mdm2 over-expression stimulates cell proliferation in the presence of estrogen in a p53-indepented manner, it is important to examine the role of Mdm2 in the presence of estrogen in a breast cancer cell line with a mutant *p53*, for example, the T-47D cell line. This cell line is $ER\alpha^{+}$, shows over-expression of Mdm2 in the presence of estrogen and has a mutant p53 (L194F). Our laboratory has begun to work on generating clonal T-47D cell lines with inducible shRNA expression toward *mdm2*. Future experiments will focus on characterizing the cell lines and determining if the effect of Mdm2 knockdown on estrogen-mediated cell proliferation in this cell line would be similar to the one we observed in the MCF-7 cell line. In addition, to test if the estrogen-mediated signaling via Mdm2 is p53-independent, it is important to examine if there is a difference in estrogen-mediated cell proliferation in cells with up-regulated function of p53. By

over-expressing a functional p53 in MCF-7 cells in the presence of estrogen it will be possible to examine if the up-regulation in p53 can attenuate the estrogen-mediated cell proliferation.

In addition, since the knockdown of Mdm2 shows that Mdm2 is required for estrogen-mediated cell proliferation, but it is not clear if Mdm2 is targeting p53 in this process, both p53 and Mdm2 should be targeted simultaneously. To achieve that, cell lines that carry inducible shRNAs for both p53 and Mdm2 should be generated. To make sure there is an efficient expression of both shRNAs in the same cells, the *p53* and *mdm2* shRNA-containing constructs should be cloned into expression vectors with different selection and expression markers. If indeed the inhibition in cell proliferation after Mdm2 knockdown is not due to activation of p53, then cell proliferation will still be inhibited after simultaneous knockdown of Mdm2 and p53. However, if we find out that the estrogen-mediated cell proliferation does not depended on p53, it is also possible that the p53 family members (p63 and p73) are involved in this process. Therefore, it is also important to determine if estrogen has any inhibitory functions with respect to p63 or p73 proteins.

4.3 Determine if MdmX plays a role in the estrogen-mediated breast cancer cell proliferation.

Gene amplification and over-expression of MdmX, an Mdm2 homolog, have been implicated in tumor development (Wade et al. 2010). Both Mdm2 and MdmX can bind the p53 protein and thus inhibit its activity (Toledo and Wahl 2007). Like Mdm2, the MdmX protein directly inhibits p53 due to its ability to bind to the transactivation domain of p53 and, thus, block the p53 transcriptional activity. But in contrast to Mdm2, MdmX is not under the transcriptional control of p53. Although MdmX contains a RING finger domain, it does not possess an E3 ubiquitin ligase activity. Mdm2 and MdmX form heterodimers through the conserved C-terminal RING finger domains of the proteins. While both proteins can form homodimers, heterodimers are preferentially formed. The heterodimer formation results in reduced auto-ubiquitination of Mdm2 and increased p53 ubiquitination and degradation (Kostic et al. 2006, Linke et al. 2008). Phosphorylation of MdmX plays a key role in its regulation and promotes MdmX degradation in response to DNA damage, which, in turn, leads to p53 stabilization and activation (Okamoto et al. 2005).

We have observed that the Mdm2 protein is required for the estrogen-mediated stimulation of breast cancer cells proliferation. Based on the literature, Mdm2 appears to function in synergy with MdmX (Wade and Wahl 2009). Therefore, it is important to determine if the MdmX protein plays a role in the estrogen-mediated breast cancer cells proliferation, and whether the Mdm2 and the MdmX proteins function together during this process. Evidently, the MCF-7 cells over-express MdmX due to genomic amplification (Danovi et al. 2004). Elevated MdmX expression has been shown to cooperate with the oncogenic Ras to transform cells (Danovi et al. 2004). Therefore, the MCF-7 cell line is a good candidate to examine the changes

in the estrogen-mediated cell proliferation after *mdmx* knockdown in the presence of estrogen. Notably, in the future experiments it will be important to determine how MdmX knockdown affects cell proliferation and what happens to the MdmX protein level when the Mdm2 protein is knocked down and vice versa.

4.3.1 Preliminary data

In our preliminary experiments, we have generated several MCF-7 cell lines (pools) which contain doxycycline-inducible shRNA for *mdmx*. An example of MdmX knockdown characterization in a clonal MCF-7 cell line with *mdmx* shRNA is shown below (Figure 40). We observed that doxycycline addition for six days, in order to induce *mdmx* shRNA expression, decreased the MdmX protein level (Figure 40, compare lanes 3 and 4), while in the vector control cell line there was no change in the MdmX protein level (Figure 40, lanes 1 and 2). This cell line will be used in future experiments to determine if MdmX plays a role in estrogen-mediated breast cancer cell proliferation.

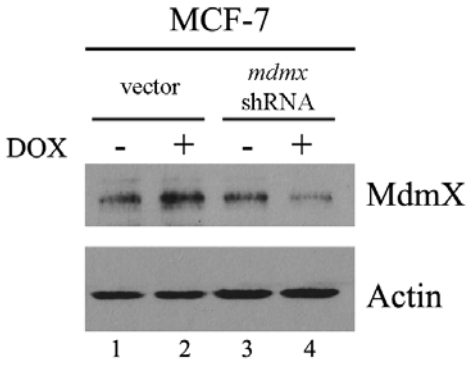

Figure 40: Characterization of the MCF-7 clonal cell line carrying the shRNA for mdmx. *MCF-7 cells with control vector or clonal MCF-7 cell line with mdmx shRNA (13023 clone D5) were treated with 2 µg/ml doxycycline for six days. Western blot analysis of MdmX and Actin protein levels from whole cell lysates.*

4.4 Determine the roles of Mdm2 and MdmX in drug-sensitivity of breast cancer cells in the presence of estrogen.

Here, we propose that the roles of Mdm2 and MdmX in drug-sensitivity of breast cancer cells in the presence of estrogen should be examined. Earlier, we observed that Mdm2 knockdown and etoposide treatment additively inhibited the proliferation of the MCF-7 cells. Several studies have shown that the Mdm2 knockdown can sensitize the breast cancer cells to chemotherapeutic agents and radiation treatments (Bianco et al. 2005, Liu T. G. et al. 2004, Wang H. et al. 2001, Zhang Z. et al. 2004c). Similarly, endogenous MdmX levels have been shown to affect the sensitivity of breast cancer cells to anti-cancer agents (Lam et al. 2010). Therefore, we will explore the strategy of targeting Mdm2 and MdmX by knockdown to increase the drug response of breast cancer cells in the presence of estrogen.

4.4.1 Preliminary data

In our preliminary experiments, we began to examine the effects of the different drugs on the MCF-7 breast cancer cells. We used drugs that differentially affect the MCF-7 cells: etoposide, Mitomycin C (MC), and 10-decarbamoyl Mitomycin C (DMC). While etoposide is known to induce DNA damage in a form of double strand breaks (van Maanen et al. 1988), MC and DMC treatments result in various DNA adducts, intra- and inter-DNA cross-links (Paz et al. 2008). We observed that all three drugs inhibited the mitochondrial activity of the cells (Figure 41A, MTT assay). However, inhibition of mitochondrial activity can be interpreted as either inhibition of cell proliferation or induction of cell death. The viability assay, which determines cell membrane integrity, showed that the cell membrane integrity was disrupted after MC treatment, but was not affected by etoposide or DMC treatments (Figure 41B).

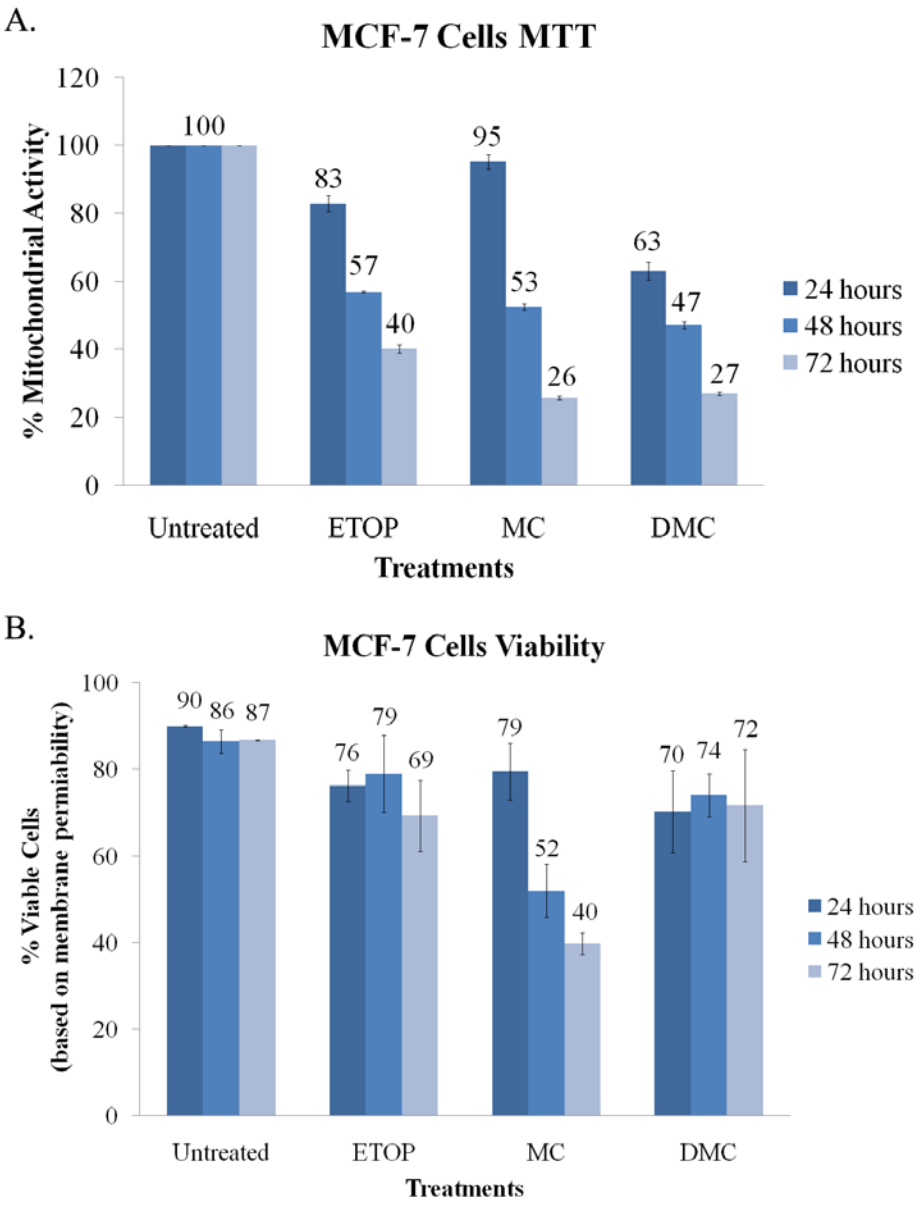

Figure 41: MCF-7 cells were differentially affected by etoposide, MC and DMC treatments. *MCF-7 cells were treated with 50 μM etoposide (ETOP), 10 μM Mitomycin C (MC), and 10 μM 10-decarbamoyl Mitomycin C (DMC) for 24, 48 and 72 hours. Graphs show averages and standard errors of two independent experiments. (A) Mitochondrial activity (which can represent both cell proliferation and cell death) was measured by the MTT assay. (B) Cell viability was determined by the Guava Viacount assay (measures membrane integrity).*

The cell cycle distribution analysis determined by the FACS assay showed that etoposide induced a G2/M cell cycle arrest (Figure 42, compare Untreated and ETOP). MC treatment increased the percent of cells in the sub-G1 phase with a concomitant decrease in the percent of cells in the G1 phase (Figure 42, compare Untreated and MC). This indicates that the MC drug induced DNA fragmentation and that the cells were undergoing apoptosis. This is supported by the observation that there were fewer cells with mitochondrial activity, as measure by the MTT assay (Figure 42A), and that cells lost their membrane integrity (Figure 42B). Interestingly, DMC treatment showed no change in cell cycle profile (Figure 42, compare Untreated and DMC), though it induced the greatest level of cell death (Figure 42A and data not shown). Recently, we have shown that the DMC drug kills cells through a p53-independent cell death pathway which involves Chk1 down-regulation (Boamah et al. 2010).

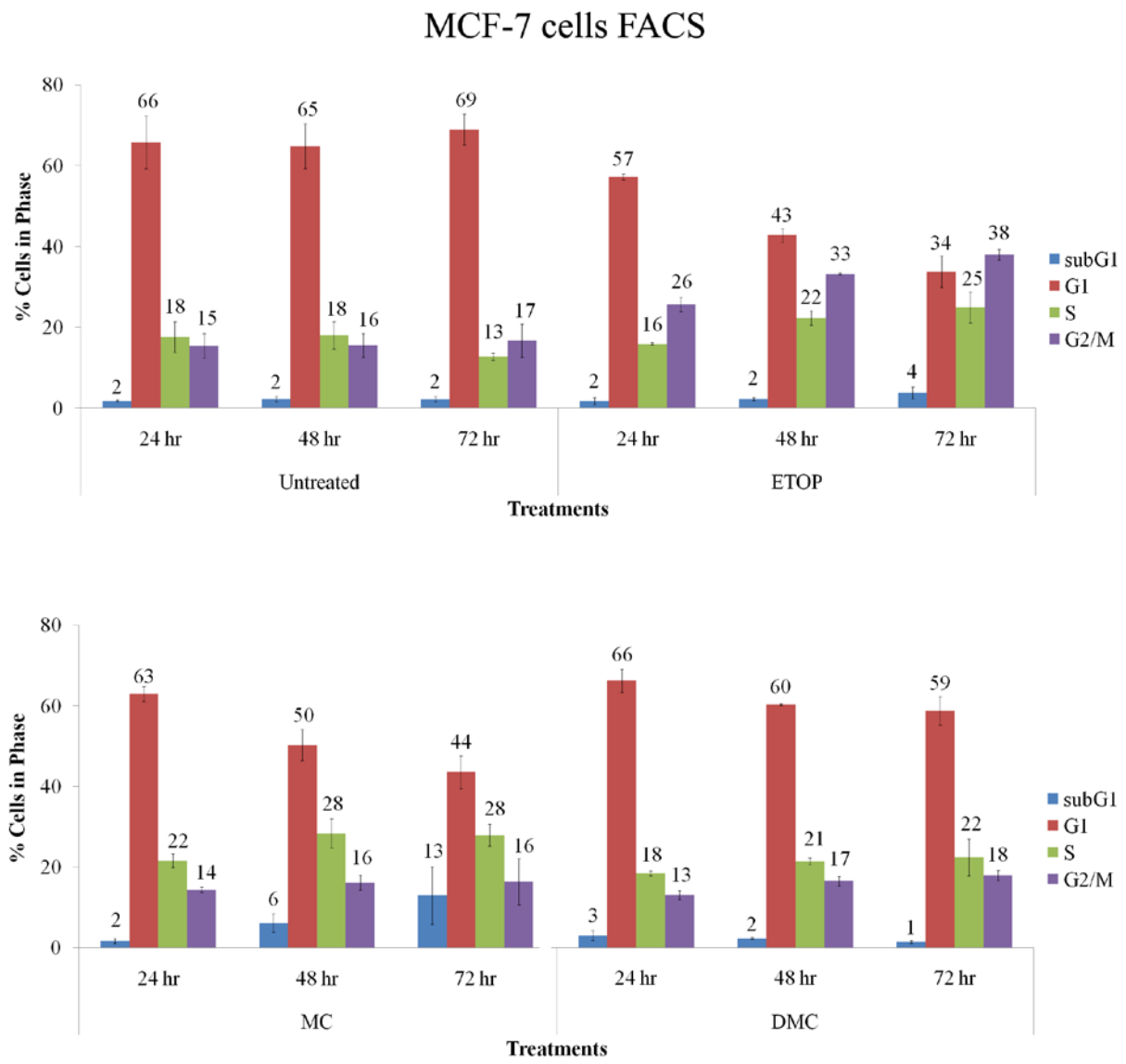

Figure 42: DMC-mediated death of MCF-7 cells did not appear to be apoptotic. *MCF-7 cells were treated with 50 μM etoposide (ETOP), 10 μM Mitomycin C (MC), and 10 μM 10-decarbamoyl Mitomycin C (DMC) for 24, 48 and 72 hours. At the end of the treatments Fluorescence activated cell sorting (FACS) was carried out. Cells were harvested, fixed in 30% ethanol, and cellular DNA was stained with propidium iodide. Graphs show averages and standard errors of two independent experiments.*

Though the DNA damaging drug, etoposide, and the Mdm2 small molecule inhibitor, Nutlin-3, did not induce MCF-7 cell death, we observed that these drugs induced PARP cleavage (Figure 43, compare lanes 1, 3 and 5). Since PARP cleavage is mediated by the caspases and is indicative of the apoptotic pathway activation, this result suggests that etoposide and Nulin-3 treatments activated the caspase cascade pathway in the MCF-7 cells. Estrogen treatment decreased drug-induced PARP cleavage (Figure 43, lanes 3-4 and 5-6), suggesting another role estrogen may play in inhibiting apoptosis in the MCF-7 cells. This observation correlates with our earlier observation that estrogen decreased the drug-induced Puma and in parallel increased the Bcl-2 protein levels. To test whether estrogen inhibits apoptosis in MCF-7 cells and whether Mdm2 plays a role in this process, the MCF-7 cells should be treated with MC (which robustly induces apoptosis) in the absence and presence of estrogen and with or without Mdm2 knockdown.

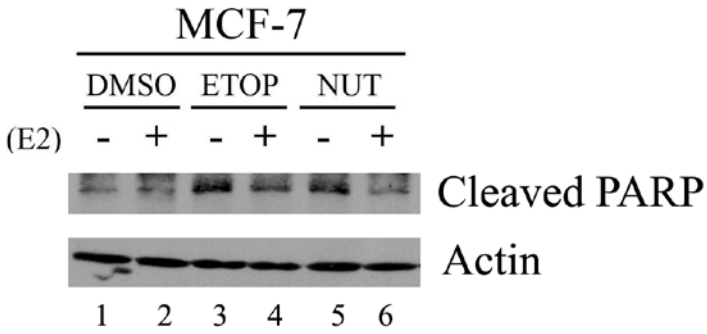

Figure 43: Estrogen decreased basal and drug-induced PARP cleavage in MCF-7 cells. *MCF-7 cells were treated with 10 nM estrogen (E2) for five days, 50 μM etoposide (ETOP) for 48 hours and 10 μM Nutlin-3 (NUT) for 24 hours. Cleaved PARP and Actin protein levels from whole cell lysates were analyzed by Western blot.*

In addition to inhibiting the etoposide-induced PARP cleavage, we also observed that estrogen treatment decreased the basal and the etoposide-induced levels of the H2AX histone phosphorylation. The H2AX phosphorylation on Ser 137 (called γH2AX) is indicative of the double stand DNA damage and is important for the recruitment of repair factors to the DNA damage sites (Paull et al. 2000). By Western blot analysis, we observed that estrogen treatment decreased the basal protein level of γH2AX in the MCF-7 cells, while the total level of the H2AX protein was not affected (Figure 44A, compare lanes 1 and 2). Similarly, estrogen treatment decreased the basal γH2AX foci accumulation (Figure 44B, DMSO). Etoposide treatment increased the protein levels of both H2AX and γH2AX (Figure 44A, compare lanes 1 and 3), but the increase of the γH2AX protein level was greater than that of the H2AX, 2.6 and 1.6 folds respectively (relative band intensities were determined by the ImageJ software). Surprisingly, we did not observe an increase in foci formation after etoposide treatment by the immunofluorescence method (Figure 44B, compare DMSO and ETOP in the absence of estrogen). Importantly, estrogen treatment dramatically decreased etoposide-induced γH2AX protein level and had no significant effect on the H2AX protein level (Figure 44A, compare lanes 3 and 4). Similarly, estrogen treatment decreased the γH2AX foci formation in etoposide-treated cells (Figure 44B, ETOP).

This result is very interesting and suggests that estrogen either blocked double strand DNA breaks formation after etoposide treatment or that estrogen blocked H2AX phosphorylation, which, in turn, reduced the recruitment of repair factors to the DNA damage sites. In the latter case, we can hypothesize that reduced γH2AX level in the presence of estrogen can lead to cell cycle progression despite the DNA damage and thus result in accumulation of genomic abnormalities and cancer development. Interestingly, earlier we observed that estrogen

increased the nuclear protein levels of the Mdm2 protein in the absence and in the presence of etoposide treatment. Therefore, it will be important to determine if the over-expression and the nuclear accumulation of Mdm2 in the presence of estrogen play a role in the inhibition of H2AX phosphorylation. By examining if estrogen can reduce the levels of γH2AX when Mdm2 is knocked down, we could determine if the decrease in γH2AX is Mdm2-dependent.

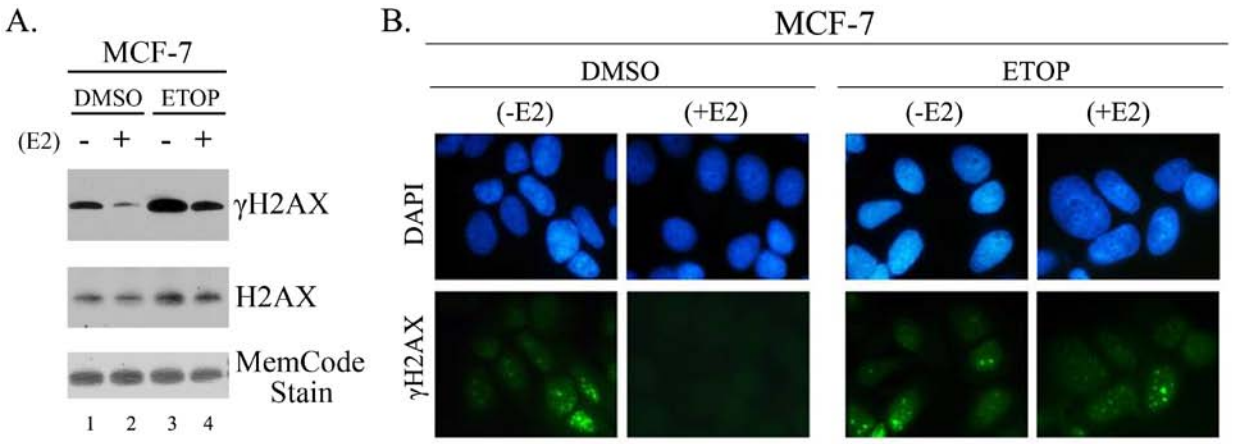

Figure 44: Estrogen decreased basal and etoposide-induced H2AX phosphorylation in MCF-7 cells. *MCF-7 cells were treated with 10 nM estrogen (E2) for five days and 50 μM etoposide (ETOP) for 48 hours. (A) γH2AX (Ser-139) and H2AX protein levels from cell lysates by histone extraction method were analyzed by Western blot. Total protein loading is shown by the MemCode Stain (reversible protein stain). (B) γH2AX nuclear staining was visualized on fluorescent microscope after indirect immunofluorescent staining of the γH2AX protein. Nuclear DNA was stained with DAPI.*

4.5 Determine the downstream pathway(s) targeted by Mdm2 in the presence of estrogen.

Because our experiments indicate that the Mdm2 protein plays an important role in the estrogen-mediated breast cancer cell proliferation, it is important to determine the downstream pathway(s) that are targeted by Mdm2 in the presence of estrogen. The growth proliferative effect of estrogen is largely attributed to the ability of estrogen to induce cell cycle progression through the G1 to S transition (Lewis-Wambi and Jordan 2009). In addition to targeting the p21 cell cycle inhibitor, it is possible that the estrogen-mediated Mdm2 over-expression positively affects cell cycle progression in the MCF-7 cells by targeting the Rb-E2F pathway. It has been shown that estrogen promotes Rb phosphorylation thus stimulating proliferation of the G1-arrested breast cancer cells (Altucci et al. 1996). In turn, the Mdm2 protein has been shown to stimulate E2F transcriptional activity (Martin et al. 1995), disrupt the Rb-E2F complex (Sdek et al. 2004) and promote Rb degradation (Sdek et al. 2005, Uchida et al. 2005). Therefore, the effect of the Mdm2 protein on the Rb-E2F pathway in the presence of estrogen should be examined.

In addition to studying the effect of Mdm2 over-expression on the Rb-E2F pathway in the presence of estrogen, a study with a more general and holistic approach should be conducted. There, it should be determined what Mdm2 signals to in the presence of estrogen to stimulate cell proliferation. To achieve that, it is important to determine what proteins (and thus pathways) are affected by the Mdm2 over-expression in the presence of estrogen. Proteins that will be identified to be affected by Mdm2 over-expression and those that gain or lose their interactions with Mdm2 in the presence of estrogen should be further characterized in reverse genetics studies and the pathways in which these proteins are involved should be further addressed.

CHAPTER 5:

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