p53-Independent Role of MDM2 in TGF-β1 Resistance

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Transforming growth factor–β (TGF-β) inhibits cell proliferation, and acquisition of TGF-β resistance has been linked to tumorigenesis. A genetic screen was performed to identify complementary DNAs that abrogated TGF-β sensitivity in mink lung epithelial cells. Ectopic expression of murine double minute 2 rescued TGF-β–induced growth arrest in a p53-independent manner by interference with retinoblastoma susceptibility gene product (Rb)/E2F function. In human breast tumor cells, increased MDM2 expression levels correlated with TGF-β resistance. Thus, MDM2 may confer TGF-β resistance in a subset of tumors and may promote tumorigenesis by interference with two independent tumor suppressors, p53 and Rb.

The TGF-β signaling pathway has been implicated in tumor suppression (*1*). Loss of TGF-β sensitivity is frequently observed in tumors derived from cells that are normally sensitive, and the extent of TGF-β resistance often correlates with malignancy (*2*). Some tumors may develop TGF-β resistance following inactivation of essential components of the TGF-β signaling pathway (*3*–*5*) or through deletion of the $p15^{INK4B}$ locus (*6*). However, such alterations cannot account for the majority of cases in which TGF-β responsiveness is lost. Therefore, TGF-β resistance must also be achieved by other mechanisms.

To identify genetic alterations that lead to TGF-β resistance in tumor cells, we screened for genes that, when overexpressed, allow cells to escape TGF-β–induced growth arrest (*7*). A cDNA library was introduced into Mv1Lu, a TGF-β–sensitive mink lung epithelial cell line, using a retrovirus-based genetic screening system (*8*). Infected cells were selected for the ability to sustain proliferation in the presence of TGF-β. We recovered three genes that conferred TGF-β resistance: *Mdm2* c-*myc*, and *NF-IX-1* (Fig. 1, top panel). When treated with TGF-β, cells expressing MDM2, c-*myc*, or NF-IX-1 formed colonies and were morphologically identical to untreated cells (Fig. 1) (*9*). MDM2 also conferred TGF-β resistance in human mammary epithelial cells (HMECs) (Fig. 1, bottom panel).

The isolation of c-*myc*, a gene previously shown to overcome TGF-β–induced arrest (*10*), validated the genetic screen. NF-IX-1 is a member of a family of transcription factors that may function in development and differentiation (*11*). The mechanism by which NF-IX-1 confers TGF-β resistance remains to be investigated. Because MDM2 is an oncogenic protein that is commonly overexpressed in a broad spectrum of tumors (*12*), we focused on understanding how this protein confers TGF-β resistance.

Activation of TGF-β signaling regulates the expression of a battery of genes. MDM2 overexpression in Mv1Lu cells did not alter the response of known TGF-β targets (for example, *PAI-1*, *p15*, c-*myc*, and *cdc25A*) (*9*), indicating that MDM2 does not confer resistance by disruption of TGF-β signaling.

MDM2 associates with and inactivates the tumor suppressor protein, p53. To test the possibility that MDM2 bypasses TGF-β–induced growth arrest through an effect on p53, we investigated whether interference with p53 activity could produce cytokine resistance. Two dominant-negative p53 alleles, p53Val135 (a temperature-sensitive mutant) or p53-175H (*13*–*15*) were introduced into Mv1Lu cells, which contain endogenous, wild-type p53 (*16*). The functionality of these p53-interfering mutants was confirmed by their ability to suppress p53-dependent transcription (*9*). Cells in which p53 had been inactivated by

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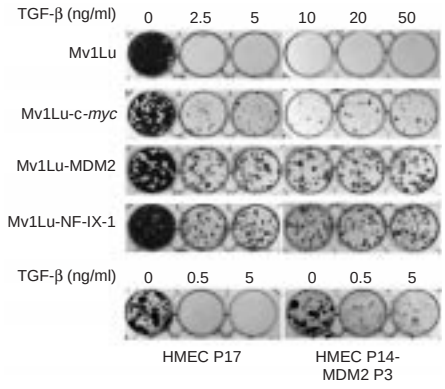

Fig. 1. *Mdm2*, c-*myc*, and *NF-IX-1* bypass TGF-β–induced growth arrest. (Top panel) Control Mv1Lu or Mv1Lu expressing c-*myc*, MDM2, or NF-IX-1 (4000 cells) were treated with TGF-β for 8 days. (Bottom panel) HMECs at passage 14 were infected with a retroviral vector that drives MDM2 expression, and infected cells were selected with hygromycin. After three more passages, HMECs expressing MDM2 or control HMECs (4000 cells) at passage 17 were treated with TGF-β for 16 days. All cells were visualized by staining with crystal violet.

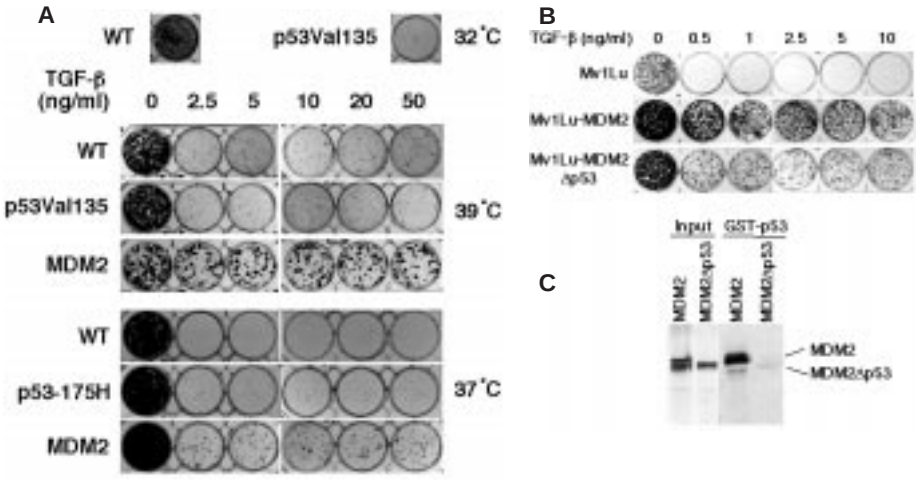

Fig. 2. MDM2 confers TGF-β resistance through a p53-independent mechanism in Mv1Lu cells. (**A**) Control Mv1Lu cells or cells expressing MDM2, p53Val135, or p53-175H were treated with TGF-β for 8 days. (**B**) Control Mv1Lu cells or cells expressing MDM2 or an MDM2 mutant that cannot bind p53 were treated with TGF-β for 8 days. (**C**) Wild-type or mutant MDM2 proteins were translated in vitro from pcDNA3 in the presence of [^{35}S]methionine and were incubated with a glutathione *S*-transferase (GST)–p53 fusion protein bound to glutathione–Sepharose 4B beads. Proteins that remained bound to beads after washing (right two lanes) were separated by 12% SDS–polyacrylamide gel electrophoresis, and radiolabeled proteins were visualized by autoradiography. A portion of each in vitro translation reaction is shown for comparison (left two lanes).

expression of either dominant-negative mutant retained TGF-β sensitivity (Fig. 2A). Furthermore, an MDM2 mutant from which the p53-binding domain had been removed failed to bind p53 (Fig. 2C) but still conferred TGF-β resistance (Fig. 2B). Thus, MDM2 overcomes TGF-β through a mechanism that is distinct from its ability to inactivate p53.

TGF-β induces G_1 arrest through effects on the Rb/E2F pathway (*17*–*19*). Because expression of human papillomavirus HPV-16 E7 protein, which abolishes Rb but not p53 function (*20*), conferred TGF-β resistance in Mv1Lu cells (*9*), we investigated the possibility that MDM2 could bypass TGF-β by interference with the RB/E2F pathway. This hypothesis is consistent with the recent finding that MDM2 can bind directly to Rb and E2F/DP transcription factors (*21*, *22*).

In control Mv1Lu cells, TGF-β treatment led to a gradual change in Rb phosphorylation status (Fig. 3A). After 24 hours (the time at which growth arrest was established), the majority of Rb had shifted from the hyperphosphorylated form to the growth-inhibitory, hypophosphorylated form. However, in MDM2- and c-*myc*–expressing cells, the majority of Rb remained in hyperphosphorylated, non–growth-inhibitory state.

E2F proteins are transcription factors that bind to unphosphorylated Rb. Rb phosphorylation releases E2F proteins in an active, growth-promoting form (*23*). The effect of MDM2 on Rb phosphorylation predicted that MDM2 would have a positive effect on E2F activity. In contrast to previous studies in other cell lines (*21*, *22*), expression of MDM2 in Mv1Lu cells did not increase the activity of an E2F-dependent reporter construct (Fig. 3B). TGF-β treatment reduced transcription of this reporter by twofold. However, MDM2 expression prevented this reduction (Fig. 3B). Alteration of E2F activity by either TGF-β treatment or MDM2 overexpression reflected changes in E2F-1 protein levels (Fig. 3C). TGF-β treatment led to a gradual decrease in E2F-1, and this decrease was prevented by ectopic MDM2 expression. These results indicate that MDM2 rescues TGF-β–induced growth arrest, at least in part, through maintenance of E2F-1 protein levels and E2F activity. Similar effects were evident in cells that ectopically express c-*myc* (Fig. 3, B and C), suggesting that c-*myc* and MDM2 may bypass TGF-β–induced arrest through overlapping mechanisms.

MDM2 is frequently overexpressed in human tumors (*12*). We identified one biological consequence of MDM2 overexpression, bypass of TGF-β–induced growth arrest. TGF-β induces growth arrest in normal human lymphocytes, melanocytes, and breast epithelial cells. However, cells from human leukemia, lymphomas, melanomas, and breast carcinomas are often TGF-β resistant (*24*–*27*). Coincidentally, MDM2 is commonly overexpressed in these tumors (for example, in 73% of human breast carcinomas) (*28*–*32*). Enforced expression of MDM2 in primary HMECs converted these TGF-β–sensitive cells to a resistant phenotype (Fig. 1, bottom panel). These observations raised the possibility that increased MDM2 expression might contribute to TGF-β resistance in tumors.

Therefore, we examined the relationship between MDM2 expression levels (Fig. 4A) and TGF-β responsiveness (Fig. 4B) in seven human breast tumor cell lines. MDM2 was expressed in T-47D, ZR-75-1, and HTB20 cells at levels comparable to those observed in cells (HMEC and Mv1Lu) that had been made TGF-β–resistant by infection with MDM2 retroviral vectors. These three cell lines were completely resistant to TGF-β–induced growth arrest. The two cell lines (MCF-7 and BT549) that were most sensitive to TGF-β treatment had very low MDM2 levels, similar to those seen in TGF-β–sensitive, normal HMECs. Thus, in several tumor cell lines, increased MDM2 expression strictly correlated with the ability to escape TGF-β–induced growth inhibition. Two other breast carcinoma cell lines (HBL100 and MDA-MB-468) exhibited partial resistance to TGF-β despite low levels of MDM2 ex-

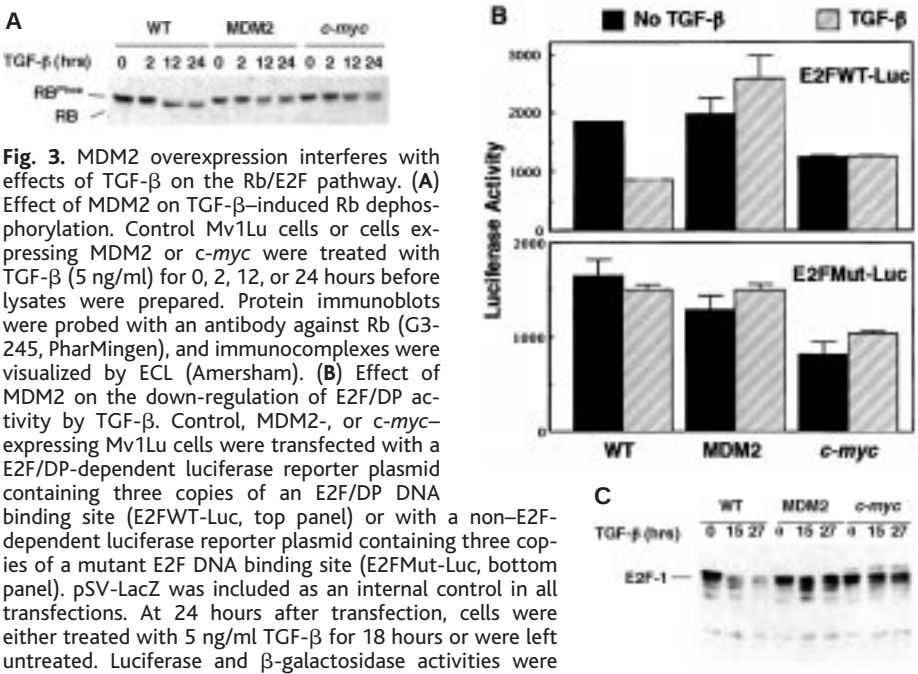

Fig. 3. MDM2 overexpression interferes with effects of TGF-β on the Rb/E2F pathway. (**A**) Effect of MDM2 on TGF-β–induced Rb dephosphorylation. Control Mv1Lu cells or cells expressing MDM2 or c-*myc* were treated with TGF-β (5 ng/ml) for 0, 2, 12, or 24 hours before lysates were prepared. Protein immunoblots were probed with an antibody against Rb (G3-245, PharMingen), and immunocomplexes were visualized by ECL (Amersham). (**B**) Effect of MDM2 on the down-regulation of E2F/DP activity by TGF-β. Control, MDM2-, or c-*myc*–expressing Mv1Lu cells were transfected with a E2F/DP-dependent luciferase reporter plasmid containing three copies of an E2F/DP DNA binding site (E2FWT-Luc, top panel) or with a non–E2F-dependent luciferase reporter plasmid containing three copies of a mutant E2F DNA binding site (E2FMut-Luc, bottom panel). pSV-LacZ was included as an internal control in all transfections. At 24 hours after transfection, cells were either treated with 5 ng/ml TGF-β for 18 hours or were left untreated. Luciferase and β-galactosidase activities were then measured (Promega). Values represent means ± SEM of luciferase activities (normalized to β-galactosidase activities) from three independent transfections. (**C**) Effect of MDM2 on the down-regulation of E2F-1. Control, MDM2- or c-*myc*–expressing Mv1Lu cells were treated with 5 ng/ml TGF-β for 0, 15, or 27 hours. Cell lysates were prepared, and protein immunoblots were probed with an antibody against E2F-1 (C20, Santa Cruz Biotechnology).

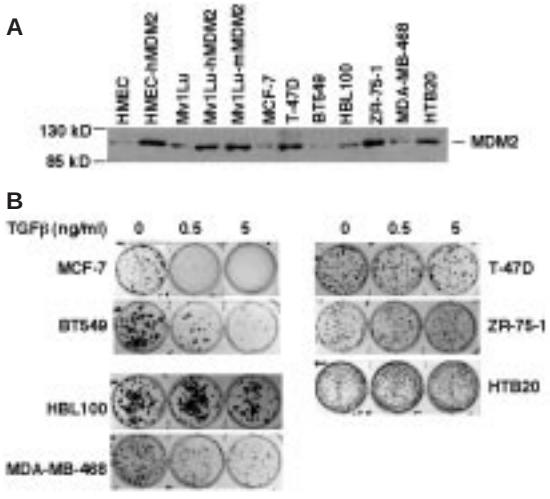

Fig. 4. MDM2 expression correlates with resistance to TGF-β in human breast tumor cell lines. (**A**) MDM2 protein levels in human breast tumor cell lines. Cell lysates were prepared from the indicated cell lines, and protein immunoblots were probed with an antibody to MDM2 (2A10). (**B**) TGF-β responsiveness of human breast tumor cell lines. Human breast tumor cell lines were treated with TGF-β at the indicated concentrations for 20 days.

pression, confirming that other mechanisms (for example, c-*myc* overexpression, receptor mutation, and so forth) must also contribute to TGF-β resistance.

As breast carcinomas and melanomas become metastatic, they secrete large amounts of TGF-β (*25*, *27*). This may enhance tumor cell invasion through effects on extracellular matrix (*27*, *33*). Thus, TGF-β resistance may be an essential adaptation to the metastatic phenotype. In accord with this notion, the extent of TGF-β resistance correlates with metastatic progression (*28*, *30*), and targeted deletion of an essential component of the TGF-β signaling cascade, *Smad3*, promotes the formation of metastatic tumors (*1*). Although TGF-β resistance can be achieved through multiple routes, increased expression of MDM2 is sufficient to confer this phenotype.

Previous work indicated that MDM2 may contribute to transformation through mechanisms that are independent of effects on p53. For example, in some human breast carcinomas and lymphomas, p53 mutation and MDM2 overexpression occur together (*31*, *32*). Recently, alternatively spliced forms of MDM2 were identified in bladder and ovarian carcinomas (*34*). These alternative forms lack the p53-binding domain but still transform NIH-3T3 cells. We have demonstrated that MDM2 can overcome growth inhibition by TGF-β through effects on the RB/E2F pathway. These results provide a potential mechanism underlying p53-independent oncogenic activities of MDM2. Thus, in tumors, MDM2 may antagonize both the Rb and p53 pathways, functioning in many respects as a cellular version of SV40 large T antigen.

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7. A library was made from Swiss 3T3 and Balb/c 3T3 cells and was cloned into a retroviral expression vector HygroMaRXII (*8*), packaged in an ecotropic virus packaging cell line LinX E (L. Y. Xie, D. Beach, G. J. Hannon, unpublished results), and used to infect Mv1Lu cells which had been engineered to express the ecotropic retrovirus receptor. We estimated that a total of 10^7 cells were infected. The infected cells were selected with hygromycin and then subjected to TGF-β treatment for 3 months. Integrated proviruses were then excised with Cre recombinase from genomic DNA isolated from plates containing resistance cells. cDNA from 38 plates of resistant cells have been recovered and sequenced thus far. Among these, seven plates contained a cDNA encoding *Mdm2*, one contained c-*myc*, and seven contained *NF-IX-1*.
8. The cDNA expression vector (HygroMaRXII; P. Sun, G. J. Hannon, D. Beach, unpublished data) was designed based on Molony murine leukemia virus (MoMLV). We included a recognition site (loxP) for Cre recombinase in a 3′ long terminal repeat (LTR) and a bacterial replicon and a bacterial selectable marker within the retroviral genome. These modifications allow easy and efficient recovery of cDNAs by Cre-mediated excision of integrated proviruses from the genome. The recovered circular plasmids contained a single LTR, and thus could be directly used to produce recombinant viruses for further studies.
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Regulation of Cocaine Reward by CREB

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Cocaine regulates the transcription factor CREB (adenosine 3′,5′-monophosphate response element binding protein) in rat nucleus accumbens, a brain region that is important for addiction. Overexpression of CREB in this region decreases the rewarding effects of cocaine and makes low doses of the drug aversive. Conversely, overexpression of a dominant-negative mutant CREB increases the rewarding effects of cocaine. Altered transcription of dynorphin likely contributes to these effects: Its expression is increased by overexpression of CREB and decreased by overexpression of mutant CREB. Moreover, blockade of κ opioid receptors (on which dynorphin acts) antagonizes the negative effect of CREB on cocaine reward. These results identify an intracellular cascade—culminating in gene expression—through which exposure to cocaine modifies subsequent responsiveness to the drug.

Cocaine causes complex molecular adaptations in brain reward systems, some of which affect its addictive qualities (*1*). For example, chronic cocaine use increases formation of adenosine 3′,5′-monophosphate (cAMP) and activity of cAMP-dependent protein kinase (PKA) in the nucleus accumbens (*2*), a neural substrate for the rewarding actions of cocaine (*3*, *4*). Stimulation of PKA in the nucleus accumbens counteracts the rewarding properties of cocaine (*5*), which suggests a neural mechanism of drug tolerance. Increased PKA activity would be expected to lead to increased phosphorylation of CREB, which mediates many of the effects of cAMP and PKA on gene expression (*6*, *7*). However, direct evidence for a role of CREB in cocaine actions has been lacking. To address this issue, we selectively induced CREB overexpression in the nucleus accumbens with microinjections of a herpes simplex virus vector (HSV-CREB) and measured alterations in the rewarding properties of cocaine with place conditioning (*8*). We performed the same experiments in other rats after overexpression of a dominant negative mutant CREB (mCREB) (*8*), which contains a single point

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