Molecular Cancer Research

Context-Dependent Bidirectional Regulation of the MutS Homolog 2 by Transforming Growth Factor β Contributes to Chemoresistance in Breast Cancer Cells

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Abstract

The TGF-β, a tumor suppressive cytokine in normal cells, is abused in cancer to promote the malignancy. In this study, we reported that TGF-β downregulated the mutS homolog 2 (MSH2), a central component of the DNA mismatch repair (MMR) system, in HER2-transformed MCF10A mammary epithelial cells and in breast cancer (BC) cells. This was mediated by a TGF-β–induced micro RNA (miRNA), miR-21, which targeted the 3′ untranslated region of MSH2 mRNA and downregulated its expression. A negative correlation between the expression of TGF-β1 and MSH2 was also detected in primary breast tumors. In contrast, TGF-β upregulated MSH2 in nontransformed cells through Smad-mediated, p53-dependent promoter activation, which was absent in BC cells with impaired p53 function. Although this upregulating mechanism also existed in MCF10A/HER2 and p53-proficient BC cells, both basal and TGF-β–induced MSH2 promoter activities were significantly lower than those in MCF10A. Moreover, the basal and TGF-β–induced miR-21 levels were markedly higher in transformed cells, suggesting that the preset levels of miR-21 and MSH2 promoter activity, which is affected by the p53 status, determine the outputs of the bidirectional regulation of MSH2 by TGF-β in a certain cellular context. We further found that by downregulating MSH2, TGF-β contributed to resistance to DNA-damaging chemotherapy agents in cancer cells. Our results indicated a regulatory antagonism between promoter activation and miRNA-mediated posttranscriptional inhibition underlying a dual effect of TGF-β on the DNA repair machinery, which may influence the genomic stability in a context-dependent manner and contribute to chemoresistance in cancer. *Mol Cancer Res; 8(12); 1633–42. ©2010 AACR.*

Introduction

A number of cell signaling pathways partaking in the maintenance of tissue homeostasis, such as the TGF-β-initiated cascade, are abused in cancer. TGF-βs are a family of multitasking cytokines that play important roles in proliferation, apoptosis, lineage determination, extracellular matrix production, and modulation of immune function (1). The ligands signal through binding to heteromeric complexes of transmembrane receptors and activating their serine/threonine kinase activity, which subsequently phosphorylates the transcription factors Smad2 and Smad3. Smad2/3 then associate with a common mediator Smad, Smad4, and translocate to the nucleus where they regulate gene transcription (2). It is recently discovered that Smad2/3 also mediates micro RNA (miRNA) maturation through interacting with the DROSHA miRNA processing complex, an event induced by TGF-β (3).

The versatile nature of TGF-β is accompanied with highly contextual cell response to this cytokine, dependent on the type of tissue and stage during development. Similarly, a transformed (cancerous) cellular context may direct a TGF-β response different from a normal context. Indeed, although TGF-β plays a tumor suppressive role in normal epithelia by potently inhibiting cell proliferation and inducing apoptosis, it accelerates progression of established cancer by multiple autocrine and paracrine mechanisms (4–6). On one hand, some cancers lose or attenuate TGF-β–mediated antimitogenic action by mutational inactivation of TGF-β receptors, Smads, or cofactors required for Smad-mediated transcriptional regulation (7–12). On the other hand, TGF-β may gain cancer-promoting functions by synergizing with transforming oncogenes. Activation of TGF-β signaling in the mammary gland of transgenic mice expressing mouse mammary tumor virus (MMTV)/ErbB2 accelerates metastases from ErbB2-induced mammary

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doi: 10.1158/1541-7786.MCR-10-0362

tumors (13–15). At the cellular level, TGF-β confers enhanced survival, motility, and invasiveness to HER2 (ErbB2)-transformed MCF10A mammary epithelial cells (MCF10A/HER2) and breast cancer (BC) cells that overexpress HER2, through inducing ligands that activate HER2-containing receptor complexes (16, 17). In a previous study, we determined the proteome-wide TGF-β response in MCF10A/HER2 cells using 2-dimensional multivariable difference gel electrophoresis (2D DIGE) coupled with MS. More than 60 protein species exhibit significant changes in abundance or charge-altering posttranslational modification in response to TGF-β (18). Among them, mutS homolog 2 (MSH2) was discovered as a novel TGF-β target and was investigated in-depth in this study.

MSH2 is a major component of the highly conserved mismatch repair (MMR) system and plays a central role in the recognition and repair of DNA replication errors, contributing to genomic integrity. In cancer cells, MSH2 recognizes DNA adducts caused by many chemotherapy drugs, which triggers further MMR-mediated processing at the damaged sites, resulting in cell-cycle arrest and apoptosis. The inability of MMR-deficient cells to recognize chemotherapy-induced DNA damages results in a damage-tolerant phenotype and drug resistance, leading to the selection of MMR-deficient cancer cells during therapy (19). We hereby, demonstrated a context-dependent, bidirectional regulation of MSH2 by TGF-β. Although TGF-β activated MSH2 promoter in a p53-dependent manner in nontransformed cells, it downregulated MSH2 expression through an miRNA-mediated mechanism in transformed cells, resulting in resistance to DNA-damaging agents. The differential regulation of the same target gene by TGF-β in different cellular context provides a model through which cancer hijacks a tumor suppressive pathway and makes it favorable to cancer malignancy.

Materials and Methods

Cell lines, plasmids, and viruses

Human BC cell lines MDA-MB-231 (MDA231), MCF7, and HCC1954; MCF10A mammary epithelial cells; and 293 embryonic kidney cells were obtained from American Type Culture Collection (ATCC) and cultured in the recommended media in a humidified 5% CO_2 incubator at 37°C. MCF10A cells stably overexpressing HER2 (MCF10A/HER2) or vector (MCF10A/vec) and primary human mammary epithelial cells (HMEC) were generated and cultured as previously described (20, 21). Recombinant human TGF-β1 was purchased from R&D Systems. The type I/II TGF-β receptor inhibitor LY2109761 was provided by Eli Lilly and Company. The HER2/EGFR small molecule inhibitor lapatinib ditosylate was purchased from LC Laboratories. The MDM2 antagonist nutlin-3 was purchased from Sigma-Aldrich. The expression construct of MSH2 was kindly provided by Dr. Jeremy Stark (City of Hope).

To construct the 3′ untranslated region (UTR) reporter plasmids, annealed oligonucleotides 5′-tcgataatggaatgaaggtaatattgataagctattgtctg-3′ and 5′-ggcccagacaatagcttatcaatattaccttcattccatta-3′ containing 11 to 47 nucleotides of MSH2 3′UTR sequence (encompassing the putative miR-21 binding site), 5′-tcgataatggaatgaaggtaatattgatccgctattgtctg-3′ and 5′-ggcccagacaatagcggatcaatattaccttcattccatta-3′ containing the mutated MSH2 3′UTR sequence (MSH2 3′UTR^{mut}; mutated nucleotides in the miR-21 binding site underlined), or 5′-tcgaagttatttgctatatattattttcaacatgagttttt-3′ and 5′-ggccaaaaactcatgttgaaaatatatatagcaaataact-3′ containing a scrambled sequence, were inserted into the *Xho*I/*Not*I sites of psiCHECK-2 vector (Promega), downstream of the luciferase encoding region. A 224-basepair promoter region (−201–+24) of human MSH2 gene (*hMSH2*) was cloned by PCR using the upstream primer 5′-atatatgctagcaggatgcgcgtctgcgggtttcc-3′ [for Smad-binding element (SBE) wild-type (wt)] or 5′-atatatgctagcaggatgcgcgatcgcgggtttcc-3′ (for SBE mt) and the downstream primer 5′-gcgcgcaagcttacacccactaagctgtttcc-3′, and inserted into the *Nhe*I/*Hin*dIII sites of pGL3-Basic vector (Promega), upstream of the luciferase reporter gene, to generate the *hMSH2* promoter reporters. The small hairpin (sh) RNA targeting the MSH2 mRNA sequence 5′-gggctggtgacagtcaattga-3′ was first constructed by inserting annealed oligonucleotides 5′-gatcgggctggtgacagtcaattgatttgtgtagtcaattgactgtcaccagccctttttgcatg-3′ and 5′-cccgaccactgtcagttaactaaacacatcagttaactgacagtggtcgggaaaaaac-3′ into the *Bgl*II/*Sph*I sites of pU6tetO vector (22; kindly provided by Dr. John J. Rossi), downstream of a doxycycline (Dox)-inducible U6 promoter. The efficiency of gene knockdown by this construct as well as other 2 constructs targeting different MSH2 sequences was examined in transiently transfected MDA231 cells, where this construct most efficiently reduced MSH2 expression as detected by Western blot (data not shown). The entire U6tetO-MSH2 shRNA region was then subcloned into the *Not*I/*Sph*I sites of lentiviral vector pTIG (pHIV7-TetR-IRES-GFP; ref. 22; kindly provided by Dr. Rossi), to generate pTIG-MSH2 shRNA. Viruses expressing MSH2 shRNA or empty vector were produced by cotransfecting 293 cells with the construct and packaging plasmids pCHGP-2, pCMV-Rev, and pCMV-G, as previously described (23), and used to infect MDA231 cells, followed by green fluorescent protein (GFP) selection.

RNA extraction, reverse transcription, and real-time quantitative PCR

RNA was isolated using the TRIzol reagent (Invitrogen) and subjected to reverse transcription (RT) using the miScript Reverse Transcription Kit (Qiagen), following the manufacturers' protocols. Gene expression was quantified by quantitative PCR (qPCR) using the miScript SYBR Green PCR Kit (Qiagen) and 25 ng of cDNA per reaction. For miR-21 detection, qPCR was carried out using the miScript Primer Assay for miR-21 or the U6 noncoding small nuclear RNA (snRNA; as a control) and the miScript Universal Primer (Qiagen). Primers used for detection of MSH2 mRNA and in chromatin immunoprecipitation (ChIP) assay (for an *hMSH2*-coding region) are 5′-tcatggctgaaatgttggaa-3′ and 5′-ttggccaaggcagtaagttc-3′, and for GAPDH mRNA 5′-accacagtccatgccatcac-3′ and 5′-tccac-

caccctgttgctgta-3′. Primers used for detection of the SBE-p53 region in *hMSH2* promoter in the ChIP assay are 5′-atatatgctagcaggatgcgcgtctgcgggtttcc-3′ and 5′-gcgcgcaagct-tacacccactaagctgtttcc-3′. An annealing temperature of 55°C was used for all the primers. PCR reactions were performed in a standard 96-well plate format with the iQ5 multicolor real-time PCR detection system (BioRad). For the data analysis of qRT-PCR, raw Ct was first normalized to U6 (for miRNA) or GAPDH (for mRNA) for each sample to obtain dCt. The normalized dCt was then calibrated to control cell samples to obtain ddCt. For the data analysis of qChIP–PCR, raw data were calibrated to the corresponding input DNA sample, which was purified from one twentieth of the cell lysate used in ChIP but without the IP procedure.

ChIP assay and Western blot analysis

Preparation of cell lysates, ChIP, and Western blot were carried out as described previously (21, 24). Smad2/4, p53, MSH2, and GAPDH antibodies were purchased from Cell Signaling.

Cell transfection and RNA interference studies

DNA **t**ransfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol, as described previously (20). The miRIDIAN miRNA hairpin inhibitor and mimic of miR-21 and the negative controls were purchased from Dharmacon. Silencer siRNA against p53 target sequence 5′-aaggaaatttgcgtgtg-gagt-3′ and the All Stars negative control siRNA were purchased from Qiagen. Micro RNA inhibitors/mimics and siRNAs were transfected into cell lines using DharmaFECT Duo Transfection Reagent (Dharmacon) according to the manufacturer's procedures. In a 6-well plate format, a final concentration of 25 nmol/L of miRNA inhibitors/mimics or 100 nmol/L of siRNAs and 6 μL of DharmaFECT Duo Transfection Reagent mixed in 2 mL of serum-free medium were used for each transfection. In the cotransfection with psiCHECK reporter plasmids, 25 nmol/L of miRNA mimics/inhibitors and 500 ng reporter plasmid DNA were added.

Luciferase reporter assay

Firefly and *Renilla reniformis* (as internal controls) luciferase activities were measured at 48 hours after cell transfection using the dual luciferase assay system (Promega) as reported previously (21).

MTT (thiazolyl blue tetrazolium bromide) cell viability assay

Cells were seeded in quadruplicate at 5,000 cells per well in 96-well plates, before drugs were added at the indicated concentrations after 24 hours. At 72 hours after drug treatment, MTT/PBS solution was added to the medium to a final concentration of 0.5 mg/mL. After 4 hours incubation at 37°C, MTT-containing medium was removed and 150 μL of 0.04N of HCl/isopropanol was added to each well. The absorbance at 570 nm (test) and 630 nm (reference) was measured on an ELISA plate reader to obtain sample signal (OD_{570}–OD_{630}), which was then compared with the signal of untreated control wells.

Statistical correlation analysis

The linear dependence between microarray-determined expression levels of TGF-β1 and MSH2 was evaluated by the Pearson correlation coefficient. The gene expression data and associated clinical data were extracted from a published 295 BC data set (25, 26).

Results

TGF-β downregulated MSH2 in transformed cells and negatively correlated with MSH2 in primary breast tumors

We identified MSH2 as a downstream target of TGF-β in a previous study using 2D DIGE to quantitatively measure the proteomic changes in TGF-β–treated MCF10A/HER2 cells (18). The MSH2 protein is reduced by greater than 60% at 24 hours and 40 hours after TGF-β treatment (Fig. 1A). A significant downregulation of MSH2 at both mRNA and protein levels was also observed in BC cell lines MDA231, MCF7, and HCC1954 (Fig. 1B and C). Consistently, inhibition of TGF-β signaling with LY2109761, an inhibitor of the TGF-β receptors, elevated MSH2 protein level in MDA231 cells (Fig. 1C). To explore whether there is a correlation between TGF-β1 and MSH2 in primary cancer tissue, we analyzed the expression levels of the 2 genes in a previously reported 295 BC data set (25, 26). A strong negative correlation ($P < 0.001$) was observed between the microarray-determined TGF-β1 and MSH2 expression levels in the whole data set. When stratifying patients by grade, the linear association is significant among patients with grade 1 ($P = 0.001$) and grade 2 ($P = 0.002$) tumors. When stratifying patients by stage, the correlation is only significant among patients with stage 1 ($P = 0.001$) and stage 2a ($P = 0.001$) tumors (Table 1), suggesting the downregulation of MSH2 by TGF-β may occur during early cancer development.

TGF-β downregulated MSH2 in transformed cells through inducing miR-21

One mechanism to downregulate gene expression at posttranscriptional level is through miRNAs, which are frequently dysregulated in human cancers (27, 28). These naturally occurring noncoding small RNA molecules play crucial functions in cells by base pairing to the 3′UTR of target mRNAs, resulting in mRNA degradation or translation inhibition. To determine if the downregulation of MSH2 involved miRNA-mediated posttranscriptional control, we interrogated the 3′UTR sequence of *hMSH2* gene transcript using TargetScanHuman 5.1 (www.targetscan.org) and miRDB (www.mirdb.org). Both programs predicted a potential miR-21 targeting site at positions 33 to 39, which was highly conserved among various species

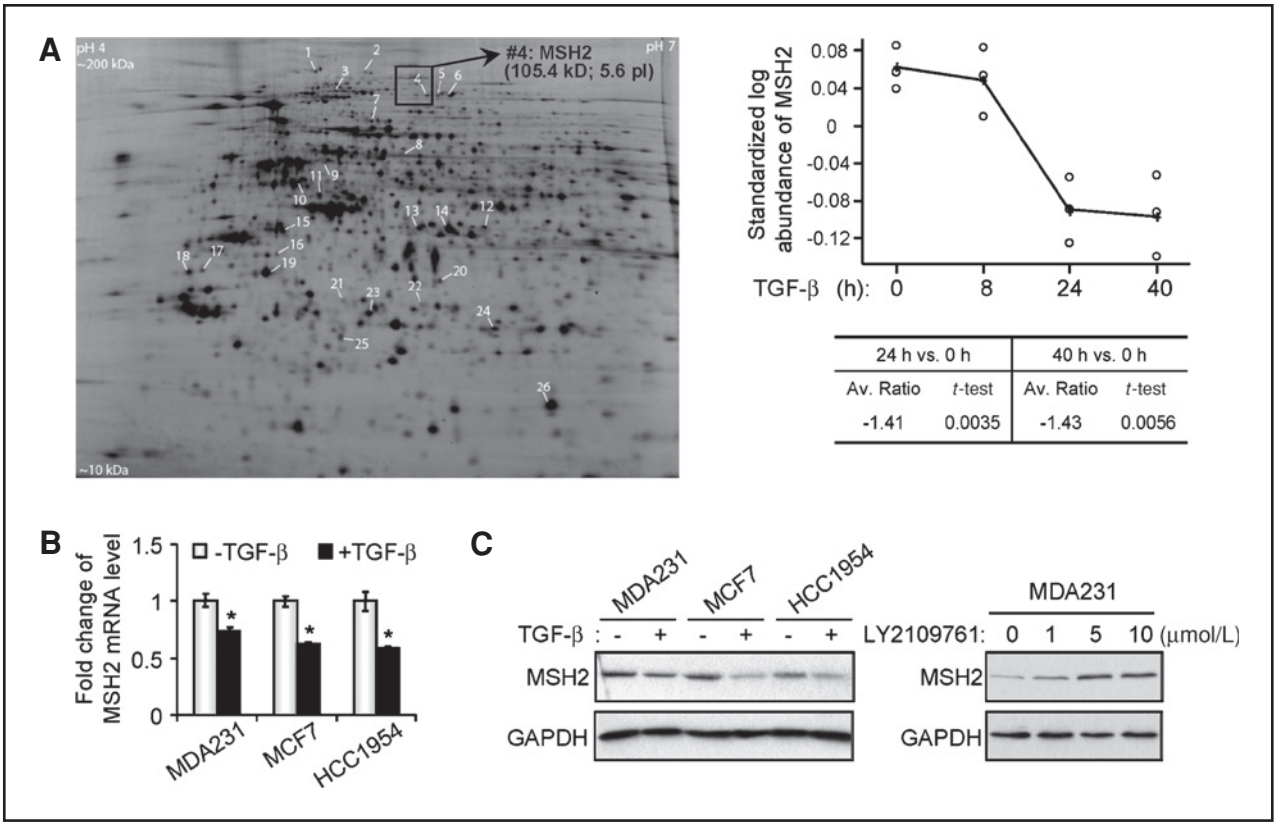

Figure 1. TGF-β downregulated MSH2 in transformed cells and negatively correlated with MSH2 in primary breast tumors. A, identification of MSH2 as a target of TGF-β by 2D DIGE and MS. This research was originally published in Molecular and Cell Proteomics. Friedman DB, Wang SE, Whitwell CW, Caprioli RM, Arteaga CL. Multivariable difference gel electrophoresis and mass spectrometry:a case study on transforming growth factor-beta and ERBB2 signaling. Molecular and Cell Proteomics 2007;6:150–69. © the American Society for Biochemistry and Molecular Biology. DIGE was carried out as previously described using the protein extracts of MCF10A/HER2 cells treated with 2 ng/mL of TGF-β for indicated time (18). Left: a representative Cy2-labeled gel image illustrating proteins resolved in the pH = 4 to 7 range and the position of MSH2 (position 4). Horizontal axis: pH gradient (4–7); vertical axis: molecular mass range. Right: the average ratio of MSH2 abundance (compared with 0 hour) and *t*-test value. B, BC cell lines were treated with TGF-β or vehicle for 24 hours. Quantitative RT-PCR was carried out to determine the MSH2 mRNA level. GAPDH was used as an internal control. In each cell line, data were normalized to control cells treated with vehicle. *, $P < 0.005$. C, cells treated with TGF-β, LY2109761 or vehicle for 24 hours were lysed and total proteins subjected to Western blot.

(Fig. 2A, top). We then cloned this putative miR-21 binding region as well as a mutated version downstream to a luciferase reporter gene in psiCHECK vector and transfected 293 cells with these constructs or a control vector containing a scrambled sequence. Cotransfection with a miR-21 mimic, but not a control miRNA, efficiently suppressed expression of the luciferase reporter followed by the wild-type MSH2 3′UTR sequence, but not the mutated or scrambled sequences (Fig. 2A, middle). Transfection of an miR-21 hairpin inhibitor increased MSH2 protein levels

Table 1. Linear correlation between TGF-β 1 and MSH2 in primary human BCs by stage and grade

Group		*n* (%)	R^a	*P*
All		295 (100)	−0.25	<0.001
Grade	1	75 (25.4)	−0.36	0.001
	2	101 (34.2)	−0.31	0.002
	3	119 (40.3)	−0.17	0.06
Stage	1	82 (27.8)	−0.35	0.001
	2a	142 (48.1)	−0.27	0.001
	2b	41 (13.9)	0.05	0.76
	3–4	30 (10.2)	−0.33	0.07

[a]Pearson correlation coefficient.

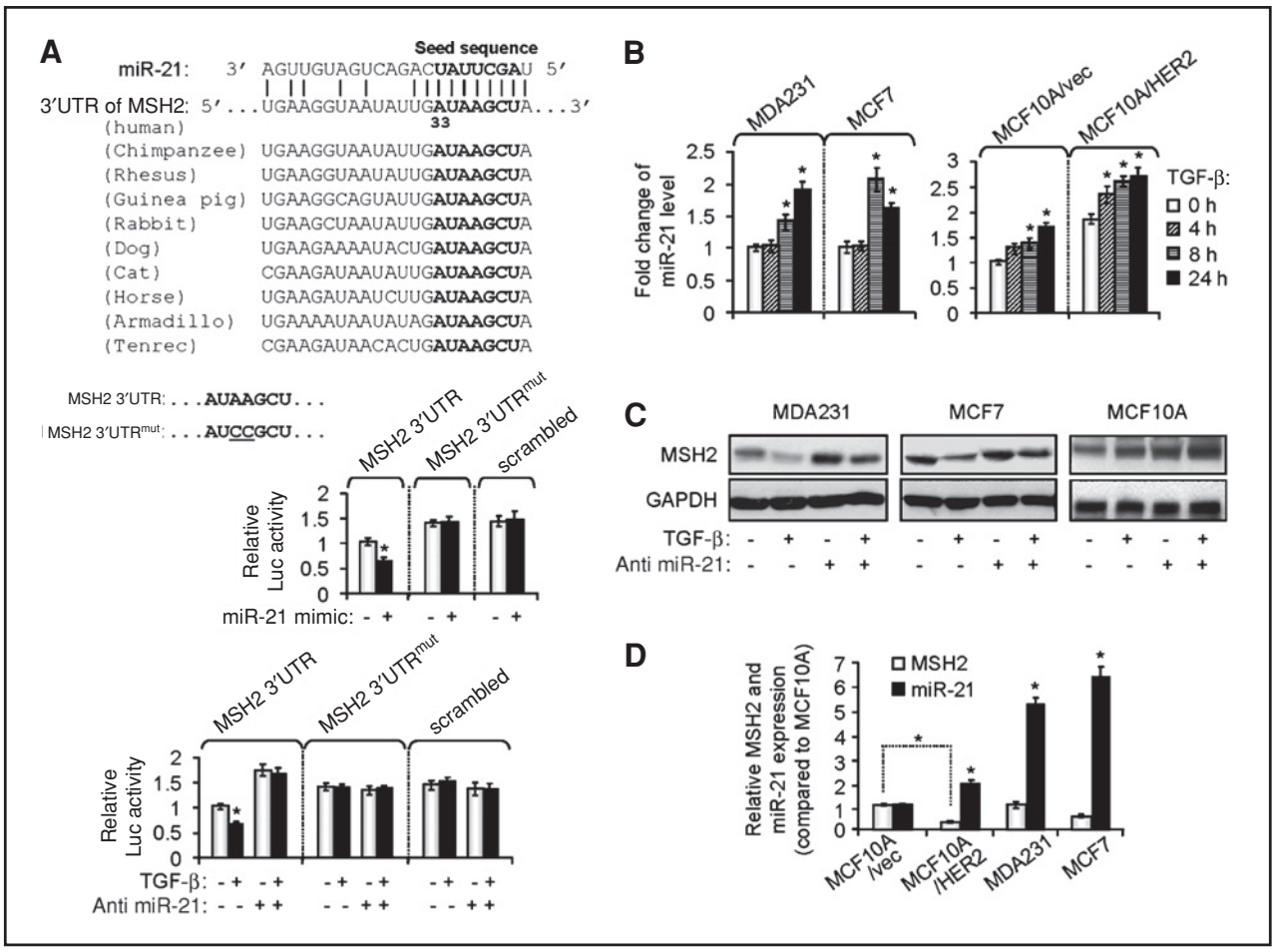

Figure 2. TGF-β downregulated MSH2 in transformed cells through inducing miR-21. A, top: the predicted miR-21 targeting site in the 3′UTR of MSH2 mRNA and the sequence conservation among various species. Middle: MDA231 cells were cotransfected with psiCHECK reporters containing the miR-21 targeting site in MSH2 3′UTR, a mutated miR-21 site (MSH2 3′UTR^{mut}) as indicated, or a scrambled sequence, together with the miR-21 mimic (or control). Bottom: MDA231 cells were cotransfected with psiCHECK reporters and miR-21 hairpin inhibitor (or control) as indicated. Luciferase activity was analyzed at 48 hours after transfection. *, $P < 0.005$. B, total RNA was isolated from cells treated with TGF-β as indicated and subjected to qRT-PCR. Data were normalized to the level of U6. *, $P < 0.005$ (compared with 0 hour). C, cells transfected with miR-21 hairpin inhibitor (or control) and treated with TGF-β (or vehicle) were subjected to Western blot. D, relative levels of MSH2 and miR-21 in transformed and nontransformed cells. The qRT-PCR data of MSH2 and miR-21 were first normalized to GAPDH and U6 in each sample, respectively, and then compared with the expression levels in MCF10A/vec cells. *, $P < 0.005$.

in various cell lines (Fig. 2C), consistent with the suppressive function of miR-21 on MSH2 expression. TGF-β has been reported to induce the maturation of miR-21 through Smad2/3, which bind to the primary transcript of miR-21 by interacting with the DROSHA miRNA processing complex and facilitate its function (3). We thereby speculated that TGF-β downregulated MSH2 by inducing miR-21–mediated posttranscriptional repression. Quantitative RT-PCR indicated that the level of miR-21 was indeed increased by TGF-β in various cell lines tested (Fig. 2B). In BC cells, treatment with TGF-β suppressed both MSH2 expression (Fig. 2C) and expression of luciferase reporter containing the miR-21 binding site in MSH2 3′UTR (Fig. 2A, bottom). These effects were impaired by transfection of the miR-21 inhibitor (Fig. 2A and C). However, in nontransformed MCF10A cells, TGF-β increased MSH2 protein level, although it was able to induce miR-21 in these cells (Fig. 2B and C). Compared with MCF10A/HER2 and BC cells, MCF10A cells expressed a lower level of miR-21 and a relatively high level of MSH2 (Fig. 2D).

TGF-β upregulated MSH2 in normal cells through Smad-mediated, p53-dependent transcriptional activation

It was intriguing that in sharp contrast to the downregulation of MSH2 in BC cells, TGF-β upregulated both MSH2 mRNA and protein levels in nontransformed MCF10A cells and primary HMEC (Fig. 3A). This effect was absent in MCF10A/HER2 cells but could be partially restored by HER2 inhibition using lapatinib, a small molecule inhibitor of HER2 kinase (Fig. 3A). Searching of the upstream promoter sequence of *hMSH2* indicated a

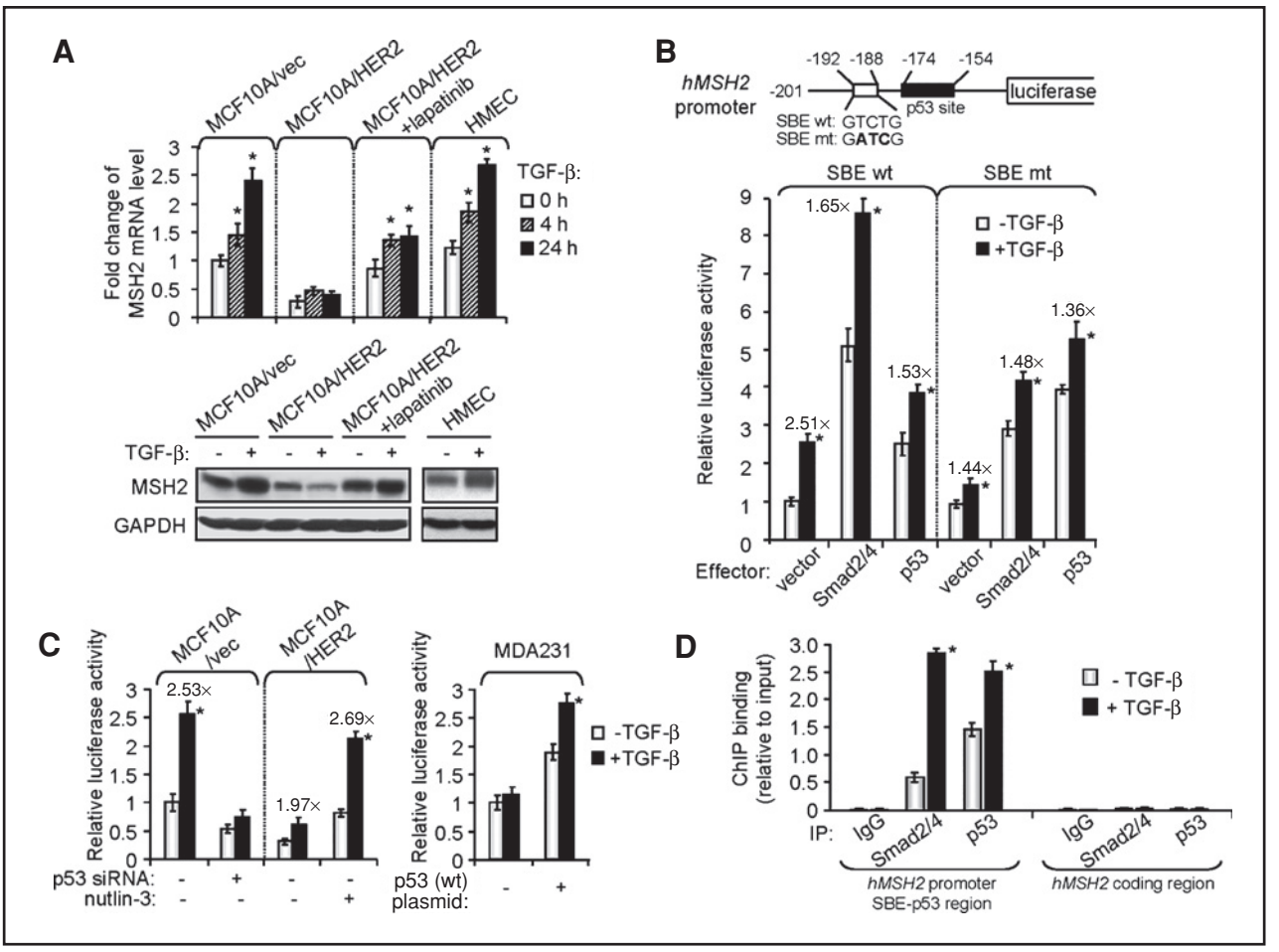

Figure 3. TGF-β upregulated MSH2 in normal cells through Smad-mediated, p53-dependent transcriptional activation. A, HMEC, MCF10A/vec, and MCF10A/HER2 cells grown in the presence or absence of lapatinib (0.5 μmol/L) for 24 hours were treated with TGF-β or vehicle. Top: qRT-PCR was carried out to determine the MSH2 mRNA levels upon TGF-β treatment. Data were normalized to untreated MCF10A/vec cells. *, $P < 0.005$ (compared with 0 hour). Bottom: Western blot of cells treated with TGF-β or vehicle for 24 hours. B, top: a schematic representative of the *hMSH2* promoter reporter indicating the positions of SBE and p53 binding site. Bottom: 293 cells were transiently cotransfected with *hMSH2* promoter luciferase reporters containing mutated or wt SBE and expression plasmids of Smad2, Smad4, p53, or vector control, and 8 hours later treated with TGF-β or vehicle. Twenty-four hours after the addition of TGF-β, the cells were harvested and tested for luciferase expression as indicated in Materials and Methods. Each bar represents the mean normalized luciferase activity ± SD calculated from 3 wells. C, left: MCF10A/vec and MCF10A/HER2 cells were transiently cotransfected with the *hMSH2* promoter reporter (SBE wt) and small interfering (siRNA) targeting p53 (or control siRNA). Eight hours posttransfection, cells were treated with TGF-β, nutlin-3 (5 μmol/L) or vehicle as indicated; 24 hours later, the cells were harvested and tested in a luciferase reporter assay. Fold change was calculated on the basis of the luciferase activity in untreated MCF10A/vec cells which was set at 1. Right: MDA231 cells were transiently cotransfected with the *hMSH2* promoter luciferase reporter (SBE wt) and the p53 expression plasmid or vector control and treated with TGF-β or vehicle for 24 hours prior to luciferase activity assay. *, $P < 0.005$. D, ChIP assay was carried out in MCF10A cells treated with TGF-β (3.5 ng/mL) or vehicle for 24 hours, using antibodies against Smad2/4, p53, or IgG (as a negative control). Quantitative PCR of the SBE-p53 region in the *hMSH2* prompter and an *hMSH2* coding region were performed using primers described in Materials and Methods. Data were compared with the input DNA control (prior to IP) in each sample. *, $P < 0.005$.

potential SBE located 14 bp upstream of a reported p53 binding site at −154 to −174 bp (Fig. 3B, top). We previously reported that TGF-β/Smad and p53 synergize to activate the expression of tumor suppressor gene *maspin*, which requires both the p53 sites and adjacent SBEs in *maspin* promoter (21). P53 can interact with Smad2 (29), providing physical evidence for a functional synergy between Smads and p53. We thereby cloned the *hMSH2* promoter region containing both SBE and p53 site to construct an *hMSH2*-luciferase reporter. Transfection assays in MCF10A cells indicated that TGF-β, or expression of Smad2/4 or p53, activated wt *hMSH2* promoter activity, whereas the promoter reporter containing mutated SBE exhibited an impaired response to TGF-β and Smad2/4, but not to p53 (Fig. 3B).

We also observed that the basal activity of transfected wt *hMSH2* promoter was significantly lower in MCF10A/HER2 cells than that in MCF10A cells (Fig. 3C, left). This suggested a lower activity of endogenous *hMSH2* promoter in HER2-transformed cells and was consistent

with the lower MSH2 mRNA and protein levels observed in these cells (Fig. 3A). Because HER2 overexpression was reported to induce downregulation of p53 in mammary epithelial cells (30), we examined that whether modulation of p53 expression/activity in transfected cells influenced MSH2 expression. Knockdown of p53 in MCF10A cells using siRNA markedly decreased basal *hMSH2* promoter activity and abolished the induction by TGF-β. Consistently, restoration of p53 in MCF10A/HER2 cells using nutlin-3, a small molecule that induces p53 stabilization by inhibiting MDM2-dependent p53 degradation (31), elevated *hMSH2* promoter activity and restored TGF-β's effect (Fig. 3C, left). MDA231 cells harbor a point mutation in codon 280 (G/A) of the *p53* gene. TGF-β failed to induce *hMSH2* promoter activity in transfected MDA231 cells, but this functional deficiency was rescued by cotransfection with a wt p53 plasmid (Fig. 3C, right). ChIP assay further confirmed the binding of Smads and p53 to the promoter region, but not a coding region of MSH2 in MCF10A cells. This binding was significantly induced by TGF-β (Fig. 3D). Thus, the Smad-mediated MSH2 promoter activation by TGF-β is dependent on an intact p53 function, and is paralyzed in some cancer cells due to p53 inactivation.

The TGF-β/miR-21/MSH2 axis regulated cancer cell response to DNA-damaging agents

MSH2 deficiency has been implicated in the tolerance to DNA damages and chemoresistance. To determine whether TGF-β contributes to resistance to DNA-damaging drugs by suppressing MSH2 in cancer cells, we tested the response of MDA231 to cisplatin, methyl methanesulfonate (MMS) and doxorubicin in the presence and absence of TGF-β. Exposure to TGF-β for 24 hours increased cell viability upon treatment with these DNA-damaging agents (Fig. 4A). In contrast, transfection of the miR-21 hairpin inhibitor further enhanced the inhibitory effect of cisplatin in MDA231 cells (Fig. 4B). Using an MDA231-derived cell line stably expressing shRNA against MSH2 under the control of a Dox-regulated promoter, knockdown of MSH2 induced resistance to both cisplatin and doxorubicin, but not docetaxel, a chemotherapy drug targeting the microtubule (Fig. 4C).

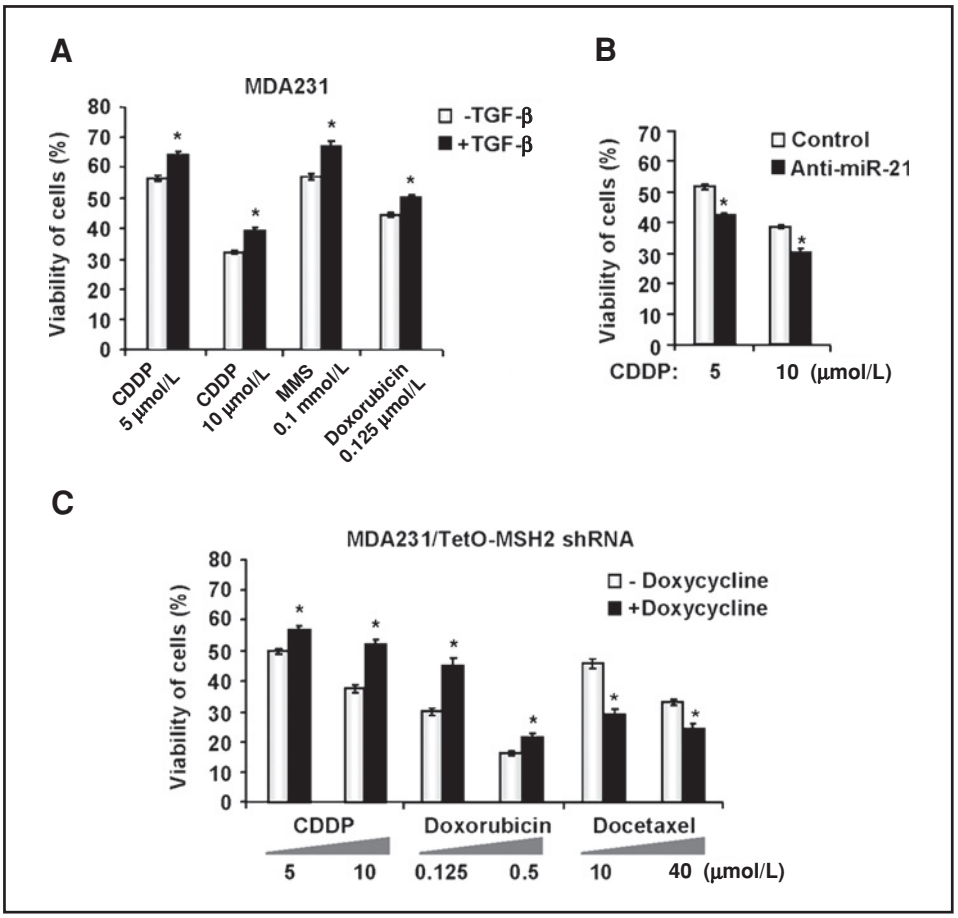

Figure 4. The TGF-β/miR-21/MSH2 axis regulates cancer cell response to DNA-damaging agents. A, MDA231 cells were treated with TGF-β (or vehicle) for 24 hours before seeded in a 96-well plate and exposed to cisplatin (CDDP), MMS, or doxorubicin in the presence or absence of TGF-β. Cell viability was assessed by MTT assay on day 3 and compared with day 0. B, MDA231 cells were transfected with miR-21 inhibitor (or control) for 24 hours before CDDP was added. MTT assay was carried out on day 3. C, MDA231/(pTIG-MSH2 shRNA) cells were treated with 1 μg/mL of Dox (or vehicle) for 24 hours before exposed to CDDP, doxorubicin or docetaxel in the presence or absence ofDox. MTT assay was carried out on day 3. *, $P < 0.005$.

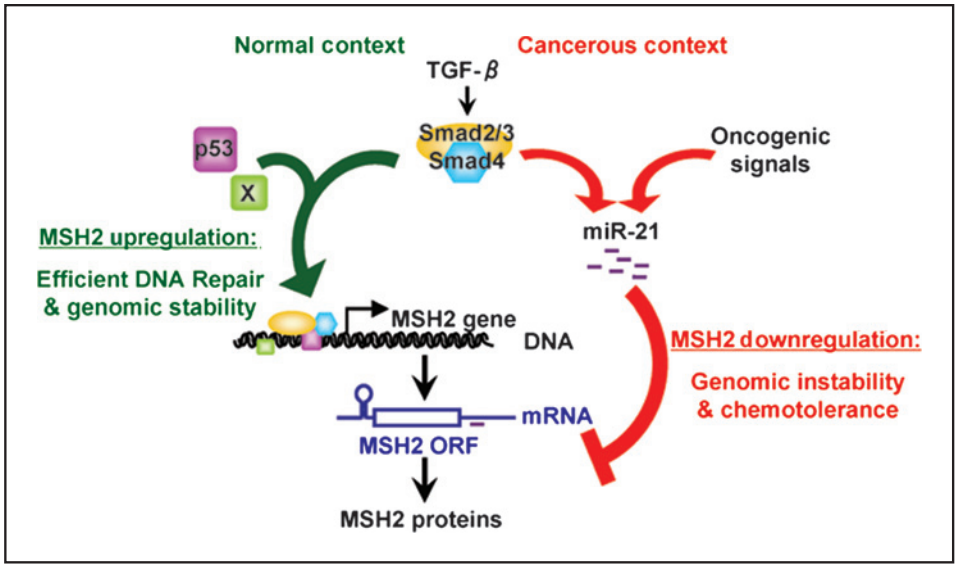

Figure 5. Bidirectional regulation of MSH2 by TGF-β. At the transcriptional level, TGF-β activates *hMSH2* promoter through a Smad- and p53-dependent mechanism, whereas at the posttranscriptional level, miR-21 induced by TGF-β targets MSH2 transcript and suppresses its expression. Thereby, in a normal cellular context with an intact p53 function and a lower level of miR-21, TGF-β predominantly upregulates MSH2 expression through promoter activation, contributing to a competent DNA repair function and the maintenance of genomic stability. In contrast, in a cancerous context that often involves p53 inactivation and miR-21 overexpression, TGF-β fails to activate *hMSH2* promoter and downregulates MSH2 expression by further inducing miR-21, resulting in genomic instability and resistance to DNA-damaging chemotherapy agents.

Discussion

TGF-β is both a tumor suppressor and a tumor promoter; its signaling output is highly dependent on the cellular context. Here, we reported a bidirectional regulatory strategy of MSH2 by TGF-β that involves both Smads and p53-dependent promoter activation and miR-21–mediated posttranscriptional inhibition. Thus, in a normal cellular context with an intact p53 function and a lower level of miR-21, TGF-β predominantly upregulates MSH2 expression through promoter activation, which may contribute to a competent DNA repair function required for genomic stability. In contrast, in a cancerous context that often involves p53 inactivation and miR-21 overexpression, TGF-β fails to activate *hMSH2* promoter and downregulates MSH2 expression by further inducing miR-21, which may result in genomic instability and resistance to DNA-damaging chemotherapy agents (Fig. 5).

The intrinsic DNA binding activity of Smads is relatively low and unspecific. Thereby, the contextual function of Smads relies on interactions with other transcriptional factors with more potent and specific DNA binding capacity. We have previously reported that TGF-β activates the promoter of tumor suppressor *maspin* by inducing binding of Smads and p53 to an adjoint SBE-p53 site (21). A number of genes are synergistically regulated by Smads and p53, and many of them are tumor suppressors. A common feature of the promoter regions of these genes is the presence of adjoint or adjacent p53 binding sites and SBEs, usually within a 100-bp segment (29). This pattern was also observed in the *hMSH2* promoter (Fig. 3B). Because the efficient regulation of these genes by TGF-β and Smads requires an intact p53 signaling, this TGF-β function is abolished in a context with deficient p53 function, which is frequently observed in cancer. Mutations or deletions of the *p53* gene are observed in 60% to 70% of human cancers; many cancers with a wt p53 lose normal p53 function, suggesting that additional factors are involved in p53 inactivation. Overexpression of HER2 has been reported to decrease p53 level via activating PI3K pathway and inducing MDM2 nuclear translocation (30). Our study also indicated that HER2 overexpression impaired p53-dependent transcriptional regulation of MSH2 by TGF-β, which could be restored by nutlin-3 (Fig. 3C). Therefore, the functional status of p53 may serve as a determinant in many cancers for their response to TGF-β–mediated gene regulation. In addition, promoter methylation is frequently observed in cancer and is another important mechanism resulting in silenced expression of tumor suppressor genes. This also results in the abolishment of TGF-β function, as in the case of *maspin*. Although the methylation status of *hMSH2* was not determined in this study, its hypermethylation has been documented in hereditary nonpolyposis colorectal cancers (32).

MiR-21 is overexpressed in many types of cancer and is known as an onco-miRNA. It is reported that TGF-β promotes the processing of primary transcripts of miR-21 (pri-miR-21) into precursor miR-21 (pre-miR-21) through the interaction of Smads and DROSHA microprocessor complex, resulting in an increase of mature miR-21 (3). In primary BCs, high miR-21 expression is positively correlated with TGF-β1 and is associated with features of aggressive disease (33). Here, we showed that TGF-β

upregulated miR-21 in both nontransformed (MCF10A) and transformed (MCF10A/HER2, MDA231, MCF7) cells (Fig. 2B). This suggests that the Smads and Drosha-mediated miR-21 upregulation is likely to be a general mechanism. However, both the basal and induced levels of miR-21 are significantly higher in transformed cells (Fig. 2B and D), consistent with the increased abundance of this onco-miRNA in primary cancers. Although the mechanism underlying this is not yet fully understood, it may involve oncogenic signaling, such as HER2, which may influence the biogenesis of miR-21. As a result, the posttranscriptional regulation of MSH2 by this miRNA, which is a fine-tuning mechanism usually weaker than transcriptional regulation (as such in nontransformed MCF10A cells), becomes a dominant regulatory mechanism in cancer. The abolished p53-dependent promoter activation and/or the promoter methylation in cancer cells also affect the equilibrium between the 2 mechanisms. The ultimate effect of TGF-β on MSH2 expression thereby reflects the result of the antagonism between the 2 regulatory pathways, both determined by factors preset in the cellular context.

The majority of chemotherapy drugs produce a cytotoxic effect by causing damage in the DNA of cancer cells, resulting in cell death or growth arrest. DNA-damaging agents are also the first drugs developed for the treatment of cancer and include several families which cause different types of DNA damage such as the alkylating agents, the platinum-based drugs, and the antitumor antibiotics (34). Chemotherapy drugs are often given in combination to optimize clinical outcomes. However, a durable response is only achieved in a fraction of cancer patients. Several mechanisms of drug resistance have been proposed including gene mutations, upregulation of drug transporters, and the involvement of cancer stem cells (35, 36). A recent work with 47 BC patients received doxorubicin (a DNA intercalating agent) or docetaxel (a microtubule-targeting agent) treatment indicates that genes related to TGF-β signaling are upregulated in doxorubicin-resistant, but not docetaxel-resistant tumors, compared with drug-sensitive tumors (37). Our study herein further suggests that downregulation of MSH2 may induce resistance to DNA-damaging agents (cisplatin and doxorubicin), but not docetaxel (Fig. 4C). These results may suggest differential functions of the TGF-β/MSH2 axis in cancer response to different chemotherapy agents and may provide clinical implications in the selection of effective chemotherapy regimens. Nonetheless, a direct association between TGF-β level/activity and drug resistance in BCs remains to be explored and will be one of our future directions, to precisely evaluate the therapeutic value of TGF-β interventions, especially when combined with conventional DNA-damaging agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. John J. Rossi and Dr. Jeremy Stark for kindly providing reagents; Dr. Shiuan Chen and Dr. Susan Kane for valuable comments; and colleagues in the Division of Tumor Cell Biology for enthusiastic support and discussion.

Grant Support

This work was supported by NCI R00 CA125892 (S. E. Wang); CBCRP 16IB-0081 (S. E. Wang).

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Received 08/09/2010; revised 09/21/2010; accepted 10/11/2010; published OnlineFirst 10/14/2010.

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