Wild-type p53 controls cell motility and invasion by dual regulation of MET expression

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Recent observations suggest that p53 mutations are responsible not only for growth of primary tumors but also for their dissemination. However, mechanisms involved in p53-mediated control of cell motility and invasion remain poorly understood. By using the primary ovarian surface epithelium cell culture, we show that conditional inactivation of p53 or expression of its mutant forms results in overexpression of MET receptor tyrosine kinase, a crucial regulator of invasive growth. At the same time, cells acquire increased MET-dependent motility and invasion. Wild-type p53 negatively regulates MET expression by two mechanisms: (i) trans-activation of MET-targeting miR-34, and (ii) inhibition of SP1 binding to MET promoter. Both mechanisms are not functional in p53 absence, but mutant p53 proteins retain partial MET promoter suppression. Accordingly, MET overexpression, cell motility, and invasion are particularly high in p53-null cells. These results identify MET as a critical effector of p53 and suggest that inhibition of MET may be an effective antimetastatic approach to treat cancers with p53 mutations. These results also show that the extent of advanced cancer traits, such as invasion, may be determined by alterations in individual components of p53/MET regulatory network.

Transcriptional factor p53 provides integrated responses to implement cell cycle arrest, senescence, differentiation, inhibition of cancer metabolism, or induction of the apoptotic cascade (1). Mutations of p53 occur in about 50% of all cancers and result in loss of its function, either by null phenotype or dominant-negative effect. Additionally, some mutations result in new activities of p53, known as gain-of-function mutations (2). Recent observations indicate that p53 mutations affect cell motility and invasion, key features of metastasis (3–8). Better understanding of mechanisms of p53-dependent effects on cell motility and invasion should lead to development of approaches aimed toward correction of aberrant p53 signaling not only for suppressing growth of primary tumors but also for preventing their dissemination.

A signaling conduit known to play a critical role in invasion and metastasis is the MET pathway (9). The MET proto-oncogene encodes a transmembrane receptor-protein tyrosine kinase, whose overexpression is associated with poor prognosis in a broad variety of cancers (10, 11). Inhibition of MET functions has been shown to be effective in animal models and is among the most promising candidates for targeted therapy (10).

Previously it has been reported that MET is overexpressed in tumors of p53-deficient mice and in Li-Fraumeni patients (12). This observation is consistent with recent reports that MET represents one of the common targets for the miR-34 family (13–15). Genes encoding for the miR-34 family have been identified as direct targets of p53 transactivation (reviewed in ref. 16). At the same time, it has been reported that mouse Met promoter has a putative p53 responsive element and that Met promoter activity is activated by p53 through DNA binding to the p53 consensus sequence (17). Thus, the role of MET in p53-dependent suppression of invasion remains uncertain.

Because many cancers are genomically unstable and separation of critical alterations from “genetic noise” may be a daunting task in cells derived from advanced stages of the disease, we have used a model of conditional p53 inactivation in the primary ovarian surface epithelium (OSE), transformation of which leads to epithelial ovarian cancer (EOC) (18, 19). This system is highly clinically relevant because p53 mutations are by far the most frequent alterations in human high-grade serous adenocarcinoma of the ovary (20), are detected in the stage 1 of those cancers and in adjacent dysplastic lesions (21, 22), and their presence correlates with metastatic potential (23). MET overexpression is also associated with poor prognosis of EOC patients and targeting the MET pathway has been reported to suppress EOC in mouse models (11).

We report that MET is a critical player in p53-mediated control of motility and invasion, and show that such control includes miR-34–independent regulation of MET expression by p53, in addition to earlier described MET targeting by miR-34. Alterations in individual components of the p53/MET regulatory network may affect the extent of cancer invasion.

Results

p53 Inactivation Leads to MET Overexpression. To evaluate immediate transcriptome changes associated with p53 inactivation, we conducted mRNA microarray analysis of primary OSE cells after acute inactivation of p53, Rb, or both p53 and Rb concomitantly (Fig. 1A and Fig. S1). Interestingly, in addition to the expected targets of p53 and Rb/E2f signaling, up-regulation of the Met proto-oncogene was detected as a consequence of p53 and p53/Rb inactivation, but not of inactivation of Rb alone (Fig. S1). According to qRT-PCR (Fig. 1B) and Western blot analysis (Fig. 1C), MET expression levels continued to rise for 72 h after gene inactivation and were particularly high in neoplastic OSE cell lines (over 40 passages) deficient for p53 (OSN2) or p53 and Rb (OSN1). Consistently, p53 knockdown in human ovarian cancer cells OVC4A33 and colon cancer cells HCT116 carrying wild-type p53 resulted in increased MET expression (Fig. S2A). To examine whether MET overexpression would be detected in vivo early after p53 inactivation, Ad-Cre was delivered to the OSE of p53fl/fl/Rbfl/fl Z/EAG mice by transoviductal injection. Consistent with the cell-culture experiments, elevated levels of MET were detected in OSE cells that had Cre-loxP–mediated recombination according to expression of EGFP reporter 72 h after Ad-Cre administration, but Ad-Blank administration did not result in detectable MET or EGFP expression (Fig. 1D). No morphologically detectable differences were observed between mutant and wild-type OSE at that time (Fig. S2B).
MET Is Essential for p53-Controlled Cell Motility and Invasion. Because increased motility and invasion are among the principal effects of up-regulated MET signaling, those features were tested in p53-deficient OSE cells. Compared to cells with wild-type p53, p53-null cells showed significantly increased cell motility in wound healing/time-lapse microscopy and migration assays, as well as increased propensity for invasion in Matrigel chambers (Fig. 2 A–D, Fig. S3A, and Movies S1 and S2). Moreover, treatment of cells with MET ligand HGF enhanced migration and invasion even further (Fig. 2A and B). The increased motility and invasion was accompanied by elevation of levels of phosphorylated MET (Fig. S3B). To test the extent of Met contributions to these properties of p53-null cells, both genes were inactivated in OSE cells derived from p53<sup>fl/fl</sup>Met<sup>fl/fl</sup> mice. Met inactivation abrogated the motility and invasion, but not proliferation phenotype associated with p53 inactivation (Fig. 2E and Fig. S4). Thus, MET signaling is essential for p53-controlled motility and invasion.

p53 Has a mir-34-Independent Mechanism of MET Regulation. Consistent with regulation of MET expression by p53, expression of p53 at levels comparable to those of endogenous activation resulted in decreased amounts of MET protein in both mouse and human neoplastic ovarian cell lines, OSN2, and SKOV-3, respectively (Fig. 3A and Fig. S5).

To test the extent of MET expression dependence on miR-34, ovarian neoplastic cells were subjected to increasing amounts of miR-34 precursor molecules in transient transfection experiments. Maximum reduction of MET expression was observed at 30-nM concentration, with no apparent effect of further increases in amounts of miR-34a precursor molecule (Fig. S6A and B) or combination of miR-34a, b, and c (15). No effect on MET promoter activity was observed after transfection of the full-length MET promoter reporter construct (pGL2-3.1MET) together with individual miR-34 family precursor molecules (Fig. S6C). These observations were in agreement with bioinformatics predictions and experimental evidences that miR-34 regulates MET expression principally by targeting MET 3′UTR (reviewed in ref. 16). However, cotransfection of miR-34a precursor together with wild-type p53 resulted in further down-regulation of MET (Fig. 3A), suggesting that p53 may have a miR-34-independent mechanism of MET regulation. Similar effects of p53 and miR-34a precursor transfection on MET down-regulation were observed in p53-null lung cancer cells NCi-H1299, indicating potential significance of these observations for pathogenesis of other types of epithelial cancers (Fig. S6D).

To directly show the presence of miR-34-independent mechanism of MET regulation by p53, we isolated OSE cells from mir-34a<sup>−/−</sup>mir-34b/c<sup>−/−</sup> (triple knockout, TKO) mice, lacking the entire mir-34 family of genes. As expected, OSE cells from TKO

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p53 Suppresses MET Promoter Activity. To test if MET promoter activity can be affected by p53 through binding of p53 consensus sequences in ovarian cells, reporter constructs containing -2619 to +353 fragments of MET upstream sequence (3.1MET) and its shorter fragments (24) were cotransfected with p53 expression vector into SKOV-3 (Fig. 4) and OSN2 cells (Fig. S7 A and B). Transfection of p53 significantly suppressed the promoter activity of the 3.1MET construct, as well as that of smaller fragments. Even when the putative p53 responsive element was mutated (0.65METm), p53 was still capable of repressing MET promoter activity comparatively to the repression by corresponding wild-type promoter construct (0.65MET), indicating that the discrepancy between a previous report (17) and our findings may be a result of cell-type–specific effects. To rule out the possibility that the observed effects of p53 on MET promoter activity were caused by global transcriptional repression by ectopically expressed p53, PG-13-huc and MG-15-huc reporter constructs, containing 13 copies of wild-type p53 consensus sequence and 15 copies of mutated p53 consensus sequence (25), respectively, were cotransfected with plasmids expressing either wild-type or DNA binding domain mutant p53. Only wild-type p53 could activate PG-13 promoter activity in SKOV3 (Fig. S7C) and OSN2 cells (Fig. S7D); all four mutant p53 constructs were unable to activate both promoter constructs.

In conclusion, p53 has been shown to inhibit hypoxia-inducible factor-stimulated transcription by destabilizing hypoxia-inducible factor 1a (HIF1A), one of the prominent regulators of MET promoter activity (26). However, p53 inactivation did not lead to accumulation of HIF1A in OSE cells (Fig. S8A), likely because of uneven sensitivity of different cell types to hypoxia (27). Consistent with cell-type–specificity, OSE cells and ovarian cancer cell lines (OVCA432, OVCA433, and OVCAR-3) did not overexpress MET under hypoxic conditions regardless of HIF1A accumulation (Fig. S8B), unlike colon cancer HCT-116 cells (Fig. S8 C and D).

Another potential mechanism for p53-dependent regulation of MET is through CD44. CD44 is known to form a multimeric complex and cooperate with MET (28). CD44 has also been recently identified as a p53 (29) and miR-34 (30) target. However, CD44 suppression by p53 knockdown in TKO OSE cells led to an increase of MET expression (Fig. 3D), confirming that p53 down-regulates MET expression in a miR-34–independent manner, in addition to miR-34–dependent mechanism (Fig. 3E).

p53 Inhibits SP1 Binding to MET Promoter. Because p53 was able to suppress activity of nonoverlapping promoter constructs 0.27MET and 0.37MET, transcription-factor binding sites common for both constructs were searched for by bioinformatics analysis. This search identified binding sites for the SP1 transcription factor (Fig. S7E). To test whether SP1 is required for MET promoter activity, SKOV-3 and OSN2 cells were treated with mithramycin A (mitA) to inhibit DNA binding of SP1 (31). MitA treatment suppressed luciferase expression under control of MET promoter in a dose-dependent manner, indicating that SP1 is required for MET promoter activity (Fig. 5A and Fig. S7F). This suppression was not increased by p53 expression, indicating that p53 and SP1 share a common molecular pathway to regulate MET promoter activity (Fig. 5B).

Coimmunoprecipitation experiments demonstrated that either ectopic or endogenous p53 physically interacts with endogenous SP1 in SKOV-3 and OVCA433 cells, respectively (Fig. 5 C–E). To test if p53 may inhibit SP1 DNA binding ability, ChIP experiments were performed with SP1 antibody and amplicons covering the distal and proximal region of the MET promoter, which contain and lack SP1 sites, respectively (Fig. 5F). SP1 binding was selectively enriched on the proximal region of the MET promoter.
MET promoter, and p53 expression resulted in significant reduction of SP1 binding (Fig. 5G and H). Conversely, as expected, p53 did not affect SP1 binding to the survivin promoter (Fig. 5G), which is repressed by p53 through recruitment of chromatin remodeling complexes (32). Consistently, in mouse OSE cells, p53 inactivation led to an increase of SP1 binding to the corresponding mouse Met promoter proximal region (Fig. 5J). Taken together, these results show that p53 is likely to suppress MET promoter activity through inhibition of SP1 DNA binding.

**Mutant p53 Proteins Interact with SP1 and Their Effects on OSE Motility and Invasion Depend on MET.** To explore the role of mutant p53 in Met regulation, four expression vectors encoding DNA binding domain p53 mutants (V143A, R175H, R249S, and R270H) were transfected with MET promoter reporter constructs into SKOV-3 and OSE2 cells. All mutant p53 vectors suppressed 0.65MET and 0.27MET constructs, albeit less efficiently when compared with wild-type p53 (Fig. 6A and B, and Fig. S9A and B). Accordingly, the mutant p53 proteins were communoprecipitated with SP1 (Fig. 6C), indicating that at least these common p53 mutants retain interactions with SP1.

Cells of human ovarian cancer cell lines OVCA432 and OVCAR-3 carrying mutant p53 did not show an increase of already high MET levels after p53 knockdown (Fig. S9C). To further explore the role of mutant p53 in motility and invasion, primary OSE cells were prepared from p53+/R270H and p53+/LSLR172H mice, which contain a conditionally activated copy of mutant p53 corresponding to human p53 R175H and R273H hot-spot mutations, respectively. Despite overall increased p53 expression after Ad-Cre infection (Fig. S10A), Met expression increased only slightly (Fig. 6D). Although loss of both p53 copies is sufficient to immortalize OSE cells (18), p53+/R270H and p53+/LSLR172H OSE cells had a very limited proliferation potential and underwent senescence (Fig. S10B), consistent with a previously reported phenotype of mouse embryonic fibroblasts (MEFs) carrying the same p53 mutations (33).

Similarly to MEF immortalization after the loss of remaining wild-type copy of p53, OSE2 cells from p53+/LSLR172H mice were easily immortalized after Ad-Cre infection. In these cells, levels of Met expression were higher than those in cells heterozygous for mutant p53 but less than those in a p53-null background (Fig. 6E). Cells carrying mutant R172H or R270H p53 also displayed increased cell invasion, although at a lesser extent than p53 null cells (Fig. 6F). Similarly to observations in p53-null cells, deletion of Met abrogated increased migration and invasion associated with mutant R172H or R270H p53 (Fig. 6G and Fig. S10C).

**Discussion**

Our work shows that p53 controls the expression of the proto-oncogene MET by two mechanisms, consisting of suppression of MET on the transcriptional level via promoter repression and on the posttranscriptional level via transactivation of miR-34. Contrary to a previous report (17), we were unable to find any canonical or novel p53 binding sites selectively responsible for either activation or repression of MET promoter. At the same time, the results of our promoter analysis, together with coimmunoprecipitation and ChIP assays, provide support for a mechanism of MET transcriptional repression based on inhibition of SP1 binding to DNA through physical interactions between p53 and SP1 rather than on direct promoter-binding by p53. Consistent with this possibility, it has been previously reported that SP1 activates MET promoter activity (34, 35) and interacts with wild-type (36, 37) and mutant p53 (2). Furthermore, p53 inhibits SP1 DNA binding to the HIV-LTR and MGMT promoter in vitro (38, 39).

Either lack of p53 or expression of its mutant forms abrogates miR-34 transactivation of miR-34 (16). Therefore, both types of
mutations result in elevation of miR-34-dependent MET expression. However, our study shows that unlike null mutations, mutant p53 protein retains MET promoter-suppressive function, albeit to a lesser degree than wild-type p53. Consistent with the lesser extent of MET expression, cells expressing mutant p53 display a lower MET-dependent motility and invasion compared with null mutants. These findings suggest that specific alterations in individual components of the p53/MET signaling network may modulate the course of pathological process. Consistent with this possibility, the poorest prognosis of p53 null mutations has been reported in some types of neoplasms, including ovarian, lung, and breast cancers (40-42).

Recent studies indicate that lack of p53 versus expression of mutant p53 proteins may have a different impact on motility and invasion in the context of particular cell types, as well as additional genomic alterations. Our observations of increased motility and invasion by p53-null OSE cells are consistent with previous studies that have shown comparable effects in p53-null immortalized fibroblasts and MEFs (3, 4). Similarly, non-small-cell lung carcinoma line NCI-H1299 cells harboring a p53-null mutation have been reported to migrate faster than those expressing R175H mutant p53 (43).

At the same time, some investigators have reported that mutant p53 protein but not null p53 mutations may drive cell motility and invasion by promoting integrin recycling (5), by forming a complex with Smad to oppose p63-mediated control of putative metastasis suppressors Sharp-1 and cyclin G2 (6) or by stabilizing the invasion promoter Slug (7). Consistent with cell-type specificity of p53 effects, mice expressing mutant p53 develop a more diverse spectrum of neoplasms compared with null mutants (33). Interestingly, in line with observations by Grugan et al. (44), knockdown of mutant p53 in ovarian carcinoma cells did not change levels of MET expression. This observation is at variance with our results on primary OSE cells carrying a conditional mutant p53 allele, which may indicate that established cancer lines and neoplastic cells at advanced cancer stages develop additional mechanisms ensuring MET signaling in cells carrying mutant forms of p53 protein. Assessment of primary ovarian cancer cells should address this problem in future studies. It should be also of interest to evaluate p53/MET signaling in the fallopian tube epithelium, another potential cell of origin of serous adenocarcinoma of the ovary (45, 46).

There are indications that some additional genetic or epigenetic alterations are required for stabilization and accumulation of mutant p53, thereby leading to a gain-of-function phenotype (47). In agreement with this possibility, our results in primary OSE from p53+/LSLR270H or p53+/R270H mice, as well as studies by others in MEFs with the same genotypes (33), show that cell immortalization is acquired only after the loss of the remaining wild-type copy of p53. Consistently, Adorno et al. (6) were able to observe increased migration of cells expressing mutant p53 only in combination with HRAS and TGF-β signaling. Notably, RAS mutations are extremely rare in high-grade serous ovarian carcinoma (23).

Development of therapies aimed at correction of the p53 pathway remains among the most coveted goals in cancer research (48). Importantly, our experiments have demonstrated that MET is a critical component of motility and invasion in cells either lacking p53 or expressing its mutant forms. Therefore, treatment of cancers with p53 mutations is likely to benefit from therapeutics aimed at MET, such as small targeting molecules (10). This finding is of particular significance because, despite successful outcomes in cell culture and animal in vivo experiments, direct reintroduction of the p53 gene failed in clinical trials, including EOC (49). Our study also indicates that approaches aimed at indirect elimination of mutant p53 protein (e.g., by p53 siRNA) should be avoided in cancers with an active p53/MET signaling network. The in-depth understanding of mechanisms by which p53 regulates MET in the context of different cell types, as well as specific p53 mutations, may be essential for future development of individualized therapeutics.

**Materials and Methods**

**Experimental Animals.** The origin and genotyping of mice with conditional alleles of wild-type and mutant p53, Rb1, Met, and reporter genes are described in the SI Materials and Methods. The mir-34a-1* miRNA generated in H.H.’s laboratory and mir-34b/c* miRNAs generated in A.Y.N.’s laboratory were crossed to obtain mir-34a-1* mir-34b/c* mice. All mice were maintained identically following recommendations of the Cornell Institutional Laboratory Animal Use and Care Committee.

**Cell Culture.** Derivation and culture conditions of primary mouse OSE cells, established mouse OSE cell lines (OSN1 and OSN2), and human cancer cell lines SKOV-3, OVCAR-3, OVCAR432, OVCAR433, NG-H1299, and HCT116, as well as proliferation and senescence assays, are described in SI Materials and Methods.

**Conditional Gene Inactivation and mRNA Microarray Profiling Studies.** Primary OSE cells carrying conditional gene alleles were passaged three times and treated with recombinant adenoviruses essentially as described previously (18, 19). Cells were collected two passages after infection and processed for...
mRNA isolation and assessment of mRNA profiles as described in SI Materials and Methods.

Transinfundibular Administration of Adenovirus, Collection of Histological Materials, and Immunohistochemistry. All procedures were performed as described previously (18). The detailed protocol MET/EGFP double immunofluorescence is provided in SI Materials and Methods.

Wound Healing, Time-Lapse Microscopy, Migration, and Invasion Assay. For wound-healing assay, OSE cells were infected with adenovirus and cultured in a 10-cm gelatin-coated dish to confluence. Cells were scraped with a 200-tip, and fresh medium supplemented. Digital images of the wound were taken at 0, 12, and 24 h after scraping. Analyses of the wound-closure and time-lapse microscopy are described in SI Materials and Methods. Migration and invasion assays were performed as described in ref. 15.

14. Chi JT, et al. (2006) Coimmunoprecipitation, Western Blotting, ChIP Assays, and Statistics. Promoter Activity Analysis and Transfection, Quantitative Real-Time RT-PCR, Communoprecipitation, Western Blotting, Chip Assays, and Statistics. All procedures were performed according to established methods as described in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Experimental Animals. Mice with floxed copies of p53, Rb1, Met, and p53 mutant allele genes and genotyping PCR information were described elsewhere (1–4). Z/EG reporter mice (5) were purchased from the Jackson Laboratory.

Cell Culture. Primary mouse ovarian surface epithelium (OSE) cells and OSE cell lines (OSN1: p53 and Rb-null; OSN2: p53-null) were isolated and cultured as previously described (6–8). Human ovarian cancer cell lines SKOV-3 (p53-null), OVCAR-3 (p53 mutant), lung cancer cell line NCI-H1299 (p53-null), and colon cancer cell line HCT116 (wild-type) were obtained from the American Type Culture Collection and maintained according to the supplier’s directions. Human ovarian cancer cell lines OVCA432 (p53 mutant) and OVCAR-3 (wild-type) were maintained as described previously (9, 10). Transfection was performed by using lipofectamine 2000 (Invitrogen), and control (sc-37007) and p53 siRNA (sc-29436 and sc-29435 for mouse and human, respectively) were purchased from Santa Cruz Biotechnology. Mithramycin A and doxorubicin were purchased from Sigma, and HGF from R&D Biosystems. In experiments with conditional gene inactivation, the efficiency of adenoviral Cre:EGFP fusion protein expression. Cre-mediated gene excision was at similar 90% according to use of adenoviral Cre:EGFP fusion protein-gene inactivation, the efficiency of adenoviral Cre:EGFP fusion protein expression. Cre-mediated gene excision was at similar 90% according to use of adenoviral Cre:EGFP fusion protein-gene inactivation, the efficiency of adenoviral Cre:EGFP fusion protein expression.

Cell Proliferation and Senescence Assays. Proliferation of OSE was assessed by BrdU incorporation, as described elsewhere (6). For detection of senescence-associated β-galactosidase, OSE cells were fixed with 3% formaldehyde, rinsed several times with PBS, and incubated in staining solution (4.2 mM citric acid, 12.5 mM sodium-phosphate, 158 mM sodium chloride, 0.21 mM magnesium chloride, 2.21 mg/mL potassium ferrocyanide, 1.68 mg/mL potassium ferricyanid, 1 mg/mL X-Gal, pH 6.0) for 24 h at 37 °C.

mRNA Isolation and Microarray Profiling. Total RNA was prepared using RNeasy lipid tissue mini kit (Qiagen). The generation of biotin-labeled cRNA fragmentation, hybridization to the Affymetrix murine U74Av2 arrays, washing, and scanning were done according to Affymetrix protocols. The gene expression signals from Affymetrix GCOS software were normalized by scaling each GeneChip to a target signal of 500. Log ratio was calculated with the average value of control groups. Analysis was performed by using Significant Analysis of Microarrays (SAM) software (http://www-stat.stanford.edu/~tibs/SAM/) with twofold setting and 1% false-discovery rate (FDR) of genes (11). Identified profile was visualized in TreeView software (http://rana.lbl.gov).

Quantitative Real-time RT-PCR. Total RNA was isolated using Qiazol (Qiagen) according to the manufacturer’s protocol. Total RNA was reverse transcribed using SuperScript III (Invitrogen) and oligo-dT primers. The PCR reactions were done in a 30-μL volume in a 96-well plate using predeveloped FAM TaqMan probes (Applied Biosystems). Mouse and human Gapdh were used as endogenous reference control (predeveloped TaqMan assay from Applied Biosystems). Cycling parameters were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Increase in real-time fluorescence was measured by an ABI7500 qPCR system and relative fold-changes were calculated using the $2^{-ΔΔCt}$ method (12).

Immunohistochemical Analysis. Double immunofluorescence staining of frozen sections was done with rabbit polyclonal antibody to EGFP (Clontech; 632377, 1:100) or to rabbit polyclonal MET (Santa Cruz Biotechnology; SP260, 1:100), followed by fluorescein (FITC)-conjugated anti-rabbit and rhodamine (TRITC)-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories; 1:50). To stain cell nuclei, sections were incubated with a 10-μg/mL solution of DAPI (Sigma-Aldrich) for 4 min.

Wound-Healing and Time-Lapse Microscopy Assays. Percentage of closed-wound area was measured by using TScratch software (13). For the time-lapse microscopy, cells were grown until confluent and then partially scratched by a 2-mm wide plastic space. Twenty-four hours after scratching, complete culture medium were replaced with CO2-independent medium supplemented with 5% FBS. Cell movements were captured using a camera on a microscope at 20x objective in a 37 °C chamber. Image of the cells was taken every 15 min for 5 h, for a total of 21 images. Individual cell movements were tracked by using ImageJ, Multitrack software.

Promoter Analysis. The pGL2-3.1MET construct consists of a 3 Kb upstream sequence and 131-bp 5’UTR of human MET gene. The pGL2-0.65MET construct consists of a 653-bp upstream sequence including 131-bp 5’UTR (14). The 0.37MET construct was cloned from −512 to −136 of MET 3.1 fragment. The pGL2-0.27MET construct consists of 136 bp of MET upstream sequence and 131 bp of 5’UTR. The corresponding human sequence of previously reported pMET binding site (15) in mouse Met promoter (from −289 to −256) was mutated. The 5’GGACGGACGGACGCGGAGGAACAGACCGT3’ sequence was changed into 5’GGACGGACGGACGCGGAGGAACAGACCGT3’. To measure luciferase activity, cells were processed with the Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions, and 10 μL of cell lysates was used to determine reporter enzyme activity using Lumat LB 9501 luminometer (Berthold). Lipofectamin 2000 reagent (Invitrogen) was used for the transfection following the manufacturer’s recommendations. pORF-hp53 was purchased from InvivoGen. Pre-miR miRNA precursor molecules of miR-34a, b, and c, as well as control pre-miR, were purchased from Ambion. Each experiment was performed in triplicate. Luciferase activities were normalized to total protein concentrations. The BCA method ( Pierce) was applied for measuring protein concentrations.

Western Blot Analysis. Cell lysates were prepared using RIPA buffer (Tris-HCl 50 mM, pH 7.4; Nonidet P-40 1%; Na-deoxycholate 0.25%; NaCl 150 mM; EDTA 1 mM; PMSF 1 mM; Aprotinin, leupeptin, pepstatin: 1 μg/mL each; Na2VO4 1 mM; NaF 1 mM), separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was incubated overnight at 4 °C with antibodies to detect MET (SP 260 for mouse and C28 for human, Santa Cruz Biotechnology; 1:1,000 dilution for both), p53 (FL 393 from Santa Cruz Bitechology; 1:1,000 dilution), CD44 (2C5; R&D Systems, Inc.), HIF1A (H1alpha67; NOVUS Biologicals), phospho-MET (Y1234/1235; Cell Signaling Technology), SP1 (Millipore), and GAPDH (Advanced Immunohistochemical Inc.; 1:3,000 dilution), followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and developed using chemiluminescence substrate (SuperSignal West Pico from Pierce).
Commmunoprecipitation. Lysates of were prepared in modified Lysis250 buffer (50 mM Tris-Cl pH 7.4, 100 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40 100 mM). The antibodies used for immunoprecipitation were polyclonal anti-SP1 (Millipore), polyclonal anti-p53 (Santa Cruz Biotechnology), and monoclonal anti-p53 (Cell Signaling Technology). Cell extracts were incubated at 4 °C overnight with 2 μg of corresponding antibodies, followed by incubation with 20 μL of Sepharose G bead (GammiBind Plus Sepharose, GE Healthcare) for 2 h at 4 °C. Immunoprecipitates were isolated by centrifugation and followed by washing five times with lysis buffer. Samples were resuspended in the 2X sample buffer, subjected to 12% SDS-PAGE, transferred onto a PVDF membrane, and the immunoprecipitated proteins were detected by Western blotting.

Chromatin Immunoprecipitation. SKOV-3 cells were grown on 10-cm dishes and transfected with control and p53 expression vector, respectively. One day after transfection, 1% formaldehyde was added for 10 min at 37 °C to cross-link proteins to DNA. Cells were washed two times with ice-cold PBS, scraped and lysed with SDS lysis buffer (Millipore) in the presence of protease inhibitors mixture. The lysates were sonicated to shear DNA to lengths between 200 and 1,000 bp. After 10-fold dilution of the inhibitors mixture, the lysates were sonicated to shear DNA to

Transformation related protein 53
Cyclin-dependent kinase inhibitor 1A (p21)
Doxide hydrolase 1, microsomal
Cyclin G1
Flecketin homology-like domain, family A, member 3
Adenylate kinase 1
Transcribed locus
Transformed mouse 3T3 cell double minute 2
Cysteine-rich C-terminal 1
Transformation related protein 53
Pyruvate dehydrogenase kinase, isoenzyme 4
Krummel-like factor 4 (out)
Glutathione peroxidase 3
Chemoattractant C-C motif ligand 17
Glycogen synthase kinase 3 beta
Killer cell lectin-like receptor, subfamily A, member 7
P53-variant (p53)
G protein-coupled receptor 137B
Protein tyrosine phosphatase 4A3
Bax and arid domain containing protein 1
Potassium channel tetramerisation domain containing 12
Profilin 2
Bcl2-associated X protein
Cyclin D2
Kinesin family member 1A
Acylphosphatase 2, muscle type
Expressed sequence HUF146242
Protease, serine. 23
Transmembrane protein 37
Sulfiredoxin 1 homolog (S. cerevisiae)
Adrenomedullin
A kinase (PRKA) anchor protein (marvin) 12
Clathrin. light chain (ebf)
Prostaadrenal E synthase
Tumor necrosis factor receptor superfamily. member 1B (osteoprotegerin)
Sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3E
Brain on-division-COM-related cell adhesion molecule
Pentidyl-thymidine hydrolase 1 homolog (S. cerevisiae)
RIKEN cDNA 2310041G17 gene
Fasciculation and elongation protein zeta 1 (zygin)
Exocyst complex component 4
Interleukin 17 receptor A
Bladder cancer associated protein homolog (human)
Glucan (1,4-alpha-1,3), branching enzyme 1
Cysteine rich protein 2
Transcribed locus
Cytoplasmic FMR1 interacting protein 2
RIKEN cDNA 2610042A20 gene
Serine (or cysteine) peptidase inhibitor, clade A, member 3N
Laminin, gamma 2
V-maf musculoaponeurotic fibrosarcoma oncogene family, protein K (avian)
Cysteine-rich C-terminal 1
Stromal interaction molecule 1
Prolactin family 7, subfamily a, member 2
Chemokine (C-X-C motif) receptor 7
Lin11 1
Tubulointerstitial nephritis antigen-like
Plexin A2
General transcription factor II H, polypeptide 1
Hydroxysteroid 11-beta dehydrogenase 1
Serine (or cysteine) peptidase inhibitor, clade A, member 3
RIKEN cDNA 6330500D04 gene
Furin peptidase P2X. G-protein coupled 2
Aldehyde dehydrogenase family 1, subfamily A7
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3
ABH1K nucleoporin (desmoplakin)
Phoshatidic acid phosphatase type 2B
Cyclin-dependent kinase inhibitor 1C (p57)
Sorbin and SH3 domain containing 1
Cholesterol 25-hydroxylase

Fig. S1. (Continued)
Fig. S1. Gene expression profile of p53 inactivated OSE cells. Gene expression profiles of OSE cells from p53\textsuperscript{fl/fl}, Rb\textsuperscript{fl/fl}, and p53\textsuperscript{fl/fl}Rb\textsuperscript{fl/fl} mouse were analyzed either Ad-LacZ or Ad-Cre infection. Two class analysis (FDR < 1%) between p53 inactivated (p53\textsuperscript{fl/fl} and p53\textsuperscript{fl/fl}Rb\textsuperscript{fl/fl}) with Ad-Cre) and control groups (Rb\textsuperscript{fl/fl} with Ad-Cre and p53\textsuperscript{fl/fl}Rb\textsuperscript{fl/fl} with Ad-LacZ) was performed. A total of 76 and 52 genes were identified to be significantly up-regulated (Upper) and down-regulated (Lower), respectively, in the p53 inactivated group. Among those genes, several were well established downstream targets of p53-mediated activation, such as p21, Mdm2, and Bax, and repression, such as cyclin B1.
**Fig. S2.** Effect of p53 knockdown on MET expression in human cancer cells expressing wild-type p53 and OSE morphology. (A) Twenty nanomolars of either control or p53 siRNA was transfected into OVCA433 and HCT116 cells and MET expression was measured by Western blotting 48 h afterward. (B) OSE (arrow) 72 h after either Ad-Cre (Left) or Ad-Blank (Right) infection. H&E staining. (Scale bar, 100 μm.)

**Fig. S3.** p53 inactivation increases cell motility along with increased MET expression and MET phosphorylation. (A) Either Ad-LacZ or Ad-Cre–infected OSE cells from p53fl/fl mouse were cultured, and then scratch was made by a p200 tip. Wound healing was monitored in 12 and 24 h. Quantitative measurements of the assay are presented in Fig. 2C. (Scale bar, 500 μm.) (B) Twenty-four hours after either Ad-LacZ or Ad-Cre infection, OSE cells from p53fl/fl mouse were treated with either blank solution (−HGF) or 20 ng/mL HGF (+HGF) and MET and phospho-MET expression were measured by Western blotting after another 24 h in cell culture.
Migration and invasion but not proliferation depends on MET presence after p53 inactivation. (A) Percentage of invasion was measured in OSE cells by using either Ad-EGFP or Ad-Cre:EGFP. (B) Cre-loxP-mediated recombination was confirmed by PCR. Floxed and recombined p53 allele were detected as 316- and 198-bp DNA fragments, respectively. Wild-type, floxed, and recombined Met allele were detected as 480-, 320-, and 600-bp fragments, respectively. (C) Adenoviral infection efficiency was monitored by fluorescent microscopy one day after infection. Over 90% of cells showed strong green fluorescence. (Scale bar, 500 μm.) (D and E) Either Ad-LacZ or Ad-Cre–infected OSE cells from p53<sup>fl/fl</sup> and p53<sup>fl/fl</sup>Met<sup>fl/fl</sup> mice were seeded onto either empty or Matrigel insert for counting migrating (D) or invading (E) cells, respectively, in the presence of HGF (20 ng/mL). (F) OSE cells from either p53<sup>fl/fl</sup> or p53<sup>fl/fl</sup>Met<sup>fl/fl</sup> mice were incubated with BrdU for 2 h, 48 h after infection with Ad-Cre. BrdU-positive cells were detected by anti-BrdU antibody and counted (n = 3). Bar, SD ; **P < 0.01.

Ectopic p53 expression is comparable to endogenous p53 expression level. pCMV-p53 and pORF-hp53 were transfected into SKOV-3. In the case of pCMV-p53, the highest concentration was used for all promoter analysis. In the case of pORF-p53, the lowest concentration or lesser amount was used. To compare endogenous p53 level, wild-type p53-harboring HCT116 cells were treated with doxorubicin (2 μg/mL) for 6, 12, or 24 h.
miR-34 and p53 effects on MET expression and promoter activity. (A and B) miR-34a precursor molecules were transfected into SKOV-3 cells at 10-, 30-, or 90-nM concentration. Note that miR-34a transfection at 30 nM is sufficient for the maximum effect on MET down-regulation. MET protein and mRNA level were measured by Western blot (A) and qRT-PCR (B) analysis. Bars, SD (n = 3). P < 0.01. (C) The effect of miR-34 family on MET promoter activity. Individual miR-34 family members and pGL2-3.1MET promoter construct were cotransfected into SKOV-3 cells. Luciferase activity was measured 24 h after transfection. Bars, SD (n = 3). (D) MET expression after transient transfection of p53 expression vector (pORF-hp53) or miR-34a precursor molecule (30 nM) into NCI-H1299 cells.
Fig. S7. MET promoter activity in OSN2 cells and involvement of SP1 in MET promoter activity. (A) p53 expression vector (pORF-hp53) and each promoter construct were cotransfected in OSN2 cells. Cell lysates were harvested and luciferase activity was measured 24 h after transfection. (B) p53 responsive element mutation does not abolish MET promoter suppression by p53. (C and D) PG13-luc, which contains 13 copies of p53 responsive elements, was cotransfected with pCMV, pCMV wild-type p53, or pCMV-mutant p53 (V143A, R175H, R249S, and R273H) into SKOV-3 (C) and OSN2 (D) cell lines. MG15-luc, which contains 15 copies of mutated p53 responsive elements, was cotransfected with either pCMV, pCMV wild-type p53, or pCMV-mutant p53 (V143A, R175H, R249S, and R273H) in SKOV-3 (C) and OSN2 (D) cell lines. (E) In the Web-based TF binding sites algorithm with 85 threshold score (default setting), five potential SP1 binding sites were identified in both pGL2-0.37MET and pGL2-0.27MET. (F) OSN2 cells were pretreated with different doses of mithramycin A 1 h before transfection with 0.27MET promoter construct. Every experiment was repeated three times. Bars, SD.
Fig. S8. Hypoxia does not affect HIF1A and MET expression, and CD44 is not affected by p53 expression in OSE cells. (A) p53<sup>fl/fl</sup> OSE cells were exposed for 24 h to either normoxic (20% O<sub>2</sub>) or hypoxic (2% O<sub>2</sub>) conditions 24 h after exposure to Ad-LacZ or Ad-Cre. MET and HIF1A were detected by Western blotting. (B) Three human ovarian cancer cell lines were exposed for 24 h to either normoxic (20% O<sub>2</sub>) or hypoxic (2% O<sub>2</sub>) conditions. (C and D) HIF1A and MET expression in the HCT-116 cell line exposed to either normoxic or hypoxic conditions. Western blotting (C) and quantitative RT-PCR (D). (E) CD44 protein levels after pORF-p53 and miR-34a precursor molecule transfection into SKOV-3 cells.
Fig. S9. The effect of mutant p53 on MET expression. (A and B) 0.65MET (A) and 0.27MET (B) promoter constructs were cotransfected with wild-type or mutant p53 into OSN2 cells. Bars, SD (n = 3). (C) Twenty nanomolars of either control or p53 siRNA was transfected into OVCA432 and OVCAR-3 cells and MET expression was measured by Western blotting 48 h afterward.
Fig. S10. The role of mutant p53 in OSE cells. (A) OSE cells from p53^+/LSLR172H or p53^+/LSLR270H mice were infected by either Ad-LacZ or Ad-Cre. Slight increase of p53 was detected, likely because of mutant p53 expression in Western blot analysis. (B) OSE cells expressing mutant p53 were not immortalized and underwent senescence. Staining for senescence-associated β-galactosidase. (Scale bar, 500 μm.) (C) Either Ad-LacZ or Ad-Cre–infected OSE cells from p53^{fl/LSLR270H} and p53^{fl/LSLR270H/Me^{fl/fl}} mice were seeded on a control insert or Matrigel insert with serum-free media and exposed to complete growth media containing 20 ng/mL HGF in the lower chamber. Migrating and invading cells were counted 20 h after staining under the microscope. Bar, SD (n = 3). **P < 0.01. N.S., not significant.

Movie S1. Movement of OSE cells with wild-type p53.