p53 Attenuates Cancer Cell Migration and Invasion through Repression of SDF-1/CXCL12 Expression in Stromal Fibroblasts

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Abstract

The p53 tumor suppressor acts as a major barrier against cancer. To a large extent, this is due to its ability to maintain genome stability and to eliminate cancer cells from the replicative pool through cell-autonomous mechanisms. However, in addition to its well-documented functions within the malignant cancer cell, p53 can also exert non-cell-autonomous effects that contribute to tumor suppression. We now report that p53 can suppress the production of the chemokine SDF-1 in cultured fibroblasts of both human and mouse origin. This is due to a p53-mediated down-regulation of SDF-1 mRNA, which can be exacerbated on activation of p53 by the drug Nutlin-3. SDF-1 promotes the migration and invasiveness of cells that express its cognate receptor CXCR4. Indeed, medium conditioned by p53-deficient fibroblasts induces cancer cells towards increased directional migration and invasiveness, which are largely reversed by CXCR4 antagonist peptides. Because SDF-1 produced by stromal fibroblasts plays an important role in cancer progression and metastasis, our findings suggest that the ability of p53 to suppress stromal SDF-1 production may be an important mechanism whereby it does its non-cell-autonomous tumor suppressor function. (Cancer Res 2006; 66(22): 10671-6)

Introduction

Tumor development is very complex; it involves not only the genetically altered cancer cells that drive the malignant process but also a collection of various noncancerous stromal cells that constitute the cancer cell microenvironment. The stroma is engaged in extensive interactions with the cancer cells, actively promoting their survival and proliferation and contributing to the growth and invasiveness of the tumor (1). These interactions are also crucial for tumor metastasis at distant organ sites, where local stroma-derived factors play a seminal role in attracting incoming tumor cells and allowing subsequent growth of the metastases.

Chemokines are cytokines that can mobilize cells to migrate along gradients within the body in a manner that requires an interaction between the chemokine and cognate receptors present on the target cell plasma membrane. In addition to their roles in normal embryonic development and postnatal tissue homeostasis, chemokines also play an important role in cancer: chemokine receptors expressed on tumor cells are involved in the migration of such cells and are associated with distant metastasis, suggesting that chemokines may control tumor dissemination. Furthermore, at least in some cases, chemokines can enhance the proliferation and survival of cancer cells, as well as promote angiogenesis and neovascularization of the growing tumor (for recent reviews on chemokines and cancer, see refs. 2–5).

Of paramount importance in this context is the chemokine stromal cell–derived factor 1 (SDF-1/CXCL12), initially characterized as a pre-B-cell growth-stimulating factor. SDF-1 is present on the surface of vascular endothelial cells and is secreted by stromal cells from a variety of tissues such as bone marrow, lung, and liver. SDF-1 exerts effects through its only physiologic cognate receptor CXCR4, which is known to mediate chemotaxis, hematopoiesis, vasculogenesis, and tumor spread and metastasis. The CXCR4/SDF-1 axis has been shown to contribute to the progression of many types of tumors, including breast, prostate, and pancreatic cancer, as well as osteosarcoma, lymphoma, and leukemia (2, 3, 5). Indeed, SDF-1 secreted by stromal cells associated with primary tumors can strongly enhance the growth of the adjacent cancer cells (6, 7). Moreover, in vivo studies showed that blocking the SDF-1/CXCR4 axis by small-molecule antagonists of CXCR4 or neutralizing antibodies to SDF-1 could significantly reduce tumor metastasis to the lung, bone marrow, and lymph nodes (reviewed in refs. 2, 3, 5).

The p53 protein is a sequence-specific transcription factor that functions as a major tumor suppressor in mammals (reviewed in refs. 8–10). In response to various types of oncogenic stress, p53 is activated to promote cell cycle exit, apoptosis, or replicative senescence, thereby preventing the propagation of incipient cancer cells. Consequently, p53 is very often found to be disabled within cancer cells, either by direct mutational activation of the TP53 gene or by alterations in other genes of which the products impinge on p53.

Whereas the effect of p53 has been widely investigated in a cell-autonomous context, much less attention has been given to its non-cell-autonomous functions. Of note, inoculation of cancer cells into p53-null mice was found to reduce the latency for tumor development (11). Furthermore, tumor reconstitution experiments showed that the fibroblastic compartment of the tumors is sufficient to modulate both the latency of tumorigenesis and the morphology of the resulting tumors in a p53-dependent manner (11). Confirming the biological importance of these observations, Hill et al. (12) showed in a mouse model of prostate cancer that epithelial tumorigenesis imposed a strong selective pressure for the loss of p53 in tumor-associated fibroblasts. Remarkably, p53-regulated factor(s) secreted by normal cells in vitro as well as in vivo have been implicated in controlling the proliferation of tumor cells (13).

In the present study, we sought to further elucidate the molecular mechanisms whereby p53 in stromal cells exerts its
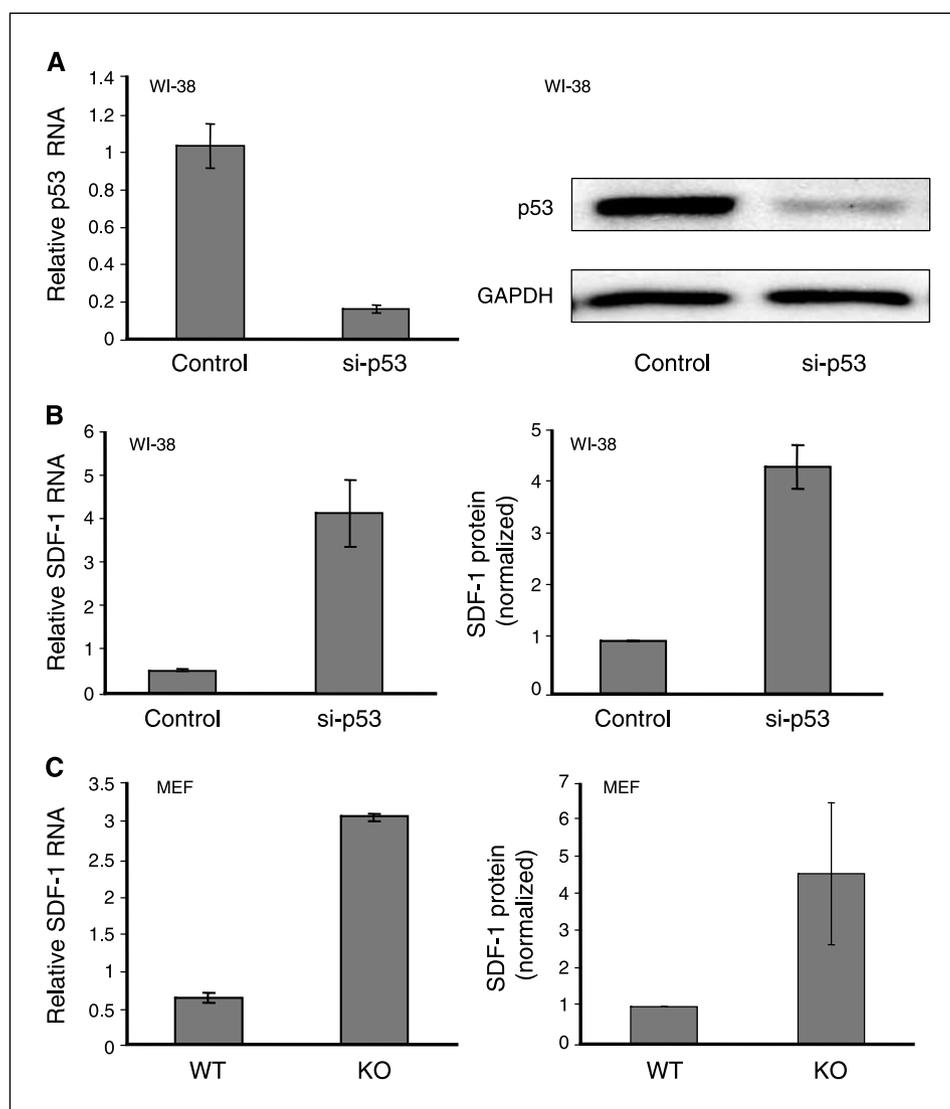
Figure 1. p53 represses SDF-1 expression. WI-38 cells stably expressing lacZ shRNA (*Control*) or p53 shRNA (*si-p53*), as well as WT MEF and p53-KO MEF, were allowed to grow in culture for 48 hours, at which time the cells were harvested for RNA and protein analysis and their conditioned media collected and subjected to SDF-1 protein analysis. *Left,* analysis of p53 mRNA in WI-38 cells (*A*) and SDF-1 mRNA in WI-38 cells (*B*) and MEF (*C*). Real-time PCR was done on cDNA prepared from total RNA using primers specific for p53, SDF-1, and HPRT. The relative levels of p53 and SDF-1 mRNA are shown after normalization for the HPRT control. *A, right,* proteins were extracted from control WI-38 cells and their p53 shRNA–expressing derivatives (*si-p53*). Aliquots containing equal amounts of total protein were subjected to Western blot analysis for p53 and GAPDH. *B* and *C, right,* analysis of SDF-1 protein levels in conditioned medium of WI-38 cells (*B*) and MEF (*C*). The conditioned medium of cells grown in 10% FCS, corrected for cell number at the harvesting time, was subjected to SDF-1 protein determination by ELISA. The amount present in the conditioned medium of the control cells in each individual experiment was taken as 1.

tumor suppressor activity over cancer cells. We report that, within both human and mouse fibroblasts, p53 can suppress the production of SDF-1. Conversely, abrogation of p53 expression in fibroblasts promotes SDF-1 secretion, augmenting the migration and invasiveness of cultured tumor cells. The suppression of SDF-1 production by p53 may attenuate tumor development and metastasis, underpinning a non-cell-autonomous tumor suppressor function of p53.

Materials and Methods

Cell culture. WI-38 human diploid embryonic lung fibroblasts were grown in MEM supplemented with 10% FCS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and antibiotics. Ecotropic retrovirus–producing 293T cells were grown in DMEM supplemented with 10% FCS and antibiotics. Osteosarcoma SJSA-1 cells were grown in RPMI supplemented with 10% FCS and antibiotics. G_2 cells, derived from precursor-B ALL cells, were grown as described (14). To prepare conditioned medium, cells were grown to 70% confluence, washed twice with serum-free medium, and fresh medium containing either 0.5% or 10% FCS was added to the dish. After 48 hours, the conditioned medium was collected, normalized to the cell number at the time of collection, filtered through a 0.45-μm filter, aliquoted,

and stored at $-20°C$. Control medium was obtained by incubation of culture medium for 48 hours in the absence of cells.

Plasmids and reagents. pBabe-si-p53-puro was kindly provided by Dr. R. Agami (Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Amsterdam, the Netherlands). Nutlin-3 (Alexis Corporation, San Diego, CA) was dissolved in DMSO and kept as a 10 mmol/L stock solution in small aliquots at $-20°C$. The CXCR4 antagonist TN14003 was kindly provided by Dr. N. Fujii (Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan). AMD3100 was purchased from Sigma (St. Louis, MO).

Retroviruses. Retroviral constructs and infection procedures have previously been described (15).

ELISA for SDF-1. ELISA for SDF-1 was done as previously described (16).

Transwell migration and invasion assays. To assess cell migration *in vitro*, SJSA-1 or G2 cells (1.5×10^5 in 100 μL serum-free medium) were placed in the top chamber of transwell migration chambers (8 or 5 μm; BD Biosciences, San Jose, CA). The lower chamber was filled with 600 μL of either conditioned medium or control medium with or without 125 ng/mL of SDF-1 (Cytolab, Rocky Hill, NJ). After 24 hours, SJSA-1 cells that had not migrated to the lower chamber were removed from the upper surface of the transwell membrane with a cotton swab. Migrating cells on the lower membrane surface were fixed, stained, and photographed. G2 cells were allowed to migrate for 4 hours, at which time migrating cells were collected from the bottom chamber of the transwell and counted for 30 seconds using

FACScan. *In vitro* invasion assays were done under the same conditions as the transwell migration assays, but in Matrigel-coated transwells (BD Biosciences). Where indicated, some of the cells were preincubated with the CXCR4 antagonist peptide TN14003 (50 and 100 nmol/L for G2 and SJSA-1 cells, respectively, or as indicated in the figure) or AMD3100 (1 μmol/L) for 15 minutes at 37°C before being placed in the transwell upper chamber.

Real-time reverse transcription-PCR analysis. Total RNA was extracted with the NucleoSpin kit (Macherey Nagel, Germany). One microgram of each RNA sample was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random hexamer primers. Real-time PCR was done on an ABI 7000 machine (Applied Biosystems, Foster City, CA) with Syber Green PCR mastermix (Applied Biosystems), mouse SDF-1 specific primers (sense, GAAAGGAAG-GAGGGTGGCAG; antisense, TCCCCGTCTTTCTCGAGTGT), human SDF-1 specific primers (sense, GATTGTAGCCCGGCTGAAGA; antisense, TTCGGGTCAATGCACACTTGT), and mouse p21 specific primers (sense, GGCCCGGAACATCTCAGG; antisense, AAATCTGTCAGGCTGGTCTGC). cDNA levels were normalized to hypoxanthine phosphoribosyltransferase (HPRT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplified with appropriate primers (mouse HPRT: sense, GCAGTACAGCCC-CAAAATGG; antisense, GGTCCTTTTCACCAGCAAGCT; human HPRT: sense, TGACACTGGCAAAACAATGCA; antisense, GGTCCTTTTCACCAG-CAAGCT; human GAPDH: sense, ACCCACTCCTCCACCTTTGA; antisense, CTGTTGCTGTAGCCAAATTCGT).

Immunoblotting. Cells were lysed in Tris-Triton lysis buffer and cell extract aliquots containing equal amounts of total protein (Bradford assay) were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Kent, United Kingdom), which were probed with the following primary antibodies: a mixture of the p53-specific monoclonal antibodies PAb1801 and DO-1 for human p53; rabbit polyclonal anti-p53 (CM5; Novocastra, Newcastle upon Tyne, United Kingdom) for mouse p53; rabbit polyclonal anti-p21 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) for detecting human p21; mouse monoclonal anti-GAPDH (MAB374; Chemicon International, Chandlers Ford, United Kingdom) for detecting mouse and human GAPDH. Western blots were developed with the enhanced chemiluminescence reagent (Amersham Pharmacia, Piscataway, NJ).

Results

p53 represses SDF-1 expression in cultured fibroblasts. In an effort to explain the ability of stromal p53 to modulate the behavior of tumor cells, and in view of the important role of SDF-1 in stroma-tumor crosstalk, we assessed the effect of p53 on SDF-1 expression in normal fibroblasts. To that end, p53 expression was knocked down in WI-38 human fetal lung fibroblasts via the use of p53-directed small hairpin RNA (shRNA; Fig. 1*A*), and the effect of this manipulation on SDF-1 mRNA and protein levels was

Figure 2. Activation of p53 by Nutlin represses SDF-1 expression. *A* and *B*, *left*, WT MEF and p53-KO MEF, maintained in culture for 48 hours, were treated with Nutlin-3 at a final concentration of 25 μmol/L. Twenty-four hours later, the cells were harvested and RNA was prepared and subjected to real-time reverse transcription-PCR (RT-PCR) analysis as in Fig. 1, employing primers specific for SDF-1, p21, and HPRT. The relative levels of p21 (*A*) and SDF-1 (*B*) mRNA are shown after normalization for the HPRT control. *A* and *B*, *right*, WI-38 cells, treated with Nutlin-3 (25 μmol/L) for the indicated time periods, were harvested and subjected to real-time RT-PCR analysis, employing primers specific for SDF-1, p21, and GAPDH. The relative levels of p21 (*A*) and SDF-1 (*B*) mRNA are shown after normalization for the GAPDH control. *C*, WT MEF and p53-KO MEF were treated with Nutlin-3 (25 μmol/L). Cells were harvested at the indicated times of Nutlin-3 treatment, and aliquots containing equal amounts of total protein were subjected to Western blot analysis for p53 and GAPDH. *D*, WI-38 cells were treated as in (*C*) and subjected to Western blot analysis for p53, p21, and GAPDH.
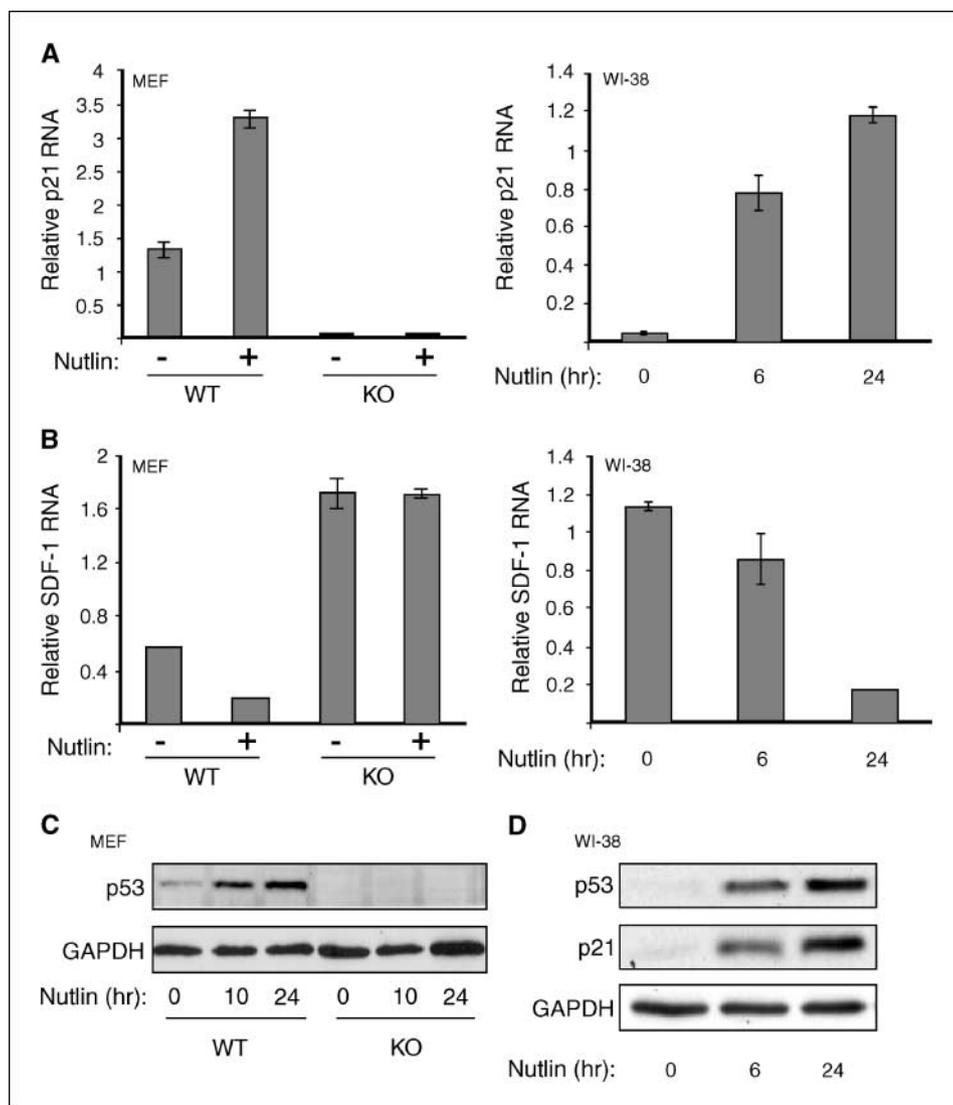
monitored. Down-regulation of p53 led to a marked increase in endogenous SDF-1 mRNA (Fig. 1B, left). Similarly, cultured mouse embryo fibroblasts (MEF) derived from p53 knock-out (KO) mice produced significantly higher amounts of SDF-1 mRNA (Fig. 1C, left) than their wild-type (WT) counterparts.

SDF-1 is a secreted protein; when produced by stromal fibroblasts, it is released into the interstitial space, where it acts in a paracrine fashion on cells in the local microenvironment to stimulate directional migration of hematopoietic and nonhematopoietic normal and malignant cells (2–5). We therefore employed an ELISA-based assay to determine whether p53 affects the amount of secreted SDF-1. Indeed, down-regulation of p53 resulted in higher SDF-1 levels in the conditioned medium of both WI-38 cells (Fig. 1B, right) and MEF (Fig. 1C, right). In both cases, the amount of SDF-1 in the conditioned medium was in the range of 50 to 1,000 pg/mL.

Collectively, these findings imply that basal p53 activity in proliferating cultured human and mouse fibroblasts can repress SDF-1 expression and secretion.

Activation of p53 further suppresses SDF-1 expression. The data described above argue that, under standard tissue culture conditions, basal p53 activity already restricts SDF-1 expression. To investigate whether this restrictive effect can be further exacerbated by elevation of stromal cell p53 activity, we took advantage of the small-molecule drug Nutlin-3. By occupying the hydrophobic p53-binding pocket on the surface of the Mdm2 molecule, Nutlin effectively disrupts p53-Mdm2 binding, leading to stabilization of p53 and activation of the p53 pathway (17).

Nutlin treatment led to a time-dependent increase in the level of p53 protein in WT MEF (Fig. 2C). As expected, this resulted in elevated p21 mRNA levels in WT MEF but not in p53-KO MEF (Fig. 2A, left), confirming that Nutlin augmented the transcriptional activity of p53 in this setting. Importantly, Nutlin caused a further 3-fold reduction in SDF-1 mRNA levels in WT MEF but not in their p53-null counterparts (Fig. 2B, left). Similar results were obtained with WI-38 cells: whereas Nutlin-3 caused a gradual increase in p53 protein (Fig. 2D), as well as in p21 mRNA (Fig. 2B, right)
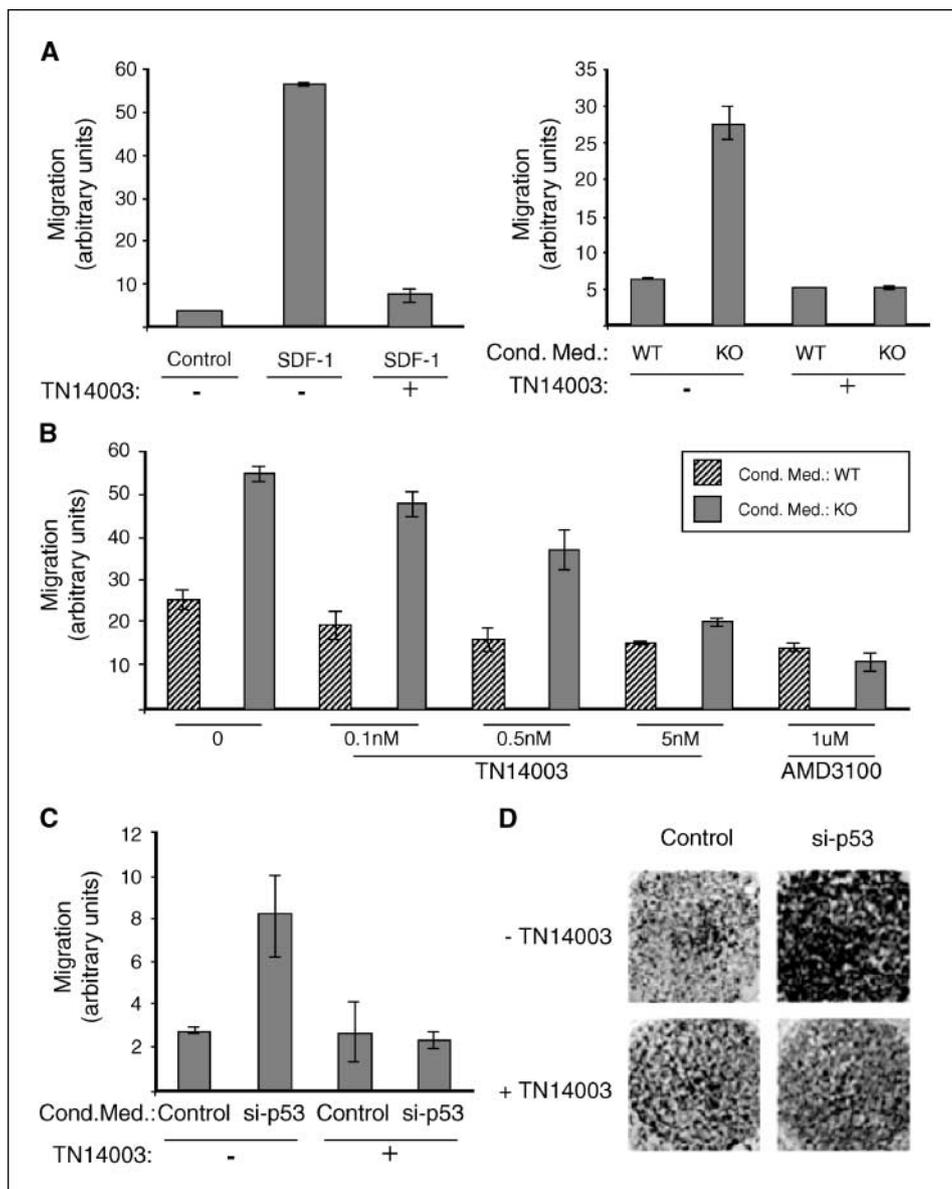
Figure 3. p53-dependent repression of SDF-1 modulates the migration of cancer cells. A to C, G2 cell migration was assessed using transwells containing culture medium either without (Control) or with added SDF-1 (A, left), or conditioned medium collected from cultures of MEF (A, right; B) or WI-38 cells (C) as described in Materials and Methods (see Fig. 1 for description of the different cell types). When indicated, G2 cells were pretreated with the CXCR4 peptide antagonist TN14003 (50 nmol/L, unless indicated otherwise) or with AMD3100. Numbers relate to arbitrary units of transwell-migrating cells, as determined by fluorescence-activated cell sorting analysis of three separate wells for each treatment. Bars, SD. Each result is representative of at least three independent experiments. In the experiment depicted in (B), concentrations of SDF-1 in the conditioned medium of WT MEF and p53-KO MEF were 142 and 590 pg/mL, respectively, as measured by ELISA. D, migration of SJSA-1 osteosarcoma cells towards conditioned medium of WI-38 cells was assayed as in (C). Low-magnification photographs of representative wells are shown; darker areas are due to higher cell density. Note the increased number of migrating cells attracted by conditioned medium from si-p53 cells in the absence of the inhibitor but not in its presence.

and protein (Fig. 2*D*), it led to a marked reduction in SDF-1 mRNA (Fig. 2*B, right*).

Hence, the extent of suppression of SDF-1 expression increases with p53 activity, suggesting that the biological effect of this phenomenon may become more pronounced under physiologic and pathologic conditions that entail extended triggering of the p53 pathway.

p53-dependent repression of SDF-1 modulates the migration and invasiveness of cancer cells. The CXCR4/SDF-1 axis plays a crucial role in the targeting of metastatic cancer cells of various origins (e.g., breast, kidney, lung, pancreas, and prostate), as well as leukemic cells, to the bone marrow, lymph nodes, and lung; this is achieved by promoting the migration of the malignant cells towards SDF-1 produced by the stroma of these target organs (2, 3, 5). To evaluate the potential biological relevance of the regulation of SDF-1 by p53, we tested whether it has an effect on the directed migration of tumor cells. Migration of leukemic cells (G2 pre-B-cell line) and SJSA-1 osteosarcoma cells was measured in a transwell assay. As shown in Fig. 3*A* (*left*), this assay revealed extensive migration of G2 cells towards recombinant human SDF-1, which was significantly impaired on inhibition of the CXCR4/SDF-1 axis by pretreating the cells with TN14003, a 14-mer peptide CXCR4 antagonist (18). Remarkably, G2 cells also exhibited substantial migration towards conditioned medium of p53-KO MEF, but not of WT MEF (Fig. 3*A, right*); this migration was effectively prevented by pretreatment with TN14003 (Fig. 3*A, right*) in a dose-dependent manner (Fig. 3*B*). Importantly, the migration of G2 cells towards conditioned medium of p53-KO MEF was also effectively repressed by another CXCR4 inhibitor, AMD3100 (Fig. 3*B*). TN14003 and AMD3100 inhibit CXCR4 by different mechanisms of action (5). The fact that both inhibitors exert a similar effect argues strongly that this effect is due to specific blockage of the CXCR4/SDF-1 axis. Similarly, migration of G2 cells was selectively induced by conditioned medium of WI-38 cells stably expressing p53-specific shRNA, but not control lacZ shRNA (Fig. 3*C*). Once again, this effect was nearly abolished by TN14003. Essentially similar results were obtained when SJSA-1 osteosarcoma cells were evaluated in such transwell migration assay (Fig. 3*D*).

Thus, in human and mouse fibroblasts the presence of functional p53 strongly compromises their ability to secrete molecules that promote the migration of cancer-derived cells. Remarkably, inhibition of CXCR4 on the target cancer cells strongly impaired their migration towards the conditioned medium of p53-deficient cells, practically eliminating the difference between p53-positive and p53-deficient cells. Hence, at least in these experimental models, SDF-1 is the major, if not the only, chemokine responsible for directional cancer cell migration, and suppression of SDF-1 production by p53 almost completely abolishes such migration.

To find out whether the repression of SDF-1 by p53 in fibroblasts also plays a functional role in stimulating tumor cell invasion, Matrigel invasion chambers were used to monitor the invasiveness of G2 cells. Administration of recombinant SDF-1 (125 ng/mL) to the lower chamber resulted in a 2-fold increase in the number of invading cells compared with medium only, and pretreatment of the G2 cells with TN14003 interfered with this effect of SDF-1 (Fig. 4). Importantly, G2 cells exhibited greater invasiveness towards conditioned medium of p53-KO MEF than towards that of WT MEF. This effect of the p53 KO MEF-conditioned medium was largely mediated by the CXCR4/SDF-1 axis because pretreatment with TN14003 eliminated it effectively (Fig. 4). Very similar
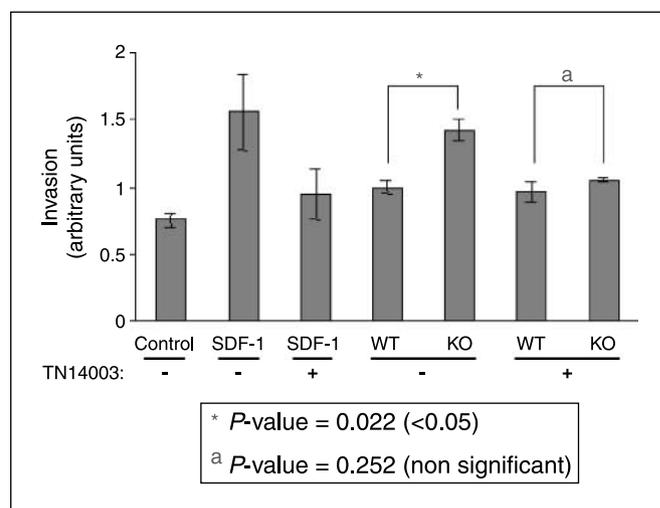
Figure 4. p53-dependent repression of SDF-1 modulates the invasiveness of cancer cells. The invasive ability of G2 cells was examined over 14 hours using transwells with Matrigel-coated filters. Numbers relate to arbitrary relative units of invading cells. *Columns,* normalized mean of two wells; *bars,* SD. Representative of two independent experiments.

results were obtained with conditioned medium of control versus p53 knocked-down WI-38 cells (data not shown).

Together, our findings show that p53 can suppress SDF-1 production by stromal fibroblasts, thereby restricting their ability to promote cancer cell migration and invasion.

Discussion

In the present study, we report that p53 can repress the production of SDF-1 by cultured normal human and mouse fibroblasts. Nevertheless, p53 is not a universal repressor of SDF-1 expression. In several WT p53–containing tumor-derived cell lines, we did not observe an effect of p53 knock-down on SDF-1 mRNA levels (data not shown), in agreement with a recent study employing glioma cells (19). Interestingly, however, p53 knock-down did increase SDF-1 mRNA expression in MCF-7 breast cancer cells, which possess exceptionally high basal levels of this transcript (data not shown).

Recent studies indicate that stromal cells in the primary tumor are an important source of SDF-1. In myofibroblasts isolated from surgically resected breast cancer specimens, SDF-1 expression is significantly up-regulated relative to myofibroblasts obtained from normal breast tissue (6, 20). Moreover, such cancer-associated fibroblasts can enhance SDF-1/CXCR4-dependent growth of human breast cancer cells *in vitro* and in a mouse model, underscoring the importance of stromal-derived SDF-1 for tumor progression. The ability to restrict the production of SDF-1 by stromal fibroblasts may therefore serve as an additional mechanism by which p53 can exert, in a non-cell-autonomous fashion, its tumor suppressor effects *in vivo*. It will be of interest to determine to what extent this ability contributes to the previously reported non-autonomous tumor suppressor activities of p53 (11). Moreover, given the documented importance of the SDF-1/CXCR4 axis in tumor metastasis, our findings may also provide a mechanistic explanation for the observation that p53 loss in the host can promote metastasis in experimental animal models (21, 22).

Levels of p53 mRNA are rather low in quiescent fibroblasts (23). Combined with the lack of p53-activating stress signals in fibroblasts residing within a normal tissue, this might result in

p53 protein amounts that are insufficient to have any effect on gene expression patterns. Conversely, when fibroblasts are driven to proliferate *in vitro*, in the presence of chronic mild stress due to "culture shock," p53 activity might become markedly augmented, to an extent that it affects the transcription of genes like *SDF-1* even without a need for added acute stress. Similar conditions may pertain *in vivo* when fibroblasts are induced into proliferation by adjacent tumor cells, particularly if p53 activity is further enhanced by types of stress that are inherent to many tumors, such as hypoxia, nitric oxide, local nutrient deprivation, etc. Indeed, in a mouse model of prostate cancer, epithelial oncogenic stress was shown to trigger a p53 response in the associated prostate mesenchyme (12). p53 expression was induced in both the epithelial cells and the stromal fibroblasts, consistent with a p53-dependent tumor suppression response in both compartments. Our data predict that, under such conditions, SDF-1 production by fibroblasts will be markedly restrained, imposing a selective pressure for attenuation of p53 activity in tumor-associated stroma. In extreme cases, the outcome might be the complete loss of p53 expression in the stromal fibroblasts, as illustrated compellingly in the mouse model of prostate cancer (12). The relevance of these observations to human cancer is underscored by the detection of p53 mutations and loss of heterozygosity at the *TP53* locus in the stromal compartment of human solid tumors (24, 25). We speculate that, in other cases, dampening of stromal p53 activation might be achieved through inhibitory influences exerted by neighboring successful tumor cells, which override the need for direct genetic alterations in the *TP53* locus of the stromal cells. In either case, inhibition of the SDF-1/CXCR4 axis is likely to reverse, at least in part, the cancer-promoting consequences of the dampened stromal p53 activity.

Furthermore, our results suggest that pharmacologic activation of p53 by molecules such as Nutlin might exert tumor-inhibitory effects *in vivo* not only by reactivation of p53 in WT p53–positive cancer cells but also by depriving those cells of the benefits offered by adjacent stromal cells. Such p53 activators may therefore prove efficacious also in the treatment of tumors harboring p53 mutations, as long as the cancerous cells within such tumors express the CXCR4 receptor and rely on stromal-derived SDF-1 for optimal growth and survival.

Acknowledgments

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