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Gain-of-function mutant p53 upregulates CXC chemokines and enhances cell migration

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The role of dominant transforming p53 in carcinogenesis is poorly understood. Our previous data suggested that aberrant p53 proteins can enhance tumorigenesis and metastasis. Here, we examined potential mechanisms through which gain-of-function (GOF) p53 proteins can induce motility. Cells expressing GOF p53 -R175H, -R273H and -D281G showed enhanced migration, which was reversed by RNA interference (RNAi) or transactivation-deficient mutants. In cells with engineered or endogenous p53 mutants, enhanced migration was reduced by downregulation of nuclear factor-kappaB2, a GOF p53 target. We found that GOF p53 proteins upregulate CXC-chemokine expression, the inflammatory mediators that contribute to multiple aspects of tumorigenesis. Elevated expression of CXCL5, CXCL8 and CXCL12 was found in cells expressing oncogenic p53. Transcription was elevated as CXCL5 and CXCL8 promoter activity was higher in cells expressing GOF p53, whereas wild-type p53 repressed promoter activity. Chromatin immunoprecipitation assays revealed enhanced presence of acetylated histone H3 on the CXCL5 promoter in H1299/R273H cells, in agreement with increased transcriptional activity of the promoter, whereas RNAi-mediated repression of CXCL5 inhibited cell migration. Consistent with this, knockdown of the endogenous mutant p53 in lung cancer or melanoma cells reduced CXCL5 expression and cell migration. Furthermore, short hairpin RNA knockdown of mutant p53 in MDA-MB-231 cells reduced expression of a number of key targets, including several chemokines and other inflammatory mediators. Finally, CXCL5 expression was also elevated in lung tumor samples containing GOF p53, indicating relevance to human cancer. The data suggest a mechanistic link between GOF p53 proteins and chemokines in enhanced cell motility.

Introduction

Loss of the normal tumor suppressive functions of the p53 protein is crucial for carcinogenesis as multiple cellular functions, including the response to genotoxic stress, are compromised (1). This can result in propagation of genetic aberrations in daughter cells due to failure to repair damaged DNA or initiate apoptosis. However, missense mutations resulting in single amino acid substitutions account for greater than half of the p53 gene mutations found in human cancer, which is considerably higher than that of other tumor suppressor genes (2,3). Furthermore, proteins encoded by these mutated genes are, in general, highly stable, resulting in overexpression. Thus, in addition to loss of normal p53 function through deletion or intragenic mutation, a class of 'gain-of-function' (GOF) mutants exists, in which the encoded proteins are endowed with oncogenic properties that actively drive tumor progression (3,4). In support of this hypothesis, it is recognized that mutant p53-containing cancers have a poorer clinical outcome than p53-null lesions (5).

In experimental systems, it has been shown that some p53 mutants can cooperate with oncogenes such as Ras and Myc to transform rodent cells (6,7), indicating dominant-negative function, whereas the work from our own laboratory has demonstrated that some p53 mutants alone are sufficient to transform immortalized fibroblasts to a tumorigenic and metastatic phenotype (8). Furthermore, cooperativity between mutant p53 and ras is reported as early events in human skin carcinogenesis (9). Other reported functional gains (reviewed in refs. 5,10) include accelerated growth, resistance to chemotherapy and radiotherapy and disruption of the spindle checkpoint (11), potentially through upregulation of Cks1 and failure to regulate the anaphase-promoting complex (12). Evidence from murine models also provides support for p53 GOF: skin tumors developing in mice that harbor the R172H allele have a higher propensity to metastasize (13), whereas models of Li-Fraumeni syndrome also demonstrate the emergence of metastatic tumors (14) as well as tissue-specific GOF (15,16). Other studies (17) indicate that R248W and R273H mutants inactivate the function of the ataxia telangiectasia protein by binding to Mre11, one of its downstream mediators in the S-phase delay pathway, thereby enhancing genetic instability.

Many different cancer types have an inflammatory component (18), largely as the result of chemokine action. Deregulated chemokine function has been shown to enhance tumor cell proliferation and migration in different types of cancer (reviewed in ref. 19). Increased CXCL1 and CXCL2 levels correlate with enhanced growth, motility, adhesion to extracellular matrix substrata, *in vitro* invasion and more aggressive *in vivo* behavior. In addition, chemokine receptors CXCR4 and CCR7 are upregulated in some breast cancers and can induce actin polymerization, migration and invasion *in vitro* and metastasis *in vivo* (20). Moreover, CXCL12-CXCR4 signaling can induce rapid dephosphorylation of ezrin–radixin–moesin proteins, key elements that link the actin cytoskeleton to peripheral cellular processes, resulting in their dissociation from actin and subsequent loss of microvilli and cell polarity (21). ELR^+ chemokines, which contain a glutamic acid–leucine–arginine motif, are key inducers of angiogenesis (22) and CXCR2 signaling in response to CXCL5 or CXCL8 facilitates migration and proliferation of endothelial cells (23). Wild-type p53 represses angiogenesis through thrombospondin-mediated mechanisms (24). However, Moskovits *et al.* (25) reported that p53 represses CXCL12 expression , perhaps representing an additional antiangiogenic mechanism. In addition, these authors demonstrated that conditioned media from p53-knockout fibroblasts could induce migration and invasion of tumor cells in a CXCL12-dependent manner.

In previous studies (8,26), we cloned and characterized p53 mutants from head and neck squamous cell carcinomas, in order to determine their biological and biochemical functions. Using immortal non-tumorigenic NIH3T3 fibroblasts, we generated stable cell lines expressing aberrant p53 proteins (8). Transplantation of these cell lines to athymic mice resulted in tumor development, whereas the expression of p53-H179L consistently induced a highly metastatic phenotype, spreading from the transplantation site to lung, mediastinum and abdominal cavity, activities that are consistent with a GOF activity (8). Furthermore, cells recultured from tumor xenografts showed elevated expression of the p53-H179L protein compared with the parental cell line, suggesting that high levels of expression are selected for *in vivo*. Separate studies from our laboratories have documented that cells expressing GOF p53 mutants have a transcriptome distinct from that of p53-null or p53-wild-type cells (27,28). Notably,

Abbreviations: GOF, gain-of-function; NF-κB2, nuclear factor-kappaB2; qRT–PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA; siRNA, small interfering RNA.

mutant p53-containing cells show elevated expression of genes whose protein products are critical for DNA replication and repair, cell cycle regulation, regulation of transcription and cell–matrix interactions (29). Although considerable evidence is available documenting potential mechanisms through which *P53* mutation deregulates cell growth and cell cycle checkpoints, the mechanisms through which mutant p53 proteins enhance tumor progression remain relatively unexplored. In the present study, therefore, we have investigated the effects of GOF p53 proteins on deregulation of cell motility. We found a novel activity for GOF p53: upregulated expression of CXC chemokines, which contributes to enhanced tumor cell migration.

Materials and methods

Cell lines and culture conditions

H1299 cells expressing GOF p53 mutants R175H, R273H and D281G and the vector control line HC5 have been described previously (27). H1299 cells expressing p53-H179L, p53-D281G/L22Q/W23S, nuclear factor-kappaB2 (NF-κB2) p52 and CXCL5 were generated similarly. Cells were cultured in RPMI supplemented with 10% fetal bovine serum and 400 μg/ml G418 at 37°C in 95% air/5% CO_2. Expression of p53 transgenes was confirmed by western blotting. NCI-H1437 and NCI-H1048 lung cancer cells (expressing p53-R267P and p53-R273C, respectively), MDA-MB-435 melanoma cells (expressing p53-R280K) and MDA-MB-231 breast carcinoma cells (expressing p53-G266E) were cultured under the same conditions.

Lung cancer RNA

RNA extracted from human non-small-cell lung cancer samples was provided by the Virginia Commonwealth University Tissue Data Acquisition and Analysis Core following approval by the institutional review board. RNA (1 μg per sample) was reverse transcribed (Superscript II; Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and used as template in quantitative real-time–polymerase chain reaction (qRT–PCR).

Antibodies, western blotting and affinity precipitation

Primary antibodies that recognize p53 (clone D-01; EMD Biosciences, San Diego, CA), Rac1 and NF-κB2 (clone 23A8 and 05-361; Upstate, Charlottesville, VA) and actin (sc-1616; Santa Cruz Biotechnology) were used in this study. Horseradish peroxidase-conjugated secondary antibodies were obtained from MP Biomedical (Aurora, OH). Analysis of protein expression was carried out essentially as described previously (30), using 50 μg aliquots of total cellular lysates for western blotting experiments. Activation of Rac1 was performed essentially as described (31). Briefly, 1 mg aliquots of whole-cell lysates were incubated with glutathione–agarose beads conjugated to the cdc42-Rac interaction and binding domain of PAK1 for 1 h at 4°C with rotation. Beads were collected by centrifugation, washed three times in lysis buffer, heated in sample buffer and bound proteins analyzed by western blotting.

Plasmid constructions and short hairpin RNA

The CXCL5 short hairpin RNA (shRNA) plasmid has been described previously (32,33). The shRNA sequence targeting p53 was designed using web-based tools (Ambion). Complementary oligonucleotides were synthesized (Sigma-Genosys, The Woodlands, TX) and annealed to form double-stranded molecules. Controls of 'scrambled' nucleotide sequences with the same base composition were similarly treated. Annealed double-stranded shRNA and control oligonucleotides were ligated into BamHI–EcoRI digested pSirenRetroQ (BD Clontech, San Diego, CA). Plasmids were sequenced as confirmation. Plasmids were transfected using Lipofectamine (Invitrogen) according to standard protocols. Stable clones were selected in the presence of 2 μg/ml puromycin, expanded and gene knockdown confirmed by western blotting. Alternatively, some gene knockdowns were performed using small interfering RNA (siRNA) (Proligo; Sigma, St Louis, MO). Cells were transiently transfected with siRNAs or non-targeting control sequences by nucleofection (Lonza, Gaithersburg, MD). Luciferase reporter plasmids containing the CXCL5 (34) and CXCL8 (35) promoters were generous gifts from Dr A.C.Keates (Harvard Medical School) and Dr N.Mukaida (Kanazawa University), respectively. The promoter region of CXCL8 was subcloned from the original pBSK vector, which included 4.5 kb of promoter sequence up to exon 4 of the CXCL8 gene. A 1.5 kb EcoRI/HindIII fragment was subcloned into the pGL3-basic plasmid (Promega Corp., Madison, WI).

Migration assays

Haptotactic migration assays were carried out essentially as described previously (32). Briefly, 1×10^4 cells resuspended in serum-free Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin were seeded in triplicate onto the upper surface of 8 μm pore size Transwell culture inserts coated on the undersurface with 10 μg/ml fibronectin. Cells were incubated at 37°C and allowed to migrate for 6 h. Cells were fixed in 100% MeOH, stained in 0.1% crystal violet, washed and the non-migrated cells removed with a cotton swab. Membranes were excised from the inserts, mounted on microscope slides and cells photographed and counted in 20 random high-power fields. 'Wound-closure' assays were carried out as described previously (33,36). Briefly, confluent monolayers (triplicate cultures, 12-well plates) were scratched with a sterile pipette tip, washed three times and the width of the denuded space measured at 0 h at three separate points per well and then again after 6–20 h incubation, depending on the cell line.

Quantitative real-time–PCR

qRT–PCR was performed using an ABI 7500 Fast system (Applied Biosystems, Rockville, MD) and an SYBR green-based procedure, as described previously (32). Oligonucleotide primers were designed using the Primerbank database (37). Primer sequences are given in Supplementary Table S1, available at *Carcinogenesis* online.

Luciferase reporter assays

To determine chemokine promoter activity, parental H1299 cells were cultured to 60% confluence in six-well plates, then transfected using Lipofectamine (Invitrogen) with 1 μg of reporter plasmids in which the promoter drives expression of Firefly luciferase, together with 0.1 μg of a 'Renilla' luciferase plasmid to facilitate normalization, and 1 μg of wild-type or mutant p53 expression plasmids or empty vector as control. Forty-eight hours later, cells were harvested, lysates prepared and luciferase activity determined by standard procedures using a commercially available kit (Dual Luciferase Assay System; Promega Corp.).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed essentially as described (33). To cross-link protein and DNA, cell cultures were incubated in 2% formaldehyde for 10 min at ambient temperature and then 200 mM glycine was added for a further 10 min. Cells were washed in cold phosphate-buffered saline, scraped and centrifuged. Pellets were resuspended in lysis buffer containing protease inhibitors and then sheared by multiple passages through a 27.5 gauge needle followed by 25 min of sonication on ice. Following centrifugation, the protein content of the supernatants was determined and equal amounts used for immunoprecipitation with antiacetylated histone H3 antibody or IgG as a control, overnight at 4°C. Immune complexes were captured using Protein A-Sepharose and then washed sequentially in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate and 1% NP-40), high salt buffer (500 mM NaCl, 50 mM Tris pH 8, 0.1% sodium dodecyl sulfate and 1% NP-40), twice in LiCl buffer (250 mM LiCl, 50 mM Tris pH 8, 0.5% sodium deoxycholate and 1% NP-40) and then twice in TE buffer. Protein was eluted from beads in fresh elution buffer (20% sodium dodecyl sulfate, 10 mM dithiothreitol and 100 mM $NaHCO_3$), cross-linking reversed overnight at 65°C in the presence of NaCl and then samples were ethanol precipitated. Following centrifugation, pellets were resuspended in TE buffer and incubated sequentially with 50 μg/ml RNase A (30 min) and 100 μg/ml proteinase K (1 h). Samples were phenol extracted, ethanol precipitated and the pellets washed in 70% ethanol, dried and resuspended in sterile water. Quantitative polymerase chain reaction was carried out as described above, using oligonucleotide primers that target the CXCL5 promoter. Nucleotide sequences are given in Supplementary Table S1, available at *Carcinogenesis* online.

Statistical analysis

Data obtained from migration and qRT–PCR assays were analyzed by *t*-test using the SPSS v.13 software package (SPSS, Chicago, IL). A value of $P < 0.05$ was considered to be statistically significant.

Results

GOF p53 enhances cell migration

Our previous studies indicated that cells expressing mutant p53 harboring a histidine to leucine substitution at codon 179 (p53-H179L) gained a metastatic phenotype when xenografted to the flanks of nude mice (8). Therefore, we determined the migratory ability of H1299 cells expressing GOF p53 proteins (Figure 1A) as increased motility is a key property of metastatic cells. Thus, cells were subjected to *in vitro* wound-closure (scratch) assays, where confluent monolayers were denuded and the width of the gap measured at the initial time and again after 10 h migration. As indicated in Figure 1B and C,

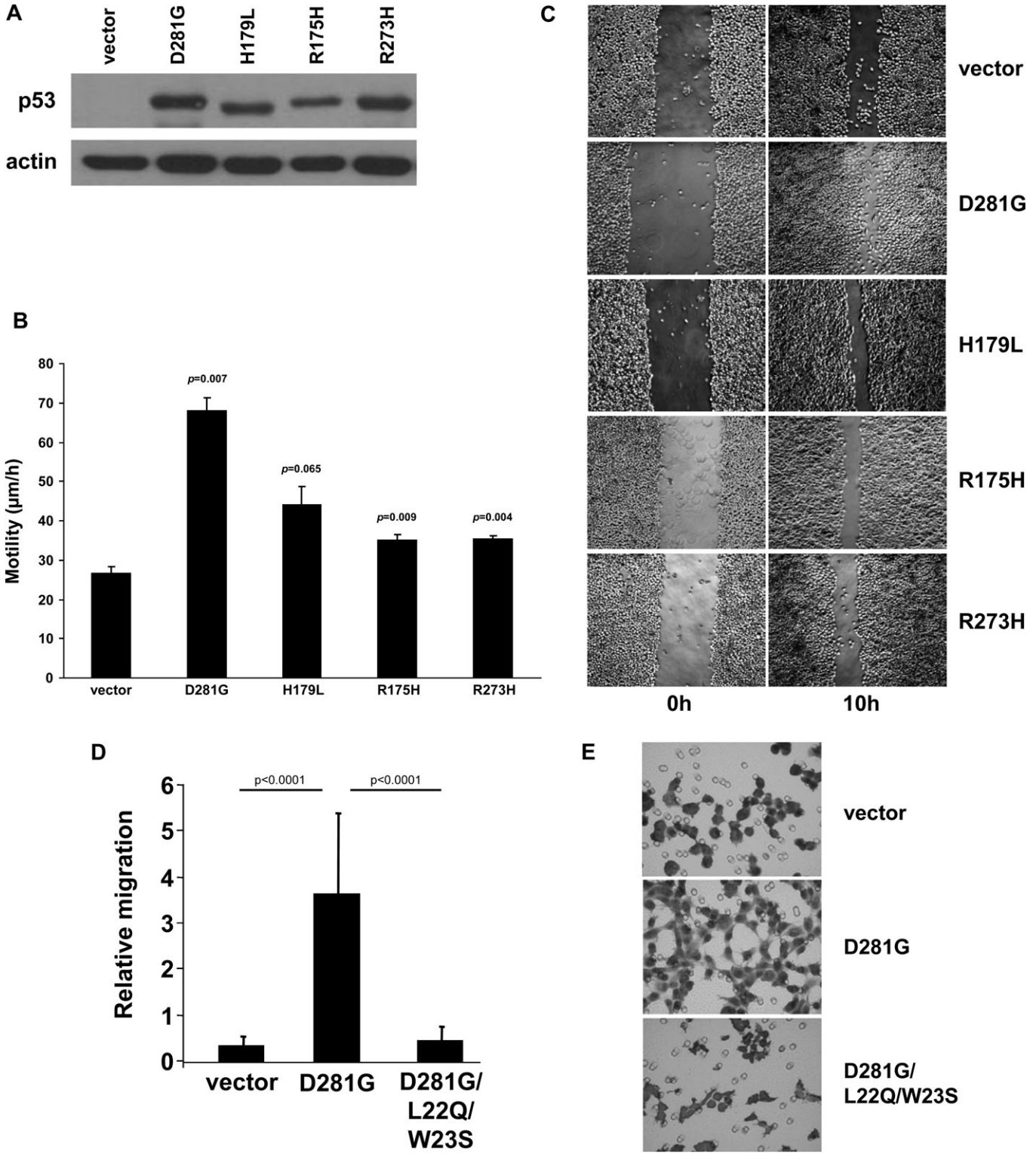
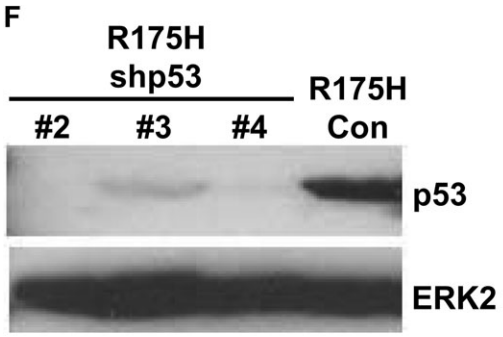


Fig. 1. Oncogenic p53 enhances cell motility. (**A**) H1299 cells, transfected as indicated, were used to prepare protein lysates and then western blotted with the indicated antibodies. (**B** and **C**) Cells described in (A) above were subjected to wound-closure assays as described in Materials and methods. Original magnification ×50. (**D** and **E**) H1299 cells, transfected as indicated, were counted and plated in triplicate in the upper chambers of Transwell filters coated on the underside with fibronectin and allowed to migrate for 6 h, after which migrating cells were stained and counted. Original magnification ×200. (**F**) H1299/R175H cells were stably transfected with shRNA or non-targeting control (Con) plasmids directed against p53 and p53 expression determined by western blotting of 50 μg aliquots of total protein lysates. Data are representative of at least three independent experiments.

expression of p53 mutants enhances H1299 cell motility. Enhanced migration was also observed in haptotactic migration assays. Cells were seeded in the upper chamber of Transwell inserts and allowed to migrate for 6 h, after which migrating cells were stained and counted. As shown in Supplementary Figure S1, available at *Carcinogenesis* online, H1299 cells expressing R175H, R273H and D281G p53 mutants exhibited enhanced migration in this assay by 3- to 4-fold compared with HC5 empty vector controls. Compared with H1299/



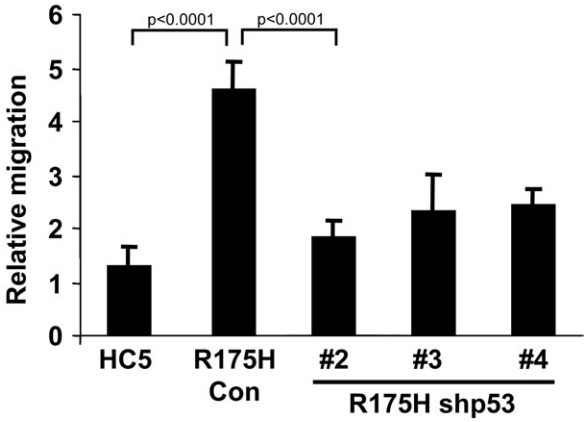


Fig. 1. (Continued)

D281G cells, expression of the D281G/L22Q/W23S transactivation-deficient mutant showed decreased migration similar to the vector control (Figure 1D and E), suggesting that transactivation functions of mutant p53 are required. To examine the contribution of GOF p53 to cell motility further, we generated stable knockdown of mutant p53 in H1299/R175H cells using shRNA (Figure 1F). When these cells were subjected to migration assays, we found that decreased expression of p53-R175H resulted in decreased cell migration (Figure 1F). Together, these data clearly indicate that GOF p53 proteins contribute directly to enhanced motility when expressed in H1299 cells. Consistent with this, we found elevated activity of Rac1 in H1299 cells expressing GOF p53 mutants compared with vector controls (Supplementary Figure S2 is available at *Carcinogenesis* Online), which is consistent with increased cell motility.

The GOF p53 transcriptome differs from that of wild-type p53-expressing or p53-null cells in many respects (28). One key difference may be mutant p53-dependent upregulation of the transcription factor NF-κB2. Indeed, our studies have already shown that these mutants are able to decrease the cellular sensitivity to chemotherapeutics, such as etoposide in an NF-κB2-dependent manner (29). Therefore, we were keen to determine whether NF-κB2 was also important for the effects of GOF p53 proteins on cell motility. To address this possibility, MDA-MB-435 melanoma cells, which express an endogenous G266E p53 substitution mutant, were transfected with either p53- or NF-κB2-directed siRNA. We performed haptotactic migration assays, as described above, and determined the relative motility of these cells. Migration was inhibited by suppression of either mutant p53 or NF-κB2 (Figure 2A), suggesting that NF-κB2 might mediate some of the motility-enhancing effects of mutant p53. As shown in Figure 2B, siRNA-mediated gene knockdown of both targets was achieved, as judged by western blotting.

Similar migration experiments were carried out using H1299/R175H cells transfected with p53 or NF-κB2 siRNA that produced similar results (Supplementary Figure S3A is available at *Carcinogenesis* Online). Conversely, ectopic expression of NF-κB2 in HC5 control cells resulted in enhanced migration in wound-closure assays (Supplementary Figure S3B is available at *Carcinogenesis* Online), whereas siRNA knockdown of endogenous NF-κB2 in HC5 cells produced a small, but statistically significant, decrease in migration (Supplementary Figure S3C is available at *Carcinogenesis* Online). Thus, NF-κB2 may also regulate migration independent of mutant p53.

GOF p53 proteins upregulate CXC-chemokine expression

We had previously found a link between CXC chemokines and tumor cell motility in squamous cell carcinomas (32,38). As the expression of these proteins has been reported to be regulated by NF-κB family members (39), we investigated whether GOF p53 proteins could upregulate chemokine expression. H1299 cells transfected with a range of GOF p53 mutants or empty vector were analyzed by qRT–PCR for

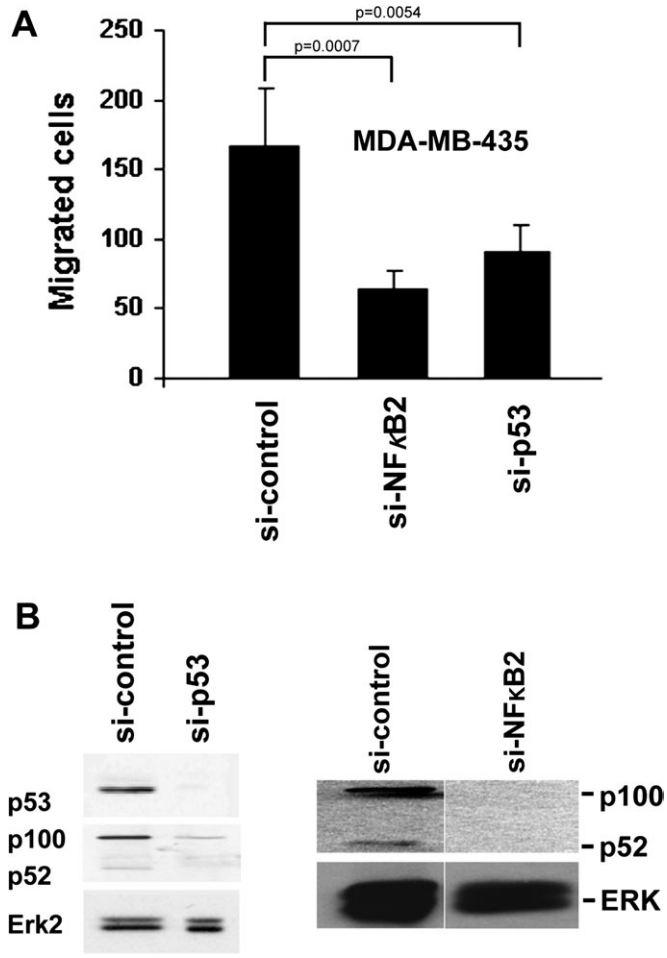


Fig. 2. Cancer cell migration is inhibited by p53 or NF-κB2 downregulation. (**A**) MDA-MB-435 cells were transfected with siRNAs directed to inhibit expression of p53 or NF-κB2 or a non-targeting control. Forty-eight hours later, standard Transwell migration assays were carried out as described in Materials and methods. (**B**) In parallel, total protein lysates were prepared and 50 μg aliquots were western blotted with anti-p53 or anti-NF-κB2 antibodies to determine the extent of gene knockdown, normalized to extracellular signal-regulated kinase 2 (ERK2).

expression of CXCL5, CXCL8 and CXCL12. Although chemokine levels were low in vector-transfected cells, mutant p53-expressing cells expressed elevated levels of CXCL5 (Figure 3A), CXCL8 (Figure 3B) and CXCL12 (Figure 3C).

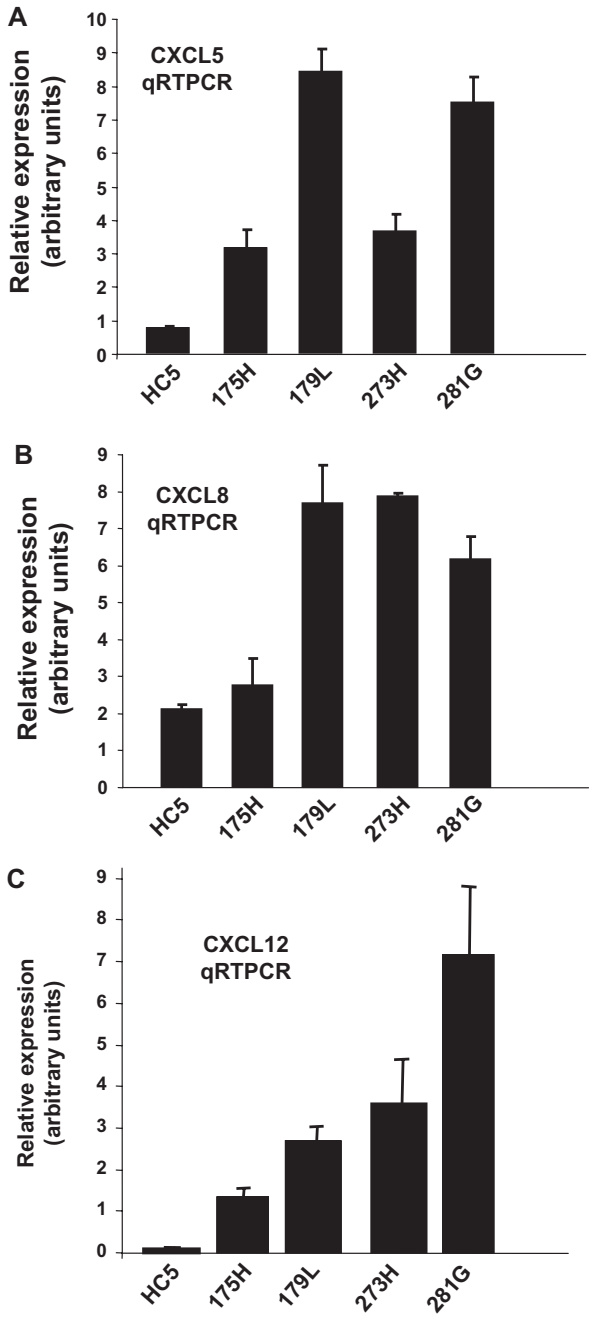


Fig. 3. Cells expressing oncogenic p53 proteins express elevated levels of CXC chemokines. RNAs isolated from the indicated cell lines were reverse transcribed and the resultant complementary DNAs used as template in qRT–PCR experiments for (**A**) CXCL5, (**B**) CXCL8 and (**C**) CXCL12. Chemokine expression data are shown normalized to actin. Bar = SD.

Using luciferase reporter assays, we tested the ability of mutant p53 to activate chemokine promoters. Analysis of CXCL5 (Figure 4A) and CXCL8 (Figure 4B) promoter activity indicated that, although wild-type p53 represses [as shown by Moskovits *et al.* (25) for CXCL12], promoter activity is stimulated by p53 mutants. We also examined the endogenous CXCL5 promoter using chromatin immunoprecipitation. As shown in Figure 4C, enhanced acetylated histone H3 was bound to the CXCL5 promoter in H1299 cells expressing mutant p53-R273H compared with control, suggesting that the promoter is in a conformation permissive for transcription. Studies using shRNA knockdown of endogenous mutant p53 (R267P and R273C, respectively) in H1437 and H1048 lung cancer cells showed significantly reduced expression of CXCL5 (Figure 4D) when p53 expression was inhibited. Together, these data indicate that chemokine expression can be upregulated in cells expressing mutant p53 proteins.

CXCL5 mediates some of the pro-migratory effects of mutant p53

To determine if CXCL5 contributed to the pro-migratory activity of mutant p53, we transfected H1299/vector and H1299/D281G cells with CXCL5 shRNA plasmid or non-targeting control plasmid. A significant reduction in motility of H1299/D281G cells was observed when these were transfected with CXCL5 shRNA plasmid (Figure 5A), whereas similar transfection of control cells did not produce any significant effect on cell motility. Knockdown of CXCL5 in cells expressing p53-D281G also reduced motility of cells in haptotactic migration assays (Figure 5B), suggesting a direct contribution of CXCL5 to motility in this system. Consistent with these results, we found that ectopic expression of CXCL5 enhanced migration of H1299 cells in the absence of mutant p53 (Supplementary Figure S4 is available at *Carcinogenesis* Online).

To extend our findings, we performed similar experiments in MDA-MB-435 melanoma cells, which harbor an endogenous G266E GOF p53 mutant. In the presence of the p53 shRNA plasmid, p53 protein was undetectable by western blot (Figure 6A) compared with non-targeting control cells and resulted in reduced cell migration (Figure 6B) as well as diminished expression of CXCL5 (Figure 6C), consistent with our data indicating a role for mutant p53 in regulation of CXCL5 expression (Figures 3 and 4). Furthermore, MDA-MB-435 cells with shRNA-reduced levels of CXCL5 showed decreased motility *in vitro* compared with controls (Figure 6D).

We also carried out microarray analysis of MDA-MB-231 cells with or without shRNA-mediated knockdown of the endogenous mutant p53-R280K. When mutant p53 levels were reduced in these cells, we found significantly lowered expression of 45 genes (including p53) and upregulation of 40 genes (Supplementary Table 2 is available at *Carcinogenesis* Online). Consistent with our observations in H1299/mp53 cells and in MDA-MB-435 cells, downregulation of mutant p53 expression led to a reduction in levels of several chemokines including CXCL1, CXCL2, CXCL8 and CCL2 as well as repression of potent stimulators of chemokine expression such as interleukin-1α, interleukin-1β and interleukin-6. Together, these data support a role for chemokines as motility factors that are regulated, at least in part, by mutant p53.

Mutant p53 and CXCL5 are co-expressed in human lung cancer

To determine whether the relationship that we had discovered between mutant p53 and chemokines was relevant to tumor development *in vivo*, we prepared RNA from archival specimens of lung cancers of known p53 status and determined CXCL5 expression by qRT–PCR. As shown in Figure 6E, expression of CXCL5 was generally low in tumors containing wild-type p53, whereas elevated expression was noted in five of seven mutant p53-containing tumors. Notably, one tumor containing a 'hotspot' p53 mutant, R248L, showed a substantial increase in CXCL5 expression. These observations are consistent with the data derived from cell line models and support a role for mutant p53 in upregulating expression of CXCL5, and likely other chemokines, during tumor development.

Discussion

Previous studies from our laboratories have demonstrated upregulated expression of NF-κB2 in cells expressing GOF p53 mutants (27–29). NF-κB proteins are well-recognized mediators of carcinogenesis, as well as playing key roles in immunity and inflammation (40–42). Hodgson *et al.* (43) reported a requirement for NF-κB activation in melanoma cell motility on collagen , and other studies indicated that elevated NF-κB expression and activity in melanoma were dependent

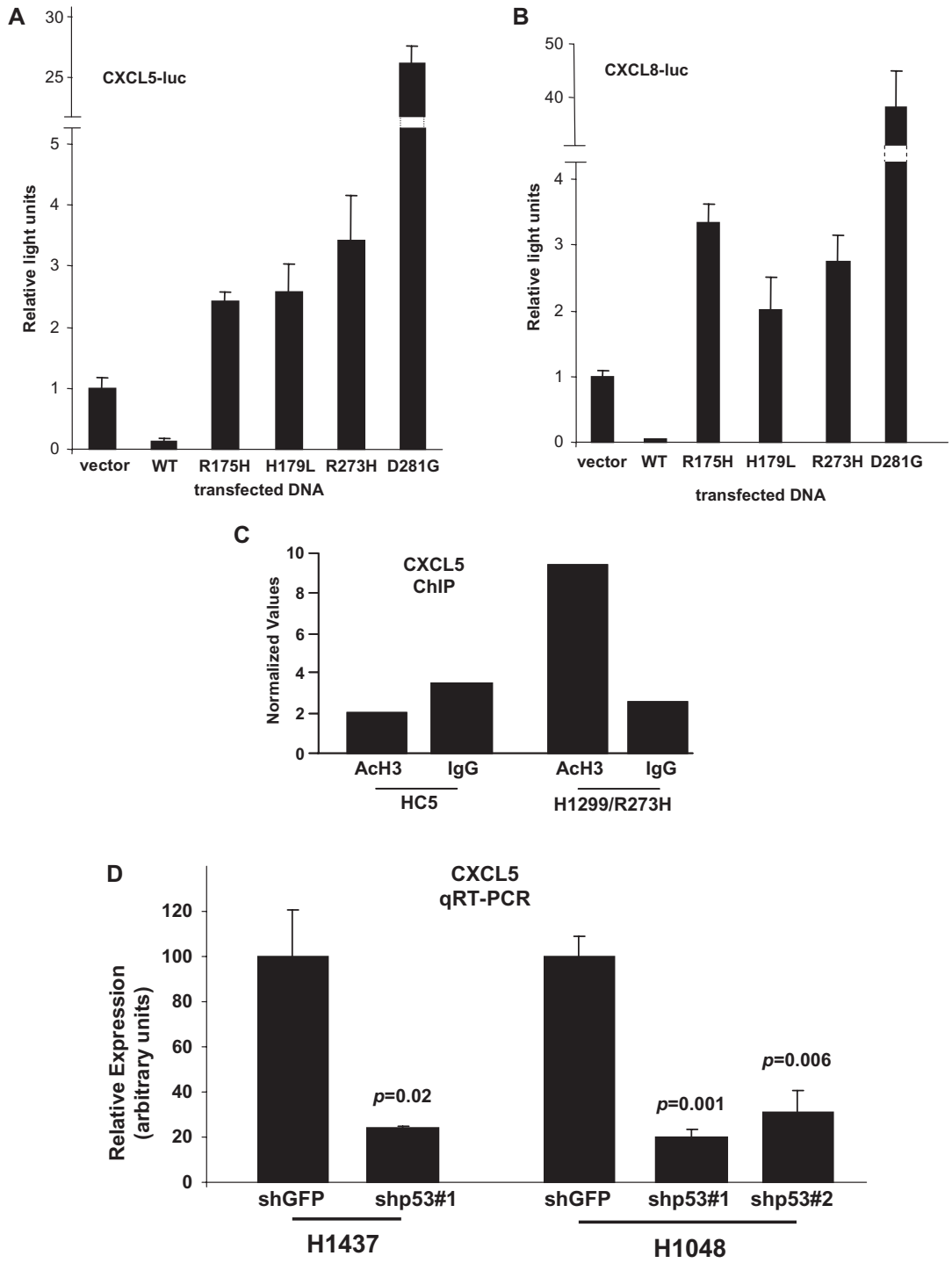


Fig. 4. GOF p53 proteins transactivate the CXCL5 promoter. (**A**) Saos-2 cells were transfected with a CXCL5 promoter-luciferase plasmid and plasmids encoding wild-type p53 (WT) or the indicated mutant p53 proteins. Forty-eight hours later, luciferase activity was determined. (**B**) Saos-2 cells were transfected as in (A) but with the CXCL8 promoter-luciferase plasmid instead of the CXCL5 promoter. Luciferase activity was determined as in (A). (**C**) Chromatin immunoprecipitation assays were carried out in vector-transfected or p53-R273H-transfected H1299 cells. Relative amounts of acetylated histone H3 on the CXCL5 promoter were determined by quantitative polymerase chain reaction as described in Materials and methods. (**D**). Endogenous mutant p53 was knocked down by shRNA in the indicated cell lines and CXCL5 expression determined by qRT–PCR as described in Materials and methods.

on Akt (44). We found that inhibition of NF-κB2 was sufficient to inhibit motility induced by expression of mutant p53, which raises the possibility of a mechanistic role for this transcription factor in p53 GOF. Also, NF-κB family proteins have been reported as key regulators of chemokine expression (45). This is consistent with our finding of elevated expression of CXC chemokines in cancer cells harboring GOF p53 mutants. It is also possible that mutant p53-mediated activation of chemokines could result in activation of an autocrine- or paracrine-positive feedback loop as CXCL8 may, in turn, activate NF-κB (46). However, although we found that GOF p53 proteins regulate NF-κB2 [Figure 2; Supplementary Figure S3 is available at *Carcinogenesis* Online; (29)], it is clear that mutant

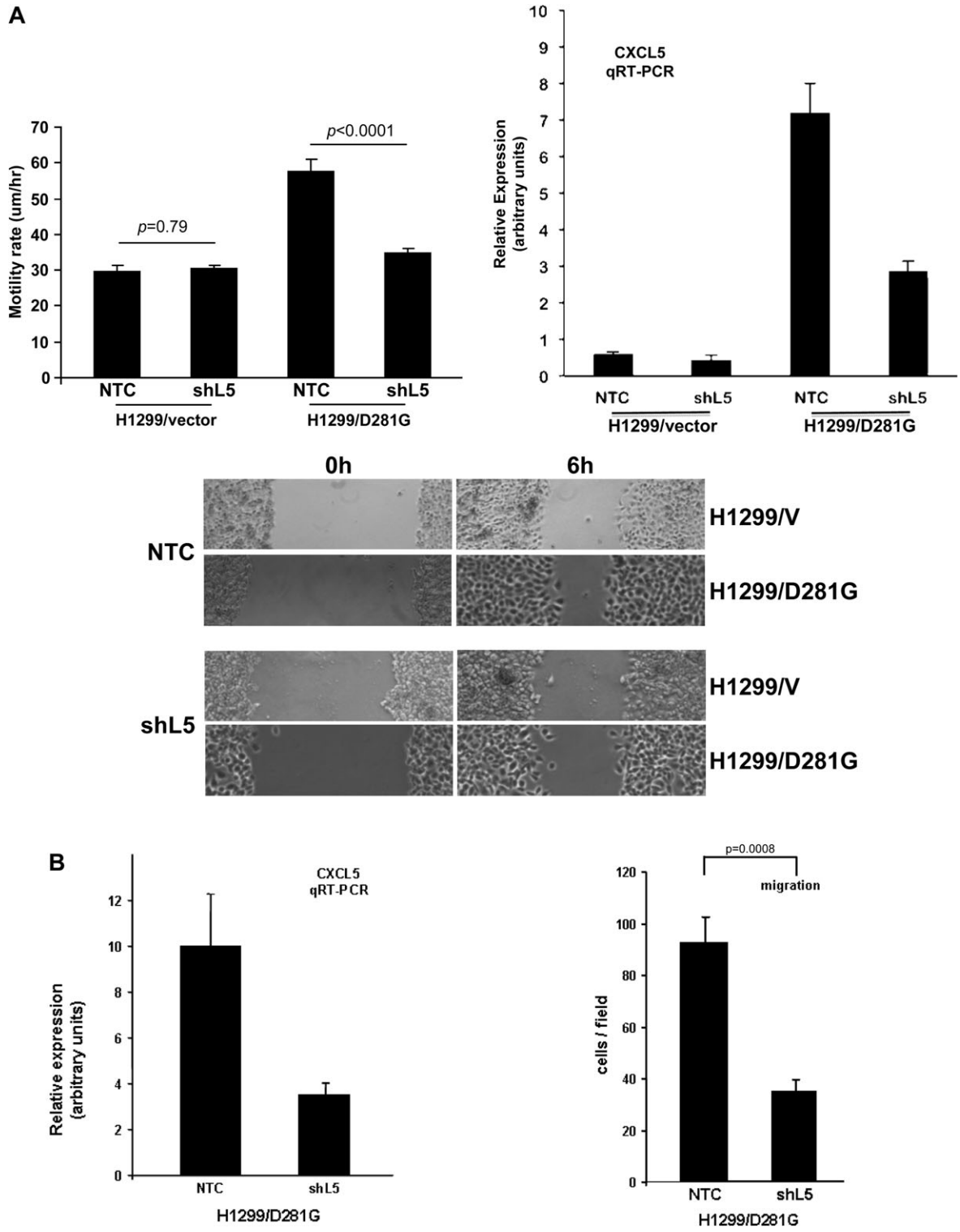


Fig. 5. CXCL5 contributes to GOF p53-induced motility. (**A**) H1299 cells expressing p53-D281G, or empty vector as control, were stably transfected with plasmids encoding CXCL5 shRNA (shL5) or a non-targeting control (NTC) and pooled populations isolated. Total RNA was prepared, reverse transcribed and CXCL5 expression determined by qRT–PCR, normalized to actin. In parallel, migration was determined by wound-closure assay. Original magnification ×50. (**B**) H1299/p53-D281G cells with or without CXCL5 knockdown were tested for migratory ability by Transwell assay on fibronectin. Migrated cells were fixed, stained and counted after 6 h. CXCL5 expression was reconfirmed by qRT–PCR (left panel).

p53-independent NF-κB2 activity is also important for cancer cell migration as we found a small but statistically significant decrease in migration of p53-null H1299 cells when NF-κB2 levels were reduced by RNA interference (Supplementary Figure S3C is available at *Carcinogenesis* Online).

In addition to NF-κB-dependent pathways, mutant p53 may up-regulate chemokine expression by alternative mechanisms. In a recent study, Yan and Chen provided evidence for direct binding of mutant p53 to the CXCL1 promoter in SW480 cells [p53-R273H and p53-P309S (47)]. Furthermore, Fontemaggi *et al.* (48)

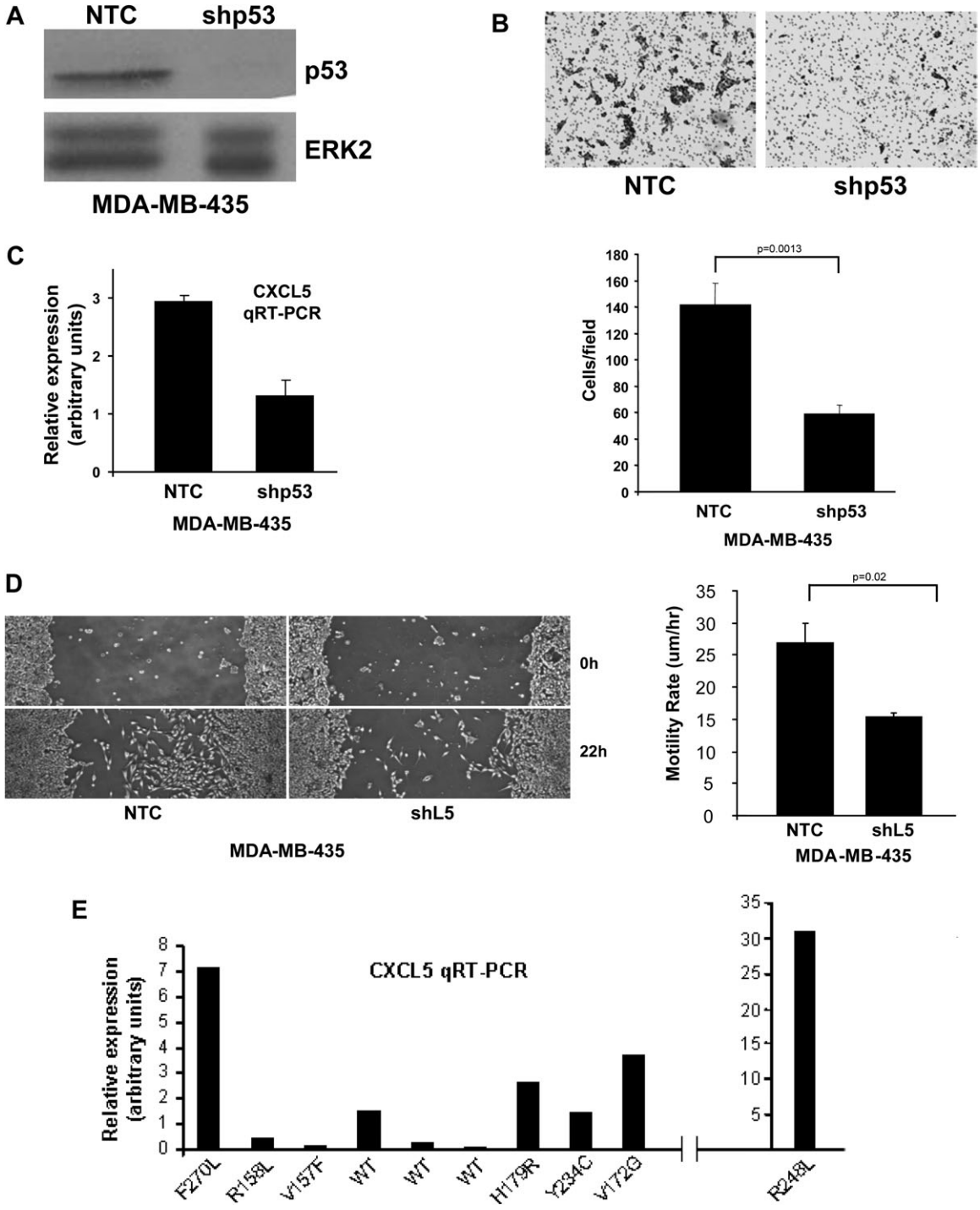


Fig. 6. Relationship between CXCL5 expression and mutant p53 in human cancer. (**A**) Protein lysates were prepared from MDA-MB-435 cells harboring p53 shRNA or a non-targeting control. Western blots (50 μg protein per lane) were probed with the indicated antibodies. (**B**) MDA-MB-435 cells were subjected to haptotactic migration assays for 6 h. Numbers of cells were counted in 20 random high-power (×200) fields. Bar = SD. (**C**) Total RNA was prepared from the indicated cells, reverse transcribed and the complementary DNA used as template in qRT–PCR experiments. Expression data are shown normalized to actin. Bar = SD. (**D**) MDA-MB-435 cells with or without CXCL5 knockdown were subjected to wound-closure assays. Bar = SD. (**E**) RNA was prepared from specimens of human lung cancers of known p53 status (as indicated), reverse transcribed and the resultant complementary DNA used as template in qRT–PCR experiments. Relative expression is shown after normalization to an internal standard (GAPDH).

identified *ID4* as a transcriptional target of p53 GOF mutants including R175H, R273H and R280K and showed that CXCL1 and CXCL8 messenger RNAs were stabilized by binding of ID4 protein, leading to enhanced expression of these chemokines. Another likely mechanism to explain mutant p53 GOF is interference with the function of other p53 family proteins, such as p63 (49,50). Indeed, preliminary studies from our laboratory are suggestive of a role for p63 inactivation in enhanced chemokine expression and motility in cells expressing mutant p53 (Yeudall,W.A., Wang,H. and Bulysheva,A.A, unpublished results). This area is under active investigation. Thus, these reports, together with our own studies presented here, support the existence of multiple biochemical mechanisms utilized by mutant

p53 to deregulate expression of chemokines that are directly involved in tumor progression, angiogenesis and metastasis.

The CXCL12/CXCR4 signaling axis is also known to be important for cellular migration (19), and wild-type p53 has been shown to suppress expression of CXCL12 (25) as well as CXCR4 (51), the latter of which inhibits CXCL12-dependent invasion *in vitro*. Our results provide evidence that wild-type p53 also represses the activity of the CXCL5 and CXCL8 promoters. Thus, loss of p53 alone would be sufficient to elevate expression of these chemokines. However, GOF p53 proteins induce expression of CXCL5, CXCL8 and CXCL12, representing a potential mechanism to explain GOF p53-dependent motility. Furthermore, although the sample size was small, we found that CXCL5 levels were elevated in the majority of lung tumors containing p53 mutations and to a high degree in a tumor containing a codon 248 mutation, a known GOF mutant (4). It should be cautioned, though, that even although we saw reduction of cell motility in the presence of the transactivation-deficient D281G/L22Q/W23S mutant, this does not necessarily imply that transcriptional transactivation by the p53 protein is required as this mutant has been reported to be incapable of binding to MDM-2 or MDM-X (52,53). This warrants further investigation.

In this study, we have provided strong evidence of a link between GOF p53 mutants and cell motility. Several previous lines of evidence suggest that wild-type p53 can act as a suppressor of migration, invasion or metastasis, in part through control of Rho-family GTPases. Together with $p14^{ARF}$, wild-type p53 has been shown to suppress activity of phosphatidylinositide 3-OH kinase–Rac1 signal transduction pathways (54), whereas filopodia formation is inhibited by p53-dependent regulation of cdc42 (55). In addition, p53-null fibroblasts were induced to invade by ectopic expression of constitutively active Rho-GTPases (56). Furthermore, in a model of hepatocellular carcinogenesis, expression of polyoma middle T antigen in the absence of p53 resulted in metastatic progression, whereas this was not observed in $p53^{+/+}$ mice (57). Here, we found that GOF p53 proteins activate Rac1, a key enzyme involved in actin dynamics and cell motility. Other recent studies indicate that loss of p53 function is sufficient to enhance motility and invasion of mouse embryo fibroblasts through a RhoA-ROCK-mediated mechanism (58), whereas wild-type p53 inhibits RhoA activation by oncogenic Ras (59). Evidence suggests that CXCR1 and CXCR2 signaling may be mediated by Rho-ROCK and Rac-dependent pathways (60). These results are not inconsistent with our own data: using p53-null cancer cells, we showed that GOF p53 proteins could enhance cell motility and activate Rac1, whereas motility is inhibited by blocking p53 expression or function. Also, we found that wild-type p53 represses chemokine transcription, whereas GOF mutants stimulate expression over and above this loss of repression. Thus, there may be two aspects to consider: repression of motility by wild-type p53 and active enhancement by mutant p53 (a true 'GOF' beyond that of the null phenotype). However, although we found a functional relationship between chemokine expression and cell motility in cells containing mutant p53, elevated chemokine messenger RNA levels did not correlate completely with elevated motility. There may be several explanations for this, including differential stabilization of chemokine messenger RNAs, different protein–protein interactions of specific p53 mutants or chemokine-independent effects of mutant p53 on motility. Our ongoing work is dissecting these possibilities.

It is well recognized that wild-type p53 is a negative regulator of angiogenesis (61), as a regulator of thrombospondin (24), whereas p53 mutation is associated with expression of the pro-angiogenic vascular endothelial growth factor and poor prognosis in breast cancer (62). Our previous studies documented the highly vascular nature of mutant p53-induced tumors (8). The data presented in this study provide a likely explanation of these observations as CXCL5, CXCL8 and CXCL12 are key mediators of neovascularization (22) that affect endothelial cell migration and vascular sprouting (63,64). Furthermore, CXCR7, a receptor for CXCL12, is expressed on blood vessels associated with breast and lung tumors but not on normal vasculature and enhances tumor progression (65). Thus, in addition to CXCR4 expressed on tumor cells providing a means of homing of metastatic cells to target organs (20), induction of CXCL12 in tumor cells expressing GOF p53 might stimulate angiogenesis directly. In summary, it is probably that GOF p53 mutants coordinate, via multiple mechanisms, a program of events that impact on a number of factors involved in tumor progression, over and above those affected by loss of the wild-type protein.

Supplementary material

Supplementary Table S1–2 and Figures S1–S4 can be found at http://carcin.oxfordjournals.org/.

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