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Mutant p53 and NOX4 are modulators of a CCL5-driven pro-migratory secretome

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Abstract

Previously, we showed wild-type (WT) and mutant (mt) forms of p53 differentially regulate ROS generation by NADPH oxidase-4 (NOX4). We found that WT-p53 suppresses TGF-β-induced NOX4, ROS production, and cell migration, whereas tumor-associated mt-p53 proteins enhance NOX4 expression and cell migration by TGF-β/SMAD3-dependent mechanisms. In this study, we investigated the role of mutant p53-induced NOX4 on the cancer cell secretome and the effects NOX4 signaling have on the tumor microenvironment (TME). We found conditioned media collected from H1299 lung epithelial cells stably expressing either mutant p53-R248Q or R273H promotes the migration and invasion of naïve H1299 cells and chemotactic recruitment of THP-1 monocytes. These effects were diminished with conditioned media from cells cotransfected with dominant negative NOX4 (P437H). We utilized immunoblot-based cytokine array analysis to identify factors in mutant p53 H1299 cell conditioned media that promote cell migration and invasion. We found CCL5 was significantly reduced in conditioned media from H1299 cells co-expressing p53-R248Q and dominant negative NOX4. Moreover, neutralization of CCL5 reduced autocrine-mediated H1299 cell mobility. Furthermore, CCL5 and TGF-beta from M2-polarized macrophages have a significant role in crosstalk and H1299 cell migration and invasion. Collectively, our findings provide further insight into NOX4-based communication in the tumor microenvironment and its potential as a therapeutic target affecting metastatic disease progression.

Keywords

NADPH oxidase; NOX4; mutant p53; CCL5; TGF-beta; secretome; tumor microenvironment

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1. Introduction:

Approximately 50% of human cancers have a missense mutation within the DNA-binding domain of the *TP53* gene, known as the hot-spot region. Such mutations usually result in a protein that loses it tumor-suppressive functions and gains pro-tumorigenic effects [1, 2]. Mutant p53 can lead to the dysregulation of several downstream events such as resistance to apoptosis, defective DNA damage responses, escape from cell cycle checkpoints, and alterations in the cell secretome, which is the production and secretion of extracellular factors that signal between tumor cells and other surrounding stromal cells comprising the tumor microenvironment (TME) [3, 4].

The tumor microenvironment is a complex environment of tumor cells and resident stromal cells such as fibroblasts, endothelial cells, and immune cells including macrophages and lymphocytes that interact and communicate by secreted factors (growth factors, cytokines, chemokines) and extracellular matrix (ECM) proteins (fibronectin, collagen). Recent studies have shown that some of the oncogenic mutant p53 proteins have a key role in modulating the tumor secretome that support a pro-tumor and pro-migratory environment [5].

Tumor promoting signaling occurs when anti-tumor immune cells lose their ability to attack and destroy cancer cells and become tumor-promoting immune cells that support tumor growth and survival. Inflammation is recognized as a hallmark of cancer. Unlike wound healing, inflammation is considered to be non-resolving throughout cancer development [6]. It is becoming more evident that elevated production of reactive oxygen species (ROS) is a common event during the initiation and progression of the inflammatory response. ROS such as superoxide, hydrogen peroxide and hydroxyl radicals can cause damage by oxidizing biomolecules such lipids, proteins and DNA. However, in recent years, it has become evident that ROS are important intracellular and extracellular signaling molecules [7]. Increased ROS from tumor cells and infiltrating immune cells in the TME has also been shown to be an important contributor to tumor progression [8].

Previously, we showed wild-type (WT) and mutant forms of p53 differentially regulate ROS generation by NADPH oxidase-4 (NOX4) [9]. We found that WT-p53 suppresses TGF-β-induced NOX4, ROS production, and cell migration, whereas tumor-associated mutant p53 proteins enhance NOX4 expression and cell migration by TGF-β/SMAD3-dependent mechanisms. We further demonstrated through analysis of expression data collected from primary tumor clinical samples from The Cancer Genome Atlas (TCGA) that a positive correlation exists between NOX4, and genes involved in EMT, cell migration, and macrophage M1 and M2 genetic signatures. Moreover, this correlation was strengthened in primary tumors with p53 hot spot mutations [10, 11].

In this study, we investigated the role of mutant p53-induced NOX4 in the cancer cell secretome. We found that conditioned media collected from H1299 lung epithelial cells stably expressing mutant p53 proteins (R248Q or R273H) promotes the migration of naïve H1299 cells and recruitment of THP-1 monocytes. These pro-migratory effects were diminished with conditioned media collected from cells that co-expressed dominant negative NOX4 (P437H) with mutant p53. Cytokine array analysis revealed tumor cell

secretion of CCL5 was regulated in a mutant p53 and NOX4-dependent manner. We found that neutralization of CCL5 with monoclonal antibodies significantly reduced autocrine-mediated H1299 cell migration. Furthermore, we found that CCL5 and TGF-beta secreted by M2-polarized macrophages have a significant role in their crosstalk promoting H1299 cell migration and invasion. Collectively, our findings provide further insight into NOX4-based regulation of communication between cells in the TME and its potential as a therapeutic target affecting metastatic disease.

2. Methods:

2.1 Cell Culture

THP-1 human monocytes, H1299 human non-small cell lung carcinoma cells, and MDA-MB-231 human breast epithelial cells were all obtained from the ATCC (Rockville, MD, USA). THP-1 and H1299 cells were maintained in RPMI-1640 and MDA-MB-231 cells were maintained in DMEM (Thermo Fisher Scientific, Asheville, NC) supplemented with 10% fetal bovine serum (HyClone/Thermo Scientific, Logan, UT) and 100 mg/ml of penicillin-streptomycin. All cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.2 Cell Transfections

H1299 were transfected with Fugene 6 (Promega, Madison, WI, USA) using manufacturer's protocols. Briefly, 1.5×10^6 cells were seeded into 10 cm tissue culture dishes (BD Biosciences, San Jose, CA, USA) 24 h before transfection. Transfection mixtures were incubated at RT for 20 min in OPTI-MEM (Thermo Fisher Scientific, Asheville, NC) and then added dropwise to cells. For empty vector control pCMV, p53-R248Q, or p53-R273H stable cell lines the culture medium was changed to RPMI supplemented 10% FBS and 800 ug/ml G418 48 hours after transfection. Stable clones were selected with G418 and analyze for mutant p53 overexpression by immunoblotting.

2.3 Harvest of Conditioned Medium

Approximately 1.5 X 10^6 of H1299 cells stably expressing control pCMV, p53-R248Q, or p53-R273H were seeded into 10 cm tissue culture dishes. Twenty-four hours after seeding, the cells were transfected with 5 μg of 3.1 Vector, NOX4-WT, or NOX4-P437H plasmids with Fugene 6 transfection reagent and incubated for 24 hours. The cells were then washed twice with 1 X PBS followed by one wash with serum-free medium and incubated in 10-12 ml of serum-free phenol red-free RPMI-1640 for 24 hours. The conditioned medium was then harvested and filtered through a 0.22 μm sterile filtered syringe and stored at −80°C.

2.4 Human Cytokine Array

Human cytokine arrays were used according to the manufacturer's protocol. Briefly, conditioned medium was collected after 24 hours in serum-free medium from H1299 cells stably expressing pCMV, p53-R248Q, or p53-R273H and co-expressing pcDNA3.1, Nox4-WT, or Nox4-P437H and incubated overnight with RayBio Human Cytokine Antibody Array #5 containing cytokine specific antibodies (RayBiotech, Peachtree Corners, GA, USA). Arrays were visualized by enhanced chemiluminescence (ECL) method using the

iBright Imaging System (ThermoFisher). Cytokine expression was determined by measuring the signal intensity of each spot normalized to an internal control with ImageJ imaging software (NIH, USA). The complete array map can be found in the supplemental materials or at https://www.raybiotech.com/cytokine-array-c5.

2.5 Polarization of THP-1 cells

THP-1 human monocytes were differentiated into naïve macrophage-like cells (M0) by treatment with phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich). Approximately 10 x 10^6 cells were seeded into a 10 cm culture dish and treated with 50 ng/ml PMA for 72 hours. Following the incubation period, the cells were treated with either LPS (50 ng/ml) and TNF-α (50 ng/ml) or IL-4 (50 ng/ml) and IL-13 (50 ng/ml) for 48 hours to induce M1 and M2 polarized cells, respectively. Conditioned medium was collected from polarized cells by washing the cells twice with 1 XPBS followed by one wash with serum-free medium and incubated with 10-12 ml of serum-free phenol red-free RPMI-1640. After 24 hours, the conditioned medium was collected and passed through a 0.22 μm sterile filtered syringe and stored at −80°C until use.

2.6 Cell Tracker Dye

H1299 and THP-1 cells were loaded with CellTracker™ Red (ThermoFisher). Briefly, the cells were collected by centrifugation and resuspended in 1 ml of prewarmed CellTracker™ Working Solution (2.5 μM). The cell solution was then incubated for 30 minutes at 37°C. After the incubation time, the cells were washed with serum-free medium and prepared for use.

2.7 Cell Migration Assay

H1299 cells were seeded (3.5 × 10^4 per well) in a 96-well tissue culture plates for monolayer scratch wound assays. Wounds were made using WoundMaker (Essen Bioscience, Ann Arbor, MI, USA) 96-pin tool, creating reproducible, uniform cell-free zones 800 μm in width. Immediately following wounding, cells were washed twice with serum-free medium then incubated with conditioned medium for 18 hours during wound closure. Calculation of % wound closure (Wounded Area at Time = 0 – Wounded area 18 hours after wounding)/ (Wounded Area at Time =0) x 100 = % closure.

2.8 Matrigel Invasion Assay

Briefly, 3 x 10^4 H1299 cells were seeded in the upper chamber of a 12-well BD Biocoat Matrigel transwell in serum-free medium. The insert was incubated in the lower chamber containing conditioned medium. After 18 h, non-migrating cells were scraped away and migrating cells were stained with Diff Stain (IMEB, San Marcos, CA, USA). Invading cells were counted from 10 random fields. Visible light images (4 × objective) of fixed and stained migrating cells were captured with a phase contrast microscope (Evos FL Cell Imaging System, Life Technologies, Carlsbad, CA, USA). Matrigel invasion experiments were repeated three times.

2.9 Immunoblotting

Cell lysates were processed for Western blotting by collecting total cell lysates in RIPA buffer containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). Fifty micrograms of unheated total cell lysates were resolved electrophoretically on 4-to-12% Bis-Tris NuPAGE gels (ThermoFisher) and subsequently transferred to polyvinylidene difluoride membranes (ThermoFisher). Western blotting was performed by sequentially blotting with the following antibodies: anti-NOX4 (AF8158) (R & D Systems Inc., Minneapolis, USA), anti-p53 (DO-1) (SC-126), and anti-HSP90 (F-8) (SC-13119) (Santa Cruz Biotechnology, USA).

2.10 Statistical Analysis

Data are represented as the mean +/− standard deviation of the results of at least three independent experiments. Student's t-test was used to calculate significant values. Significance values are indicated as * *P*-value < .05, ** *P*-value < .01, or *** *P*-value < .001.

3. Results:

3.1 Identification of mutant p53 and NOX4-mediated pro-migratory secretory effects

Figure 1A displays an overview of our experimental approach determining whether NOX4 has a role in the mutant p53 secretome. We used 24-hour conditioned medium (CM) to analyze secreted cytokine profiles, as well as migration and invasion, and monocyte recruitment. We examined the p53 and NOX4 protein expression in cell lysates collected from p53-null H1299 lung adenocarcinoma cells stably overexpressing mutant p53 (R248Q or R273H) and transiently overexpressing either NOX4-WT or NOX4-P437H dominant-negative inactive mutant (Figure 1B). Western blotting analysis shows robust transient expression of both wildtype and mutant forms of NOX4 after 48 hours of transfection. Interestingly, heterologous expression of NOX4-WT or NOX4-P437H resulted in molecular weights at approximately 80 kDa and 55 kDa, whereas the endogenous band was detected around 65 kDa. Previous studies have reported molecular masses of Nox4 protein by western blot analysis ranging from 55 to 80 kDa [12-17]. This range of molecular weights could be attributed to a previously reported putative signal peptide cleavage site or predicted glycosylation sites.

Approximately 24 hours after the cells were transfected, the cells were washed and incubated for an additional 24 hours in serum-free medium. This second 24-hour conditioned media was collected from the transfected cells to study the effects of the secretome. We conducted scratch-wound closure (migration) assays using the 96-pin WoundMaker to create uniform wounds approximately 800 μm in width in a 96-well plate. Figure 1C-D shows the effect of conditioned medium on wound closure of H1299 p53-null cells after approximately 18 hours of incubation with CM. We found that wounded cells treated with CM-pCMV/NOX4-WT showed a robust migratory response in comparison with CM- pCMV/3.1 Vector control, which was significantly reduced in wounded cells treated with CM-pCMV/NOX4-P437H (dominant-negative). Moreover, cells incubated with either CM-R248Q/3.1 Vector or CM-R273H/3.1 Vector showed significantly enhanced wound closure in comparison to cells incubated with CM- pCMV/3.1 Vector control. The

promigratory secretome effects of the p53 mutants also appear to involve NOX4 since CM from cells co-expressing dominant-negative NOX4-P437H showed diminished effects on migration. This suggests that NOX4 is also involved in the pro-migratory effect of the mutant p53 secretome. Interestingly, CM from mutant p53 cells overexpressing NOX4-WT did not augment the migratory effects of mutant p53 alone. Thus, tumor cell expression of either mutant p53 or NOX4 induces a pro-migratory secretome that stimulates cell migration involving NOX4.

3.2 The mutant p53/Nox4 secretome can recruit THP-1 monocytes

It is widely known that human tumors can recruit leukocytes including monocytes and macrophages that can infiltrate into malignant tumors that in some instances can comprise up to 50% of the cell tumor mass [18]. To determine whether the mutant p53/NOX4 secretome was involved in the recruitment of monocytes, we suspended THP-1 monocytes in the upper chamber of uncoated 3μm porous transwell inserts and incubated with H1299 CM in the lower chamber, as described in Figure 2. We found that the secretome from H1299 cells expressing either mutant p53 R248Q or R273H, or NOX4-WT could significantly increase the recruitment of THP-1 cells from the upper chamber to the lower chamber of the transwell, whereas CM from dominant-negative NOX4-P437H expressing cells could only minimally recruit THP-1 cells (Figure 2 A and B). Lower chambers containing either 0%, 5%, or 10% serum were used as chemoattractant controls. Together, these data suggest NOX4 has an important role in the mutant p53 secretome of tumor cells by affecting a pro-migratory response by monocytes in the TME.

3.3 Mutant p53 tumor cells secrete CCL5 in a NOX4-dependant manner.

It was previously reported that H1299 cells expressing tumor-associated mutant p53 proteins upregulated chemokine expression and enhanced cell migration [19]. We sought to explore which soluble factors in the mutant p53 secretome are NOX4-dependent using a membrane-based cytokine immunoassay to screen and compared the cytokine/chemokine expression profiles of our CM samples. Image analysis and quantification of signal intensity from three independent experiments are shown in Figure 3A and 3B. Conditioned medium from both p53-R248Q and p53-R273H expressing cell lines displayed a significant increase in the CCL5 chemokine compared to control CM that was downregulated by transfection of dominant-negative NOX4-P437H. While there were some minor changes in other cytokines detected in CM of mutant p53 expressing cells, including Angiogenin and NT-3, CCL5 was the only cytokine/chemokine that was significantly regulated in both a mutant p53- and NOX4-dependent manner when compared to the CM collected from mutant p53/3.1 control and pCMV/3.1 vector control cells. Considering CCL5 is a well-known inducer of migration and recruitment, these data provided insight into what could be the driving force of a mutant p53/NOX4 pro-migratory secretome.

3.4 Neutralization of CCL5 reduces mutant p53/NOX4- mediated tumor cell migration.

To confirm the relevance of enhanced tumor cell secretion of CCL5 has on autocrine signaling, we explored the effects of conditioned medium containing CCL5 monoclonal neutralizing antibodies on cell migration. Figure 4A and 4B show that wound closure was diminished in cells treated with CM-pCMV/NOX4-WT/+CCL5 Mab and CM-R248Q/3.1

Vector/+CCL5 Mab. Interestingly, CM from cells expressing R248Q together with transient overexpression of NOX4 was enough to overcome decreased migration in the presence of CCL5-neutralizing antibodies. We further confirmed our findings in another cell model. We previously reported that TGF-beta-dependent migration of MDA-MB-231 breast epithelial cells involves NOX4 and p53-R280K which is endogenously expressed [9]. We found that MDA-MB-231 cells treated with MDA-MB-231 CM containing CCL5 neutralizing antibodies resulted in significant reduction in cell migration when compared to control cells treated with CM lacking CCL5 neutralizing antibodies (supplemental S2). Together, these data suggest mutant p53 and NOX4 together are involved in a tumor cell pro-migratory secretome driven by CCL5.

3.5 TGF-beta and CCL5 from polarized macrophages induce tumor cell migration and invasiveness.

CCL5 can be chemotactic for different types of immune cells. Previous reports have shown that the secretion of CCL5 from lung adenocarcinoma cells resulted in the recruitment of immunosuppressive M2-polarized macrophages to the tumor microenvironment and increased tumor growth [20]. Moreover, NOX4 also has been associated with CCL5 expression and M2 macrophage recruitment. In a recent report by Mongue-Din et al., cardiomyocytes of transgenic mice overexpressing NOX4 altered the baseline inflammatory environment of the myocardium with increased expression of CCL5 and recruitment of M2 polarized macrophages [21]. We have previously shown that TGF-beta is a potent inducer of NOX4 in several cell types [9, 10, 22, 23].

Next, we wanted to determine whether TGF-beta and/or CCL5 secreted from polarized macrophages influence the mobility of p53-null H1299 tumor cells. We collected conditioned medium from THP-1 cells that were either non-polarized (M0), or pro-inflammatory, classically activated (M1), or anti-inflammatory, alternatively activated (M2) macrophage like cells. Wounded H1299 cells were incubated with CM-M0, CM-M1, or CM-M2 in the presence or absence of TGF-beta or CCL5 neutralizing antibodies. Figures 5A and 5B demonstrate that 24-hour treatment of CM-M2 with either TGF-beta or CCL5 neutralizing antibodies, or both in combination, decreased the percent of wound closure of H1299 cells compared to wounded cells treated with CM-M2 in the absence of neutralizing antibodies. We then sought to determine whether TGF-beta and CCL5 had similar effects on the invasive potential of CM from polarized macrophages on H1299 cells. We seeded H1299 cells in the upper chamber of a Matrigel transwell that was then incubated in a lower chamber containing CM with or without neutralizing antibodies for approximately 18 hours. In Figure 5C and 5D, we found TGF-beta and CCL5 neutralizing antibodies alone or in combination significantly reduced the number of cells invading through the Matrigel basement membrane. These data suggest TGF-beta and CCL5 secreted from M2 polarized macrophages can crosstalk with tumor cells in the TME and promote cell migration and invasion.

4. Discussion:

During cancer progression, chemokines are induced and secreted by tumor or stromal cells to regulate the directional migration of monocytes and macrophages to the tumor site. It is well established that chemokines have pro-oncogenic effects in several cancer types [24]. While CCL5 is typically relevant to inducing an immune response, CCL5 in the TME can promote cancer progression and metastasis. In many tumors including breast, lung, gastric, and prostate the CCL5/CCR5 axis is associated with increased invasion and metastasis [25, 26].

Here, we demonstrate tumoral NOX4 can act through mutant p53 to promote a pro-migratory and pro-invasive secretome driven by CCL5 (Figure 6). Tumor cell secretion of CCL5 induced autocrine-mediated tumor cell migration and recruited THP-1 monocytes in a paracrine fashion. We also showed that the lung tumor cells were responsive to TGF-beta and CCL5-derived from a polarized M2 macrophage secretome showing an increase in migration and Matrigel invasion compared to an M1 or M0 secretome.

When we analyzed CCL5 mRNA and protein expression in the tumor cells, we did not observe significant changes between mutant p53 vs. control or NOX4 vs. control transfected cells (unpublished data). However, we observed changes in CCL5 that were both mutant p53 and NOX4-dependent when analyzing the cell secretome by cytokine array, indicating mutant p53 and NOX4 together regulate CCL5 at the secretory level. Previous studies have shown mutant p53 was involved driving a pro-metastatic cell secretory pathway. A recent study by Capaci et al., showed mutant p53 proteins induced the expression of miR-30d, which increased the expression of several genes involved in vesiculation of the Golgi apparatus resulting in enhanced release of soluble factors that favored a more tumorigenic microenvironment [27]. Moreover, a previous report demonstrated ER-localized NOX4 interacting with calnexin, which is involved in the quality control of protein folding and the secretory pathway [28]. Collectively, these data suggest mutant p53 and NOX4 are regulators of the CCL5 secretory pathway.

We have previously reported how tumor-associated mutant p53 proteins are involved in the epigenetic regulation of NOX4 in a TGF-beta/SMAD3-dependent manner in tumor cells [10]. Our analysis of a large pan-cancer cohort of 23 tumor types in The Cancer Genome Atlas (TCGA) also showed a high correlation between mutant p53 status and NOX4 gene expression in primary clinical tumor samples [11]. Furthermore, in the context of mutant p53 bearing tumors, higher NOX4 expression was correlated with several cancer progression gene expression programs as well as poorer patient survival. While we and others have reported on NOX4 expression in tumor cells, there are multiple reports that indicate NOX4 in non-tumor stromal fibroblast cells is also a contributor to the TME and cancer progression [29-31]. Since ROS have a pivotal role in cellular homeostasis, it is likely NOX4 is the ROS-generator of several cell types that make up the TME. Several recent reports have indicated that pathways involved in crosstalk between the tumor and surrounding stroma may be promising therapeutic targets. Sampson et al., reported tumor cell secretion of TGF-beta induced NOX4 in peri-tumoral fibroblast stromal tissue resulting in myofibroblast activation and ECM production that sustain the TME [29].-Moreover, in another study, Ford

et al., suggested CAFs expressing NOX4 protect tumor cells from killing by infiltrating $CD8^+$ T-cells; here, targeted knockdown or pharmacological inhibition of NOX4 in CAFs promoted infiltration of $CD8^+$ T-cell, allowing them to attack and kill tumor cells [30].

NOX4 has also been detected in monocytes and macrophages. Helfinger et al. reported that NOX4 deficiency reduces M2 or M(IL4+IL13) polarization and forces expansion of M1 or M(LPS + IFN-γ) macrophages in a murine inflammation-driven fibrosarcoma model [32]. They demonstrated how NOX4 deficiency promotes expression of pro-inflammatory genes and cytokines, which is accompanied by increased numbers of proinflammatory $Ly6C^+$ macrophages in tumors [32]. This model provides evidence that NOX4 expression results in an anti-inflammatory response. Here we demonstrate that tumoral CCL5 under the influence of mutant p53 and NOX4 is not pro-inflammatory, but rather pro-migratory and pro-invasive.

Collectively, our data provides new insight into the role of mutant p53 /NOX4/CCL5 -dependent signaling axis that affects both tumor and immune cell migration in the tumor microenvironment. Future studies should explore whether targeting components of this novel signaling pathway would provide an effective strategy to combat metastatic disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mutant p53 and NOX4 together induce a tumor cell pro-migratory secretome driven by CCL5.
- Conditioned media (CM) collected from tumor cells expressing mutant p53 proteins promotes the migration of naïve tumor cells and recruitment of THP-1 monocytes.
- CCL5-induced tumor cell migration is mediated in an autocrine manner.
- Tumor cells are responsive to TGF-beta and CCL5 derived from a polarized M2 THP-1 cell secretome, causing increased tumor cell migration and invasion.

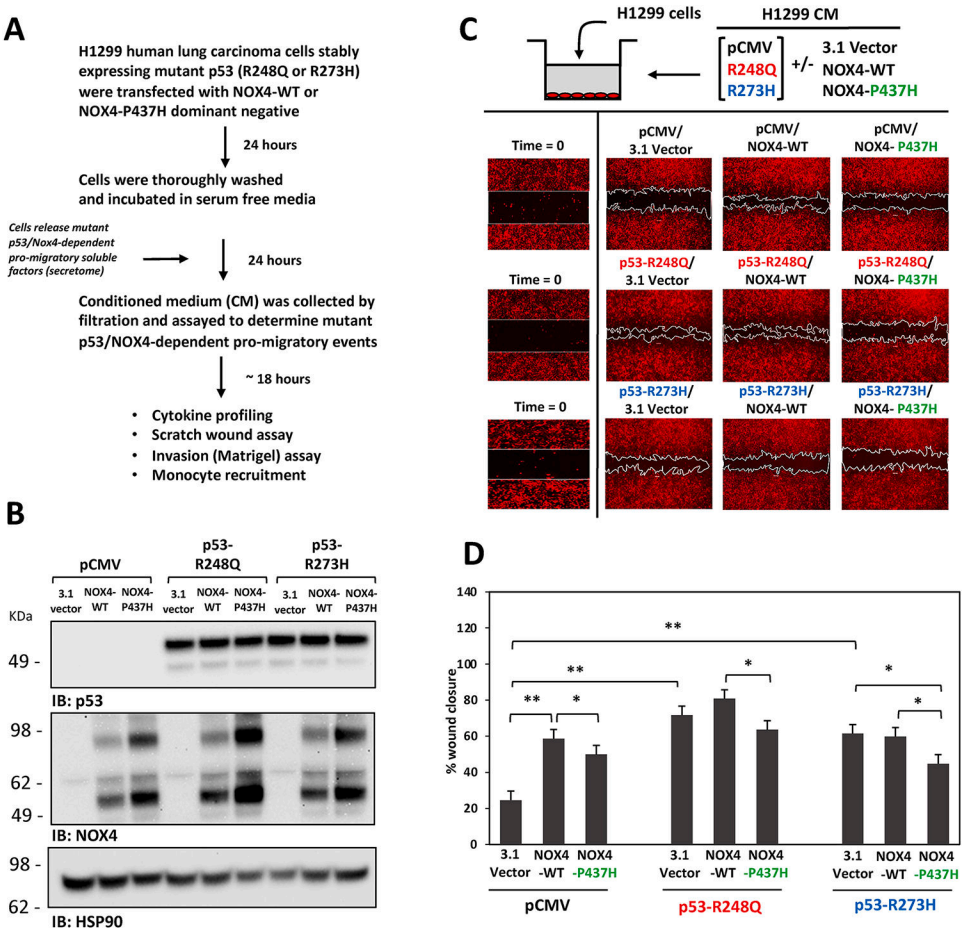

Fig 1.

Mutant p53 and NOX4 are modulators of a pro-migratory secretome in human non-small lung carcinoma cells. (**A**) Overview of timelines of cell treatments and collection of conditioned medium (CM). (**B**) Western blotting analysis of 50 micrograms of total H1299 cell lysates probed sequentially with antibodies against NOX4, p53, and HSP90. (**C**) Scratch-wound closure assays of naïve H1299 cells treated with CM for approximately 18 hours before the migrating cells were imaged. The images presented are representative of n=3, in triplicate. (**D**) Calculation of % wound closure (Wounded Area at Time =0 – Wounded area 18 hours after wounding)/ (Wounded Area at Time =0) x 100 = % closure. Significance values are indicated as * P-value < .05 or ** P-value < .01.

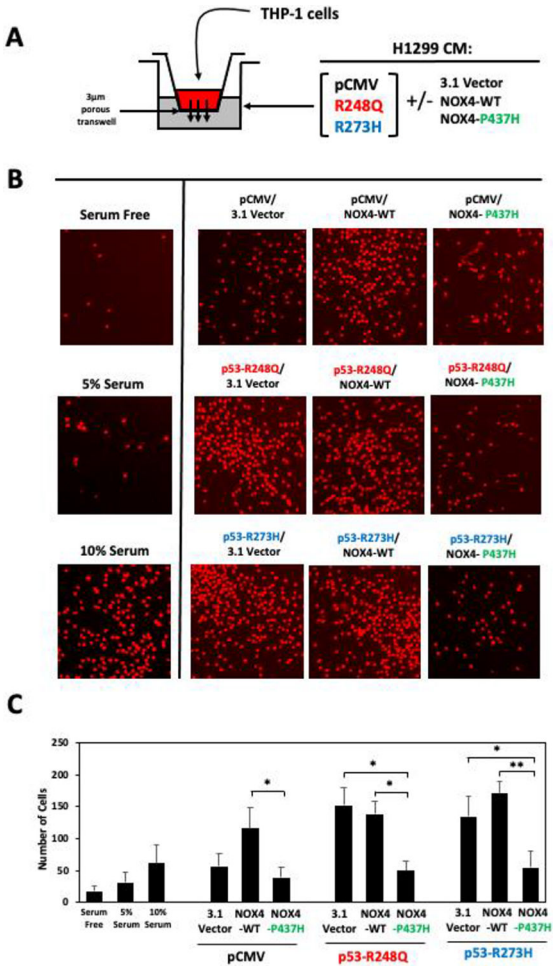

Figure 2.

The mutant p53/NOX4 pro-migratory secretome recruits THP-1 monocytes. (**A**) Experimental design schematic of transwells used to monitor chemotactic recruitment of THP-1 monocytes. THP-1 cells were seeded into the upper chamber of a transwell with a 3 µm porous membrane. The upper chamber was then placed in a lower chamber containing described CM for 18 hours. (**B**). Cell tracker images of migrating cells detected on the lower surface of transwells at 18 hours post-migration. (**C**) Total number of THP-1 cells that migrated through the porous membrane. The images are representative of n=3, in triplicate. Significance values are indicated as **P*-value < .05 or ***P*-value < .01.

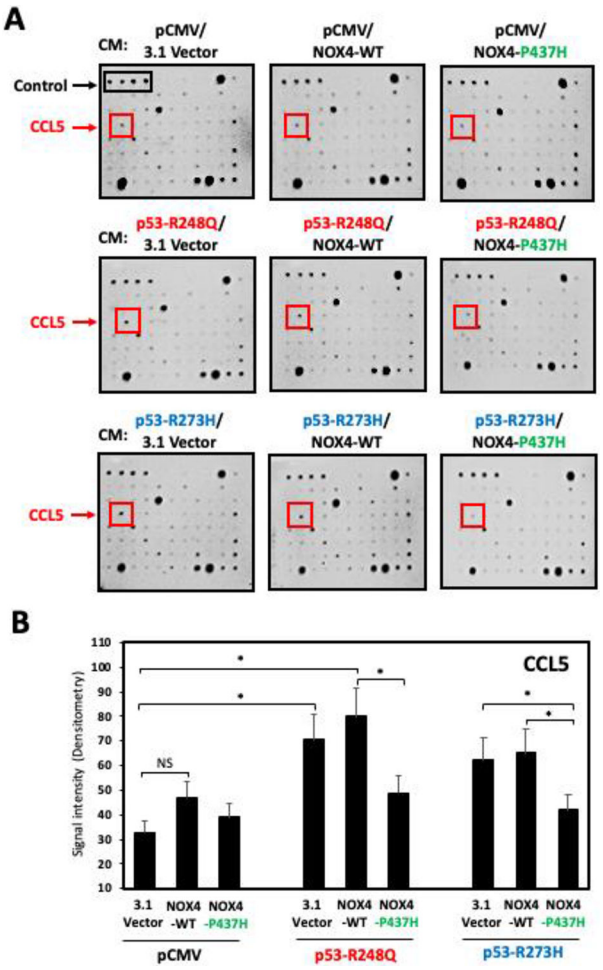

Figure 3.

Mutant p53-dependent secretion of CCL5 is regulated by NOX4. (**A**) Cytokine antibody array analysis of conditioned medium. Representative images are shown for array membranes. (**B**) Quantification of CCL5 array signal intensities normalized to array control (n=3). Significance values are indicated as *P-value < .05. Not significant (NS).

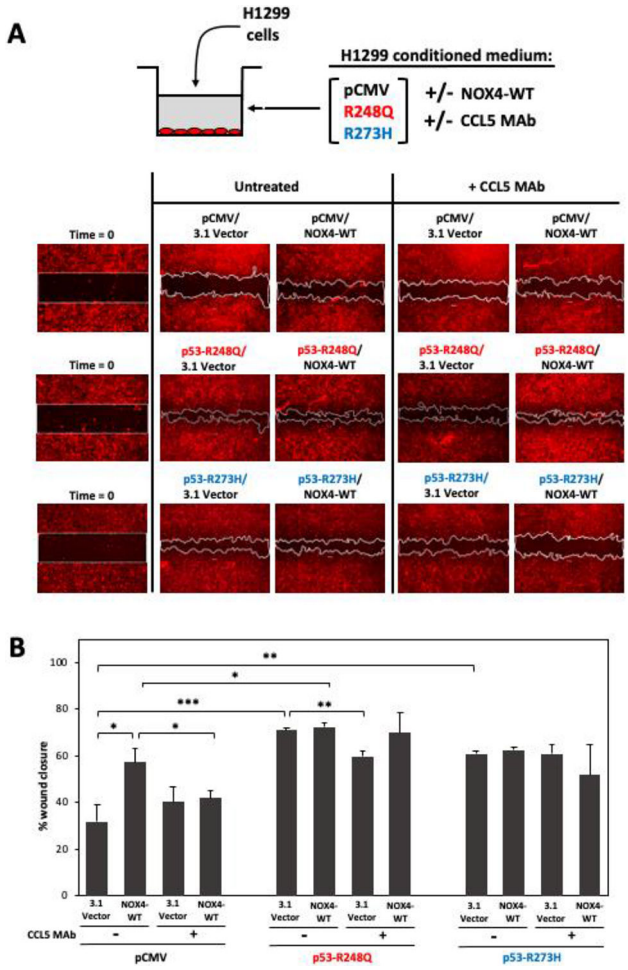

Figure 4.

Neutralization of CCL5 reduces mutant p53/NOX4-mediated tumor cell migration. (**A**) H1299 cells were seeded into a 96-well tissue culture plate (3.5×10^4 per well) and grown to confluence. The cells were wounded and treated with CM and either left untreated or treated with CCL5 Mab (2ug/ml). Approximately 18 hours after treatment, the migrated cells were imaged. The images presented are representative of n=3, in triplicate. (**B**) Calculation of % wound closure (Wounded Area at Time =0 – Wounded area 18 hours after wound)/ (Wounded Area at Time =0) x 100 = % closure. Significance values are indicated as **P*-value < .05, ***P*-value < .01, or ****P*-value <.001

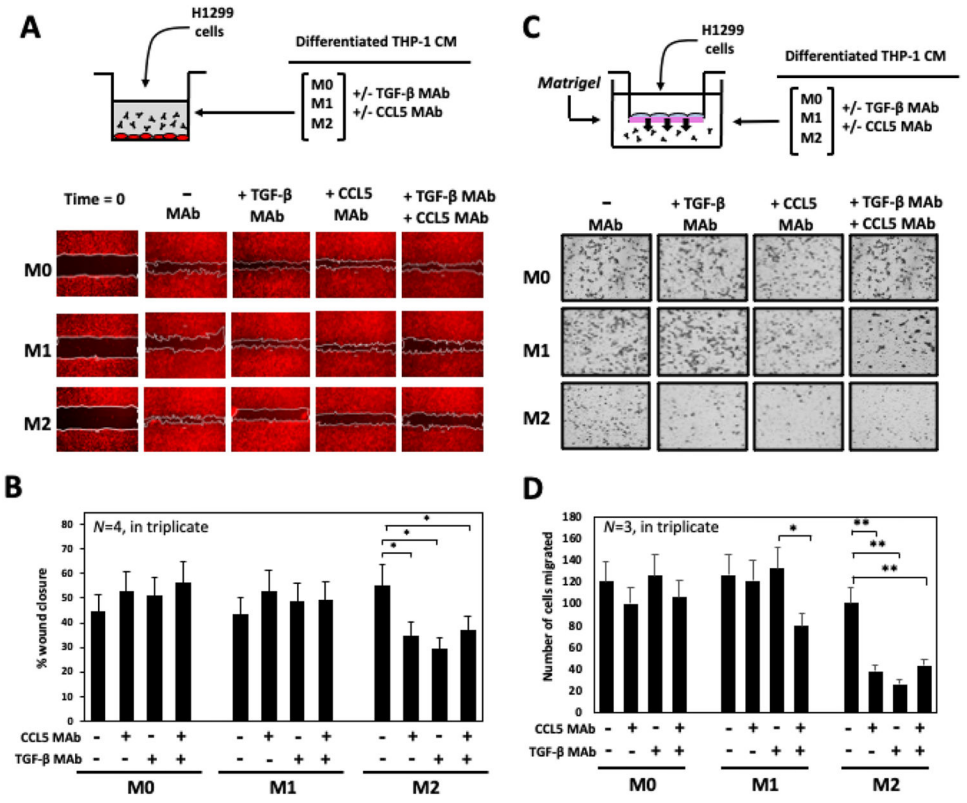

Figure 5.

TGF-beta and CCL5 secreted by M2 polarized macrophages induce tumor cell migration and invasiveness. (**A**) H1299 cells were grown to confluence before being wounded and treated with M0, M1, or M2 differentiated THP-1 CM. The THP-1 cell differentiated CM was collected as described in the materials and methods section. The cells were also either untreated or treated with TGF-beta Mab (2μg/ml), CCL5 Mab (2μg/ml), or combination of TGF-beta and CCL5 Mab. Cells were imaged approximately 18 hours after wounding. The images presented are representative of n=4, in triplicate. (**B**) Calculation of % wound closure. Significance values are indicated as * P-value < .05. n=4, in triplicate (**C**) H1299 cells were seeded into the upper chamber of a Matrigel transwell membrane. The upper chamber was then placed in a lower chamber containing polarized M0, M1, or M2 as described in panel A and incubated for 18 hours. The invading cells were then fixed and images of three random fields were collected. (**D**) Total number of H1299 cells that migrated through the porous membrane. The images are representative of n=3, in triplicate. Significance values are indicated as ** P-value < .01.

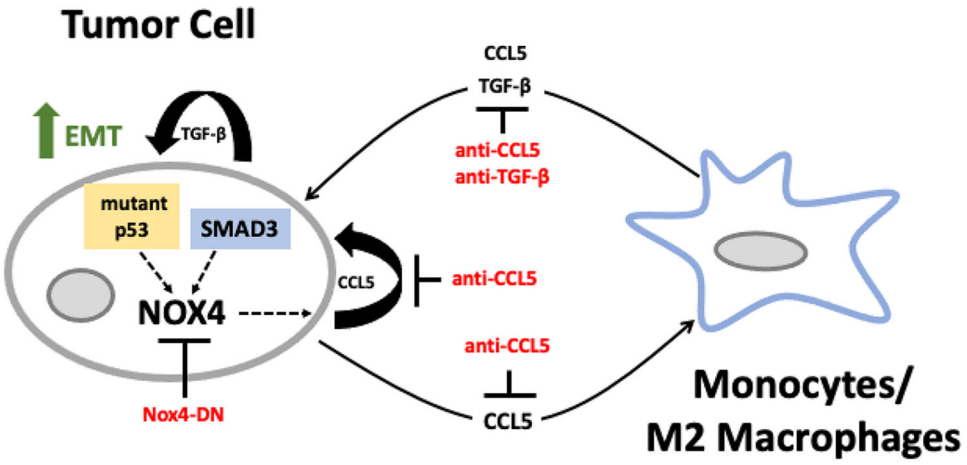

Figure 6.

NOX4 and mutant p53 are mediators of crosstalk in the tumor microenvironment. An overview of mutant p53/NOX4-mediated CCL5 release from tumor cells induces tumor cell migration in an autocrine fashion and can recruit monocytes. TGF-beta and CCL5 released from M2 polarized macrophages induce EMT in surrounding tumor cells. TGF-beta signaling with mutant p53 induce NOX4 expression further promoting EMT and tumor cell invasiveness.