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Original Articles

Mutant p53^{R175H} promotes cancer initiation in the pancreas by stabilizing HSP70

Kishore Polireddy[a], Kanchan Singh[a], Melissa Pruski[a], Neal C. Jones[a], Naveen V. Manisundaram[a], Pavani Ponnela[b], Michel Ouellette[c], George Van Buren[d], Mamoun Younes[e], John S. Bynon[d], Wasim A. Dar[d], Jennifer M. Bailey[a,*]

[a] Division of Gastroenterology and Hepatology, Department of Internal Medicine, The University of Texas McGovern Medical School, Houston, TX, 77030, USA
[b] Division of Immunology and Organ Transplantation, Department of Surgery, The University of Texas McGovern Medical School, Houston, TX, 77030, USA
[c] Division of Gastroenterology, Department of Internal Medicine, The University of Nebraska Medical Center, Omaha, NE, 68198, USA
[d] Division of Surgical Oncology, Department of Surgery, Baylor College of Medicine, Houston, TX, 77030, USA
[e] Division of Pathology and Laboratory Medicine, The University of Texas McGovern Medical School, Houston, TX, 77030, USA

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ABSTRACT

Pancreatic cancer remains a highly lethal malignancy. We have recently shown that simultaneous expression of *Kras* and mutant *Tp53^{R175H}* promotes invasive ductal adenocarcinoma from pancreatic ductal cells. We hypothesized specific mutations in *TP53* have divergent mechanisms of transforming ductal cells. In order to understand the role of mutant *TP53* in transforming pancreatic ductal cells, we used a lentiviral system to express mutant *TP53^{R175H}* and *TP53^{R273H}*, two of the most frequently mutated *TP53* alleles in pancreatic cancer patients, in immortalized, but not transformed, pancreatic ductal epithelial cells carrying a *KRAS* mutation (HPNE:*KRAS^{G12D}*). Mutant *TP53* expression enhanced colony formation and an RPPA assay results revealed *TP53^{R175H}* uniquely induced HSP70 expression in HPNE:*KRAS^{G12D}* cells. In the context of *TP53^{R175H}* expression; we observed nuclear localization of HSP70. We performed immunoprecipitation experiments to show mutant p53^{R175H} binds to HSP70. We also provide evidence mutant p53^{R175H} is important for HSP70 stability and, more importantly, HSP70 is required for mutant p53 stability. These data are critical in the context of events leading to cellular transformation in the pancreas.

1. Introduction

Pancreatic ductal adenocarcinoma (PDA) is the third leading cause of cancer related deaths in the United States [1]. PDA remains challenging to treat as most patients have few recognizable symptoms. To complicate potential therapeutic options, more than 60% of patients are diagnosed with metastatic disease, eliminating a possibility for surgical resection [2]. Genomic sequencing analysis of pancreatic tumors has revealed more than 93% of human PDA have mutations in the *KRAS* gene [3–6]. Other prominent mutations occur in *TP53* (60–70%), *SMAD4* (50%) and *CDKN2A* (> 50%) [3]. Less frequent mutations are present in *ATM, BRCA2, MLL3, ARID1A* and *SMARCA1* [3]. Sequencing data of low and high grade pancreatic intraepithelial neoplasia (PanIN) has shown *TP53* mutations occur in late stage PanIN lesions, indicating gain of function mutations in *TP53* are important for transformation [7]. Due to its high incidence of mutation in human PDA, *KRAS* remains an attractive target for therapeutic testing. However, there are no effective strategies to target *KRAS*. Therefore, deeper understanding of the biology of the earliest initiating events in PDA, especially defining the mechanisms responsible for cancer initiation from pancreatic epithelial cells, will provide important insight for the development of novel and effective therapeutic drugs.

We recently reported expression of p53R175H promotes cancer formation in pancreatic epithelial cells [8]. We have now expanded upon this finding to study how mutations in *TP53* regulate ductal cell transformation. Our data reveal a gain of function mutation in p53, (*TP53^{R175H}*) increases the abundance and nuclear localization of Heat shock protein 70 (HSP70). Heat shock proteins are chaperones that function to protect cells in the context of injury [9,10]. They are dysregulated in pancreatic diseases including pancreatitis and pancreatic cancer [11–14]. In animal models of pancreatitis, HSP70 has been shown to protect pancreatic acinar cells from injury and in pancreatic cancer, inhibition of HSP70 using Triptolide has shown HSP70 is important for cancer cell survival and invasion [11–13]. Despite an

* Corresponding author.
E-mail address: Jennifer.M.Bailey@uth.tmc.edu (J.M. Bailey).

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increased understanding regarding the significance of HSP70 expression in pancreatic diseases, the molecular events that promote HSP70 expression and stabilization remain an area of investigation.

2. MATERIALS and METHODS

2.1. RPPA assay

HPNE:*KRAS*^{G12D} cells expressing either vector control (VC) or p53 mutants (R175H and R273H) were lysed using lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl2, 1mMEGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na3VO4, 10% glycerol, containing freshly added protease and phosphatase inhibitors (Sigma-Aldrich,St. Louis, MO) and protein concentration was measured by bicinchoninic acid (BCA) method. Protein concentrations of samples were adjusted to 1 mg/ml with lysis buffer. Cell lysates were serially diluted two-fold for 5 dilutions (from undiluted to 1:16 dilution) and arrayed on nitrocellulose-coated slides in an 11 × 11 format. Samples were probed with antibodies by tyramide-based signal amplification approach and visualized by DAB colorimetric reaction. The slides were analyzed and protein expression quantitated with the use of Array-Pro Analyzer. All the data points were normalized for protein loading and transformed to linear value, designated as "Normalized Linear".

2.2. Cell culture

PANC-1, BxPC-3, MIA PaCa-2, Capan2, CFPA, HPNE, T-HPNE, HPNE:*KRAS*^{G12D}cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin/streptomycin at 37 °C in 5% CO_2. All hTert-HPNE cell lines (HPNE) were obtained from Dr. Ouellette and were cultured in Medium D as described previously [15–17]. Cell lines have been authenticated using ATCC.

2.3. Cell proliferation assay

Cell growth was measured in logarithmically growing HPNE:*KRAS*^{G12D}cells stably expressing either vector control or mutant p53 (p53^{R175H} or p53^{R273H}). Cells were plated in six well plate at initial seeding density of 20000 cells/well and cell viability was measured at 24, 48, 72 and 96 h using a hemocytometer. Live and dead cells were differentiated using tyrpan blue dye.

2.4. Heat shock treatment

HPNE:*KRAS*^{G12D} cells expressing either vector control or mutant p53 were exposed to 42 °C for 1 h and then were allowed to recover at 37 °C for 6 h. Immediately after recovery, cell lysates were prepared and a Western blot experiment was performed.

2.5. Soft agar assay

Clonogenic growth of HPNE:*KRAS*^{G12D} cells expressing either vector control or mutant p53 was measured using colony formation assay in a 2-layer soft agar assay with top layer containing 0.3% agar, and bottom layer containing 0.5% agar. Cells were seeded in 6 well plates at 5000 cells/well. Media containing either DMSO or HSP70 inhibitor, triptolide was added on top of the agar layer. After 21 days colonies were counted using GelCount (Oxford OPtronix).

2.6. Generation of human pancreas model of neoplasia

Four human mutant *TP53* cDNAs containing gain of function R175H and R273H sequences were cloned, sequence-validated, and packaged as lentiviral particles described previously [18]. All lentiviral constructs were purchased from Addgene (Cambridge, MA). Vector control plasmid (pLenti6/V5-GW/LacZ) was obtained from Thermo Fisher Scientific (Grand Island, NY). These mutations represent the most common point mutations of *TP53* in human pancreatic cancer tissue. Stable cell lines harboring either vector control or mutant p53 were generated by transduction of lentiviral particles, followed by selection using blasticidine. Stable cell lines were maintained in 1 μg/ml blasticidine (InvivoGen, San Diego, CA). P53 over expression was confirmed by western blotting. The transduced mutant *p53* constructs were V5 epitope-tagged to discriminate between exogenous mutant p53 and endogenous wild-type p53 protein in HPNE cells.

2.7. Transfection and western for HSP70 knockdown

HSP70 siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid and siRNA transfections were performed according to the manufacturer's instructions using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Grand Island, NY). In brief, 1 μg of plasmid DNA was suspended in 125 μl of OPTIMEM or 0.05 μM of HSP70 siRNA was mixed with equal volume of OPTIMEM containing 5 μl of lipofectamine 3000. Transfection mix was incubated for 5 min at room temperature and then added to cells in 6 well plates. After transfection, cells were washed with PBS and total cell lysate was collected by directly lysing the cells in lysis buffer (Cell Signaling Technologies, Danvers, MA) for western blotting analysis.

2.8. Real-time PCR and western blotting

For western analysis, cell lysates were prepared by directly lysing the cells in cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitor. Samples were centrifuged at 14,000 g for 10 min. Supernatant fraction was collected and protein concentration was determined by BCA method. Equal amount of protein was analyzed by polyacrylamide gel electrophoresis (PAGE) followed by transfer to Nitrocellulose membrane. Blots were probed with mono and/or polyclonalanti-V5 antibody, anti-γ-tubulin (Abcam, Cambridge, MA), anti-HSP40, anti-HSP90, anti-HSF1, anti-HSF2, anti-HSF4, anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA) anti-histone 3, anti-pERK, anti-ERK, anti-pAKT, anti-AKT, anti-vinculin (Sigma, St.louis, MO), anti-HSP70 (Biolegend, San Diego, CA), (Mitosciences, Eugene, OR) antibodies. We detected the secondary antibody by using a chemi-iluminescence detection kit (Genesee Scientific, San Diego, CA). For qPCR, all isoforms mapped to chromosome 6p21.3 were analyzed. HSP isoform primers include: HSPA1b Set1F TCA GGC CCT ACC ATT GAG GA; HSPA1b Set1R CCT TGA GTC CCA ACA GTC CA; HSPA1b Set2F TGT AAC CCC ATC ATC AGC GG; HSPA1b Set2R TCC CAA CAG TCC ACC TCA AAG; HSPA2 Set1F TTG GAC GGA AAT TCG AGG ATG; HSPA2 Set1R AAA TAG GCC GGG ACC GTT ATG; HSPA2 Set2F GGT CCC GGC CTA TTT CAA CG; HSPA2 Set2R GGA CAC GTC GAA AGT GCC A; HSPA5 Set1F CAT CAC GCC GTC CTA TGT CG; HSPA5 Set1R CGT CAA AGA CCG TGT TCT CG; HSPA5 Set2F CAC GGT CTT TGA CGC CAA G; HSPA5 Set2R CCA AAT AAG CCT CAG CGG TTT; HSPA9 Set1F CTT GTT TCA AGG CGG GAT TAT GC; HSPA9 Set1R GCA GGA GTT GGT AGT ACC CAA A; HSPA9 Set2F GGA AGG TAA ACA AGC AAA GGT GC; HSPA9 Set2R CCA ACA AGT CGC TCA CCA TCT; HSPA8 Set1F ACC TAC TCT TGT GTG GGT GTT; HSPA8 Set1R GAC ATA GCT TGG AGT GGT TCG; HSPA8 Set2F ACT CCA AGC TAT GTC GCC TTT; HSPA8 Set2R TGG CAT CAA AAA CTG TGT TGG T; HSPA6 Set1F CAA GGT GCG CGT ATG CTA C; HSPA6 Set1R GCT CAT TGA TGA TCC GCA ACA C; HSPA6 Set2F CAT CGC CTA TGG GCT GGA C; HSPA6 Set2R GGA GAG AAC CGA CAC ATC GAA; HSPA1L Set1F CTA CTG CCA AGG GAA TCG CC; HSPA1L Set1R GCC GAT CAG ACG TTT AGC ATC A; HSPA1L Set2F TAA ACG TCT GAT CGG CAG GAA; HSPA1L Set2R GCA CGG TAA TCA CTG CAT TGG; Hikeshi-F Set 1 TAG GAT TTG TCA CGA ATG GGA AG; Hikeshi-R Set 1 AGC AAC AGA TGG AGT TCG GAC; HSP70—F Set1 AGC TGG AGC AGG TGT GTA AC; HSP70-r Set1 CAG CAA TCT TGG

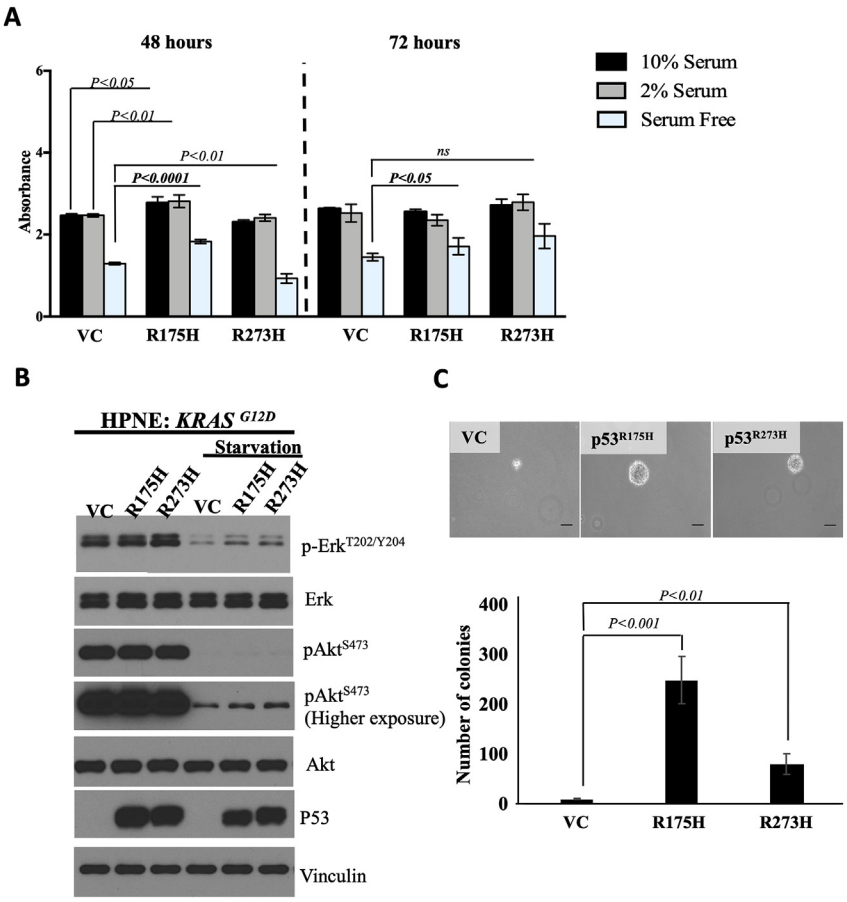

Fig. 1. Mutant p53 promotes survival and neoplastic growth of normal pancreatic ductal cells. A. An MTT assay reveals mutant $p53^{R175H}$ promotes proliferation in 10% Serum, 2% Serum and Serum Free Conditions. Mutant $p53^{R273H}$ also promotes proliferation, but in serum free conditions at 72 h, the increase is not significant. A 2-way Anova using Prism Graphpad software was used to test for significance. B. Western blot for extracellular-regulated kinase p-$Erk^{T202/Y204}$ and Akt^{S473} phosphorylation. In conditions of serum deprivation, expression of mutant 53 sustained p-$Erk^{T202/Y204}$ C. Images of colonies in soft agar assay. D. Ability of mutant p53 to induce anchorage independent growth was measured using soft agar assay. A Students t-test using Prism software was used to test for significance.

AAA GGC CC.

2.9. Isolation of nuclear fraction

Nuclear fraction from HPNE:$KRAS^{G12D}$ cells expressing either vector control or mutant p53 were isolated using the following protocol [19]. Briefly, equal number of HPNE:$KRAS^{G12D}$ cells expressing either vector control or different mutants were washed with ice cold PBS and then suspended in 1 ml of lysis buffer (PBS containing 0.1% NP40) and titrated using p1000 micropipette. A few microliters of total cell lysate was saved and the rest of the fraction was centrifuged at maximum speed for 30 s. Supernatant was saved as cytosolic fraction. Nuclear pellet was washed twice using lysis buffer. Nuclear pellet was suspended in lysis buffer and sonicated using microprobe at 15% power for 20 s (10 s on followed by 5 s off on ice). BCA method was used to determine the protein concentration.

2.10. Immunohistochemistry

Paraffin-embedded tumor sections (5 μM thick) were deparaffinized and rehydrated by serial incubation in xylene, 100%, 95% ethanol, and water. Endogenous peroxide was blocked with 3% hydrogen peroxide at room temperature for 10 min. Antigen retrieval was performed by boiling citrate buffer (Vector labs Cat no. H-3300-250) for 10 min at 50% power followed by 2 min at 20% power in microwave oven. Sections were blocked using 10% FBS in 1xPBST. Anti-HSP70 and anti-TP53 (R175H) primary antibodies (Biolegend, San Diego, CA and ewEast Biosciences) were incubated overnight at 4 °C. Biotinylated secondary antibody and DAB were used to develop the sections (Vectastain ABC–AP kit, Vector Laboratories, Burlingame, CA). All the sections were counterstained with hematoxylin. For analysis of mutant $TP53^{R175H}$ expression in human PDA specimens, individual IHC samples were imaged and defined as positive or negative based on positive immunoreactivity (presence of brown stain after DAB) in tumor cells. For scoring HSP70 expression, a scale of 1–3 was designated with 1 indicating low immunoreactivity (light brown stain), 2 indicating moderate immunoreactivity (medium brown intensity) and 3 indicating high reactivity (dark brown intensity). To determine the intensity of HSP70 expression in $p53^{R175H}$ positive versus negative samples, we scored the arrays individually then correlated the intensity score with a positive or negative classification of $p53^{R175H}$. Two columns were then defined using Prism Graphpad software and individual intensity scores were added to each column. A student's *t*-test was performed to determine if patients with $p53^{R175H}$ positive expression had a higher intensity score for HSP70. Representative IHC and analysis are shown in Fig. 4.

To analyze the percentage of HSP70 + nuclear accumulation, we quantified the percentage of cells with nuclear expression of HSP70 and divided by the total number of malignant cells per field analyzed. Two columns were then defined using Prism Graphpad software and percentage of cells with nuclear expression of HSP70 were added to each column (either p53R175H positive or p53R175H negative). A student's *t*-test was performed to determine if patients with $p53^{R175H}$ positive expression had increased nuclear expression of HSP70. Representative IHC and analysis are shown in Fig. 4.

2.11. Immunoprecipitation

Logarithmically growing HPNE:$KRAS^{G12D}$ cells expressing either vector control or different p53 mutants (R175H and R273H) were suspended in 500 μl of cell lysis buffer (CST) and then incubated on ice for 10 in. Samples were sonicated for 15 s (15 AMP power, 3 cycles, 5 Sec each). Samples were centrifuged at 14,000 × *g* for 3 min and supernatant was collected for immunoprecipitation reaction. 200 μg of

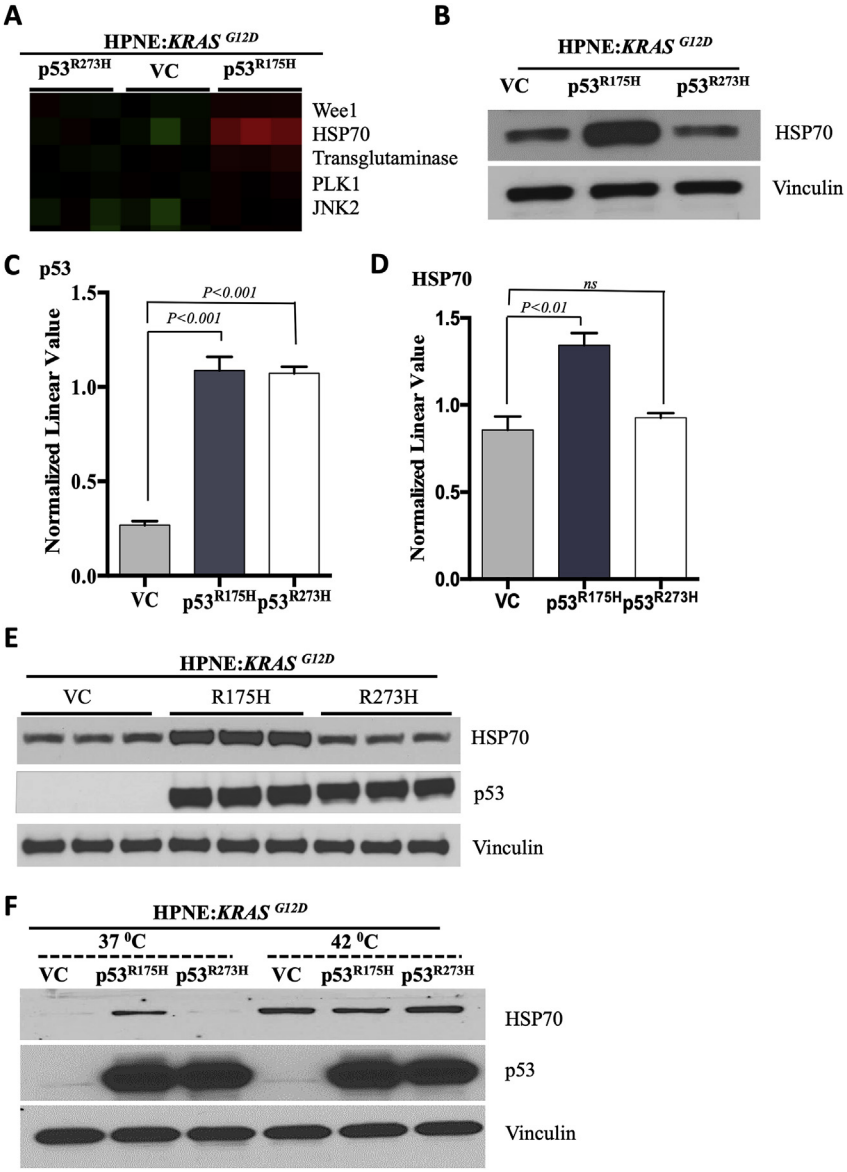

Fig. 2. A functional proteomics screen reveals mutant $p53^{R175H}$ increases expression of HSP70 A. Heat map showing differential expression of proteins upon either vector control or mutant p53 over expression in HPNE:$KRAS^{G12D}$ cells. B. RPPA assay samples were used to validate the expression of HSP70 using western blotting. C,D. Normalized RPPA data in linear values was used to draw the bar graphs showing the expression of p53 and HSP70. E. Western blot analysis to detect HSP70 expression in total cell lysate from stable cell lines in culture. F. HPNE:$KRAS^{G12D}$ cells expressing either vector control or mutant p53 exposed either 37 °C or 42 °C for 1hr and then recovered at 37 °C for 6 h. Total cell lysate was prepared for Western blot analysis and then HSP70 was detected. A students t-test was used to evaluate significance.

protein was precleared with 20 µl of protein A/G beads for 1 h at 4 °C on a shaker. Precleared protein samples were incubated with 1 µg of rabbit IgG or V5 antibody at 4 °C for overnight on a shaker. After overnight incubation, 20 µl of protein A/G beads were added to the immunoprecipitation reaction and incubated at 4 °C for 2 h on a shaker. Immunoprecipitated proteins were eluted directly into 30 µl of 2X laemmlli buffer and boiled for 5 min. Samples were centrifuged and supernatant was subjected to western blotting.

2.12. Patient samples

Human tissues studied were approved for collection and study by the UTHSC CPHS under an approved IRB protocol. All samples are de-identified. Human PDA tissue arrays were also purchased through US Biomax.

2.13. Statistical analysis

All statistical analysis was done using either a 2-way Anova comparison or student's t-test. Both tests were done using Prism Graphpad software for analysis.

3. Results

3.1. Mutant TP53 promotes survival and neoplastic features in normal pancreatic ductal cells

Mouse models of pancreatic cancer have shown gain of function mutations in *TP53* rapidly accelerate PDA initiation and metastasis [8,20,21]. In models of pancreatic cancer, mutant p53 ($p53^{R172H}$; $p53^{R175H}$ in humans) relieves growth arrest/senescence caused by $KRAS^{G12D}$ [22]. We tested the ability of 2 common mutant p53 alleles, $p53^{R175H}$ and $p53^{R273H}$, to induce proliferation in immortalized hTert-HPNE cells expressing $KRAS^{G12D}$ (HPNE:$KRAS^{G12D}$) cells using an MTT proliferation assay. For this experiment, we tested the ability of p53 mutations to promote cellular proliferation in the context of 10% Serum, 2% Serum or Serum Free media. Our results revealed expression of $p53^{R175H}$ induced a significant increase in cell proliferation in HPNE:$KRAS^{G12D}$ cells when cells were cultured in 10% Serum or 2% Serum for 48 h (Fig. 1A). Under Serum Free conditions, expression of mutant p53 enhanced the proliferative capacity of HPNE:$KRAS^{G12D}$ cells at 48 h and at 72 h, expression of $p53^{R175H}$ had the greatest effect on cell proliferation (Fig. 1A). Consistent with *in vitro* cell proliferation results, when HPNE:$KRAS^{G12D}$ cells were cultured in serum free media,

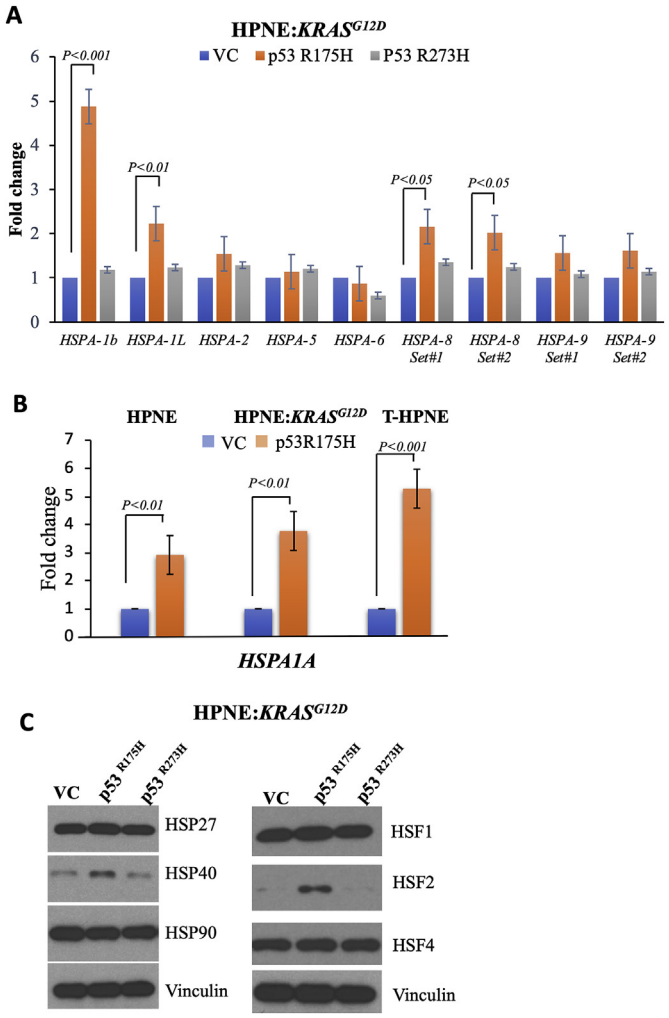

Fig. 3. Mutant p53^{R175H} increases mRNA levels of multiple HSP70 isoforms A. Real time PCR was carried out to detect gene expression changes in different HSP70 isoforms with mutant p53 over expression. Mutant p53^{R175H} significantly increased expression of HSPA-1b, HSPA-1L and HSPA-8. B. Real time PCR was used to confirm mutant p53^{R157H} increases mRNA levels of HSP70 in HPNE cells, *HPNE:KRAS^{G12D}* and T-HPNE cells, which indicated mutant p53^{R175H} exerts an effect on HSP70 independent of mutant *KRAS* C. Western blot analysis to detect HSP27, 40, 90 and HSF1, 2, 4 in HPNE:*KRAS^{G12D}* cells expressing either vector control or mutant p53. Mutant p53^{R175H} increased expression of HSP40 and HSF2. A student's *t*-test was used to calculate statistics.

we observed higher levels of expression of p-Erk$^{T204/Y204}$ in mutant p53 expressing HPNE:*KRAS^{G12D}* cells relative to vector control cells suggesting mutant p53 sustains downstream RAS/MAPK signaling in conditions of cellular stress. The ability of cells to form colonies and survive in anchorage independent conditions is one method to study the neoplastic capacity of cells. Furthermore, colony formation in soft agar assay strongly correlates with tumorigenicity of cancer cells, especially in comparison to mouse PDA xenograft models [23]. Therefore, we tested the ability of p53^{R175H} and p53^{R273H} to induce colony formation in HPNE:*KRAS^{G12D}* cells in serum free conditions. While both mutants enhanced colony formation relative to vector control (VC) transduced cells, mutant p53^{R175H} had the greatest capacity for sphere formation (Fig. 1C).

3.2. Mutant *TP53^{R175H}* stabilizes HSP70 in normal ductal epithelial cells

We employed a functional proteomics experiment to define how expression of mutant p53^{R175H} in an immortalized human pancreatic epithelial cell promoted cancer initiation. Reverse phase protein array (RPPA) was performed at the MDAnderson RPPA functional proteomics core facility to quantify proteomic changes upon mutant p53^{R175H} and p53^{R273H} expression in HPNE:*KRAS^{G12D}* cells. The protein array was probed with 304 antibodies, which recognize specific proteins in major signaling pathways. RPPA data was generated from three independent biological samples. We observed a significant increase in HSP70 with p53^{R175H} mutant expression in HPNE:*KRAS^{G12D}* cells (Fig. 2A–D). Using Western blot as a secondary assay, we validated HSP70 expression in RPPA samples and in HPNE:*KRAS^{G12D}* cell lines in culture (Fig. 2B). Transient transfection of *p53^{R175H}* also resulted in increased expression of HSP70 (Fig. 2E) and, notably, wild type p53 and p53^{R273H} failed to induce expression of HSP70 (Fig. 2D and E). To expand our approach, we tested the effect of p53^{R175H} expression on human pancreatic cancer cell lines. In the context of transient overexpression, p53^{R175H} increased the expression of HSP70 in AsPC-1, BxPC-3 and MIA PaCa-2 cells. However, p53^{R175H} did not induce expression of HSP70 in Capan-2 and CFPAC cell lines (Supplementary Fig. 1). Since HSP70 is a heat shock protein, we exposed HPNE:*KRAS^{G12D}* cells expressing VC, p53^{R273H}, or p53^{R175H} to heat shock conditions to determine if mutant p53 had any effect on heat shock response (42 °C for 1 h and recovery at 37 °C for 6 h). Using Western blot detection methods, we determined heat shock treatment increased HSP70 expression and mutant p53 did not alter this response (Fig. 2F). Notably, p53^{R175H} expression induced similar levels of HSP70 as observed in heat shock conditions (Fig. 2F).

3.3. Mutant *p53^{R175H}* increases expression of multiple isoforms of HSP70

Recent sequencing studies have shown *TP53* mutations occur in late human PanIN, which has heightened our interest in studying the mechanism by which p53^{R175H} increases expression of HSP70 as an early event in the initiation of PDAC [7,14,24]. The HSP70 family consists of 13 homologous proteins (HSPA1A, 1B, 1L, 2, 5, 6, 7, 8, 9, 12A, 12B, 13, 14). While sharing homology, HSP70 family members differ from each other by cellular localization, amino acid sequence, and expression levels. HSPA-1A and HSPA-1B, collectively known as HSP70-1, are a major stress inducible complex, frequently altered in a broad range of cancers [9]. We performed Real Time (qRT-PCR) analysis to detect expression levels of other HSP isoforms in the context of increased p53^{R175H}. Our data revealed p53^{R175H} specifically increased expression of *HSPA1A, HSPA1B*, *HSPA1L* and *HSPA8*, but not *HSPA2, 5, 6* and *9* (Fig. 3A). Interestingly, in direct contrast, expression of p53^{R273H} had no effect on the abundance of HSP70 isoforms, indicating expression of HSP70 is directly regulated by p53^{R175H} (Fig. 3A). We also tested to see if *KRAS^{G12D}* is required for mutant p53^{R175H} mediated expression of HSP70-1. Transient expression of p53^{R175H} in HPNE and transformed (T-HPNE) cells enhanced expression of HSP70 mRNA (Fig. 3B), suggesting that *KRAS^{G12D}* is not required for mutant *p53^{R175H}* mediated expression of HSP70-1. As we observed increased expression of HSP isoforms, we wanted to determine if mutant p53^{R175H} also increased the abundance of HSP transcription factors known to promote expression of HSP isoforms. Using western blots, we observed increased expression of Heat Shock Factor 2 (HSF2), but not HSF1 or HSF4 in HPNE:*KRAS^{G12D}* expressing cells (Fig. 3C). We performed Western blot analysis to detect other HSPs (HSP27, 40, and 90). p53^{R175H} increased expression of HSP40 but did not increase expression of HSP90 or HSP27 (Fig. 3C).

3.4. Mutant p53 promotes HSP70 nuclear localization

Various cellular proteins, including HSP70, undergo cytoplasmic to nuclear translocation under stress conditions [10,25]. Therefore, we studied the effect of mutant p53 on HSP70 nuclear localization. HPNE:*KRAS^{G12D}* cells that are stably expressing p53^{R175H} had higher nuclear HSP70 levels. In contrast, cells expressing either VC or p53^{R273H} failed to induce nuclear localization (Fig. 4A). We tested the sub

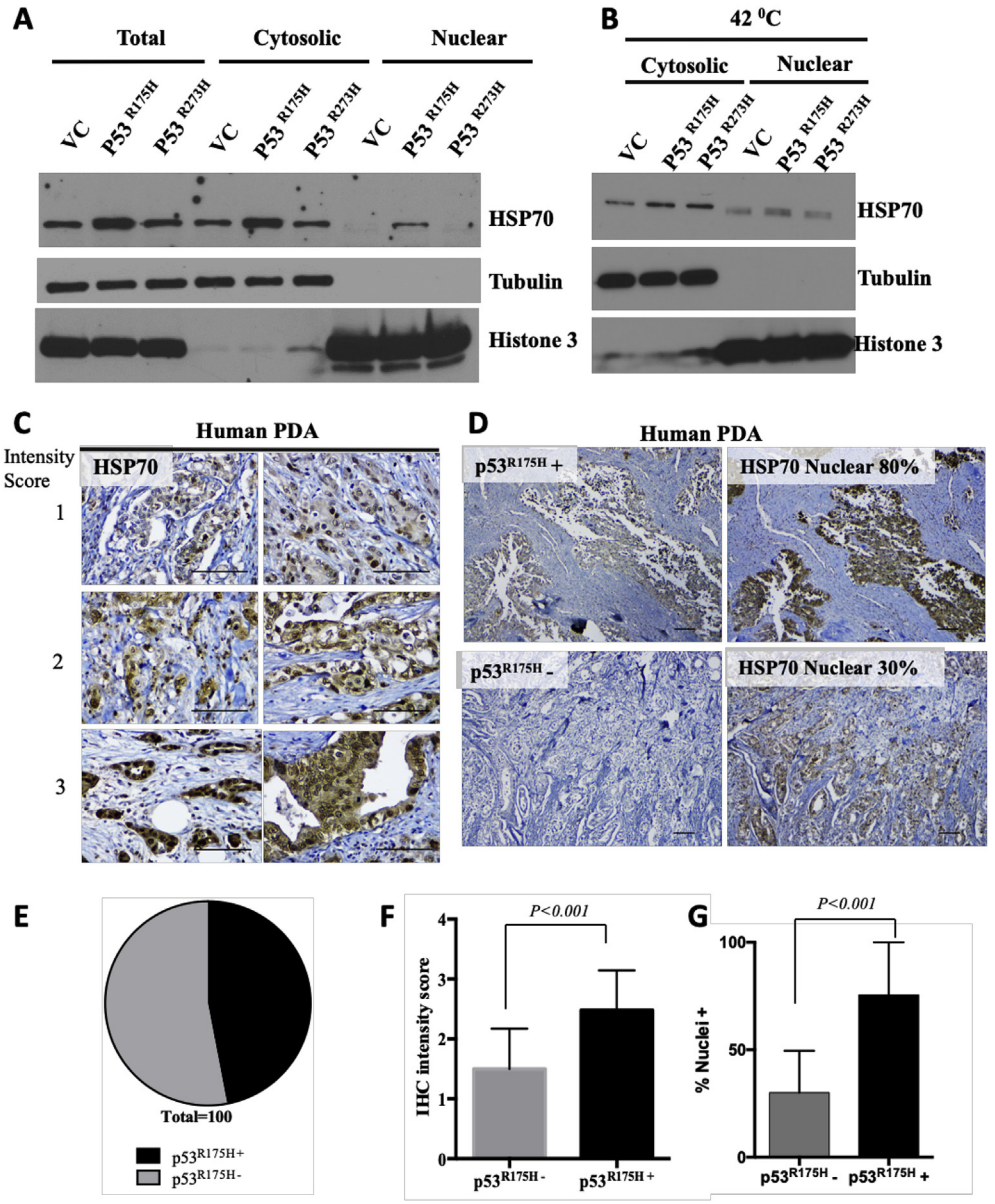

Fig. 4. Mutant p53^{R175H} promotes nuclear accumulation of and stabilizes HSP70. A. Western blot analysis was performed to detect HSP70 expression in isolated cytosolic and nuclear fractions from HPNE:*KRAS*^{G12D} cells that are stably expressing either vector control or different p53 mutants. Histone 3 was used as a nuclear marker and α-tubulin was used as a cytosolic markers. B. HPNE:*KRAS*^{G12D} cells that are stably expressing either vector control or different p53 mutants subjected to heat shock treatment and then HSP70 detected in cytosolic and nuclear fractions. C. Representative IHC showing intensity scores of staining used to define the levels of HSP70 in each human PDA sample analyzed. D. A p53^{R175H} specific antibody was used to evaluate mutant p53 status in a human PDA array. Representative HSP70 IHC in p53R175H mutant positive versus negative samples are also shown. E. Pie graph showing the percentage of human PDA samples that stained positive for mutant p53^{R175H} (47%). F. Quantification of HSP70 Intensity Score (1, 2 or 3) as a function of mutant p53^{R175H} status. IHC analysis revealed a significant increase in the intensity of HSP70 in patients with mutant p53^{R175H}. G. Quantification of percentage of cells per field with positive nuclear localization of HSP70. We observed a significant increase in the percentage of tumors cells with positive accumulation of HSP70 in patients with mutant p53. A student's t-test using Prism software was used for all analysis.

cellular distribution of HSP70 in HPNE:*KRAS*^{G12D} cells under heat shock stress conditions. Heat shock treatment promoted nuclear localization of HSP70 independent of p53 mutational status (Fig. 4B). Our nuclear preps showed either very low or lack of cytosolic marker protein, α-tubulin, eliminating the possibility of cytosolic contamination. In addition to our *in vitro* experiments, we stained a human PDA tissue array and additional clinical samples for HSP70 and a TP53^{R175H} mutant specific antibody to determine if there is a clinical correlation between TP53^{R175H} and HSP70. Using IHC, we scored human clinical samples using an intensity of staining scale from 1 (low intensity/expression of HSP70) to 3 (high intensity/expression of HSP70). Representative staining intensity is shown in Fig. 4C. In serial sections, we stained for a TP53^{R175H} specific antibody and labeled each sample as positive or negative (Fig. 4D and E). At the conclusion of our analysis, we observed 47% of the clinical samples we analyzed had TP53^{R175H} expression (Fig. 4E) and samples positive for TP53^{R175H} had significantly increased intensity of staining for HSP70 (Fig. 4F). To determine if TP53^{R175H} expression was associated with nuclear accumulation of HSP70, we quantified the percentage of neoplastic cells with nuclear localization of HSP70. We observed a significant increase in the percentage of cells with nuclear expression of HSP70 as in patients with mutant TP53^{R175H} (Fig. 4G).

3.5. HSP70 is required for the stability of mutant p53

In normal tissues, negative regulators such as MDM2 and CHIP suppress p53 levels. In response to stress, p53 is released from negative regulators and stabilized to induce cell cycle arrest, senescence, and apoptosis [26–29]. Consistent with its known role in regulating wild type p53, MDM2 promotes degradation of mutant p53 in normal and premalignant tissues [30]. As other factors regulating mutant p53 stability remain unknown [31–34], we wanted to determine if HSP70 has any role in the stabilization of p53 in the context of PDA initiation. First, we performed an immunoprecipitation experiment to detect HSP70 and mutant p53 interactions. Our results showed that p53^{R175H}, not p53^{R273H} interacts with HSP70 (Fig. 5A). We studied the role of HSP70 in mutant p53 stabilization using Triptolide, an HSP70 inhibitor with known therapeutic potential in human and murine PDA [13,35,36]. HPNE:*KRAS*^{G12D} cells expressing either vector control or p53^{R175H} were treated with Triptolide and Western blot analysis was performed to detect p53 expression. Triptolide treatment reduced the levels of mutant p53 and increased wild type p53 expression in vector

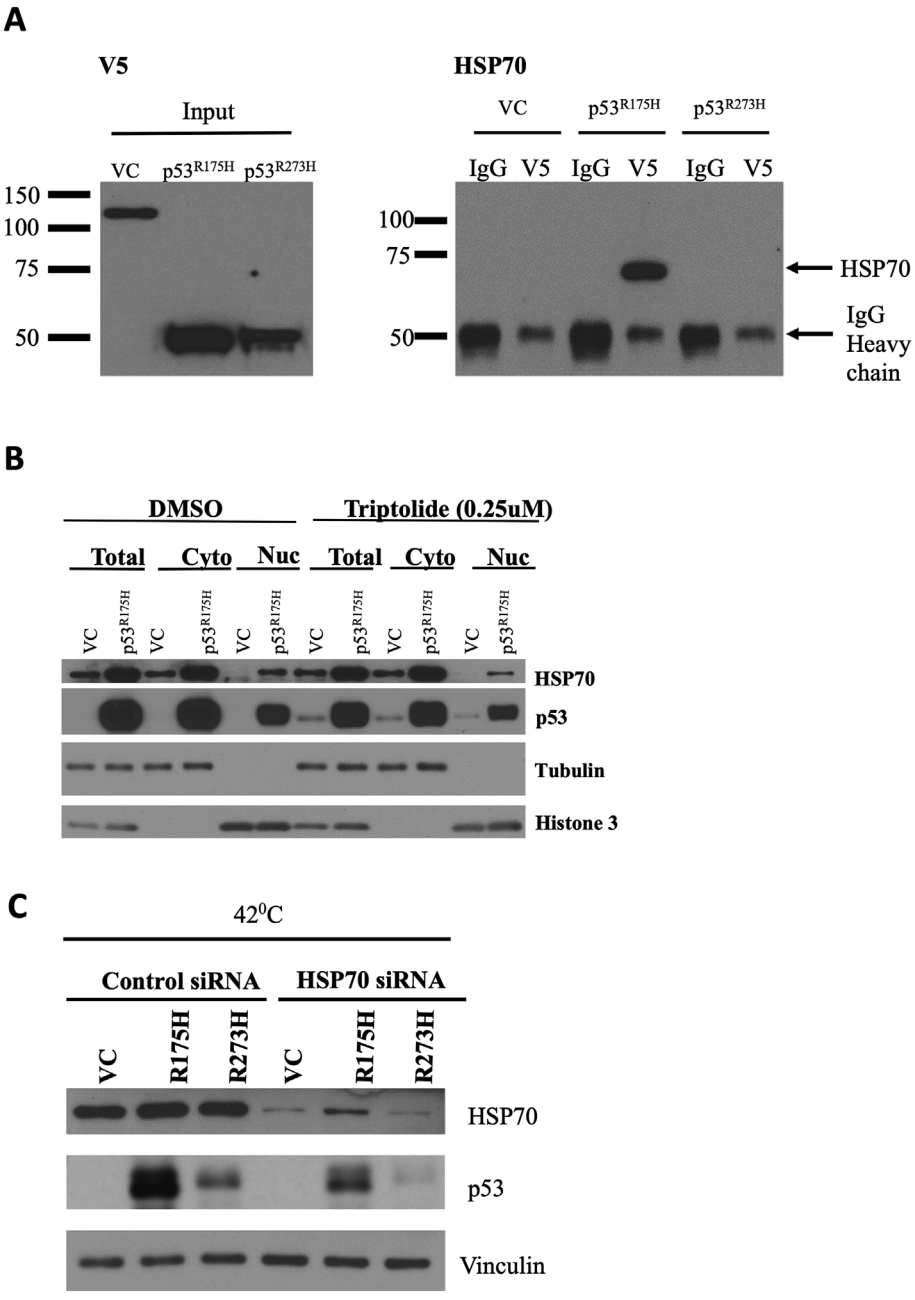

Fig. 5. HSP70 interacts with and stabilizes mutant p53. A. Western blot analysis of immunoprecipitated samples for HSP70 antibody. HPNE:*KRAS*^{G12D} cells stably expressing either vector control or mutant p53 were immunoprecipitated with V5 antibody. B, D. Western blot for p53 and HSP70 in the cytosolic and nuclear fractions after Triptolide treatment in HPNE:*KRAS*^{G12D} cells stably expressing either vector control or mutant p53. C. Immunoblot analysis of HSP70 and p53 expression after scrambled siRNA or HSP70 specific siRNA transfection, followed by heat shock treatment in HPNE:*KRAS*^{G12D} cells stably expressing either vector control or mutant p53.

control cells (Fig. 5B). As a reciprocal experiment, we wanted to determine if p53^{R175H} increased stability of HSP70. We exposed HPNE:*KRAS*^{G12D}-VC, -p53^{R175H} and -p53^{R273H} cells to heat shock conditions to induce expression of HSP70 and treated one group with siRNA targeting HSP70. We then probed for expression of HSP70 and p53. Our data revealed p53^{R175H} specifically inhibits siRNA-targeted degradation of HSP70 and, thus, is vital to HSP70 stability. Furthermore, this experiment highlighted the specificity and efficacy of our HSP70 siRNA targeting construct (Fig. 5C; see also Supplemental Fig. 2).

3.6. *HSP70 is required for mutant p53^{R175H} induced sphere formation*

The therapeutic potential for using Triptolide, an HSP70 inhibitor, to treat patients with pancreatic cancer has been described previously [11,37,38]. We wanted to determine if Triptolide would have a similar effect on the sphere forming capacity of HPNE:*KRAS*^{G12D} cells expressing specific gain of function mutations in p53. Our results revealed Triptolide significantly reduces the sphere forming capacity of p53 mutant HPNE:*KRAS*^{G12D} cells (Fig. 6A). To further study the mechanism by which Triptolide reduces sphere formation, we used a dose response and treated vector control and p53^{R175H} expressing HPNE:*KRAS*^{G12D} cells with Triptolide. In vector control cells expressing LacZ-V5 fusion protein, Triptolide treatment had no effect on V5 expression. Notably, in a dose dependent manner (Fig. 6B), we observed expression of wild type p53 in vector control treated cells, indicating in the absence of mutant p53, wild type p53 is expressed in response to Triptolide treatment, as has been previously reported [38]. In mutant p53^{R175H}–V5 fusion protein expressing cells, V5 expression was significantly reduced with Triptolide treatment, confirming Triptolide specifically promotes mutant p53 degradation (Fig. 6B).

To determine more precisely if HSP70 is required for sphere forming capacity, we used a specific siRNA targeting HSP70. Our initial data indicated the sphere forming capacity of HPNE:*KRAS*^{G12D} cells expressing mutant p53^{R175H} is dependent on HSP70 (Fig. 6A and B). We observed similar results when HSP70 was knocked down using siRNA

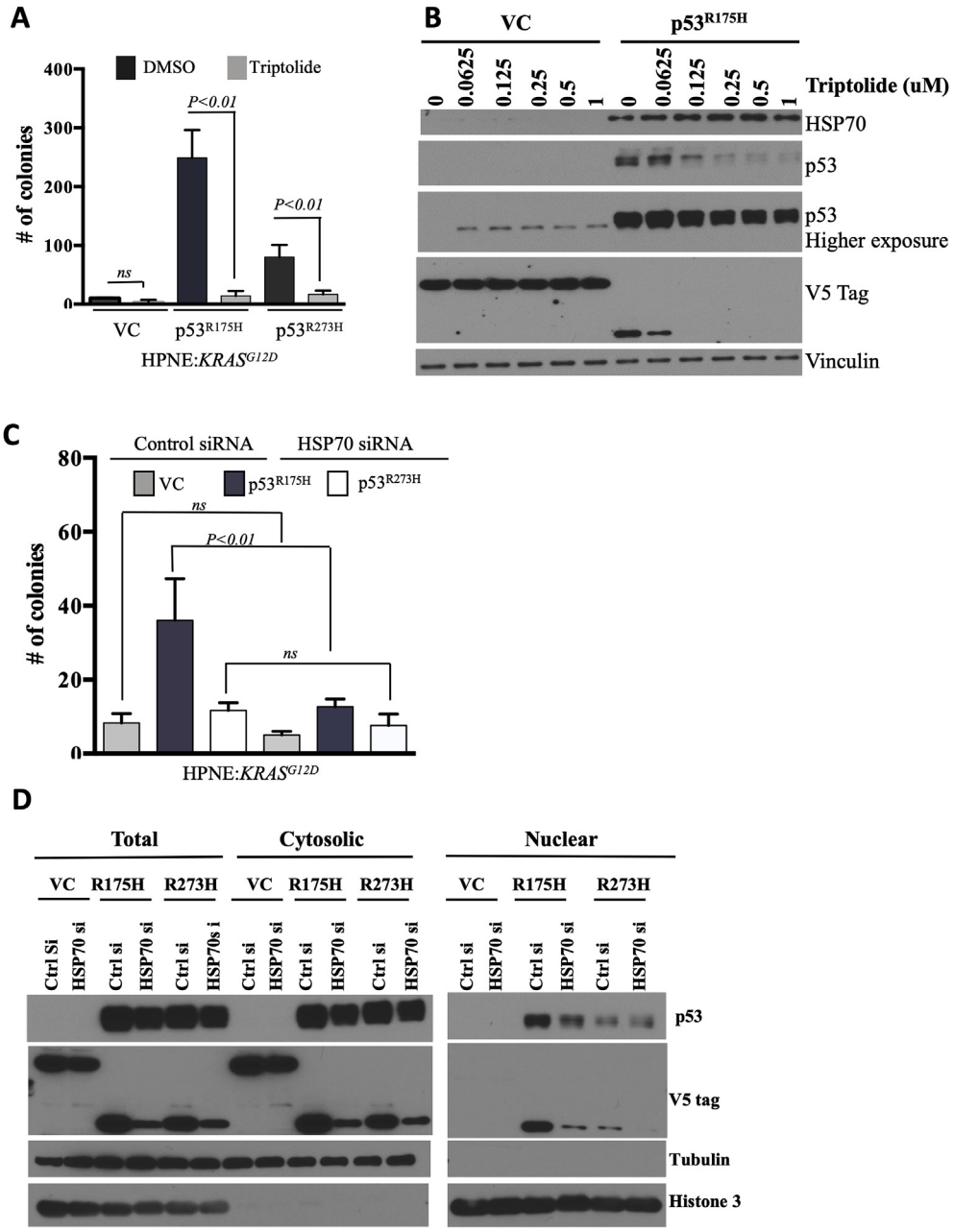

Fig. 6. HSP70 is required for mutant p53 mediated cellular transformation: A. Bar graph showing the number of colonies from HPNE:*KRAS* ^{G12D} cells that are stably expressing either vector control or mutant p53^{R175H} treated with DMSO or Triptolide in a soft agar assay. B. Western blot analysis of wild type and mutant p53 expression levels in HPNE:*KRAS*^{G12D} cells treated with a dose response C. Bar graph showing the number of colonies from HPNE:*KRAS*^{G12D} cells that are stably expressing either vector control or mutant p53 transfected with scrambled siRNA or HSP70 specific siRNA in soft agar assay. D. Western blot analysis showing siRNA guided deletion of HSP70 decreases the stability of mutant p53.

(Fig. 6C). More importantly, we show siRNA knockdown of HSP70 decreases the abundance of mutant p53, as evidenced by reduced V5 tag expression (Fig. 6D). These results reveal targeting HSP70 and mutant p53 are important for inducing anti-neoplastic effects. Previous work has shown Triptolide can reduce the levels of HSP70 in human PDA cells, but not normal pancreatic epithelial cells [38]. Our cells expressing mutant *TP53* and *KRAS*^{G12D} are experimentally represent c a neoplastic cell type with genetic features of high-grade PanIN lesions. In our experiment, the HSP70 inhibitor, Triptolide, reduced cytoplasmic levels of HSP70 at higher dosages (Fig. 6B), destabilized mutant p53, increased expression of wild type p53 and reduced nuclear accumulation of HSP70 (Fig. 5B).

4. Discussion

In mouse models of pancreatic cancer, cooperation between Kras and mutant p53 is associated with high frequency of invasion, metastasis, colony growth and reduced survival [8,21]. In this current study, we used a human pancreatic epithelial cell model to better understand how mutations in p53 promote cancer initiation. Specifically, we were interested in critical mechanisms by which *KRAS*^{G12D}, in cooperation with mutant TP53, promotes cancer initiation in pancreatic ductal cells. We show that expression of a specific gain of function mutation in TP53, *p53*^{R175H}, in HPNE:*KRAS*^{G12D} cells increases HSP70 expression, which is important for clonogenic growth. We also observe increased nuclear HSP70 in p53^{R175H} expressing cells and increased expression of HSF2, an important transcription factor for HSP70 mRNA expression.

We also observed mutant p53^{R175H} directly interacts with HSP70 and is important for HSP70 stabilization. When we introduced an siRNA targeting HSP70, we observe stabilized expression HSP70 in p53^{R175H} expressing cells. Not only did we observe mutant p53 is important for HSP70 stabilization, but we also observe HSP70 is important for p53^{R175H} stability. Our data using siRNA to target HSP70 revealed HSP70 regulates the stability of mutant p53. When siRNA was used to decrease the abundance of HSP70, we observed reduced expression of mutant p53. These data are complimentary to previous studies showing

HSP70 can inhibit MDM2-dependent degradation of $p53^{R172H}$ in mouse embryonic fibroblasts [39].

CRediT authorship contribution statement

Kishore Polireddy: Conceptualization, Data curation, Formal analysis. **Kanchan Singh:** Data curation. **Melissa Pruski:** Data curation. **Neal C. Jones:** Data curation, Funding acquisition. **Naveen V. Manisundaram:** Data curation. **Pavani Ponnela:** Data curation. **Michel Ouellette:** Resources. **George Van Buren:** Resources. **John S. Bynon:** Resources. **Wasim A. Dar:** Resources. **Jennifer M. Bailey:** Conceptualization, Formal analysis, Funding acquisition, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.047.

Conflicts of interest

The authors have no competing financial interests to disclose.

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