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p53-dependent regulation of growth, epithelial-mesenchymal transition and stemness in normal pancreatic epithelial cells

Andreia V. Pinho,[1] Ilse Rooman[2] and Francisco X. Real[1,3,*]

[1]Programa de Patología Molecular; Centro Nacional de Investigaciones Oncológicas; Madrid, Spain; [2]Cancer Research Program; Garvan Institute of Medical Research; Sydney Darlinghurst, NSW Australia; [3]Departament de Ciències Experimentals i de la Salut; Universitat Pompeu Fabra; Barcelona, Spain

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Abbreviations: aPTF1, adult-type PTF1 complex; Cpa1, carboxypeptidase A1; EMT, epithelial-mesenchymal transition; E-cad, E-cadherin; ePTF1, embryonic-type PTF1 complex; EYFP, enhanced yellow fluorescent protein; Krt, keratin; PDAC, pancreatic ductal adenocarcinoma; P, passage; PTF1, pancreas transcription factor complex 1; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; Vim, vimentin

Pancreatic acinar cells acquire in vitro a pancreatic progenitor phenotype associated with activation of p53, growth arrest and senescence. A similar programme is also activated in chronic pancreatitis. To assess the mechanisms involved in this process, we cultured pancreatic acinar cells from wild type, $p53^{-/-}$, $p16^{-/-}$ and $p21^{-/-}$ mice. Cultures from $p53^{-/-}$ mice, but not those from $p16^{-/-}$ or $p21^{-/-}$ mice, display an enhanced proliferation and can be expanded continuously for more than 20 passages. $p53^{-/-}$ cells also display features of stemness such as enhanced sphere formation, increased expression of pancreatic multipotent progenitor markers (Ptf1a, Pdx1, Cpa1, c-myc, Sox9 and Hnf1b) and of the stemness regulators Bmi1 and Klf4. Upon subculture, $p53^{-/-}$ cells undergo an epithelial-mesenchymal transition (EMT) and express high levels of vimentin and of the transcriptional regulators Snai1, Snai2, Twist, Zeb1 and Zeb2. Genetic lineage tracing unequivocally demonstrates the epithelial origin of the cells with mesenchymal phenotype. These cells express the endodermal markers Hhex, Pdx1, Sox9, Hnf1b, Foxa2, Gata6 and Sox17 and the stem cell markers c-myc, Bmi1 and Klf4. Cultures from $p53^{+/-}$ mice display intermediate levels of the transcription factors involved in EMT but do not surpass the growth arrest. Our findings support the notion that p53 controls both growth and epithelial cell differentiation in the pancreas. These observations have important implications regarding the mechanisms through which p53 inactivation in tumors may be associated with aggressive biological behavior.

Introduction

Cellular plasticity has emerged as an important mechanism that contributes to the understanding of disease and can be exploited therapeutically. In the pancreas, several studies using rodent and human tissue have strongly suggested that acinar cells display plasticity and can acquire a ductal phenotype on the basis of marker analysis.[1-3] In addition, genetic tracing analyses have unequivocally demonstrated the acinar origin of cells with a ductal phenotype in vitro[4] and in vivo[5-7] in mice; lectin-tracing and genetic tracing studies have also shown that rodents cells with endocrine features can originate from acinar cells.[8-10] Acinar cells can also undergo transdifferentiation to hepatocytes.[11,12] The best evidence that pancreatic cells can acquire features of mesenchymal derivatives comes from the analysis of mice in which c-myc has been inactivated in the pancreas, where epithelial cells transdifferentiate to adipocytes.[13] Importantly, *Kras2* oncogene activation in acinar cells can give rise to pancreatic ductal adenocarcinomas (PDAC) in mice.[5]

We have shown recently that, when cultured in suspension, murine adult acinar cells are unable to maintain their differentiation program—characterized by high level expression of the transcription factors Ptf1a and Rbpjl [tissue-specific components of the adult Pancreatic Transcription Factor 1 complex (aPTF1)],[14] and their target genes (i.e., digestive enzymes). Upon dedifferentiation, they acquire an embryonic type Pancreatic Transcription Factor 1 complex (ePTF1) and express markers characteristic of pancreatic progenitors. Using chromatin immunoprecipitation, ePTF1 components can be found on the promoters of embryonic target genes. In these cells, a strong activation of the Ras-MAPK pathway and of a senescence-like program characterized by induction of p53, p21, p16, Dec1 and positive senescence-associated β galactosidase staining is observed.[15] These findings suggest that p53 and/or p16 might impose a permanent growth

*Correspondence to: Francisco X. Real; Email: preal@cnio.es
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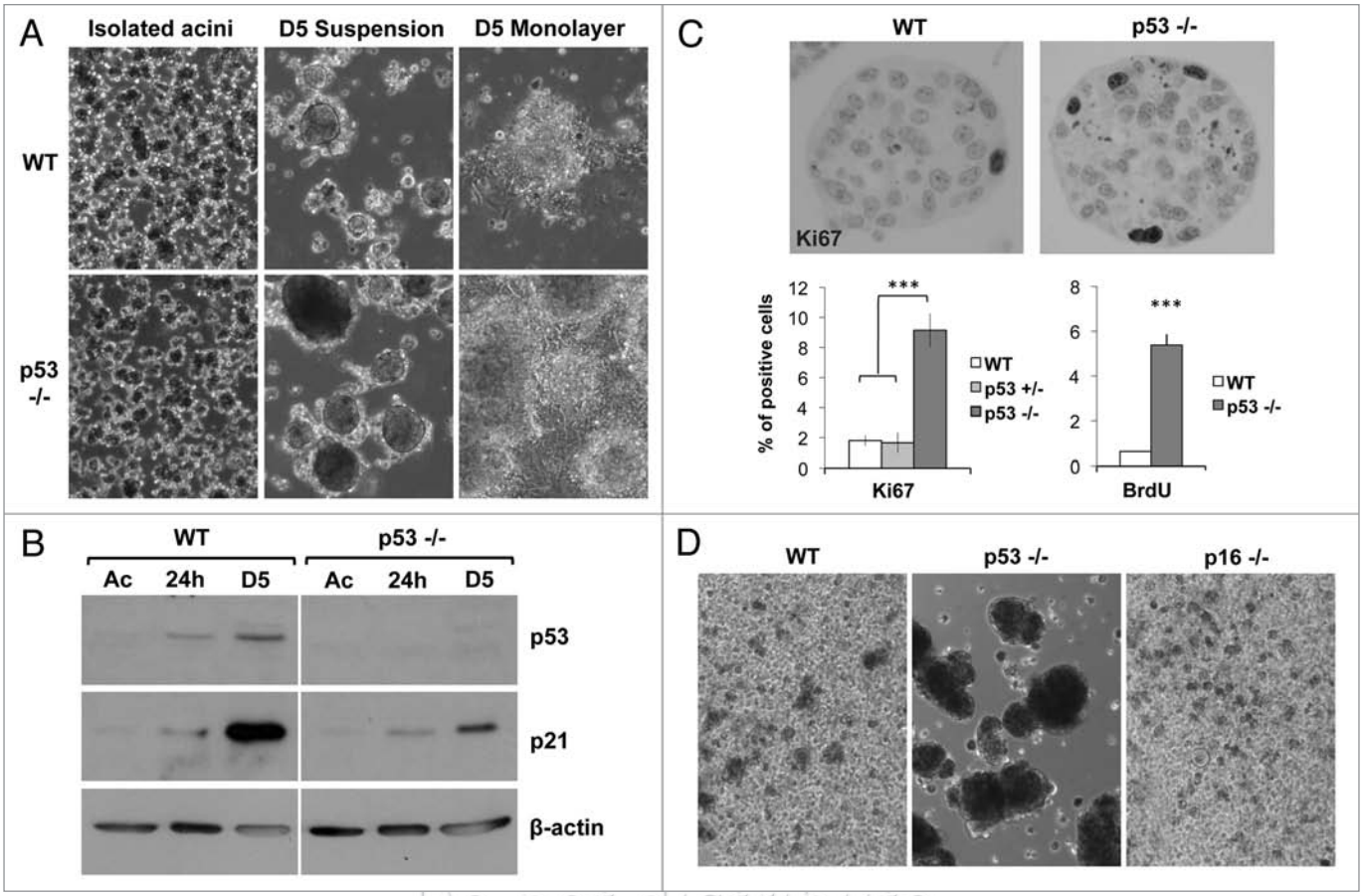

Figure 1. Acinar cell cultures from p53$^{-/-}$ mice display enhanced proliferative capacity. (A) Acinar fractions from wild type and p53$^{-/-}$ mice were cultured in suspension or in monolayer. Cultures from p53$^{-/-}$ mice—but not those from wild type—showed enhanced primary sphere formation and growth as monolayers. (B) Expression of p21 and p53 in freshly isolated acinar cells (Ac) and in primary suspension cultures at 24 h and D5. (C) Ki67 immunohistochemical staining of D5 spheres from wild type and p53$^{-/-}$ mice (upper part). Quantification of proliferative cells in wild type, p53$^{+/-}$ and p53$^{-/-}$ D5 suspension cultures by Ki67 immunohistochemistry and 24 h-BrdU incorporation (lower part). Results are shown as mean ± SEM, n = 3–6, ***p < 0.001. (D) D5 suspension cultures of wild type, p53$^{-/-}$ and p16$^{-/-}$ mice were dissociated and replated in suspension. Passage 1 (P1) cells from p53$^{-/-}$ mice, but not those from wild type or p16$^{-/-}$ mice, were able to form secondary spheres.

arrest upon cells with embryonic features when they are outside of the physiological niche. The potential pathophysiological relevance of this process is underlined by the occurrence of cells displaying similar features upon induction of chronic experimental damage and a chronic pancreatitis-like lesion in mice through caerulein administration or pancreatic duct ligation.[15] Activation of permanent growth arrest is consistent with the induction of tumor suppressive mechanisms[16] and their evasion might favor tumor development. Consistent with this notion is the finding that inactivation of p53 and p16 are very common—if not universal—events involved in the development of pancreatic ductal adenocarcinoma.[17]

To determine whether these tumor suppressor genes are required for growth arrest upon acinar cell dedifferentiation, we cultured the exocrine fraction from knockout mice and analyzed the growth capacity and phenotype of pancreatic epithelial cells using a combination of lineage tracing, RT-qPCR and immunofluorescence. In the absence or p53, epithelial cells not only become spontaneously immortalized and express stem cell markers but also undergo an epithelial-mesenchymal transition (EMT) while preserving expression of endodermal markers.

Results

Acinar cell cultures from p53$^{-/-}$ mice display enhanced proliferative capacity and life span. To assess the role of p53 in growth arrest, we cultured acinar fractions from mice in which the gene had been inactivated. Acinar cell fractions from p53$^{-/-}$ mice displayed enhanced proliferative capacity compared to wild type cells, as shown by larger sphere formation in primary cultures and increased proliferation in monolayer cultures (**Fig. 1A**). In agreement with p21 being a p53 target gene, p53$^{-/-}$ cultures expressed low levels of p21 (**Fig. 1B**). In contrast, proliferation and primary sphere formation was similar in cultures from wild type mice and from p53$^{+/-}$, p21$^{-/-}$ or p16$^{-/-}$ mice (**Sup. Fig. 1** and data not shown). Proliferation was assessed in D5 suspension cultures of wild type, p53$^{+/-}$ and p53$^{-/-}$ pancreata (**Fig. 1C**); p53$^{-/-}$ cultures had an increased proportion of Ki67^{+} (9.1 ± 1.1% vs. 1.8 ± 0.3%, p < 0.001) and BrdU-labeled cells (5.4 ± 0.5% vs. 0.64

± 0.02%, p < 0.001) in comparison with wild type mice. The proliferative properties of cells from $p53^{+/-}$ mice were similar to those from controls (**Fig. 1C**).

Day 5 cultures of wild type, $p53^{-/-}$ and $p16^{-/-}$ mice were dissociated and replated in suspension (**Fig. 1D**). Cells from wild type mice or from mice deficient in p16 could not be maintained in culture beyond day 10 and were unable to form secondary spheres or to grow in monolayer after dissociation. By contrast, those from $p53^{-/-}$ mice could be subcultured for at least six passages in suspension (**Sup. Fig. 2**) and for more than 20 passages in monolayer without signs of growth arrest.

Proliferative cells in $p53^{-/-}$ cultures express pancreatic progenitor cell markers. To determine the nature of proliferative cells in D5 $p53^{-/-}$ cultures, double labeling was performed for Ki67 and pancreatic markers (**Fig. 2A**). $Ki67^{+}$ cells were epithelial since they expressed Krt19; in addition, they co-expressed Ptf1a, Pdx1 and CpA, markers of pancreatic progenitors.[18] Furthermore, proliferating cells were also labeled by Bmi1 and Cd133, two established pancreatic stem cell markers.[19,20] At D5, $p53^{-/-}$ cultures displayed increased expression levels of transcripts coding for the ductal keratins Krt19 and Krt7 (2.6 ± 0.3 and 2.7 ± 0.5-fold, respectively) and for the pancreatic progenitor markers Ptf1a, Pdx1, Cpa1, c-myc, Sox9 and Hnf1b[14] (1.8 ± 0.2, 1.6 ± 0.1, 1.9 ± 0.2, 2.1 ± 0.3, 2.6 ± 0.4 and 1.7 ± 0.4-fold, respectively) (**Fig. 2B,** upper). Furthermore, $p53^{-/-}$ cells presented increased expression of the endodermal markers Foxa2, Gata6 and Sox17,[21] (1.4 ± 0.1, 1.6 ± 0.2 and 1.6 ± 0.2-fold, respectively) but not of Hhex. $p53^{-/-}$ cells also expressed increased levels of mRNA for the stem cell markers Bmi1, Klf4, Cd133 and Abcg2 (1.8 ± 0.2, 1.4 ± 0.1, 1.7 ± 0.3 and 3.4 ± 0.7-fold, respectively) (**Fig. 2B,** lower).

Acinar cultures from $p53^{-/-}$ mice undergo a rapid epithelial-mesenchymal transition in culture.

To assess the phenotype of the subcultured cells, we first analyzed expression of E-cadherin (E-cad) and vimentin (Vim) in primary spheres and in P2 spheres. As shown in **Figure 3A**, the phenotype of primary spheres was uniformly $E\text{-}Cad^{+}$ and Vim^{-}. By contrast, >95% of cultured cells were $E\text{-}cad^{-}$ Vim^{+} and less than 5% of P2 cells expressed E-cad (**Fig. 3A**).

To determine whether the cells with proliferative capacity and a mesenchymal phenotype were derived from pancreatic epithelial cells, lineage tracing was performed using $Ptf1a^{Cre}$;R26R-LSL-EYFP;$p53^{-/-}$ mice in which all pancreatic epithelial cells are $EYFP^{+}$ and mesenchymal-derived cells are $EYFP^{-}$ (**Sup. Fig. 3A**). Primary spheres from $Ptf1a^{Cre}$;R26R-LSL-EYFP;$p53^{-/-}$ mice contained >95% labeled cells (**Fig. 3B**), in agreement with prior studies using Ela^{CreERT} mice demonstrating the predominant contribution of acinar epithelial cells.[15] To establish pure $EYFP^{+}$ cell lines, primary cultures were dissociated and sorted (**Sup. Fig. 3B**) and $EYFP^{+}$ cells were expanded for phenotypic analyses. To eliminate contaminating cells that were not the progeny of pancreatic epithelium, $EYFP^{+}$ cells were again sorted at P12 (**Sup. Fig. 3C**) and the expression of Vim and Krt19 was analysed by immunofluorescence 48 h after plating (**Fig. 3C** and upper). All cells had mesenchymal morphology and expressed vimentin, with <5% of cells being Vim^{+} $Krt19^{+}$. E-cad was expressed at low or undetectable levels and was absent from cell-cell contacts (**Fig. 3C** and lower). These results indicate that, in the absence of p53, the proliferative progeny of pancreatic epithelial cells undergoes an EMT.

To assess whether the EMT phenotype was already activated early after placing cells in vitro, RNA from D5 cultures was analyzed. qRT-PCR analysis indicated that primary cultures from $p53^{-/-}$ mice at D5 presented a significant increase in the levels of transcripts coding for Twist, Snai1, Snai2, Zeb1 and Zeb2 (3.7 ± 0.8, 4.5 ± 0.5, 4.7 ± 0.8, 3.4 ± 0.4 and 4.5 ± 0.8-fold, respectively). Importantly, increased transcript levels were also present in cultures from $p53^{+/-}$ mice (**Fig. 3D**). D5 suspension cultures from $Ptf1a^{Cre}$;$R26R^{EYFP}$;$p53^{-/-}$ mice were dissociated, $EYFP^{+}$ cells were sorted and used for RT-qPCR analysis at subsequent passages. Expression levels of the transcripts coding for the EMT transcription factors increased markedly in P1 (20 to 100-fold compared to D5 primary cultures), remaining very high at subsequent passages (**Fig. 3E**). Vimentin transcript levels were also upregulated (10.5 ± 1.1-fold at P1) and E-cad transcript levels were reduced to 0.06 ± 0.009-fold at P1, in comparison with D5 primary cultured cells, further indicating that pancreatic epithelium-derived cultures from $p53^{-/-}$ mice had undergone an EMT.

$p53^{-/-}$ mice have not been reported to harbor any specific alterations in the pancreas. However, detailed studies have not been published. Therefore, we analyzed whether acinar cells achieved full maturation by assessing the expression level of transcripts coding for digestive enzymes and found no differences in comparison with the pancreas of wild type mice. In addition, we did not find differences in the expression of the transcription factors involved in EMT or in pancreatic progenitor markers (data not shown). These findings indicate that the effect of the lack of p53 on EMT is context-dependent.

Acinar cultures from $p53^{-/-}$ mice displaying mesenchymal phenotype retain expression of endodermal markers. Our previous analyses indicated that cultures from wild type acinar cells acquired at D5 a pancreatic progenitor phenotype characterized by expression of Ptf1a, Pdx1, Sox9, Foxa2 and Hnf1b.[15] Therefore, we analyzed the expression of these markers—as well as other endodermal markers—in the cultures obtained from $p53^{-/-}$ mice. D5 suspension cultures were dissociated, $EYFP^{+}$ cells were sorted at P1 and mRNA was analysed in subsequent passages (**Fig. 4 and Sup. Fig. 4**). Unlike in D5 cultures, Ptf1a transcripts and protein became undetectable in P1 cells and at subsequent passages (data not shown). From P1-P4, these cells retained high expression levels of pre-pancreatic endoderm markers (Sox9, Hnf1b, Foxa2, Gata6, Sox17 and Hhex) at the mRNA level, as well as of Pdx1. The stemness regulators Bmi1, c-myc and Klf4 also remained highly expressed, although Klf4 levels were reduced to 40% in P1 compared to D5 (**Fig. 4A**). Pdx1, Sox9, Foxa2 and c-myc proteins were uniformly expressed at P12 in >95% of $EYFP^{+}$ cells with mesenchymal morphology (**Fig. 4B**).

Discussion

p53 is involved in a wide variety of physiological processes such as cell cycle regulation, apoptosis, DNA repair and the control of

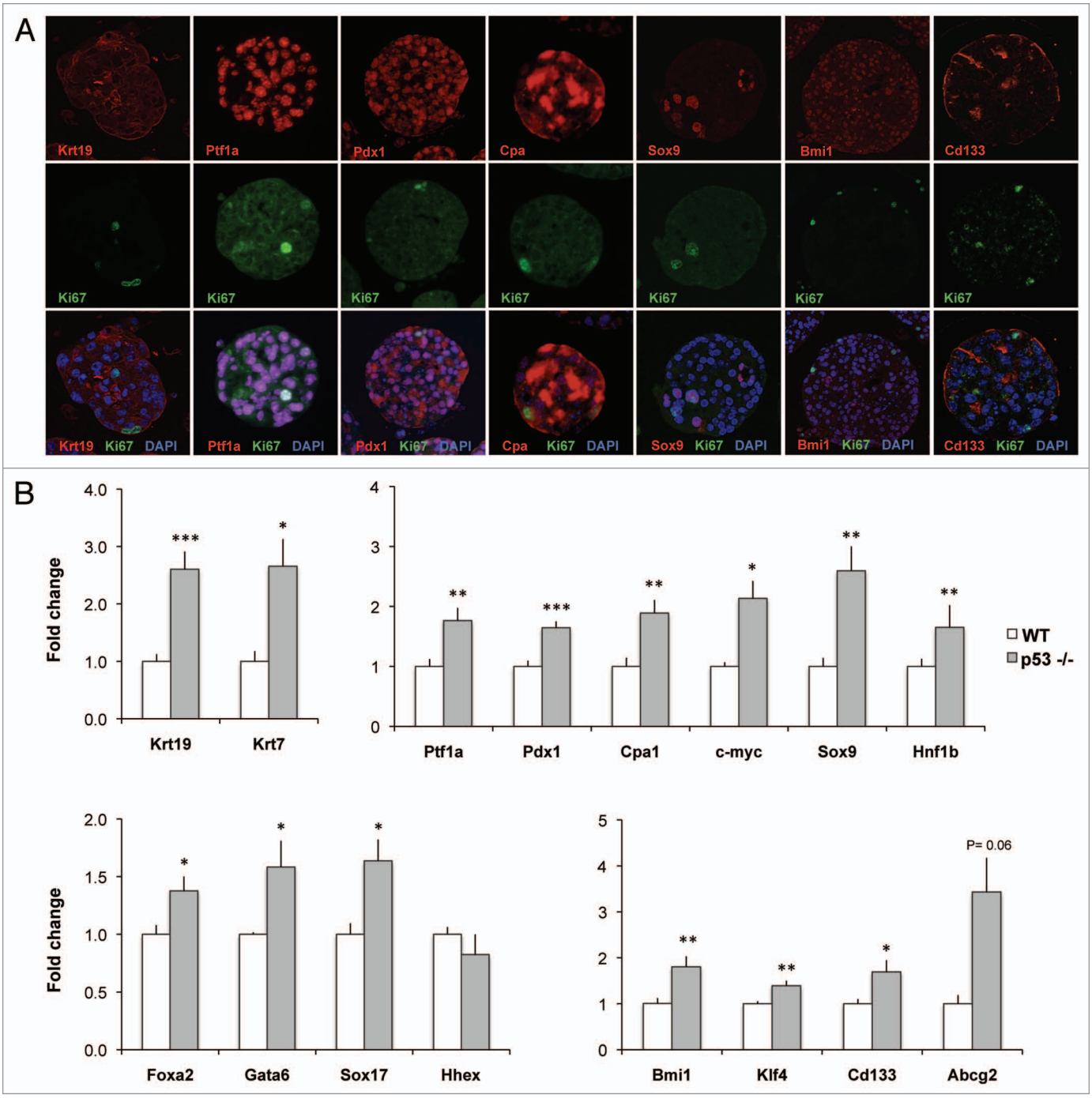

Figure 2. Proliferative cells in acinar cultures from p53$^{-/-}$ mice express pancreatic progenitor markers. (A) Double labeling with Ki67 and pancreatic progenitor markers in D5 suspension cultures isolated from p53$^{-/-}$ mice. (B) RT-qPCR analysis of pancreatic markers in D5 suspension cultures from wild type and p53$^{-/-}$ mice. Expression of transcripts coding for ductal keratins (upper left part), pancreatic progenitor markers (upper right part), endodermal markers (lower left part) and stem cell markers (lower right part). Results are shown as mean ± SEM, n = 3–13, *p < 0.05, **p < 0.01, ***p < 0.001.

genomic stability.[22] However, there is little evidence that it plays a role in the control of cell differentiation in normal cells. Here, we report that in primary cultures of normal exocrine pancreas, loss of p53 is permissive for the unlimited expansion of cells in which an EMT takes place in association with the acquisition of properties of stem cells.

Our studies were prompted by the fact that when pancreatic acinar cells are placed under stress (i.e., in vitro culture or chronic injury), they dedifferentiate and acquire an early pancreatic progenitor phenotype, concomitant with activation of the Ras-Mapk pathway and of a senescence program.[15] While the precise mechanisms involved in these processes need to be unveiled, the

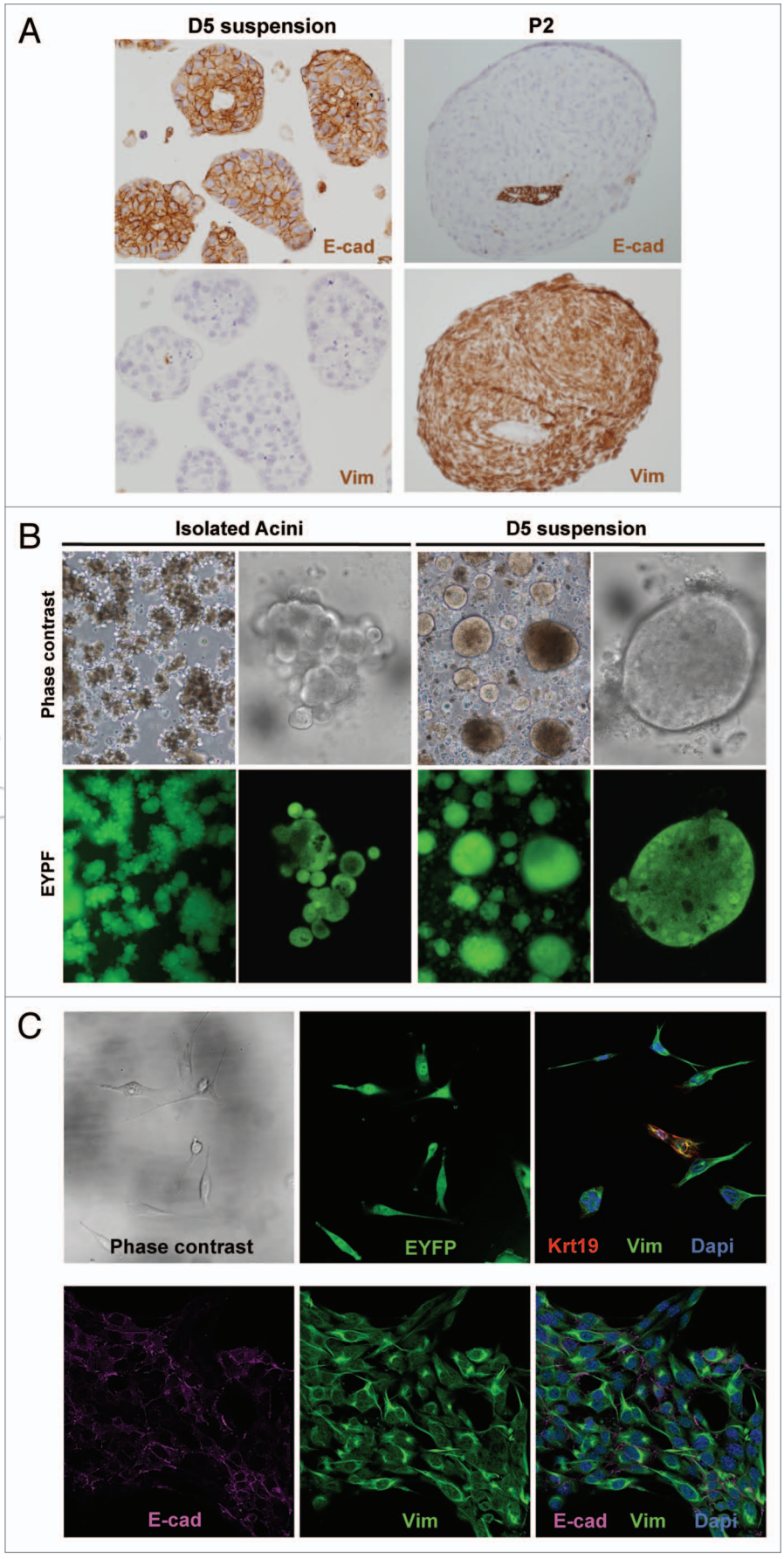

Figure 3A–C. Acinar cultures from p53$^{-/-}$ mice undergo a rapid epithelial-mesenchymal transition in culture. (A) D5 suspension cultures from p53$^{-/-}$ acinar cells are uniformly E-Cad^{+} and Vim^{-}. By contrast, after two passages the majority of the cells are E-cad^{-} and Vim^{+}; a small fraction of E-cad-expressing cells is retained. (B) Lineage tracing analysis of acinar cultures from Ptf1a^{Cre};R26R-EYFP;p53$^{-/-}$ mice shows that >95% of cells that form the pancreatic spheres have a pancreatic epithelial origin. (C) At passage 12 (P12), EYFP^{+} cells were sorted (98% positive at sorting) and fluorescence analysis was performed 48 h after sorting: all cells are Vim^{+} and <5% are double positive for Vim and Krt19 (upper). Immunofluorescence analysis using P12 cells shows very low expression of E-cad, which is not found at cell-cell contacts, and strong vimentin staining in all cells (lower).

activation of p53 suggested a role for this protein in the growth arrest. To explore this possibility, we established primary acinar cultures from p53$^{-/-}$ mice: analysis at D5 showed that acinar cultures were able to bypass the cell cycle arrest and showed increased proliferation. p53$^{-/-}$ acinar-derived cells continued to proliferate vigorously for >20 passages and can therefore be considered as spontaneously immortalized. Interestingly, pancreatic cultures from mice deficient in p21—a known downstream cell cycle effector of p53—or p16—a well established regulator of senescence—had growth properties similar to those of wild type cultures. Furthermore, we found that in the absence of p53, primary cultures also displayed increased expression of pancreatic progenitor and stem cell markers. These markers were detectable in a very high proportion of cells in the cultures. Another important finding is that in the first days of culture, an upregulation of transcripts coding for EMT regulators was observed, which was followed by an overt EMT.

EMT results from the action of a highly interconnected network of transcriptional factors that may act co-ordinately through both epistatic and parallel regulatory processes.[23,24] Many evidences suggest that deregulation of EMT might contribute to cancer progression and dissemination. Twist, one of the major players in EMT, has oncogenic properties: it inhibits p53-mediated apoptosis through Arf down-regulation,[25] prevents oncogene-induced senescence, cooperates with activated Ras to trigger a full EMT and promotes invasion.[26] Furthermore, Twist prevents premature

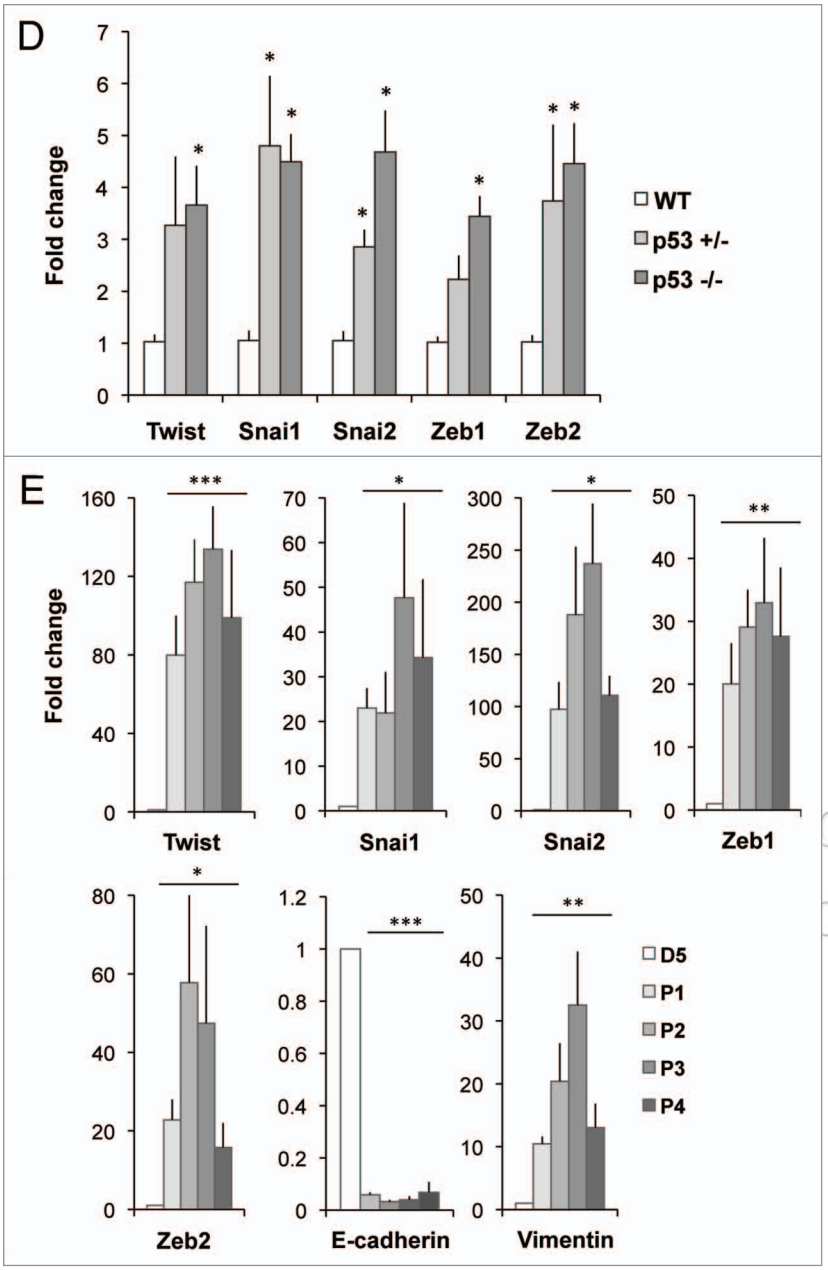

Figure 3D and E. Acinar cultures from p53$^{-/-}$ mice undergo a rapid epithelial-mesenchymal transition in culture. (D) Levels of mRNAs coding for transcription factors involved in EMT are significantly upregulated in D5 suspension cultures from p53$^{-/-}$ mice. Increased transcript levels are also present in cultures from p53$^{+/-}$ mice. Results are shown as mean ± SEM, n = 4, *p < 0.05 when compared with wild type cultures. (E) RT-qPCR analysis of transcripts coding for transcription factors involved in EMT, E-Cad and Vim in cultures from p53$^{-/-}$ mice at different passages. EYFP^{+} cells were sorted on P1. Results are shown as mean ± SEM (n = 4) *p < 0.05, **p < 0.01, ***p < 0.001, when compared with D5 suspension cultures.

senescence of pancreatic ductal cells through the repression of p16.[27] Other transcription factors involved in EMT can also modulate growth and senescence. For example, murine embryonic fibroblasts from Zeb1 mutant mice express high levels of p15 and p21 and undergo premature senescence.[28] A link between the EMT transcriptional regulators and p53 has been suggested in a few prior studies: Snai1 confers resistance to cell death upon growth factor deprivation[29] and Snai2 is a direct transcriptional target of p53 in hematopoietic progenitor cells.[30] In addition, the p53-mdm2 complex interacts with Snai2, favoring its degradation and providing a mechanism through which wild type p53 suppresses the EMT programme.[31] More recently, mutant p53^{R175H} has been shown to induce expression of Twist, thus promoting EMT in immortalized prostate cells.[32]

We find that, in the absence of p53, pancreatic epithelial cells undergo a spontaneous reprogramming to a mesenchymal phenotype, retaining expression of markers of pre-pancreatic endoderm and undergoing an upregulation of stemness regulators such as Bmi1, Klf4 and c-myc. Other studies have shown that the EMT is often linked with the acquisition of stem cell features. In mammary cancer cells, induction of EMT leads to the acquisition of stem cell properties.[33,34] Zeb1 can promote a stem cell phenotype and tumorigenic capacity in pancreatic cancer cells[35] and Twist regulates the expression of Bmi1, both proteins being required for the EMT and stemness in head and neck squamous cell carcinomas.[36] Our results further support a link between EMT and stemness and reveal the contribution of p53 to suppress these pathways in normal cells. A role of p53 in the self-renewal of embryonic stem cells after DNA damage[37] and in the suppression of somatic cell reprogramming into induced pluripotent stem cells (iPSCs) has also been described.[38-41] In the mammary gland, lack of p53 also results in increased mammosphere formation and enhanced capacity to reconstitute tissue upon transplantation[43,44] and p53 regulates the polarity of stem cell division[43] and restricts stem cell potential through activation of Notch.[44] Altogether, these results indicate that the tumor suppressive functions of p53 would involve not only cell cycle regulation but also the maintenance of a differentiated state in normal cells and tissues.

Our work does not address the mechanisms through which p53 impacts on the phenotype of pancreatic epithelial cells. While this manuscript was being prepared, Chang et al. have reported that p53 regulates EMT and stemness in mammary epithelial cells by direct transcriptional activation of the miR200c promoter. In turn, miR200c negatively regulates Zeb1 and N-cadherin, thus being a crucial mediator of the effects of p53 on the regulation of epithelial vs. mesenchymal differentiation programmes.[45] Chang et al. show that p53 regulates miR-200c, which favors epithelial differentiation through ZEB1 repression. ZEB1 and ZEB2—in turn—repress the expression of all five miR-200 family members that target Bmi1 and possibly, Klf4 and Sox2, which are involved in promoting stemness.[35] miR-200c overexpression

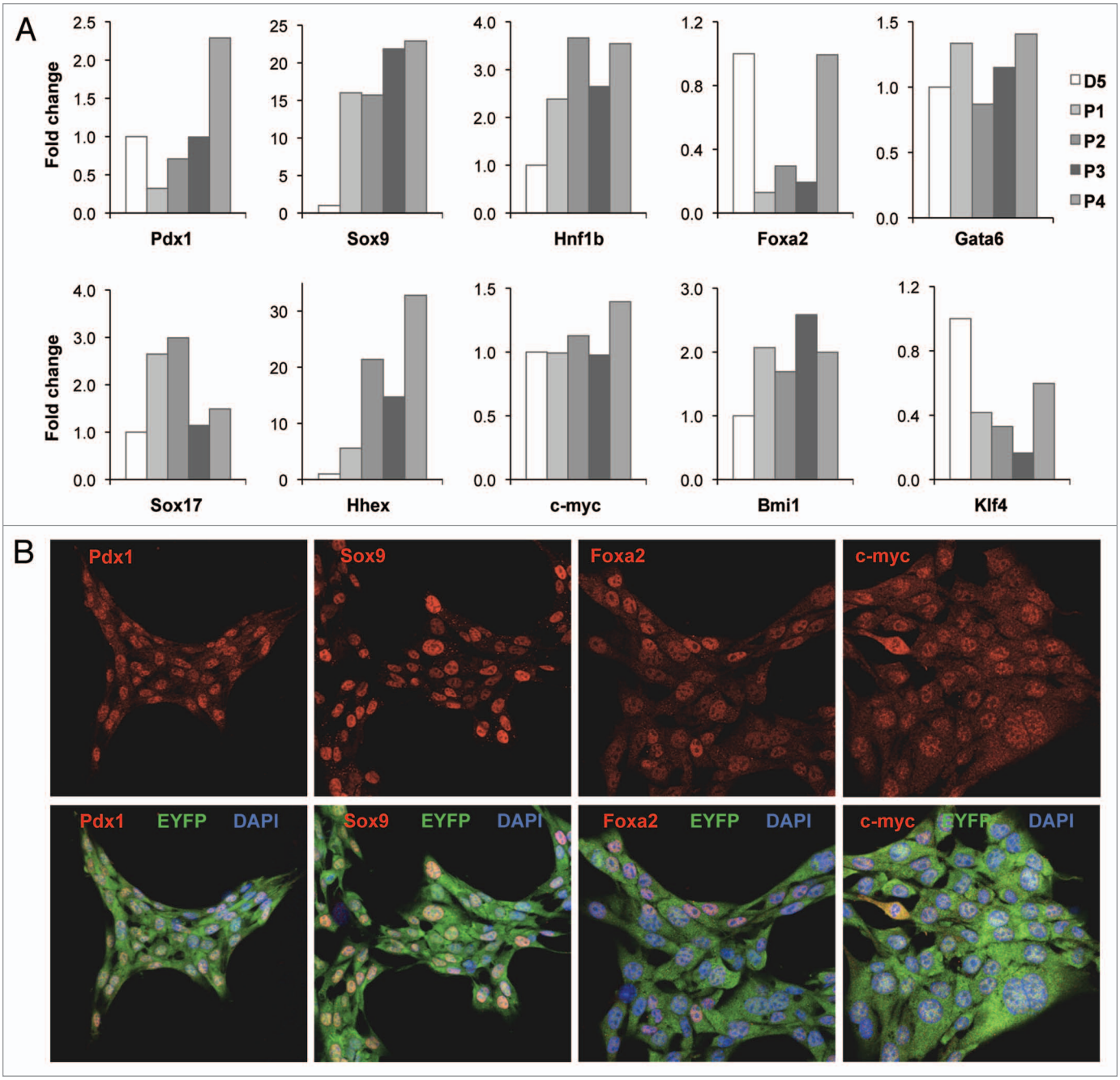

Figure 4. Acinar cultures from p53$^{-/-}$ mice with mesenchymal phenotype retain expression of endodermal markers. (A) RT-qPCR analyses of transcripts coding for markers of pancreatic progenitors, endoderm and stem cells in acinar cultures from p53$^{-/-}$ mice at different passages. EYFP^{+} cells were sorted at P1 and mRNA was analysed at each passage. Results from one representative cell line, out of 4, are shown. (B) Immunofluorescence analysis of pancreatic progenitor markers (Pdx1, c-myc and Sox9) and the endodermal marker Foxa2, using P12 cells.

also reduces clonogenic and tumor initiating activity. It will be important to determine whether additional mechanisms contribute to drive the EMT in response to p53 inactivation in pancreatic cells.

Chang et al. show that in vitro knockdown of p53, rather than its complete inactivation, was able to induce changes in miR-200c expression and its downstream effects.[45] In agreement with this, we find that in pancreatic cells p53 heterozygosity is sufficient to upregulate the expression of mRNAs for EMT transcription factors during the first five days of culture although it does not affect proliferation. These observations suggest that distinct thresholds of p53 exist for its various functions during tumor development/progression. In several cancer types, including PDAC, *Tp53* loss of heterozygosity can precede gene mutation[46] indicating that cells with one allele may be more prone to acquire migratory properties. In mutant *Kras2*-driven mouse models of PDAC, genetic loss of p53 supports escape from cell cycle arrest/senescence whereas mutant p53 favors metastasis.[47]

Our findings and those of Chang et al. support the notion that p53 plays an important role in the control of cell differentiation in a context-dependent manner, namely under stress conditions as occurs in cultured cells. In normal tissues, this effect is not apparent since young $p53^{-/-}$ mice display normal histology in most organs and the completion of the acinar differentiation programme appears unaffected. Detailed studies are required to determine whether the response to acute or chronic injury is also modulated by *Trp53* gene dosage.

One question raised by these studies is the reversibility of the effects of p53 inactivation on EMT and stemness. Our preliminary data indicate that when pancreatic $EYFP^+$ cells with a mesenchymal phenotype are placed in three-dimensional Matrigel cultures, they can form epithelial cysts. These findings suggest that the extracellular matrix can contribute as one of the contextual factors modulating the response to p53 loss of function. The potential to revert the EMT induced by lack of p53 supports its therapeutic exploitation, particularly in relationship to tumor development and progression.

Materials and Methods

Animal strains. Pancreata were isolated from 8–12 week old mice of the following mouse strains: C57BL/6JOlaHsd (Harlan Europe), referred to as wild type; *Trp53* null,[48] referred to as $p53^{-/-}$; $p21^{Waf1/Cip1}$ null,[49] referred to as $p21^{-/-}$; and $p16^{Ink4a}$ null,[50] referred to as $p16^{-/-}$. For lineage tracing, $Ptf1a^{Cre}$;R26R-LSL-EYFP;$p53^{-/-}$ mice were used.[51,52] All experiments were performed in accordance with institutional ethical committees and national guidelines and regulations.

Pancreatic acinar cell isolation. Pancreatic acinar cells were isolated as described previously in references 4 and 15. Briefly, total mouse pancreas was digested with a collagenase P (Roche) solution (0.33 mg/mL) for 20 min at 37°C. Digested pancreata were washed twice in Hank's balanced salt solution (HBSS) (Gibco BRL) supplemented with 5% fetal bovine serum (FBS) (Sigma) and filtered over 500 μm and 100 μm meshes (Spectrum Laboratories). Viable cells were recovered after low speed centrifugation over 30% FBS.

Cell culture. Isolated pancreatic acinar cells were cultured for 5 days (D5) in suspension to allow dedifferentiation into pancreatic progenitors, as described in reference 15. Isolated acini were cultured in RPMI 1640 glutamax medium (Gibco BRL) supplemented with 10% FBS, penicillin (75 μg/mL), streptomycin (100 μg/mL), geneticin sulphate (25 μg/mL) and soybean trypsin inhibitor (0.1 mg/mL) (all from Sigma) on untreated plastic (Sterilin). For monolayer culture, cells were placed in tissue culture-treated 6-well plates (Corning).

On D5, cellular aggregates were dissociated into a single cell suspension by incubation in StemPro Accutase (Invitrogen) at 37°C and cells were subcultured either in suspension or monolayer. From P1 on, cells were placed in defined medium, previously described to be suitable for pancreatic progenitor cells,[53] consisting of DMEM/F-12 (Gibco) supplemented with 3% FBS, 1x N2 supplement (Invitrogen), 0.5x B27 supplement (Invitrogen), 20 ng/mL EGF (R&D Systems), 20 ng/mL FGF2 (R&D Systems), 10 μg/mL heparin (Sigma), 100 μM β-mercaptoethanol (Gibco), 1x non-essential amino acids (Gibco), 1x penicillin/streptomycin (Gibco), 10^6 U/L Esgro-LIF (Millipore) and Rock inhibitor Y-27632 (10 μM). When applicable, geneticin sulphate (50 μg/mL) was added to eliminate contaminating fibroblasts.

BrdU incorporation of D5 suspension cultures was performed by adding BrdU (Sigma) to the culture medium (50 mM) 24 h prior to cell fixation.

Fluorescence-activated cell sorting (FACS). D5 suspension aggregates or monolayers from $Ptf1a^{Cre}$;R26R-LSL-EYFP;$p53^{-/-}$ acinar cultures were dissociated into a single cell suspension by incubation with StemPro Accutase at 37°C. After washing with DMEM/F12 supplemented with 3% FBS, cells were resuspended in PBS supplemented with BSA (0.1%) (Sigma) and EDTA (3 mM) (Sigma) at 10^6 cells/mL. To analyze cell viability, 4',6-diamidino-2-phenylindole (DAPI) (100 ng/mL) (Sigma) was added to the sorting buffer. $EYFP^+$ cells were sorted using a FACS ARIA IIu (Becton Dickinson) instrument and placed in culture in defined medium (above).

Western blotting. Proteins were extracted in Laemmli buffer (10% glycerol, 2% SDS, 0.125 M Tris-HCl pH 6.8) supplemented with protease inhibitors (Complete, EDTA-free protease inhibitor cocktail, Roche Diagnostics) and phosphatase inhibitor cocktail 3 (Sigma). Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The following antibodies were used: mouse anti-β-actin (A5441, Sigma), rabbit anti-p53 (SC-6243, Santa Cruz Biotechnology) and mouse anti-p21 (sc-6246, Santa Cruz Biotechnology).

Immunocytochemistry. Cell pellets and tissues were fixed in buffered 4% formaldehyde and processed for paraffin embedding. Antigen retrieval of paraffin sections was performed by steam heater boiling in citrate buffer pH 6.0. For immunohistochemistry, the avidin-biotin complex method was applied when using peroxidase with diaminobenzidine as substrate (Dako). Histological images were acquired with a Nikon TE2000E microscope.

Monolayer-cultured cells were stained after fixation with 4% paraformaldehyde. Cells were permeabilized with methanol at -20°C or with 0.5% Triton X-100 in PBS. Immunofluorescence images were acquired using a confocal Leica TSC-SP2_AOBS-UV ultra-spectral microscope.

The following antibodies were used: rabbit anti-Ki67 (NCL-Ki67p, Leica Biosystems), mouse anti-BrdU (G3G4) and rat anti-keratin 19 (Troma III) (Developmental Studies Hybridoma Bank), rabbit anti-Ptf1a (B. Bréant, U. Paris VI, Paris, France), rabbit anti-Pdx1 (A. Skoudy, IMIM, Barcelona, Spain), mouse anti-carboxypeptidase A (C2163, Sigma), rabbit anti-Sox9 (ab5535, Chemicon), mouse anti-Bmi1 (05-637, Millipore), mouse anti-Cd133 (ab19898, Abcam), chicken anti-vimentin (AB5733, Millipore), mouse anti-E-cadherin (610181, BD Transduction Laboratories), mouse anti-GFP (11814460001, Roche), rabbit anti-GFP (ab6556, Abcam), goat anti-Foxa2 (SC-9187, Santa Cruz Biotechnology) and rabbit anti-c-myc (ab51154, Abcam).

RT-qPCR. Total RNA was isolated using the GenElute Mammalian Total RNA kit (Sigma) and RNA integrity was assessed using an Agilent 2100 Bioanalyzer. Following DNAse

treatment (DNAfree, Ambion), cDNA was reverse-transcribed (Taqman Reverse Transcription Reagents kit, Applied Biosystems) and 20 ng RNA-equivalent was used for PCR with specific primers (available on request) in the presence of SYBR GreenER (Invitrogen) using the 7900H Fast Real Time PCR System (Applied Biosystems). All analyses were done in duplicate. A melting curve analysis was performed for each reaction to control for product quality and specificity. The expression levels were normalized to individual Hprt expression.

Statistical analyses. In vitro data were analyzed by two-tailed Student t-test, Mann Whitney or one-way Anova and statistical significance was accepted at a confidence interval <0.05 (GraphPad Prism). Results are shown as mean ± SEM. The number of independent experiments is indicated in the text as "n."

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References

1. Hall PA, Lemoine NR. Rapid acinar to ductal transdifferentiation in cultured human exocrine pancreas. J Pathol 1992; 166:97-103.
2. Vila MR, Lloreta J, Real FX. Normal human pancreas cultures display functional ductal characteristics. Lab Invest 1994; 71:423-31.
3. Rooman I, Heremans Y, Heimberg H, Bouwens L. Modulation of rat pancreatic acinoductal transdifferentiation and expression of PDX-1 in vitro. Diabetologia 2000; 43:907-14.
4. Means AL, Meszoely IM, Suzuki K, Miyamoto Y, Rustgi AK, Coffey RJ Jr, et al. Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. Development 2005; 132:3767-76.
5. Guerra C, Schuhmacher AJ, Cañamero M, Grippo PJ, Verdaguer L, Pérez-Gallego L, et al. Chronic Pancreatitis Is Essential for Induction of Pancreatic Ductal Adenocarcinoma by K-Ras Oncogenes in Adult Mice. Cancer Cell 2007; 11:291-302.
6. Habbe N, Shi G, Meguid RA, Fendrich V, Esni F, Chen H, et al. Spontaneous induction of murine pancreatic intraepithelial neoplasia (mPanIN) by acinar cell targeting of oncogenic Kras in adult mice. Proc Natl Acad Sci USA 2008; 105:18913-8.
7. De La OJ, Emerson LL, Goodman JL, Froebe SC, Illum BE, Curtis AB, et al. Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia. Proc Natl Acad Sci USA 2008; 105:18907-12.
8. Baeyens L, Bonne S, Bos T, Rooman I, Peleman C, Lahoutte T, et al. Notch signaling as gatekeeper of rat acinar-to-beta-cell conversion in vitro. Gastroenterology 2009; 136:1750-60.
9. Minami K, Okuno M, Miyawaki K, Okumachi A, Ishizaki K, Oyama K, et al. Lineage tracing and characterization of insulin-secreting cells generated from adult pancreatic acinar cells. Proc Natl Acad Sci USA 2005; 102:15116-21.
10. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008; 455:627-32.
11. Shen CN, Slack JM, Tosh D. Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol 2000; 2:879-87.
12. Wu SY, Hsieh CC, Wu RR, Susanto J, Liu TT, Shen CR, et al. Differentiation of pancreatic acinar cells to hepatocytes requires an intermediate cell type. Gastroenterology 2010; 138:2519-30.
13. Bonal C, Thorel F, Ait-Lounis A, Reith W, Trumpp A, Herrera PL. Pancreatic inactivation of c-Myc decreases acinar mass and transdifferentiates acinar cells into adipocytes in mice. Gastroenterology 2009; 136:309-19.
14. MacDonald RJ, Swift GH, Real FX. Transcriptional control of acinar development and homeostasis. Prog Mol Biol Transl Sci 2010; 97:1-40.
15. Pinho AV, Rooman I, Reichert M, De Medts N, Bouwens L, Rustgi AK, et al. Adult pancreatic acinar cells dedifferentiate to an embryonic progenitor phenotype with concomitant activation of a senescence programme that is present in chronic pancreatitis. Gut 2011; In press.
16. Collado M, Serrano M. Senescence in tumours: evidence from mice and humans. Nat Rev Cancer 10:51-7.
17. Real FX, Cibrian-Uhalte E, Martinelli P. Pancreatic cancer development and progression: Remodeling the model. Gastroenterology 2008; 135:724-8.
18. Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA. A multipotent progenitor domain guides pancreatic organogenesis. Dev Cell 2007; 13:103-14.
19. Oshima Y, Suzuki A, Kawashimo K, Ishikawa M, Ohkohchi N, Taniguchi H. Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-Met by flow cytometric cell sorting. Gastroenterology 2007; 132:720-32.
20. Sangiorgi E, Capecchi MR. Bmi1 lineage tracing identifies a self-renewing pancreatic acinar cell subpopulation capable of maintaining pancreatic organ homeostasis. Proc Natl Acad Sci USA 2009; 106:7101-6.
21. Pan FC, Wright C. Pancreas organogenesis: From bud to plexus to gland. Dev Dyn 2011; 240:530-65.
22. Vousden KH, Prives C. Blinded by the Light: The Growing Complexity of p53. Cell 2009; 137:413-31.
23. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 2007; 7:415-28.
24. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. Proc Natl Acad Sci USA 2010; 107:15449-54.
25. Maestro R, Dei Tos AP, Hamamori Y, Krasnokutsky S, Sartorelli V, Kedes L, et al. Twist is a potential oncogene that inhibits apoptosis. Genes Dev 1999; 13:2207-17.
26. Ansieau S, Bastid J, Doreau A, Morel AP, Bouchet BP, Thomas C, et al. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. Cancer Cell 2008; 14:79-89.
27. Lee KE, Bar-Sagi D. Oncogenic KRas suppresses inflammation-associated senescence of pancreatic ductal cells. Cancer Cell 2010; 18:448-58.
28. Liu Y, El-Naggar S, Darling DS, Higashi Y, Dean DC. Zeb1 links epithelial-mesenchymal transition and cellular senescence. Development 2008; 135:579-88.
29. Vega S, Morales AV, Ocana OH, Valdes F, Fabregat I, Nieto MA. Snail blocks the cell cycle and confers resistance to cell death. Genes Dev 2004; 18:1131-43.
30. Wu WS, Heinrichs S, Xu D, Garrison SP, Zambetti GP, Adams JM, et al. Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. Cell 2005; 123:641-53.
31. Wang SP, Wang WL, Chang YL, Wu CT, Chao YC, Kao SH, et al. p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. Nat Cell Biol 2009; 11:694-704.
32. Kogan-Sakin I, Tabach Y, Buganim Y, Molchadsky A, Solomon H, Madar S, et al. Mutant p53(R175H) upregulates Twist1 expression and promotes epithelial-mesenchymal transition in immortalized prostate cells. Cell Death Differ 2011; 18:271-81.
33. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008; 133:704-15.
34. Morel AP, Lievre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One 2008; 3:2888.
35. Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A, et al. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat Cell Biol 2009; 11:1487-95.
36. Yang MH, Hsu DS, Wang HW, Wang HJ, Lan HY, Yang WH, et al. Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat Cell Biol 2010; 12:982-92.
37. Lin T, Chao C, Saito S, Mazur SJ, Murphy ME, Appella E, et al. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol 2005; 7:165-71.
38. Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M, et al. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 2009; 460:1132-5.
39. Kawamura T, Suzuki J, Wang YV, Menendez S, Morera LB, Raya A, et al. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 2009; 460:1140-4.
40. Li H, Collado M, Villasante A, Strati K, Ortega S, Canamero M, et al. The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature 2009; 460:1136-9.
41. Marion RM, Strati K, Li H, Murga M, Blanco R, Ortega S, et al. A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature 2009; 460:1149-53.
42. Utikal J, Polo JM, Stadtfeld M, Maherali N, Kulalert W, Walsh RM, et al. Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. Nature 2009; 460:1145-8.
43. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, et al. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell 2009; 138:1083-95.
44. Tao L, Roberts AL, Dunphy KA, Bigelow C, Yan H, Jerry DJ. Repression of mammary stem/progenitor cells by p53 is mediated by Notch and separable from apoptotic activity. Stem Cells 2011; 29:119-27.

45. Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, et al. p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat Cell Biol 2011.
46. Luttges J, Galehdari H, Brocker V, Schwarte-Waldhoff I, Henne-Bruns D, Kloppel G, et al. Allelic loss is often the first hit in the biallelic inactivation of the p53 and DPC4 genes during pancreatic carcinogenesis. Am J Pathol 2001; 158:1677-83.
47. Morton JP, Timpson P, Karim SA, Ridgway RA, Athineos D, Doyle B, et al. Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. Proc Natl Acad Sci USA 2010; 107:246-51.
48. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, et al. Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994; 4:1-7.
49. Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature 1995; 377:552-7.
50. Krimpenfort P, Quon KC, Mooi WJ, Loonstra A, Berns A. Loss of $p16^{Ink4a}$ confers susceptibility to metastatic melanoma in mice. Nature 2001; 413:83-6.
51. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 2002; 32:128-34.
52. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 2001; 1:4.
53. Rovira M, Scott SG, Liss AS, Jensen J, Thayer SP, Leach SD. Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. Proc Natl Acad Sci USA 2010; 107:75-80.