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14. ABSTRACT
The purpose of this project is to define the role of p63 in the development and maintenance of the prostate epithelium by utilizing both in vivo and in vitro models. In the first two years of work, we have constructed the targeting vector for the generation of the p63-Cre-ERT2 knock-in mice. The p63-Cre-ERT2 vector has been electroporated in the ES cells. p63-Cre-ERT2 ES clones with successful targeting events have been obtained and injected in host blastocysts, resulting in the production of 5 high percentage p63-Cre-ERT2 chimeras, which are currently being bred. To date, two F1 p63-Cre-ERT2 knock-in pups have been generated. We have also continued to work on the identification of the molecular mechanisms through which p63 regulates development of the prostate epithelium. Specifically, the use of siRNA against p63 has been optimized in various cell lines and, most importantly, p63 shRNA inducible cell lines (including iPrEC) have been generated. Our results show that downregulation of p63 in iPrEC cells consistently causes a decrease in cell viability due to induction of apoptosis. Moreover, our data demonstrates that p63 modulates AKT and MAPK activation in iPrEC cells.

15. SUBJECT TERMS
p63, prostate, cancer, basal cells, stem cells, development

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INTRODUCTION

Basic cancer research has focused on identifying the genetic alterations that cause cancer. This has led to major advances in our understanding of the molecular and biochemical pathways that are involved in tumorigenesis. However, since most of the work focused on the effects of particular molecular changes on the proliferation and survival of model cells, such as fibroblasts or cell lines, it is not clear what the effects of such changes will be on the actual cells involved in particular cancers. Thus, a clear understanding of the molecular abnormalities underlying the development of human malignancies, including prostate cancer, cannot be achieved without the identification of the cell type(s) involved in neoplastic transformation. Various cell types (i.e. secretory, intermediate, and stem cells) have been proposed as potential targets for prostate carcinogenesis [1-8]. However, a major limitation for the identification of the cell type(s) involved in the development and propagation of prostate cancer is that the identity of prostate stem cells and their differentiation programs remain unclear.

The scientific community has long debated the hierarchical relationship between basal and secretory cells in the prostate. The basal cell marker p63 is selectively expressed in the basal cells of several epithelia, including the prostate [9, 10]. We previously demonstrated that p63-deficient (p63-/-) mice present defects in prostate buds development [9]. Our recent work shows that when such developmental defects are abolished by complementing p63-/- blastocysts with p63+/+ ES cells, only p63+/+ cells compose the normal prostate epithelium of 7-weeks old chimeric mice [11]. These results indicate that prostate secretory cells of young adult mice derive from p63-positive progenitor cells that constitute the prostate buds. In addition, our UGS transplantation experiments show that p63 expression is required in progenitor cells to restrict development to the prostate cell lineage. As a whole, our preliminary data demonstrate that p63 is a key regulator of prostate development. On the basis of these data we hypothesize that 1) secretory cells forming throughout the entire lifespan derive from p63-positive stem/progenitor cells; 2) p63-positive cells of the adult prostate retain stem cell capabilities and thus function as adult stem cells; 3) p63 controls the development of prostate basal cells by regulating the expression of specific target genes.

Results from the proposed research project are likely to provide fundamental knowledge about the way the normal prostate epithelium develops and is renewed *in vivo.* Importantly, such knowledge is very critical for the advancement of the prostate cancer field.

BODY

Research accomplishments based on the approved Statement of Work

Specific Aim 1: **To demonstrate that p63 is required for the development of secretory cells throughout the lifespan**.

In this aim we plan to utilize the p63-/-;ROSA26 chimera model that we recently developed. To demonstrate that secretory cells forming throughout the entire lifespan originate from p63-positive progenitors, the contribution of p63-/- and p63+/+ cells to the secretory cell compartment will be assessed and compared in p63-/-;ROSA26 chimeras sacrificed at different ages (7 weeks and 12 months).

We are working on the generation of the p63-/-;ROSA26 chimeras by injecting p63-/- ES cells into ROSA26 hemizygous blastocysts. We have been working on the selection of the optimal ES clones to be used for the generation of chimeric animals. We utilized real time quantitative PCR to determine both the genotype and the sex of 16 ES cell clones obtained from pre-implantation embryos (blastocysts) derived from p63+/- crosses. The karyotype of two p63+/- and two p63-/- male ES cell clones was subsequently determined in order to rule out the presence of chromosomal abnormalities. Results from these analyses are summarized in Table 1. Clone #1 (p63+/-) and clone #7 (p63-/-) were selected and are currently utilized for the generations of the p63-/-;ROSA26 and p63+/-;ROSA26 chimeras. A first experiment in which clone #1 and clone #7 have been injected into ROSA26 hemizygous blastocysts has been performed. Unfortunately, a small number of blastocysts could be isolated from the ROSA26 mice. As a consequence, when the foster mothers were sacrificed at 18.5 dpc, only a total of 7 chimeras where obtained. Such chimeras did not present any gross abnormalities and beta-gal staining showed no significant contribution from the p63-/ ES clones. These preliminary results are not very encouraging and suggest that this novel approach for the generation of p63-/-;ROSA26 is less efficient than anticipated.

ES cells ID	Sex	Genotype	Karyotype
1	M	Het	46 XY
2	M	Het	46 XY/92XXYY
3	M	Het	-
4	F	WT	-
5	M	Hom	-
6	M	Het	-
7	M	Hom	46 XY
8	M	Het	-
9	M	Het	-
10	M	Hom	46 XY
11	M	Het	-
12	F	Het	-
13	M	Hom	-
14	F	Hom	-
15	M	Het	-
16	F	Het	-

Table 1

Specific Aim 2: **To assess if p63-positive basal cells of the adult prostate sustain the renewal of secretory cells and thus represent/include adult prostate stem cells**.

This aim will be achieved by performing genetic lineage tracing experiments. We plan to generate mice expressing inducible Cre recombinase (Cre-ER^{T2}) under the control of the p63 promoter by knocking-in the Cre-ER^{T2}

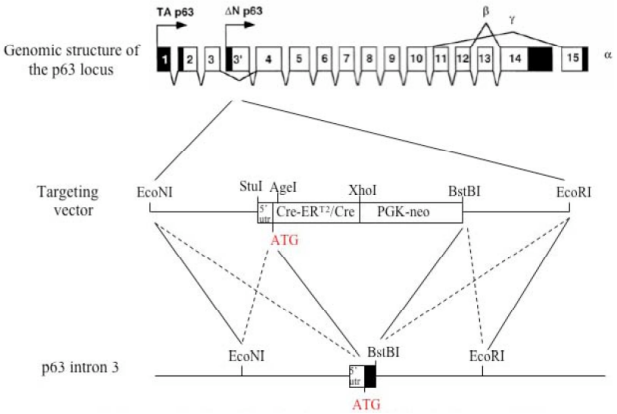

Fig. 1. Strategy for knocking–in Cre-ER^{T2} and Cre in the ΔNp63 locus.

cDNA into the p63 locus. These mice will be then crossed with R26R reporter mice to generate double mutant p63- Cre-ER^{T2};R26R mice. Analysis of the prostate of the double mutant p63-Cre-ER^{T2};R26R mice after Tamoxifen administration will allow us to determine if p63-positive basal cells of the adult prostate sustain the renewal of secretory cells and thus function as adult prostate stem cells.

To date, the construction of the targeting vector for the generation of the p63-Cre-ER^{T2} knock-in mice has been completed. The p63-Cre-ER^{T2} vector has been electroporated in the ES cells and chimeric mice have been obtained. Chimeras have been crossed with wild-type female mice and p63-Cre-ER^{T2} knock-in mice have been very recently generated.

1. Construction of the targeting vector

For the presence of two different promoters, located upstream of exon 1 and within intron 3, the p63 gene transcribes two isoforms, TA and ΔNp63. Since the ΔNp63alpha isoform is selectively expressed at high levels in basal cells of various epithelia, including the prostate, the p63-Cre-ER^{T2} knock-in mice are constructed by inserting a Cre-ER^{T2}-PGKneo cassette immediately downstream from the endogenous ΔNp63 promoter in intron 3. Specifically, after homologous recombination in the ES cells the start codon (ATG) of Cre-ER^{T2}/Cre will replace the start codon of the ΔNp63 transcript (Fig. 1).

The targeting constructs contain the diphtheria toxin A (DTA) cDNA, a homologous region upstream from the ΔNp63 ATG (5' arm), the Cre-ER^{T2}/Cre cDNA, the mouse neomycin phosphotransferase (neo) gene driven by the phosphoglycerate kinase (PGK) promoter, and a homologous region downstream from the ΔNp63 ATG (3' arm). The neo gene provides antibiotic resistance (neomycin) to the embryonic stem cells in which the homologous recombination has occurred successfully. This cassette is flanked by two FLP sites that will allow its excision from the ΔNp63 locus by crossing this mouse with an Frt mouse. The presence of the DTA cDNA is aimed at reducing the random genomic incorporation of the targeting construct.

The optimal homologous recombination rate is obtained by using ES cell lines derived from the 129 Sv/Ev mouse strain. Therefore, these ES cells are being used for the generation of the p63-Cre-ER^{T2} knock-in mice. Since even small gaps in homology due to sequence polymorphisms between mouse strains can dramatically reduce the efficiency of homologous recombination, the genomic DNA used for the construction of the targeting vectors was derived from the same strain of mouse as the ES cells, i.e. 129 Sv/Ev. To obtain the 129 Sv/Ev genomic clones containing the ΔNp63 locus, we screened a set of "dot-blot" membranes representing arrayed genomic clones of 129 Sv/Ev DNA in Bacterial Artificial Chromosomes (BAC), that have a 5-fold coverage of the entire genome. These membranes were obtained from the Dana-Farber Cancer Institute (DFCI) Gene Targeting facility directed by Dr. Ronald DePinho. Once the genomic clones containing the ΔNp63 locus were obtained, the targeting vector was constructed by the following steps:

1) A 9.3kb fragment containing the 5' and 3' homologous recombination arms was excised from the BAC and inserted into the polylinker of plasmid pSL301, creating plasmid pSL301-9.3. The identity and structure of the ΔNp63 locus in the clone was verified by end-sequencing, restriction digestion, Southern Blot and PCR.

2) A fragment of the 5' recombination arm (2861 bp) was subcloned from plasmid pSL301-9.3, into plasmid pKOII, creating plasmid pKOII-5A.

3) A PGKNeo cassette was inserted into plasmid pKOII-5A, creating plasmid pKOII-5A.Neo. After subcloning, the structure of the inserted fragment was verified by thorough restriction enzyme digestion and full-length sequencing.

4) A DNA fragment of 302 bp, including the downstream fragment of the 5' arm, was amplified by PCR using plasmid pSL301-9.3 as template. In order to replace the start codon (ATG) of the ΔNp63 transcript with the start codon of Cre-ER^{T2}, a sequence of 46 nucleotides, corresponding to the 5' sequence of Cre-ER^{T2} followed by the AgeI restriction site was included in the reverse primer. The PCR product was subcloned in the StuI-AgeI sites of pKOII-5A.Neo, creating pKOII-5B.NeoC. This step completed the subcloning of the 5' recombination arm (3122 bp). Thorough restriction enzyme digestion and full-length sequencing verified the structure of the inserted fragment.

5) The fragment of Cre-ER^{T2} cDNA downstream from the AgeI site followed by a stop codon, was then subcloned in the AgeI-XhoI restriction sites of pKOII-5B.NeoC, creating pKOII-5B.NeoCre-ER^{T2}A. Restriction digestion and full-length sequencing verified the structure of the inserted fragment. This step completed the subcloning of the Cre-ER^{T2} cDNA.

6) Finally, the 3' recombination arm (3409 bp), was subcloned, in the NotI-SalI restriction sites of pKOII-5B.NeoCre-ER^{T2}A, creating the final targeting vector pKOII-5B.NeoCre-ER^{T2}B (13338 bp). Once completed, the structure of the targeting construct was verified by extensive restriction digestion and sequencing.

2. Screening of ES cells for homologous recombination

Electroporation of the linearized targeting vectors in the 129Sv/Ev ES cells is currently performed by the core facility directed by Dr. DePinho at DFCI.

The targeted ES clones that have correctly integrated the planned modifications by homologous recombination will be identified by both drug selection (neomycin and diphtheria toxin), and Southern blot analysis. Unique probes and restriction enzyme sites (SphI) that lie outside the homology regions have been used in Southern blot analysis to identify correctly recombined neomycin resistant ES clones (Fig. 2). Six p63-Cre-ER^{T2} ES clones with successful targeting event have been generated (Fig.3).

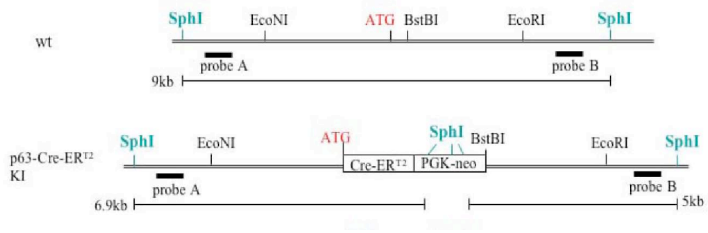

Fig.2 Southern Blot approach for screening of homologous recombination of the p63-Cre-ER^{T2} targeting vector in ES cells.

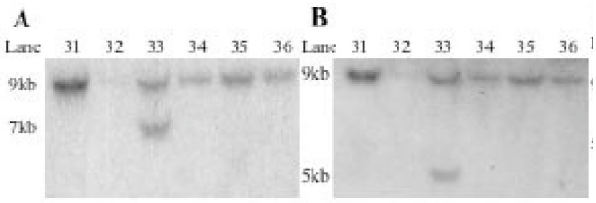

Fig. 3. Examples of Southern blot analysis of targeting events. Screening of genomic DNA originating from neomycin selected ES cells electroporated with p63-Cre-ER^{T2} targeting vector. DNA was digested with SphI and after blotting, hybridized with probe A (A) for 5'arm homologous recombination (HR) screening. The membrane was then stripped and rehybridized with probe B (B) for 3'arm HR screening. The 9 kb band represents the wt allele, the 7 kb band represents the 5' arm targeting event and the 5 kb band represents the 3' targeting event. The DNA sample in lanes 33 (in A and B) show the expected bands, indicating successful HR.

4. Generation of heterozygous knock-in mice

The p63-Cre-ER^{T2} clones with homologous recombination have been kariotyped to exclude chromosomal alterations and none of them presented abnormalities. Three targeted ES clones have been injected in host blastocysts, resulting in the production of 5 high percentage p63-Cre-ER^{T2} chimeras (90-100% of chimerism), which are being bred. To date, we have obtained four F1 pups with agouti coat color (characteristic of the 129 ES cells). DNA analysis (by PCR) shows the presence of the

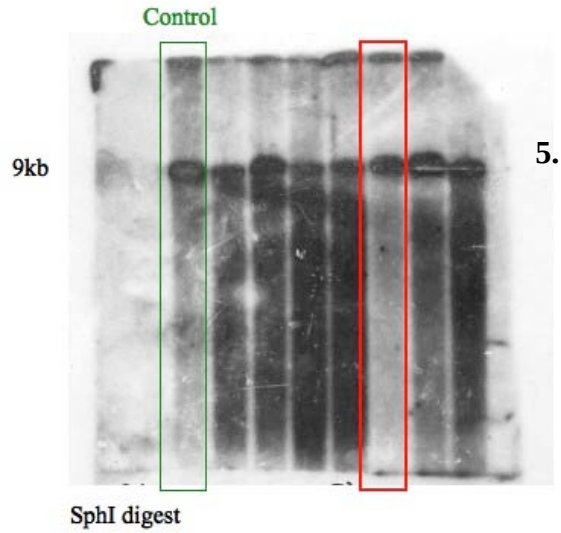

Fig. 4. Example of Southern blot analysis showing specific recombination of Cre in the p63 locus. Screening of genomic DNA originating from neomycin selected ES cells electroporated with p63-Cre-ER^{T2} targeting vector. DNA was digested with SphI and after blotting, hybridized with probe internal to CreER^{T2}. The 9 kb band represents the knock-in allele. The DNA sample in the first lane (highlighted in green) represents a positive control, the DNA sample in the sixth lane (highlighted in red) also clearly shows the expected bands, indicating successful HR of Cre solely in the p63 knock-in (KI) allele in 2/4 pups (Fig. 5). These two animals are likely to represent knock-in mice. Results are currently being confirmed by Southern Blot analysis.

5. O5. Generation of p63-Cre-ER^{T2het}/R26R^{het} double mmutant mice

p63-Cre-ER^{T2} mice are being be crossed with the ROSA26 reporter mice (R26R), which carry a reporter construct (LacZ gene) in the ubiquitously expressed ROSA26 locus. In the double mutant mice (p63-Cre-ER^{T2het}/R26R^{het}), the LacZ gene will be exclusively expressed after the Cre mediated deletion of a floxed stop codon that precedes it. Since Cre-ER^{T2} is selectively expressed in p63-positive basal cells, the recombination event will lead to the expression of the LacZ gene exclusively in these cells and their progeny. X-gal histochemistry will be utilized to identify cells expressing the LacZ gene. To date we have generated 27 male mice of which 15 are p63-Cre-ER^{T2het}/R26R^{het}. The specific expression of Cre in the basal cells of p63-CreER^{T2} knock-in mice is currently being determined by immunohistochemistry and in situ hybridization.

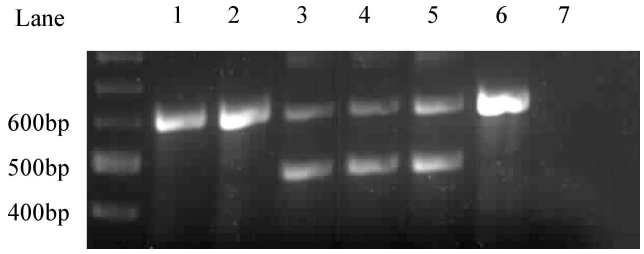

Fig. 5. PCR screening of F1 pups. Genomic tail DNA (lanes 1-4) was analyzed by PCR using locus-specific primers. DNA samples from targeted (lane 5) and WT (lane 6) ES cells were used as controls. Lane 7 is the no template control. The 600 bp band (lanes 1-6) represents the WT allele. The 470 bp band (lanes 3, 4, and 5) represents the KI allele. Two heterozygous KI pups (lanes 3 and 4) are identified.

Cre-ER^{T2} becomes active solely after exogenous estrogens administration, therefore leading to the expression of the reporter gene. To test for possible leakiness of such system, we screened double mutant litters, along with controls prior to estrogens administration by X-gal histochemistry. As expected, no expression of the reporter gene was observed prior to Tamoxifen administration.

Pilot experiments are currently being run to optimize the dose of Tamoxifen producing the highest levels of recombination in basal cells without causing significant toxicity.

Specifically, 8 seven weeks old male mice have been treated with i.p. (intra-peritoneal) injection of Tamoxifen at a dose of 7mg/40g in corn oil every other day, for up to 12 injections. In addition, four 7 weeks old male mice have been treated with i.p. injection of Tamoxifen at a dose of 7mg/40g in corn oil for 5 consecutive days. Along with the treated mice, untreated p63-Cre-ER^{T2het}/R26R^{het} mice have been also analyzed to rule out constitutive (leaky) Cre activity. We observed lethality in both experimental approaches, ranging from 25 to 50%. Preliminary beta-gal staining of the skin of the p63-Cre-ER^{T2het}/R26R^{het} treated with Tamoxifen revealed a focal beta-gal expression (not shown), indicating recombination. No staining was observed in the untreated animals.

It should be noted that the targeting vector utilized to generate p63-CreER^{T2} knock-in mice contains the neomycin (neo) resistance gene expressed from the PGK promoter. As described above, the targeting vector was constructed by flanking the neo cassette by two Flippase Recognition Target (FRT) sites. To avoid that the PGK promoter interferes with the activity of the of the endogenous ΔNp63 promoter, before further expanding the p63-CreER^{T2} mouse colony for analysis, we have decided to cross the p63-CreER^{T2} knock-in mice with the commercially available mice that ubiquitously express Flippase recombination enzyme (Flp-mice) to excise the FRT-flanked PGK-neo cassette. p63-CreER^{T2}/Flp mice in which the excision of the PGK-neo cassette has been confirmed by PCR analysis are currently being crossed with R26R mice. Double mutant p63-CreER^{T2}/R26R mice without the PGK-neo cassette will then be then be administered Tamoxifen and analyzed for beta-gal expression.

Specific Aim 3: To identify p63 target genes mediating p63 function in prostate development.

To identify the molecular mechanisms through which p63 regulates development of the prostate epithelium, we are utilizing immortalized prostate epithelial cells (iPrEC) obtained from Dr. William Hahn laboratory at DFCI. The SCC 9 cell line (squmaous cell carcinoma) was also utilized.

We have worked on silencing the expression of endogenous p63 in iPrEC as well as other cell lines cells using RNA interference. The effects of p63 silencing on cell proliferation, cell death, and activation of various signaling pathways is currently being assessed.

1. Development of siRNA against p63

We designed two oligo siRNAs against p63: 1) Tp63 siRNA, which knocks down all the p63 isoforms 2) Dp63 siRNA, which is designed in the untranslated region of DNp63 and specifically knocks down only DNp63 isoforms.

In our preliminary experiments, we optimized the concentration of the oligo siRNA to get efficient knock down of the p63 protein levels in SCC9 cells. We assayed different concentrations of the siRNA and found that 40 nM siRNA (both Tp63 and Dp63 siRNAs) induces a 80% knock down in p63 levels compared to the control siRNA (Fig. 6A). In all our experiments using oligo siRNA, we used scramble siRNA (Dharmacon) as a control. For the transfection of siRNA in SCC 9 cell line we used Oligofectamine (Invitrogen). Unfortunately, iPrEC cell could not be efficiently transfected with liposomal transfection reagents. By using the Amaxa's Nucleofector technology, we were also unsuccessful in silencing p63 expression iPrEC cell. In order to overcome this problem, we switched to a Tetracycline-inducible lentiviral siRNA expression system. Specifically, we

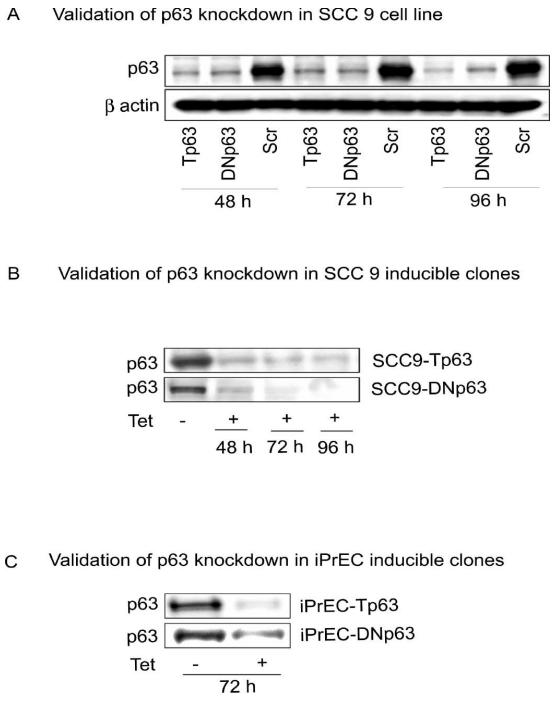

Fig 6. Silencing of p63 expression in SCC 9 and iPrEC cell lines A, SCC 9 cells were transfected with Tp63 and Dp63 siRNA. Cells were harvested at 48h, 72h, and 96h and equal amounts of protein were subjected to western blot analysis. Decrease in the protein expression was observed with both Tp63 and Dp63 siRNA compared to Scramble control. B, Decrease in p63 expression in SCC9-Tp63 and SCC9-DNp63 clones upon treatment with 5 μg/ml tetracycline for several time periods. C, Decrease in the p63 protein expression was observed in iPrEC-Tp63 and iPrEC-

used pBLOCK-iT inducible RNAi lentiviral expression system from Invitrogen. This system has several advantages including: a) Lentiviral transduction is very efficient in the cell lines that are difficult to transfect with other protocols b) the use of Tet-inducible clones allows a relatively easy manipulation of the experimental strategy.

2. Generation of p63 shRNA inducible cell lines. The mechanism of tetracycline regulation in the system is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the shRNA of interest. In the system, expression of short hairpin RNA (shRNA) is repressed in the absence of tetracycline and induced in its presence. We constructed the Tet-inducible cell line by the following procedure:

a. We designed the shRNAs based on the sequences of Tp63 siRNA and Dp63 siRNA.

b. We inserted Tp63 and Dp63 shRNAs in pENTER/H1/TO entry vector and screened for the efficiency of the knockdown by measuring p63 proteins levels after transient transfection in SCC 9 cell line. p63 protein levels were assesses at 72h and 96h time points by western blotting.

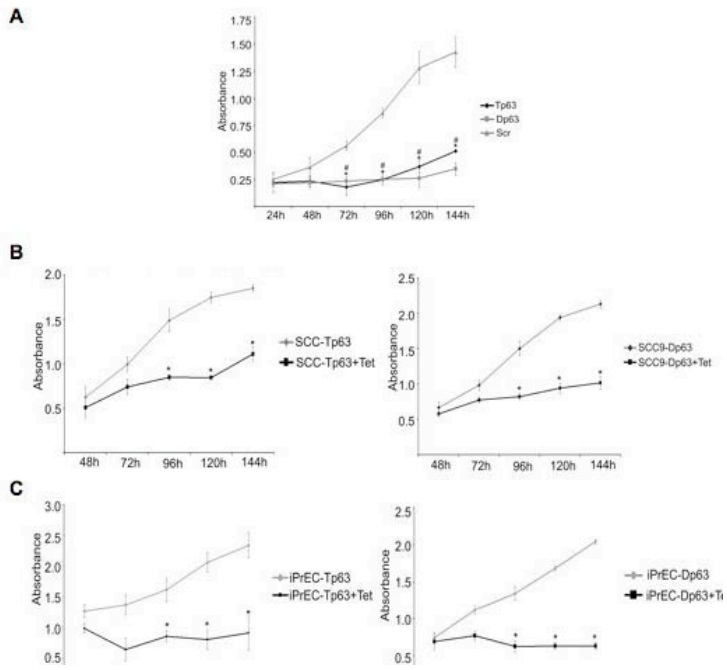

Fig 7. Knockdown of either Total or ΔN specific p63 isoforms causes a decrease in cell viability. A, SCC9 cells were transfected with Tp63, Dp63 and Scr siRNAs and cell viability was assessed at various time points by MTT reagent. B, C, SCC9-Tp63 and SCC9-Dp63 cells (B) as well as iPrEC-Tp63 and iPrEC-Dp63 cells (C) were treated with 5 µg/ml of Tet for several time points and viability was measured by MTT assay. The data shown are representative of three independent experiments.

c. For developing stable clones expressing shRNA, we recombined the pENTR/H1/TO-Tp63 and pENTR/H1/TO-Dp63 entry constructs with pLenti4/BLOCK-IT DEST to generate pLenti4/BLOCK-IT-Oli3 and pLenti4/BLOCK-IT-DNp63 shRNA constructs.

d. Generation of Tet repressor expressing cell lines: For the tight regulation of the expression of shRNA, high levels expression of Tet repressor (Tet R) is crucial. We transduced the cell lines SCC 9 and iPrEC with the pLenti6/TR lentiviral expression construct and generated the stable clones by taking the advantage of the antibiotic resistance (Blasticidin). We screened for the clones with the high levels of Tet dc R protein and clones with high expression levels were selected and named accordingly (SCC9 TR and iPrEC TR).

e. Finally, we generated the Tet- inducible cells lines by infecting SCC9 TR and iPrEC TR, with pLenti4/BLOCK- IT-Tp63 and pLenti4/BLOCK-IT-Dp63 shRNA constructs. We isolated the clones by antibiotic selection (zeocin) and screened for those showing at least 50% knock down upon addition of tetracycline (5ug/ml) (Fig 6B and 6C).

3. Knockdown of either Total or ΔN specific p63 isoforms causes a decrease in cell viability

Downregulation of p63 expression in both SCC9 and iPrEC cells resulted in a significant decrease in cell viability. More specifically, knockdown of p63 in SCC 9 cells caused a decrease in the number of viable cells as early as 48 hours after p63 siRNA transfection as compared to the scramble control (P=0.02, two-tailed *t*-test) (Fig. 7A). The experiments effects on cell viability became even more evident at the later time points. By day 6 (144 h), there was a dramatic reduction in the cell viability in cells transfected with either Tp63, (P=0.006, two-tailed *t*-test) or Dp63 (P=0.0009. two-tailed *t*-test) siRNAs as compared to cells transfected with scramble siRNA. Similar results were observed upon p63 silencing in the inducible SCC9 and iPrEC clones (Fig. 7B and 7C).

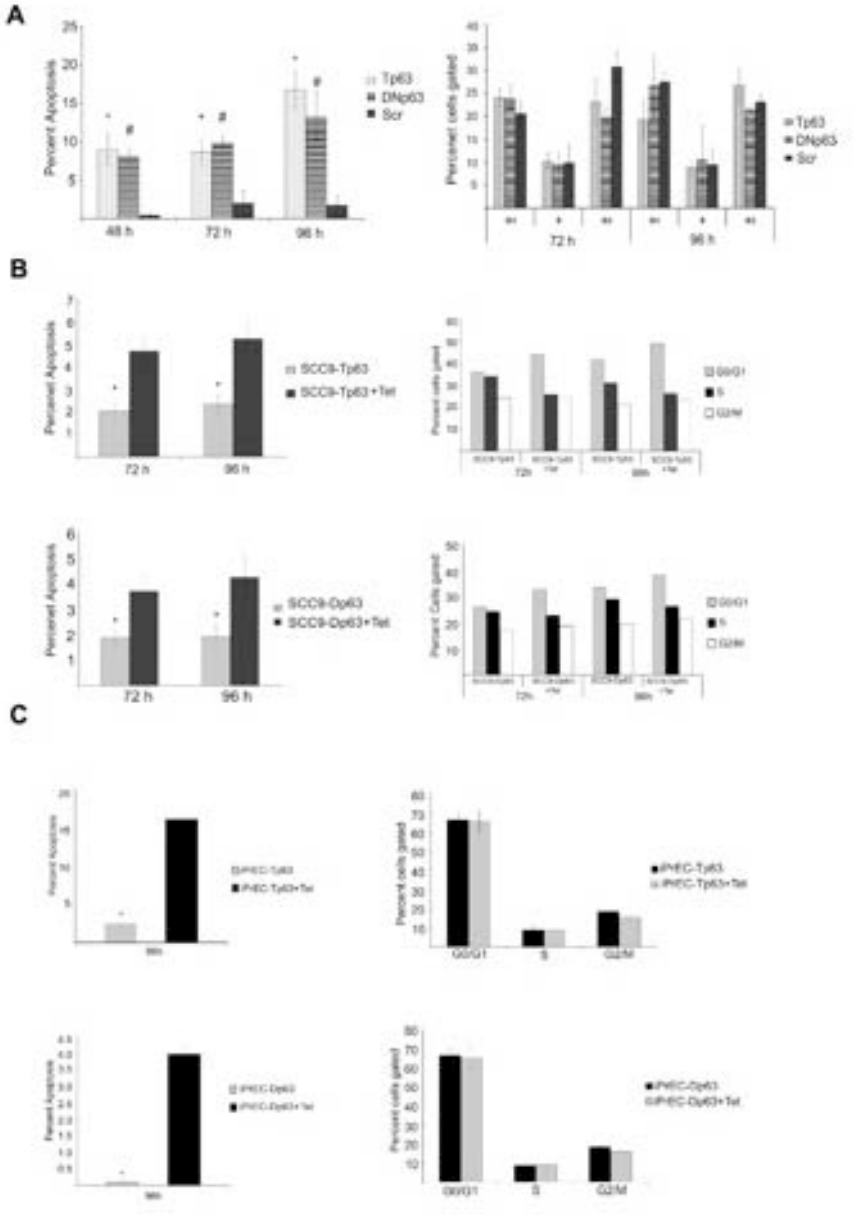

Fig 8. Silencing of either total or ΔN p63 isoforms induces apoptosis with no significant changes in cell proliferation. A, SCC9 cells were transfected with Tp63, Dp63 and Scr siRNAs. After several time periods, cells were supplemented with BrdU (100 µM) for 2 h at several time periods and fixed with 70% ethanol. B&C, SCC9-Tp63, SCC9-Dp63 and iPrEC- Dp63, iPrEC-Tp63 cells were treated with 5 µg/ml of tetracycline for 72 hr and supplemented with BrdU (100 µM) for 2 h and fixed with 70% ethanol. Fixed cells were processed for flow cyometric analysis as described in the materials and methods section. In each group, left panels represent the sub G1 population measured by PI staining and

4. Knockdown of either Total or DN specific p63 isoforms induces apoptosis without affecting the cell cycle

We next assessed whether the decrease in cell viability observed upon p63 downregulation was a result of apoptosis, cell cycle arrest, or both. We addressed this question by performing flow cytometric analysis

in both SCC 9 and iPrEC cell lines after p63 silencing. We found that p63 knockdown of either all p63 isoforms or ΔNp63 isoforms caused a significant increase in the sub G1 population of cells when compared to the control ($P<0.05$ at 48, 72, and 96 h, two-tailed *t*-test) (Fig 8A-8C, left panels). In line with these data, knockdown of p63 in both SCC 9 and iPrEC cells resulted in an increase in the cleaved caspase 3 levels and a concomitant decrease in the expression total caspase 3. Accordingly, cleaved poly (ADP-ribose) polymerase (PARP) protein levels were increased after p63 knockdown (Fig. 9). These data provide compelling evidence that p63 plays a role in the survival of both transformed and immortalized epithelial cells. Moreover, the observation that Tp63 and Dp63 siRNAs produced similar results suggests that p63 anti-apoptotic function is mostly mediated by ΔNp63 isoforms.

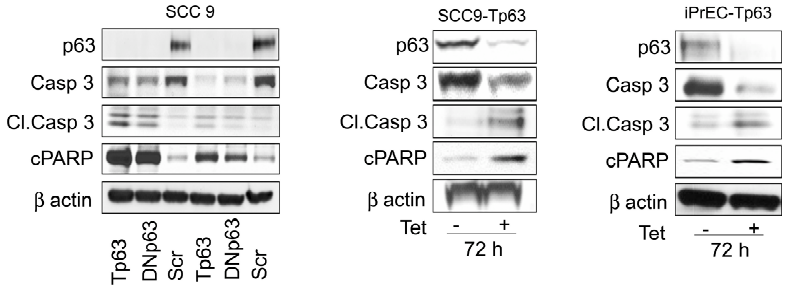

Fig 9. p63 silencing activates apoptotic pathways. SCC 9 cells were transfected with siRNA's TP63, Dp63, and Scr for 48h and 72h. Clones SCC9-Tp63 and iPrEC-Tp63 cells were treated with 5 µg/ml tetracycline for 72h. Equal amounts of protein were subjected to Western blot analysis using antibodies against p63, caspase 3, cleaved caspase3, cleaved PARP, and beta actin. Immunoreactive bands were visualized using a peroxidase-coupled secondary antibody and a chemiluminescent Western blot analysis system.

In contrast to the effects on apoptosis, we did not observe any noticeable changes in the cell cycle distribution of the p63-silenced cells compared to their control (Fig 8A-8C, right panels). These results indicate that p63 promotes cell survival without significantly affecting cell proliferation.

5. p63 modulates AKT and MAPK activation

Mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (MAPK ERK1/2) and

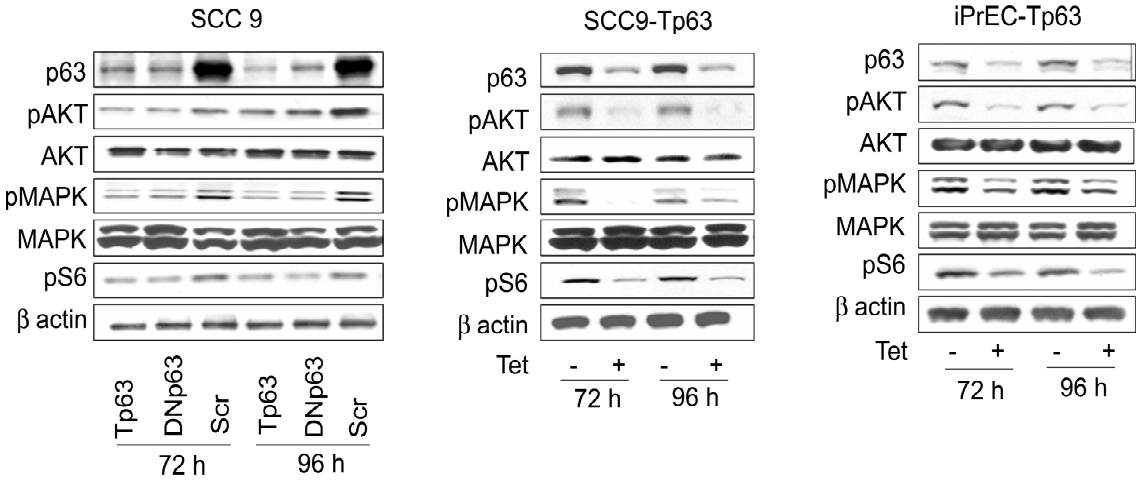

Fig. 10. p63 modulates AKT and MAPK activation. SCC 9 cells were transfected with siRNA's TP63, Dp63, and Scr for 72 h and 96h. Clones SCC9-Tp63 and iPrEC-Tp63 cells were treated with 5 µg/ml tetracycline for 72h. After each time period, cells were harvested and total cellular extracts were prepared. Equal amounts of protein were subjected to western blot analysis using antibodies against Phospho-AKT (Ser 473), total AKT, Phospho-MAPK, total MAPK, Phospho-S6, and Beta-actin. Immunoreactive bands were visualized using a peroxidase-coupled secondary antibody and a chemiluminescent Western blot analysis system. The data shown are representative of three independent experiments.

phosphatidylinositol-3′ -kinase (PI-3′ K)/protein kinase B (AKT) signaling have been extensively implicated in cell survival. Therefore, we also analyzed the role of p63 in modulating these pathways in both transformed and immortalized epithelial cells. Silencing of p63 in both SCC9 and iPrEC cells induced a significant decrease in the phosphorylation of AKT without causing any changes in total AKT levels. In a similar fashion, we noticed a consistent decrease in p44/p42MAPK phosphorylation after p63 knockdown. In addition, we observed that p63 silencing resulted in a significant decrease in phosphorylation of ribosomal protein S6, which is a downstream effector of the both AKT and MAPK pathways (Fig. 10).

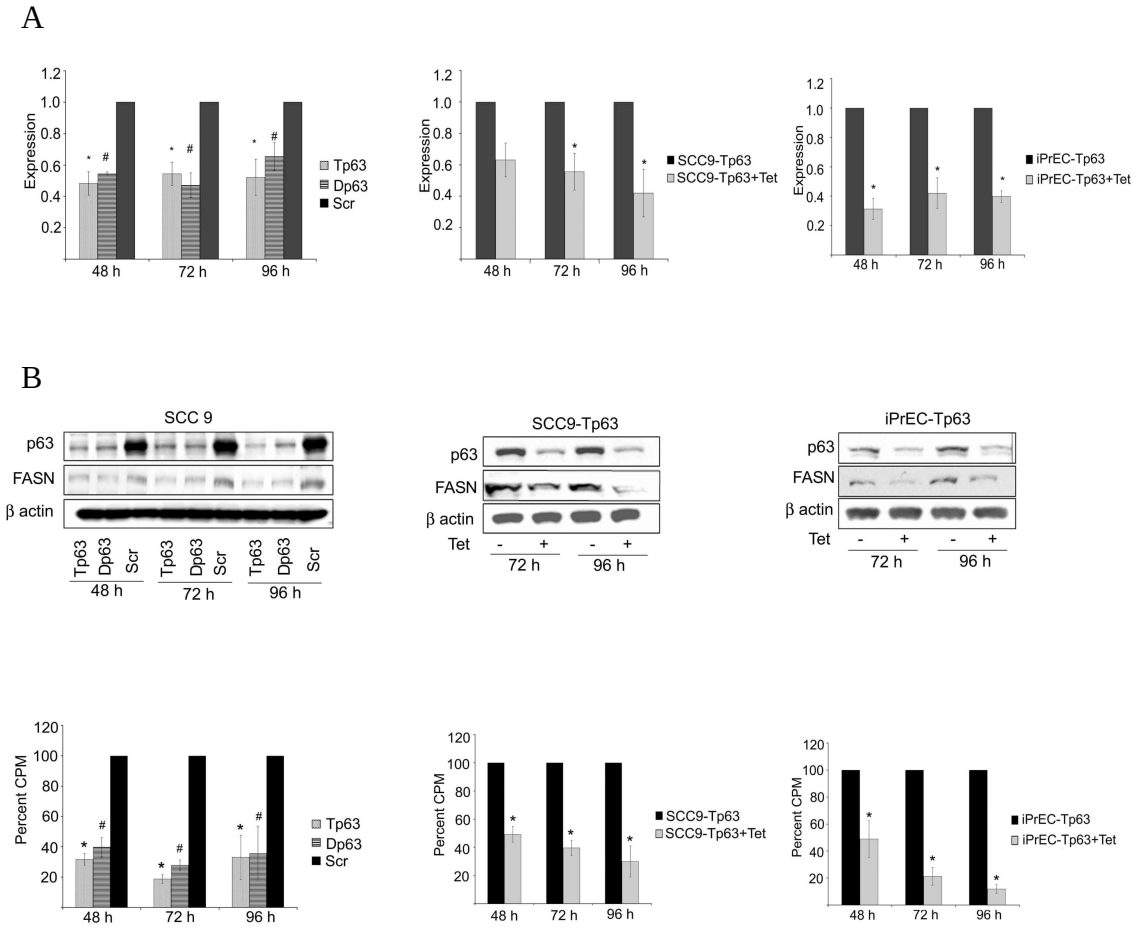

Figure 11. p63 silencing induce a decrease in FASN expression and enzymatic activity. A. Quantitation of FASN mRNA by real time RT-PCR in SCC9 cells transfected with Tp63 or Dp63 siRNA relative to cells transfected with Scr siRNA (left panel). Quantitation of FASN mRNA in SCC9-Tp63 and iPrEC-Tp63 cells treated with tetracycline relative to the correspondent untreated cells (middle and right panels, respectively). Data are shown as means +/- standard error and are representative of three independent experiments. B. Immunoblot analysis of FASN in SCC 9 cells transfected with Tp63, Dp63 or Scr siRNAs (left panel), in SCC9-Tp63 cells with or without treatment with tetracycline (middle panel) and in iPrEC-Tp63 cells with or without treatment with tetracycline (right panel). C. Quantitation of C14 incorporation within cellular lipids in SCC9 cells transfected with Tp63 or Dp63 siRNA relative to cells transfected with Scr siRNA (left panel) and in SCC9-Tp63 and iPrEC-Tp63 cells treated with tetracycline relative to the correspondent untreated cells (middle and right panels, respectively). Data are shown as means +/- standard error and are representative of three independent experiments.

6. p63 modulates Fatty Acid Synthase (FASN) levels and activity

Fatty acid synthase (FASN) is a key enzyme that synthesizes long-chain fatty acids and is involved in both embryogenesis and cancer It has recently been suggested that FASN is a conserved target of p53 family members, including p63 and p73. Therefore, we assessed whether p63 regulates FASN expression by evaluating the effects p63 downregulation on FASN mRNA and protein levels. Knockdown of p63, with either Tp63 or Dp63 siRNAs decreased the mRNA expression of FASN by approximately 50% in the SCC 9 cell line (Fig 11A, left and middle panels). In addition, we observed a similar decrease in FASN transcripts levels after p63 knockdown in iPrEC cells (Fig 11A, right panel). Consistent with these results, we detected a significant decrease in FASN protein expression upon knockdown of p63 in both SCC 9 and iPrEC cell lines (Fig 11B). These data confirm that p63 modulates FASN expression at both mRNA and protein levels in both transformed and immortalized epithelial cells.

In order to explore the functional consequences of FASN downregulation induced by p63 silencing, we investigated fatty acid synthesis in the p63-silenced cells by assessing C14 acetate incorporation into cellular lipids. In the SCC 9 cell line, p63 knockdown resulted in a significant decrease in fatty acid synthesis compared to the control ($p=0.004$, two-tailed t-test). Such decrease was observed by 48 hr after transfection with siRNAs and persisted up to the measured time point of 96 h (Fig 11C). Overlapping results were obtained in SCC9-Tp63 ($p=0.009$, two-tailed t-test, 120h) and iPrEC-TP63 ($p=0.015$, two-tailed t-test, 120h) cells. Taken together, our data demonstrate that p63 silencing causes a significant re C on in both FASN levels and FASN enzymatic activity.

7. p63 and FASN co-localize in the urogenital sinus (UGS) and developing prostate.

We have previously shown that p63 is required for the development of prostate basal cells. The observation that p63 modulates FASN expression and activation in basaloid iPrEC cells, prompted us to investigate a possible association between p63 and FASN expression in the developing prostate. To this end, transversal sections of the UGS from a mouse embryo at day E18.5 were double-immunostained for p63 and FASN. Co-localization of the two proteins was observed in the lower and intermediate layers of the UGS epithelium as well as in the prostate buds (Fig. 12). These results suggest that FASN may be an important mediator of p63 function during prostate development.

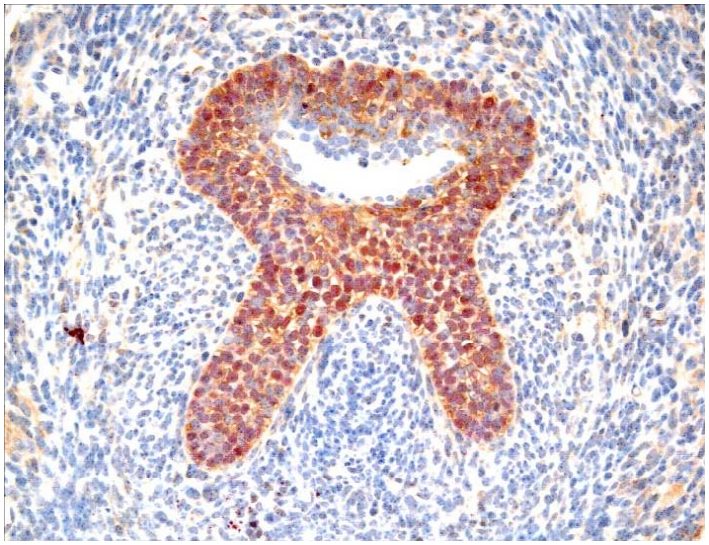

Fig. 12. Co-localization of p63 and FASN in the UGS epithelium and prostatic buds. A coronal section of the UGS from a wild-type E18.5 embryo was double immunostained for p63 (red) and FASN (brown). Expression of both proteins is observed in the vast majority of the cells. The cells lining the UGS lumen are consistently negative for both FASN and p63.

KEY RESEARCH ACCOMPLISHMENTS

Aim 1

a. We have been working on the selection of the optimal p63-/- ES clones to be used for the generation of p63-/-;ROSA26 chimeras.
b. Preliminary results are not very encouraging and suggest that this novel approach for the generation of p63-/-;ROSA26 is less efficient than anticipated.

Aim 2

a. The construction of the targeting vector for the generation of the p63-Cre-ER^{T2} knock-in mice has been completed.
b. The p63-Cre-ER^{T2} vector has been electroporated in the ES cells
c. p63-Cre-ER^{T2} ES clones with successful targeting event have been obtained.
d. Three targeted ES clones have been injected in host blastocysts, resulting in the production of 5 high percentage p63-Cre-ER^{T2} chimeras.
e. Chimeras have been bred.
f. p63-Cre-ER^{T2} knock-in mice have been generated.
g. p63-Cre-ER^{T2} knock-in mice have been bred with R26R mice to obtain p63-Cre-ER^{T2het}/R26R^{het} double mutant mice.
h. p63-Cre-ER^{T2het}/R26R^{het} double mutant mice have been analyzed for beta-gal activity to rule out unspecific Cre expression.
i. We are currently working on the optimization of Tamoxifen-mediated induction of Cre activity in the p63-Cre-ER^{T2het}/R26R^{het} double mutant mice.
j. p63-CreER^{T2} knock-in mice have also been crossed with the commercially available mice that ubiquitously express Flippase recombination enzyme (Flp-mice) to excise the FRT-flanked PGK-neo cassette.
k. p63-CreER^{T2}/Flp mice in which the excision of the PGK-neo cassette has been confirmed by PCR analysis are currently being crossed with R26R mice. Double mutant p63-CreER^{T2}/R26R mice without the PGK-neo cassette will then be administered Tamoxifen and analyzed for beta-gal expression.

Aim 3

a. The use of siRNA against p63 has been optimized in various cell lines.
b. p63 shRNA inducible cell lines (including iPrEC) have been generated.
c. Knockdown of either Total or ΔN specific p63 isoforms in iPrEC cells (and other cell lines) consistently results in a decrease in cell viability
d. Knockdown of either Total or ΔN specific p63 isoforms in iPrEC cells (and other cell lines) induces apoptosis without affecting the cell cycle
e. p63 modulates AKT and MAPK activation in iPrEC cells (and other cell lines).
f. p63 modulates Fatty Acid Synthase (FASN) levels and activity in iPrEC cells (and other cell lines).

REPORTABLE OUTCOMES

Manuscripts sponsored by the W81XWH-06-1-0365 award:

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CONCLUSIONS

We have constructed the targeting vector for the generation of the p63-Cre-ER^{T2} knock-in mice. The p63-Cre-ER^{T2} vector has been electroporated in the ES cells. p63-Cre-ER^{T2} ES clones with successful targeting events have been obtained. Three targeted ES clones have been injected in host blastocysts, resulting in the production of 5 high percentage p63-Cre-ER^{T2} chimeras, which have been bred to generate the p63-Cre-ER^{T2} knock-in mice. These mice have been with the R26R mice to obtain p63-Cre-ER^{T2het}/R26R^{het} double mutant mice. Optimization of Tamoxifen-mediated induction of Cre activity in the p63-Cre-ER^{T2het}/R26R^{het} double mutant mice is currently being performed. In addition, p63-CreER^{T2} knock-in mice have been crossed with the commercially available mice that ubiquitously express Flippase recombination enzyme (Flp-mice) to excise the FRT-flanked PGK-neo cassette. p63-CreER^{T2}/Flp mice in which the excision of the PGK-neo cassette has been confirmed by PCR analysis are currently being crossed with R26R mice. Double mutant p63-CreER^{T2}/R26R mice without the PGK-neo cassette will then be administered Tamoxifen and analyzed for beta-gal expression.

We have also continued to work on the identification of the molecular mechanisms through which p63 regulates development of the prostate epithelium. Specifically, the use of siRNA against p63 has been optimized in various cell lines and, most importantly, p63 shRNA inducible cell lines (including iPrEC) have been generated. Our initial results show that downregulation of p63 in iPrEC cells consistently causes a decrease in cell viability. The decrease in cell viability observed upon p63 downregulation is a result of apoptosis. Moreover, our data demonstrates that p63 modulates AKT and MAPK activation in iPrEC cells. Importantly, p63 also modulates Fatty Acid Synthase (FASN) levels and activity in iPrEC cells and p63 and FASN are co-expressed in the developing urogenital sinus epithelium and the developing prostate.

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