The Role of p53 Tumor Suppressor in Canine Kidney Tubulogenesis and Tumorigenesis

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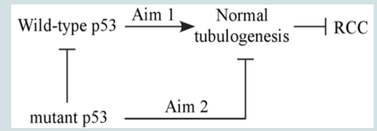
Background

- The tumor suppressor gene p53, known as the "guardian of the genome", negatively regulates cell growth through cell-cycle arrest, induction of apoptosis and DNA repair in response to stress. p53 alterations have been identified in both human and dog cancers. Such mutations can either lead to the loss of tumor suppressor function or gain of oncogenic function by inactivating wild-type p53 and promoting tumorigenesis.
- Renal cell carcinoma (RCC) is the most common type of kidney cancer in both humans and dogs and carries a poor prognosis. The tumor cells originate from the proximal renal tubular epithelium and tend to be highly invasive and metastatic. Recent studies have uncovered that p53 is a frequently mutated gene in RCC and implicated in the progression of malignancy. Inactivation in p53 is also suggested to be associated with the high resistance to radiation and chemotherapy of RCC, possibly due to attenuation of apoptosis. However, the mechanism by which loss of p53 promotes the tumor progression and metastasis in RCC is not well understood.
- Renal epithelial tubular cells retain high capacity for tubular regeneration after renal injury. Recent studies have revealed a transcriptional similarity between these progenitor-like tubular cells and tumor cells in some subtypes of RCC, suggesting that RCC may derive from malignant conversion of the tubular progenitors. Given the possible connection, we will use MDCK cells in the present study. MDCK cells were derived from the kidney tubules of a cocker spaniel. They can form polarized cysts and then develop into branching tubules in three-dimensional (3-D) culture upon stimulation with hepatocyte growth factor (HFG). This process, which resembles tubulogenesis in *vivo*, allows us to examine how knockout of wild-type p53 or knockin of a mutant p53 alters cell polarity, motility, and cell-cell adhesion.
- This study will shed light on the underlying mechanism in which wild-type p53 and mutant p53 modulate tubulogenesis and provide new insights into developing novel therapeutics for dog renal cancer, such as therapeutic strategies restoring p53 activity or targeting epithelial-mesenchymal transition (EMT) for dog renal cancer.

Question and Aims

Is p53 required for canine kidney tubular formation and does mutant p53 disrupt tubulogenesis and promote kidney tumor formation?

- **Aim 1:** To determine the role of wild-type p53 in tubulogenesis using p53-knockout Madin-Darby canine kidney (MDCK) cell lines generated by CRISPR/Cas9 genome editing system.
- **Aim 2**: To determine the role of mutant p53 in tubulogenesis and tumorigenesis by using mutant p53-R163H-knockin MDCK cell lines generated by CRISPR/Cas9 system.



Experiments

- Generated MDCK cell lines in which wild-type p53 was knocked out using CRISPR/Cas9 system. To generate p53-knockout (p53-KO) cells, we transfected a vector integrating the Cas9 gene, puromycin selection marker, along with a guide RNA that target the canine p53 gene into MDCK cells. The resulting cells were selected with puromycin.

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1921 cctctggctt tgggacctcc ctgacctgtg gcttctcaat agtctgtagg ctttggctct
1981 acgtaggatg agggtggcta ggagtcagtg gggcccacca ccctcacggc cccctgcttc
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2101 gtgcatgtcc cgggagagac cgccggactg aggaggagaa tttccacaag aaggggagc
2161 cttgtcctga gccacccccc gggagtacca agcgaggtaa gcaagcaaga caagaggagg
2221 tgaaggaggg acacctgggt ggctcagggg ttgagcatca ggcatgatcc
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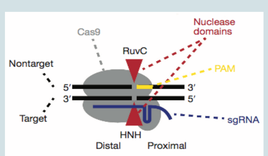
p53 KO primer-Forward : AATAGTCTGTAGGCTTT
p53 KO primer-Reverse: ATCATGCCTGATGCTCAA

- The deletion of p53 gene in selected clone was confirmed by genotyping, sequencing, and western blot.
- Cell proliferation and migration activities were examined by colony formation assay and wound healing assay in 2-D cultures.
- The level of p21 and PUMA was determined by Western blot.
- The p53-KO cell lines were also incubated in 3-D culture and stimulated with HFG. The morphology of cyst formation was assessed.
- Generated MDCK cell lines with mutant p53-R163H (equivalent to R175H in human), a mutation frequently found in canine cancer using CRISPR/Cas9 technique. To do this, we generated a vector consisting of the HDR donor, puromycin selection marker, and sgRNA targeting p53.
- Tested epithelial-mesenchymal transition (EMT) by measuring the expression of EMT markers (E-cadherin, β -catenin, laminin V) in the p53-KI cell line.



p53 R163H KI primer-1: 5'-TCATTTTCCTTCACTTGTTC-3'
p53 R163H KI primer-2: 5'-TTCATGGTGGGGGCAATGTC TTACAACCTCTGTCACGAACT -3'
p53 R163H KI primer-3: 5'- AGAAGTCGGAGTTCGTGACA GAGGTTGTAAGACATTGCCCCCACCAT -3'
p53 R163H KI primer-4: 5'-CCTGTAGTCTATCAGCCT-3'

Source: Richardson, C. et al. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nature Biotechonology*. 34: 339-345 (2016)

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 781 tttcctgtca tggggcaaag ggactcttct tcattttcct tcacttgttc attccttggc
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Results

Figure 1. Knockout of wild-type p53 altered MDCK cell morphology in 2-D culture

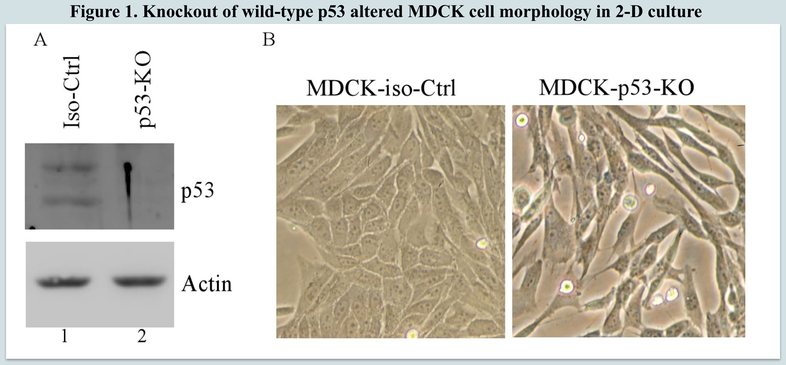

Figure 1. Knockout of wild-type p53 altered MDCK cell morphology in 2-D culture. A. Generation of MDCK cell lines in which wild-type p53 is knocked out by crispr/Cas9. Whole cell lysates were collected from MDCK isogenic control cells and p53-KO MDCK cells. The level of p53 was determined by western blotting. **B.** Representative images of MDCK isogenic control cells and MDCK cells with p53 knockout in 2-D culture (200X).

Figure 2. Knockout of wild-type p53 disrupted tubular formation in 3-D culture

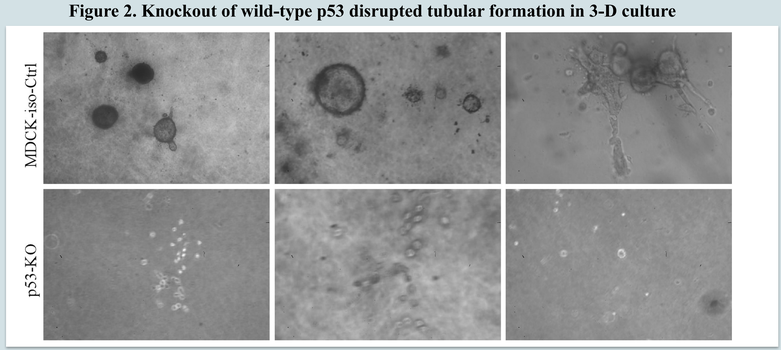

Figure 2. Knockout of wild-type p53 disrupted tubular formation in 3-D culture. Representative images of MDCK isogenic control cells and p53-KO MDCK cells in 3-D culture for 12 days.

Figure 3. Knockout of p53 promoted cell proliferation and migration

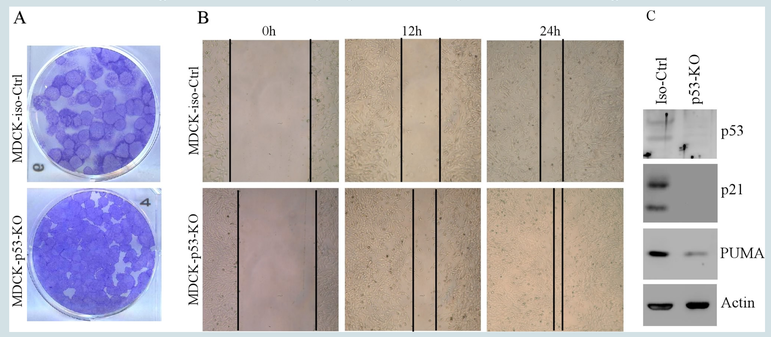

Figure 3. Knockout of p53 promoted cell proliferation and migration. A. Colony formation assay was performed with isogenic control and p53-KO MDCK cells. Cells were split into 6 well plates and then incubated for 14 days. **B.** Wound healing assay was performed with isogenic control and p53-KO MDCK cells. Cell migration was determined by visual assessment of cells migrating into the wound in 12 h and 24 h using a phase-contrast microscopy. **C.** Western blot were performed with extracts from isogenic control and p53-KO MDCK cells. The blots were probed with antibodies against p53, p21, PUMA and actin, respectively.

Figure 4. EMT markers are modulated by knockout of p53 in MDCK cells

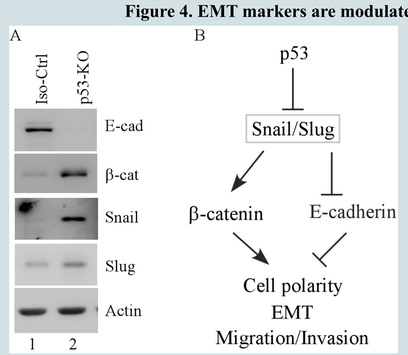

Figure 4. EMT markers are modulated by knockout of p53 in MDCK cells. A. Whole cell lysates were collected from isogenic control cells (lane 1) and p53-KO MDCK cells (lane 2). The levels of E-cadherin, β-catenin, Snail, slug and actin were measure by western blotting with their respective antibodies. **B.** Proposed model for the role of wild-type p53 in MDCK cell polarity, motility, and cell-cell adhesion.

Conclusions

- Wild-type p53 regulates MDCK cell growth and migration activities.
- Knockout of p53 disrupts cyst formation in 3-D culture of MDCK cells.
- Knockout of p53 promotes EMT marker expression.

Acknowledgement

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