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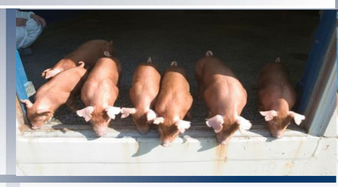


Characterization of an inducible transgenic p53/Kras oncopig model for cancer.

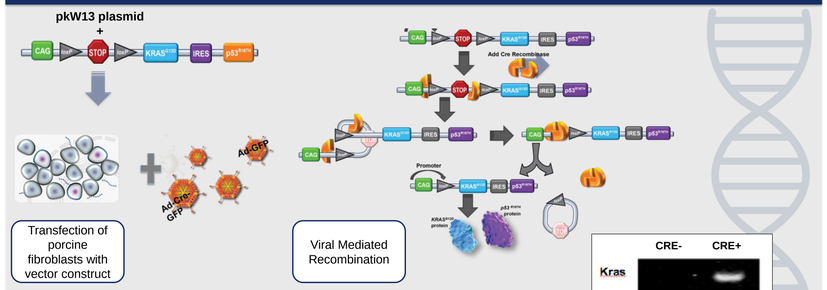
L. A. Rund[1], T. Collares[2], F. K. Seixas[2], K. Begnini[2], C.M. Counter[3], and L. B. Schook[1],

[1]Department of Animal Sciences, University of Illinois, Urbana, IL, United States
[2] Technology Development Center, Biotechnology Unit, Federal University of Pelotas, Pelotas, RS, Brazil
[3] Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, NC, United States;

Introduction

Given a number of limitations of rodent-based cancer models, coupled with the fact that pigs share many genetic and physiological similarities with humans, we investigated the potential of developing genetic porcine models of cancer. In this regard, we previously reported that activation of oncogenes such as Ras in conjunction with inhibiting tumor suppressor pathways like p53 were required, in part, to convert normal porcine cells to a tumorigenic state. To this end, pigs were created by cloning to contain oncogenic *KRAS*^{G12D} and dominant-negative *p53*^{R167H}, two commonly mutated genes in human cancers. They were cloned downstream of a LoxP-polyA (STOP)-LoxP sequence (LSL) and CAG promoter, such that exposure to Cre-recombinase would induce their expression in any desired tissue.

1 Vector construction and validation



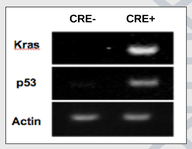
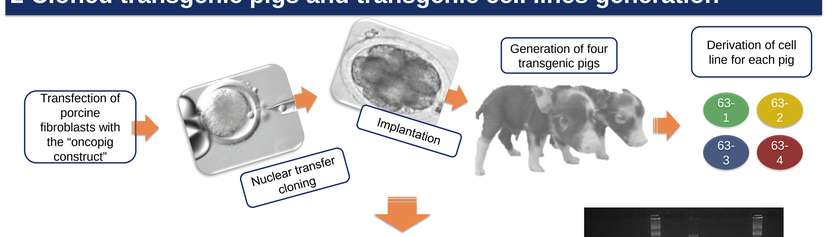


Figure 1: Validation of the deletion of LSL sequence and induction of Kras^{G12D}-p53^{R167H} expression.

Cre recombinase-mediated Kras^{G12D} and p53^{R167H} expression was significantly induced in porcine fibroblasts transfected with Ad-Cre-GFP virus (CRE+) compared with Ad-GFP (CRE-) control, which provides an *in vitro* proof of functional test of the "oncopig" construct (Figure 1).

2 Cloned transgenic pigs and transgenic cell lines generation



The four cloned piglets were born on May 21st 2012. The four transgenic fibroblast cell lines (63-1; 63-2; 63-3; and 63-4) generated from each present the "oncopig" construct containing both p53 and Kras mutant genes (Figure 2).

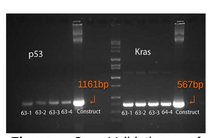
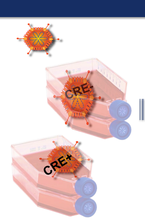


Figure 2: Validation of presence of the "oncopig" construct in the transgenic cell lines.

3 In vitro assays

Each cell line was infected with Ad-Cre-GFP(CRE+) to induce recombination and Ad-GFP (CRE-) as the viral treated, non-recombined control.



Expression

KrasG12

p53R167H

Phenotypic changes

Morphology	**Cell cycle length**
Migration	**Soft agar Assay**
Drug Resistance	

a. Fibroblast cell strains generated from four such clones were infected with adenovirus vector (CRE+) encoding Cre recombinase and GFP protein or control vector (CRE-) with GFP alone. Upon infection with CRE+, but not CRE-, all four cell strains expressed *KRAS*^{G12D} and *p53*^{R167H} mRNA, as assessed by RT-PCR (Figure 3a). Prelimnary RNASeq data show that *KRAS*^{G12D} and *p53*^{R167H} reads increase by 300 fold in the CRE+ cell lines, while wildtype message remains the same.

b. CRE+ treated cells start changing morphology at about 3 days post infection. They became small and round, while the CRE- treated cells maintain the pretreatment characteristics (Figure 3b).

c. *In vitro* migration capability of CRE+ treated cells was significantly greater than control cells. In a migration time of 24h, the mean cell number in the wound area for the CRE+ cells was 184 as for the CRE- cells was only 67 (p-value ≤ 0.01) (Figure 3c).

d. Within a 73h time period, CRE+ cells divided twice as many times than CRE- cells (p-value ≤ 0.01) (Figure 3d).

e. Control cells were unable to form colonies in soft agar, while the CRE+ cells formed over than 100 colonies (p-value ≤ 0.05).

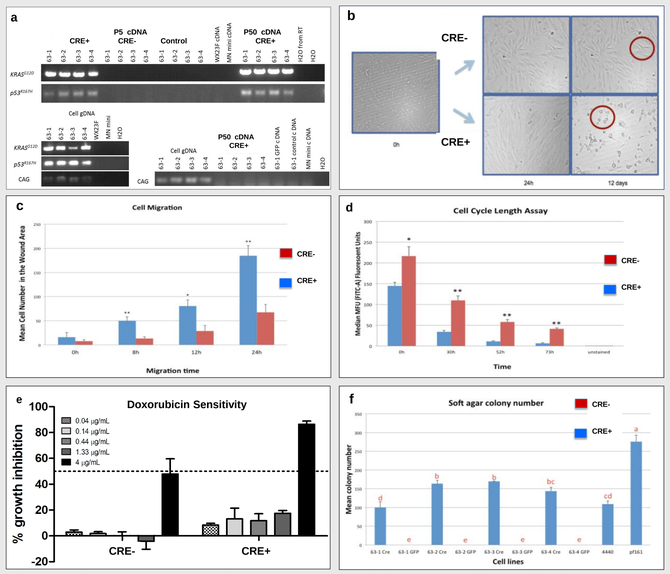


Figure 3: **a)** RT-PCR expression of KrasG12D-p53R167H following CRE+ infection **b)** Cell morphology changes triggered by CRE+ infection; **c)** Wound Assay (*p-value ≤ 0.05; **p-value ≤ 0.01; all data points are the mean of the 4 cell lines); **d)** Cell Cycle Length Assay (* p-value ≤ 0.05; ** p-value ≤ 0.01); **e)**Comparison of the sensitivity of cell line 63-3 CRE- and CRE+ to Doxorubicin; **f)** Soft Agar Assay (a,b,c,d,e : p-value ≤ 0.05; and a>b>c>d>e - colony number of the cell lines)

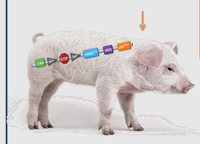
4 In vivo assays

Tumor Growth in Mice

Each CRE+ and CRE- cell line (5x10^6 in Matrigel™) were injected subcutaneously into **NOD.CB17-*Prkdc*^{scid}/J** mice (Jax stock 001303).

Tumor Growth in Pigs

Future experiments



Production and characterization of various porcine models of human cancer

Tumor Growth: Four cell lines were injected into immunodeficient mice to test for tumorigenicity. Mice had been euthanized when tumors reached the size of approximately 3000mm^2 and the tumors collected for histopathology, culture and expression analysis (Figure 4.2). Tumors from the CRE cell lines developed in the mice (13/14) while no tumors developed from the GFP lines. All the tumors contained *KRAS*^{G12D}, *p53*^{R167H}, *CAG* in gDNA and have *KRAS*^{G12D} and *p53*^{R167H} expression in cDNA (Figure 4.2). Histopathological analysis revealed the tumors to be sarcomas, which were non-encapsulated, densely cellular and locally infiltrative with marked cellular and nuclear pleomorphism. (Figure 4.3).

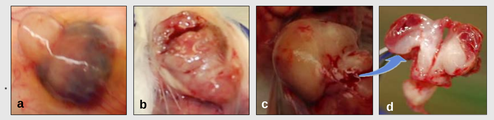


Figure 4.1: Tumors developed in the mice injected with the Ad-Cre-GFP cell lines. **a)** Mice injected with the cell line 63-1. Tumor reached the size of 2880mm^2 at d51 post injection. Adhered to the skin with no effacement of body wall. **b)** Mice injected with the cell line 63-3. Ulceration was observed when tumor reached the size of 2050mm^2 at d51 post injection. All attached to the skin with no effacement of body wall; **c)** Cell line 63-4. Tumor reached 2016mm^2 at 90 days post infection and was highly involved both outside and inside the body wall. **d)** Same mouse from Figure 4.1.c. Tumor was found invading the kidney. No other organs presented malignant cells.

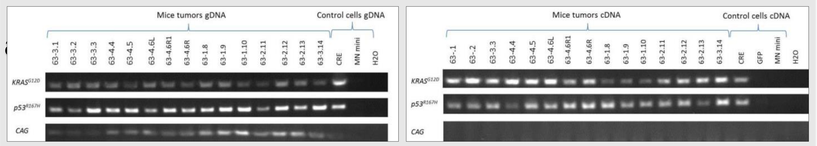


Figure 4.2 : PCR and RT-PCR results for tumors. All the tumors contained *KRAS*^{G12D}, *p53*^{R167H}, *CAG* in gDNA and have *KRAS*^{G12D} and *p53*^{R167H} expression in cDNA, it also proved tumors developed from the CRE+ cell lines not from the CRE- lines.

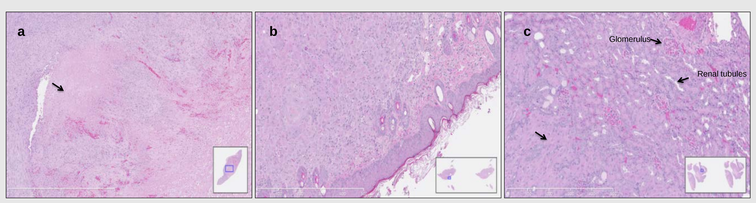


Figure 4.3: Samples were stained with H&E. **a) Sarcoma**. Developed from cell line 63-1. Presence of a nonencapsulated, densely cellular, and locally infiltrative neoplasm with central necrosis (arrow) and acute hemorrhages. **b) Sarcoma.** Developed from cell line 63-3. The dermis is expanded and effaced by an infiltrative neoplasm **c) Sarcoma with renal infiltration** Tumor from cell line 63-4. Presence of infiltrative neoplasm. Neoplastic cells are effacing the renal parenchyma (arrow).

Conclusions and Future Implications

Present results demonstrate that the onco-pig construct is functional. Moreover, demonstrates that the induction of the transgenes in these porcine cells triggered a tumorigenic phenotype. In addition, 2 clones have reached 1year 10 months of age with no development of tumors or other abnormalities demonstrating that the transgeen expression remains suppressed without cre-recombination.

In the future, offspring of these founder pigs will be monitored for tumor incidence following site-specific transgene induction. Such an approach could provide a porcine model to study cancer etiology and the development of anticancer drugs and therapies.

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