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Laboratory of

Comparative Genomics

Development of an Inducible Transgenic Onco-Pig Model

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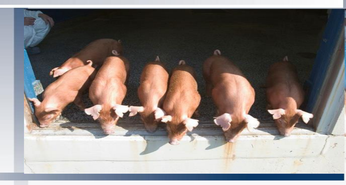
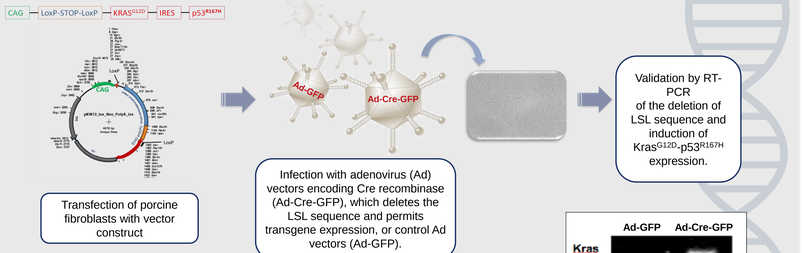


Introduction

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.

1 Vector construction and validation



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

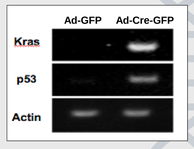
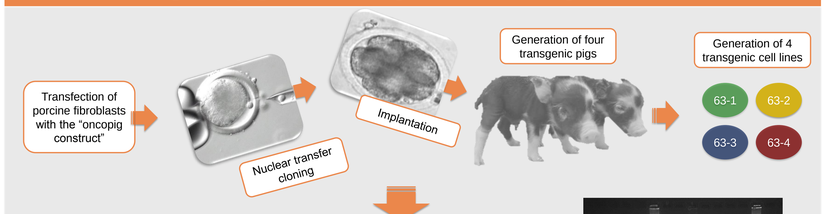


Figure 1: Validation of the deletion of LSL sequence and induction of Kras^{G12D}-p53^{R167H} expression.

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).

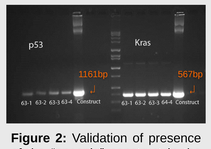
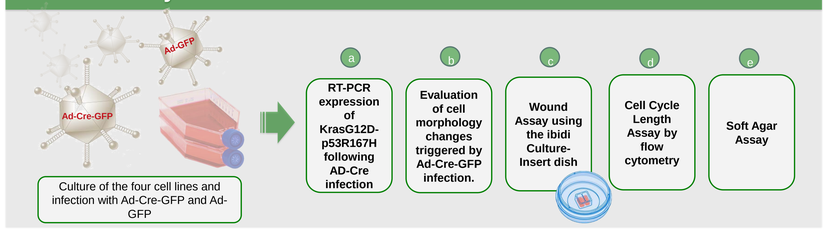


Figure 2: Validation of presence of the "oncopig" construct in the transgenic cell lines.

3 In vitro assays



a. Fibroblast cell strains generated from four such clones were infected with adenovirus vector (Ad-Cre-GFP) encoding Cre recombinase and GFP protein or control vector (Ad-GFP) with GFP alone. Upon infection with Ad-Cre-GFP, but not control Ad-GFP, all four cell strains expressed *KRAS*^{G12D} and *p53*^{R167H} mRNA, as assessed by RT-PCR (Figure 3.a.)..
b. Ad-Cre treated cells start changing morphology at about 3 days post infection. The Ad-Cre cells become small and round, while the Ad-GFP treated cells maintain the pretreatment characteristics (Figure 3.b.).
c. *In vitro* migration capability of Ad-Cre-GFP treated cells was significantly greater than Ad-GFP control cells. In a migration time of 24h, the mean cell number in the wound area for the Ad-Cre-GFP cells was 184 as for the Ad-GFP cells was only 67 (p-value ≤ 0.01) (Figure 3.c.).
d. Within a 73h time period, Ad-Cre-GFP cells divided twice as many times than Ad-GFP cells (p-value ≤ 0.01) (Figure 3.d.).
e. Ad-GFP cells were unable to form colonies in soft agar, while the Ad-Cre-GFP cells formed over than 100 colonies (p-value ≤ 0.05). As the 4440 and PF161 positive control cells (both transgenic cells expressing 6 oncogenic genes), the Ad-Cre-GFP cells are malignant transformed (Figure 3.e.).

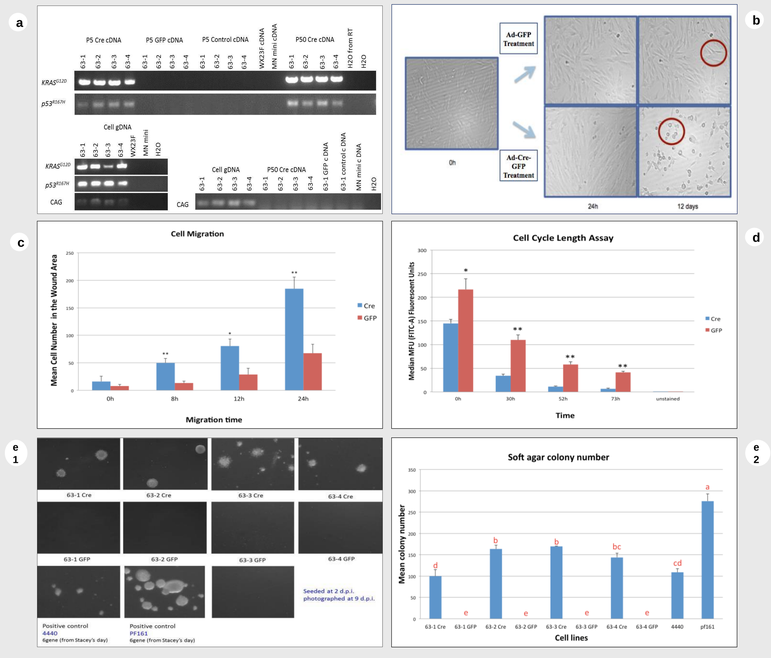


Figure 3: **a)** RT-PCR expression of KrasG12D-p53R167H following AD-Cre infection **b)** Cell morphology changes triggered by Ad-Cre infection; **c)** Wound Assay (* p-value ≤ 0.05; ** p-value ≤ 0.01; all data points are the mean of the 4 cell lines); **d)** Cell Cycle Length Assay (* p-value ≤ 0.05; ** p-value ≤ 0.01); **e1)** the colonies images in Soft Agar Assay; **e2)** Soft Agar Assay (a,b,c,d,e : p-value ≤ 0.05; and a>b>c>d>e - colony number of the cell lines)

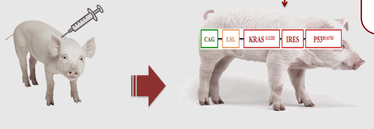
4 In vivo assays

Tumor Growth in Mice

5 ×10^6 Ad-Cre-GFP and Ad-GFP cells in Matrigel™ were injected subcutaneously into **NOD.CB17-*Prkdc*^{scid}/J** mice (Jax stock 001303).

Future experiments

Tumor Growth in Pigs



Production of the inducible transgenic porcine model for human cancer

Tumor Growth in mice: 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).

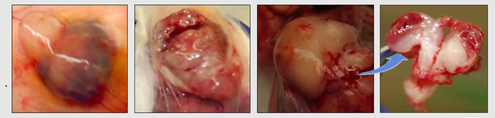


Figure 4.1: Tumors developed in the mice injected with the Ad-Cre-GFP cell lines. **a)** Mice injected with the cell line 63-1. Tumor reached the size of 2880mm^2 at 51 days post injection. All attached to the skin with no effacement of body wall. **b)** Mice injected with the cell line 63-3. Ulceration was observed when tumor reached the size of 2050mm^2 at 51 days 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.

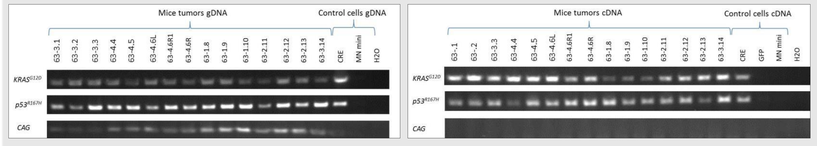


Figure 4.2 : PCR and RT-PCR results for tumors. All the tumors contained *KRAS*^{G12D}, *p53*^{R167H}, *CAG* in gDNA and have *KRAS*^{G12D} and *p53*^{R167H} expression in cDNA, it also proved tumors developed from the CRE cell lines not from the GFP lines.

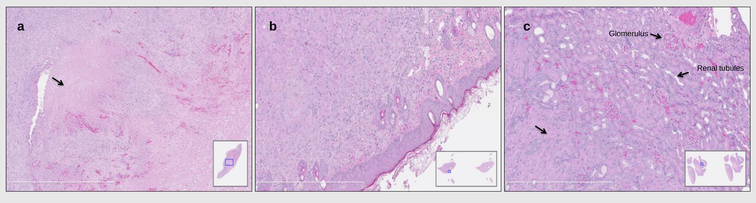


Figure 4.3: Histopathological analyses. Samples were stained with H&E. **a) Sarcoma**. Developed from cell line 63-1. Presence of a nonencapsulated, densely cellular, and locally infiltrative neoplasm with central necrosis (arrow) and acute hemorrhages. **b) Sarcoma.** Developed from cell line 63-3. The dermis is expanded and effaced by an infiltrative neoplasm (as described in Figure 4.2. a.) **c) Sarcoma with renal metastasis.** Tumor from cell line 63-4. Presence of infiltrative neoplasm (as described in Figure 4.2. a.). Neoplastic cells are effacing the renal parenchyma (arrow).

Conclusions and Future Implications

Present results demonstrate that the "oncopig" construct is functional. Moreover, demonstrate that the induction of the transgenes in these porcine cells triggered a tumorigenic phenotype.

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 therapies.

References

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