

ORIGINAL ARTICLE

Genetic induction of tumorigenesis in Swine

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The transition from basic to clinical cancer research for a number of experimental therapeutics is hampered by the lack of a genetically malleable, large animal model. To this end, we genetically engineered primary porcine cells to be tumorigenic by expression of proteins known to perturb pathways commonly corrupted in human cancer. Akin to human cells, these porcine cells were quite resistant to transformation, requiring multiple genetic changes. Moreover, the transformed porcine cells produced tumors when returned to the isogenic host animal. The ability to now rapidly and reproducibly genetically induce tumors of sizes similar to those treated clinically in a large mammal similar to humans in many respects will provide a robust cancer model for preclinical studies dependant on generating large tumors.

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Introduction

Preclinical studies of imaging, as well as hyperthermia, radiation or photodynamic therapy of tumors is limited by the lack of suitable animal models. On one hand, mice are ideal systems to genetically induce tumors or to grow xenografted human cancer cells of almost any given tissue (Fiebig and Burger, 2000; Van Dyke and Jacks, 2002), but the organs and tumors are typically too small for the devices used for these aforementioned studies. For example, it is virtually impossible to do intensity-modulated radiation therapy on mice owing to the small tumor size and the energy of clinical accelerators; high resolution intensity modulated treatment in rodents is hampered by the same problems; and devices used for hyperthermia treatment cannot be scaled down to be useful for studies in rodents. On the

other hand, companion animals with spontaneous tumors are ideal for studies of imaging and the above-described therapies, but it is very difficult and quite expensive to accrue enough animals with the desired tumor types for clinical trials. Moreover, tumors in these animals are genetically undefined and in some cases too rare to be studied (Dewhirst *et al.*, 2000). *In vivo* cancer studies requiring large tumors would therefore be greatly aided by a genetically defined large mammal model of cancer.

The swine could represent such an animal. Like humans, the incidence of cancer in pigs is rare, with a prevalence of the childhood cancer Wilm's tumors (Anderson and Jarrett, 1968) in young pigs and a more broad spectrum of cancers in adults (Brown and Johnson, 1970). The parallels in cancer biology between humans and pigs are even conserved at the molecular level. For example, in both species telomerase is suppressed in a number of tissues but reactivated during cancer (Pathak *et al.*, 2000; Stewart and Weinberg, 2000). Tumors can also reach sizes seen in humans, which is particularly relevant for many preclinical applications. Additionally, pigs have the added advantage of being more similar to humans in a number of aspects compared to other commonly used animal models in cancer biology. For example, the size, anatomy and physiology of the swine mirrors that of humans in many respects, and as such the pig is often the primary biomedical model for a number of diseases, surgical research and organ transplantation (Swanson *et al.*, 2004). The genomic sequence homology between humans and pigs is also very high (Swanson *et al.*, 2004) and the porcine pregnane X receptor protein that regulates p450 cytochrome CYP3A, which metabolizes almost half of prescription drugs in humans, is more similar to humans than, for example, mice (Xie and Evans, 2002). At the practical level, pigs have well-established animal husbandry, produce large litters, reach sexual maturity within a year, and have a short gestation period. Hence pigs can be economically bred for experimental cancer medicine. Indeed, Libechov minipigs (Vincent-Naulleau *et al.*, 2004) as well as other breeds (Oxenhandler *et al.*, 1982; Pathak *et al.*, 2000; Perez *et al.*, 2002) are already being employed as a model to study spontaneous melanoma formation. Even though porcine cancer models are currently confined strictly to melanomas; pigs could nevertheless provide an attractive model to study cancer if, as in rodent

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models, tumorigenesis could be genetically induced. We now report a rapid and reproducible method to genetically induce large tumors in the pig.

Results

Generation of porcine cells harboring genetic changes commonly found in human cancers

To establish a genetically malleable porcine model of cancer, we reasoned that normal porcine cells could be genetically engineered in culture to recapitulate changes commonly found in human cancers, and when returned back to the host (isogenic) swine, would form tumors. In humans, normal somatic cells can be driven to a tumorigenic state via the enforced expression of viral proteins that disrupt the tumor suppressors p53 and Rb and activate the proto-oncoprotein c-Myc, in conjunction with the mammalian oncogenic protein Ras and the hTERT telomerase catalytic subunit (Hahn *et al.*, 1999; O'Hayer and Counter, 2006), all reflecting alterations commonly found in human cancers (Bos, 1989; Weinberg, 1991; Levine, 1997; Nesbit *et al.*, 1999; Shay and Wright, 2002). More recently, we and others have demonstrated that expression of mammalian proteins can achieve the same feat (Kendall *et al.*, 2006). Specifically, inactivation of p53 and Rb pathways by expression of a dominant-negative p53 protein (p53^{DD}) and an activated cyclin-dependent kinase (CDK)/cyclin complex (cyclin D1/CDK4^{R24C}) in conjunction with activation of c-Myc, Ras and hTERT pathways via expression of oncogenic forms of c-Myc (c-Myc^{T58A}) and H-Ras (H-Ras^{G12V}) and hTERT is sufficient to drive human kidney cells, mammary epithelial cells and myoblasts to a tumorigenic state (Kendall *et al.*, 2005), whereas human keratinocytes (Goessel *et al.*, 2005) and melanocytes (Chudnovsky *et al.*, 2005) require slightly fewer changes. As coexpression of hTERT, p53^{DD}, cyclin D1, CDK4^{R24C}, c-Myc^{T58A} and H-Ras^{G12V} is tumorigenic in different types of human cells (Kendall *et al.*, 2005), it should be possible to similarly drive normal porcine cells to a tumorigenic state by the enforced expression of the same mammalian proteins.

Primary skin cultures, confirmed to be vimentin-positive via Western blot and displaying a fibroblast morphology (data not shown), were established from an ear notch biopsy of a healthy mixed Yorkshire/Duroc female pig (animal # 6510). Fibroblasts were chosen primarily because of the ease with which they can be isolated, the existence of established culture conditions for these cells, and the fact that human fibroblasts can be converted to a tumorigenic state by disrupting the aforementioned p53, Rb, c-Myc, Ras and telomerase pathways (Hahn *et al.*, 1999). These early passage porcine fibroblasts were sequentially and rapidly infected over the course of 6 days with three retroviruses coexpressing pairs of available human and murine versions of the complementary DNA (cDNA)s for hTERT and p53^{DD}, cyclin D1 and CDK4^{R24C}, and lastly, c-Myc^{T58A} and H-Ras^{G12V}. RNA was isolated from the

resulting polyclonal population, termed 6510T, and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with one primer anchored in the transgene and another in an accompanying vector-specific untranslated region to specifically amplify the transgenic messenger RNAs. Using this approach, all six transgenes were verified to be expressed in the 6510T polyclonal population (Figure 1a).

Porcine cells require multiple hits for cellular transformation

We next addressed whether these genetic changes endowed porcine cells with the ability to grow in an anchorage-independent manner in soft agar, one of the most rigorous *in vitro* assays for the transformed growth characteristic of cultured cancer cells. 6510T cells, or as a negative control, cells serially infected with vectors lacking these transgenes, were seeded in soft agar and 3 weeks later colony growth was assessed. Only cells expressing all six transgenes were capable of robust transformed cell growth (Figure 1b).

Human fibroblasts have diminished growth in soft agar if one of the TERT, Rb, p53, c-Myc or Ras pathways is not appropriately altered (Hahn *et al.*, 1999, 2002; Lim and Counter, 2004; Rangarajan *et al.*, 2004) whereas murine cells can grow in a transformed manner upon expression of far fewer transgenes (Rangarajan *et al.*, 2004). To address the requirements of transformation for porcine cells, all combinations of five of the six transgenes were introduced into primary normal 6510 cells by sequential infection with two retroviruses encoding four different transgenes, followed by infection with a retrovirus encoding only one transgene. The resultant six polyclonal populations (denoted by indicating the missing transgene) were confirmed by RT-PCR to express the desired combinations of five transgenes (Figure 1a). When these populations were assayed for growth in soft agar, loss of any one transgene was found to reduce the number of colonies, minimally, by a factor of five (Figure 1b). To confirm these results, a culture of normal porcine fibroblasts (vimentin-positive, fibroblast morphology, data not shown) from a pig of a different background (pure Duroc, animal # 214) was infected with empty vectors as a negative control, vectors encoding all six of the described transgenes, or combinations of five of these transgenes (Figure 1c). As with cells from the first animal, expression of all six transgenes was again found to be required for robust transformed cell growth in soft agar (Figure 1d). Lastly, we verified the resistance of porcine cells to become transformed by a completely different approach – carcinogen treatment. As noted by others (Wargovich *et al.*, 1991), treatment of the skin of pigs with 7,12-dimethylbenz[*a*]anthracene/12-*O*-tetradecanoylphorbol-13-acetate, which commonly induces tumors in mice, did not induce papillomas, at least within the timeframe of the experiment (8 months, data not shown). Thus, like human cells, porcine cells are quite resistant to transformation (Hamad *et al.*, 2002; Rangarajan *et al.*, 2004).

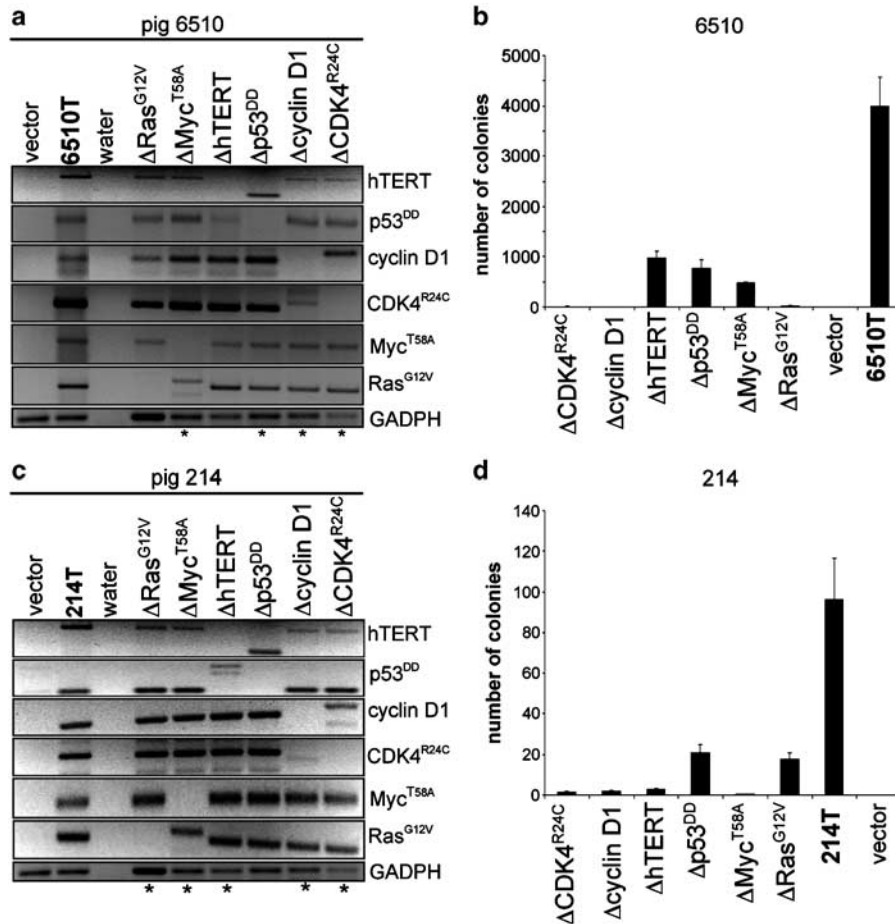


Figure 1 Genetic conversion of normal porcine cells to a tumorigenic state by disrupting pathways commonly altered in human cancers. Confirmation of appropriate transgene expression by RT-PCR of primary fibroblast cultures established from pigs (a) 6510 and (c) 214 that were infected with retroviruses encoding hTERT, p53^{DD}, cyclin D1, CDK4^{R24C}, c-Myc^{T58A} and H-Ras^{G12V}, (cell lines 6510T and 214T) or combinations of five of these six cDNAs (cell lines ΔhTERT, Δp53^{DD}, Δcyclin D1, ΔCDK4^{R24C}, ΔMyc^{T58A} and ΔRas^{G12V}, which lack the indicated transgene) or as a negative control, empty vectors (vector). Water, negative control; GAPDH, loading control; *products having alternate mobilities owing to the use of different primers to amplify transgenes from cells infected with retroviruses encoding five cDNAs. Average and standard error of the number of colonies growing in soft agar of (b) 6510 or (d) 214 cells expressing all six transgenes (T) or 5 of the six transgenes (ΔhTERT, Δp53^{DD}, Δcyclin D1, ΔCDK4^{R24C}, ΔMyc^{T58A} and ΔRas^{G12V}) assayed in triplicate.

Genetic conversion of normal porcine cells to a tumorigenic state

To determine whether expression of the described six transgenes was sufficient to drive normal porcine fibroblasts to a tumorigenic state, the 6510T cells stably expressing hTERT, p53^{DD}, cyclin D1, CDK4^{R24C}, c-Myc^{T58A} and H-Ras^{G12V} were injected subcutaneously into four immuno-compromised mice, and tumor growth was monitored over time. These cells rapidly formed tumors at the site of injection, reaching maximum tumor burden within 10 days in all animals, whereas vector control cells failed to grow in this manner (Figure 2a and Table 1). These cells maintained transgene expression *in vivo*, as assessed by RT-PCR, and formed vascularized tumors characterized by poorly differentiated cells with large nuclei and multiple nucleoli (Figure 2b and c).

To address the requirements for tumorigenic growth, we next injected porcine cells expressing five of the six

transgenes into immuno-compromised mice. These cells did not form tumors in the absence of cyclin D1, c-Myc^{T58A} or H-Ras^{G12V}, but could still form tumors when one of p53^{DD}, hTERT or CDK4 proteins was omitted (Table 1). Thus, multiple transgenes are also needed for tumor growth, although interestingly, not as many as required for robust transformed growth *in vitro*. Nevertheless, given the fact that all six transgenes were potently transforming and promoted tumor growth, we tested whether expression of these six proteins was tumorigenic in a larger sampling of porcine fibroblasts. Porcine fibroblasts isolated from ten unrelated outbred pigs infected with retroviruses encoding the same six transgenes were shown to all form tumors in mice, with an average latency period of 3 days (Table 1). We also tested whether expression of these six proteins would drive other types of porcine cells to a tumorigenic state. The porcine embryonic kidney cell strain SK-RST, the adult porcine kidney epithelial cell strain PK(15), and

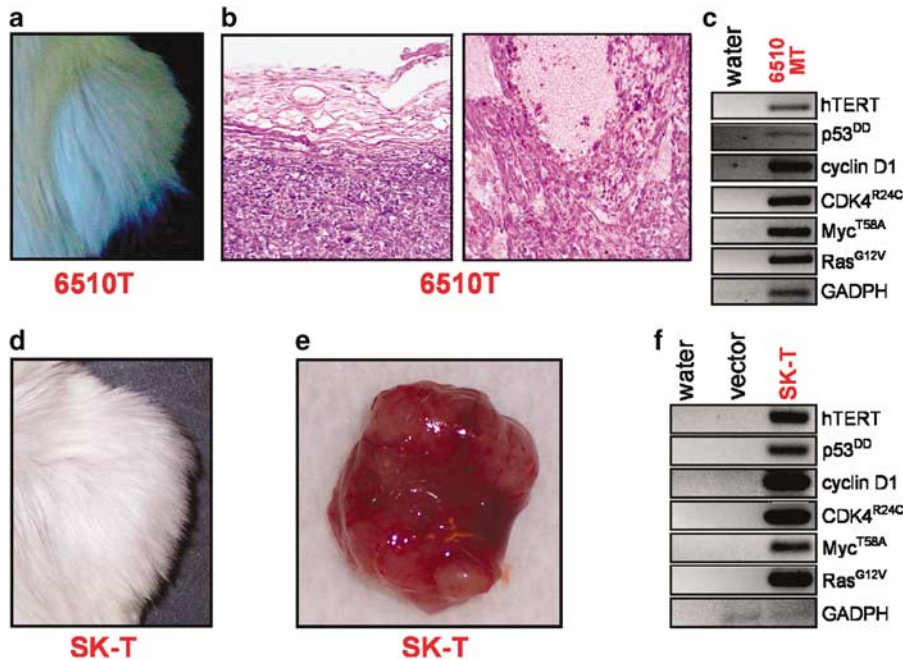


Figure 2 Genetic conversion of normal porcine dermal fibroblasts and embryonic kidney cells to a tumorigenic state by disrupting pathways commonly altered in human cancers. Fibroblasts from pig 6510 were serially infected with retroviruses encoding hTERT, p53^{DD}, cyclin D1, CDK4^{R24C}, c-Myc^{T58A} and H-Ras^{G12V}, generating the cell line 6510T. 6510T cells were then injected subcutaneously into mice and developed visible tumors that (a) were easily visible, (b) when stained with H&E were found to be composed of small, undifferentiated cells with multiple nuclei, which minimally invaded the surrounding tissue (left panel) and had a necrotic center with apoptotic cells and/or necrotic debris (right panel) (magnification $\times 200$), and finally (c) were shown to express all six transgenes, as assessed by RT-PCR. Water served as a negative control; GAPDH served as loading control. SK-RST cell strain from ATCC was serially infected with retroviruses encoding the same six transgenes, generating the cell line SK-T. SK-T injected subcutaneously into mice formed tumors in mice that (d) were easily visible, (e) when excised showed a large, vascularized tumor and (f) expressed all six transgenes via RT-PCR. Primary cells infected with empty vector and water serves as negative controls; GAPDH served as loading control.

the porcine testis cell strain ST were infected with the six transgenes to generate the cell lines SK-T, PK-T and ST-T, respectively, which were found to similarly induce tumors when injected into mice (Figure 2d and e and Table 1). We conclude that inactivation of the tumor suppressors p53 and Rb concomitant with the activation of c-Myc, Ras and telomerase is reproducibly tumorigenic in multiple porcine cell types, even when the cells are isolated from an outbred population.

Genetically defined tumor growth in pigs

To test whether such genetically defined porcine tumor cell lines could also form tumors in isogenic pigs, the 6510T cell line was injected back into the donor animal. As a combination of human and murine transgenes was used to transform these cells, the immune system of the host animal was partially suppressed to allow tumor growth. When the 6510T cells were injected orthotopically into the subcutaneous tissue of both ears, tumors rapidly formed at these two sites and maintained transgene expression *in vivo* (Figure 3a and e). Tumors continued to grow aggressively, reaching maximum allowable tumor burden (8000 mm³) at day 15 post-injection. These tumors consisted of solid sheets and infiltrative cords of markedly anaplastic and polymorphic

oval and polygonal cells. The cells varied markedly in size and shape; had prominent large oval to polygonal nuclei with large single central or multiple eccentric nucleoli and abundant peripherally dispersed chromatin; and plentiful finely granular to reticulated reddish-blue (amphophilic) cytoplasm (Figure 3c). Cell borders were indistinct; the mitotic rate was low. Tumor cells infiltrated between subcutaneous skeletal muscle fibers and into associated subcutaneous connective tissue, but were not seen invading blood or lymphatic vessels. There was also an apparent innate immune response characterized by large numbers of inflammatory cells admixed with abundant dying tumor cells and dead tumor cell debris (i.e., cytoplasmic proteins and nuclear chromatin). Immunohistochemical analysis of this tumor showed that most tumor cells were vimentin-positive (Figure 4a), desmin-negative and lysozyme-negative (data not shown), consistent with the fibroblastic origin of the initial cells. However, scattered tumor cells ($\approx <5\%$ of total tumor cells), particularly in areas of necrosis and inflammation, were cytokeratin-positive (Figure 4c), and the hematoxylin and eosin (H&E) morphology was most consistent with an epithelial origin, although undifferentiated sarcomas may also have similar morphologic characteristics. Taking all of these findings into account, the most

Table 1 Tumor growth of porcine cells in mice

Porcine cell lines	Tumor-bearing mice/mice injected
<i>Dermal fibroblast cell lines</i>	
6510T	4/4
6510-vector	0/3
6511T	4/4
6512T	4/4
6513T	4/4
2310T	3/3
2311T	3/3
4400T	3/3
4400BT	3/3
5023T	2/2
214T	4/4
214T-ΔhTERT	4/4
214T-Δp53 ^{DD}	4/4
214T-ΔCDK4 ^{R24C}	4/4
214T-Δcyclin D1	0/4 ^a
214T-ΔMyc ^{T58A}	0/4 ^a
214T-ΔH-Ras ^{G12V}	0/4
<i>Porcine kidney cell lines</i>	
SK-RST-vector	0/3
SK-T	3/3
PK(15)-vector	0/3 ^a
PK-T	6/6
<i>Porcine testis cell line</i>	
ST-vector	0/3 ^a
ST-T	3/3

Note: Cell lines serially infected with three retroviruses encoding the six described transgenes (T, tumor) or no transgenes (vector). ^aPalpable mass detected that did not grow any further over the course of the experiment (45 days).

consistent interpretation is that this is a poorly differentiated sarcoma with regions of cells expressing markers of epithelial origin. Identical results were found when another cell line generated in a similar manner from a different outbred pig was injected orthotopically into the donor animal (not shown). Thus, reproducible tumors were induced subcutaneously in pigs.

To test if tumors would grow in other sites, primary fibroblast cultures from two other pigs (4400T and 4400BT) were similarly infected with the retroviruses encoding the six transgenes and then injected into the mammary fat pad of the isogenic host animals. Again, upon immunosuppression of the pigs, tumors similar to those described above (vimentin-positive, scattered cytokeratin-positive, general pathology of a highly undifferentiated sarcoma) formed in the mammary gland (Figures 3b, d and 4b), which were confirmed to express all six transgenes (Figure 3e). Thus, this approach can be used to model tumor growth at different anatomic sites.

Discussion

We now show that porcine cells genetically engineered to perturb pathways commonly altered in human

cancers are tumorigenic when returned back to the host animal, providing the first method of genetically inducing tumors in a large mammal. Although this approach is limited because the animals need to be immuno-suppressed for tumors to grow (akin to xenograft mouse models), pigs nevertheless have a number of clear advantages that make them ideal for preclinical studies of imaging, as well as hyperthermia, radiation or photodynamic therapy of tumors. First, the changes needed to make porcine cells tumorigenic were more similar to those needed in human compared to murine cells, hence tumorigenesis in pigs may be more similar to the process in humans. Second, like companion animal models of cancer, the resultant tumors in pigs could be grown to very large sizes, ideal for a number of preclinical applications. Third, unlike these companion animal models, the porcine tumors were genetically engineered, and hence it is possible to tailor-make tumors of a defined background. Fourth, as different cell types injected at different anatomical sites all yielded tumors, it should be possible to generate many different types of tumors potentially anywhere in the body. Fifth, the entire procedure is quite simple and nearly as rapid as existing mouse xenograft models. The ability to now genetically generate experimentally malleable tumors of sizes and genetic makeup akin to those found in humans rapidly, and at will, in the pig can now be exploited with relative ease as a relevant model for preclinical studies of human cancers.

Materials and methods

Plasmids

The retroviral vectors encoding a single transgene, pBabepuro-cyclinD1●HA, pBabehydro-CDK4^{R24C}●HA, pBabeblasto-c-Myc^{T58A}, pBabeneo-p53^{DD}, pBabepuro-flag●H-Ras^{G12V} and pBabepuro-flag●hTERT, have been described previously (Hahn *et al.*, 2002; Yeh *et al.*, 2004). The retroviral vectors encoding two transgenes were constructed based on these plasmids: pBabe-hTERT + p53^{DD}, was created by subcloning the hTERT and PCR cloning the p53^{DD} transgenes into the *EcoRI-SalI* and *HindIII-ClaI* sites of pBabepuro, respectively, thereby replacing the puromycin selection marker; pBabe-cyclinD1 + CDK4^{R24C} was created by PCR cloning cyclin D1 and CDK4^{R24C} into the same *EcoRI-SalI* and *HindIII-ClaI* sites; and pBabe-c-Myc^{T58A} + H-Ras^{G12V} was created by PCR cloning the H-Ras^{G12V} transgene into the *HindIII-ClaI* sites of pBabeblasto-c-Myc^{T58A}, replacing the blastocidin selection marker. All cDNAs generated by PCR were confirmed correct by sequencing.

Porcine cell lines

At 1 day old, ears of five 3/4 Yorkshire 1/4 Duroc pigs (denoted as 6510, 6511, 6512, 6513, 5023, 4400, 4400B, 2310 and 2311) and one pure Duroc pig (denoted as 214) were sterilely/ascetically notched for identification purposes, and the resultant tissue cultured in DMEM/F10 media supplemented with 15% fetal calf serum and 1% antibiotics to establish a primary culture of fibroblasts using established methodologies. As soon as these cultures were established they were serially infected every 48 h with one of each of the retroviruses derived from the plasmids

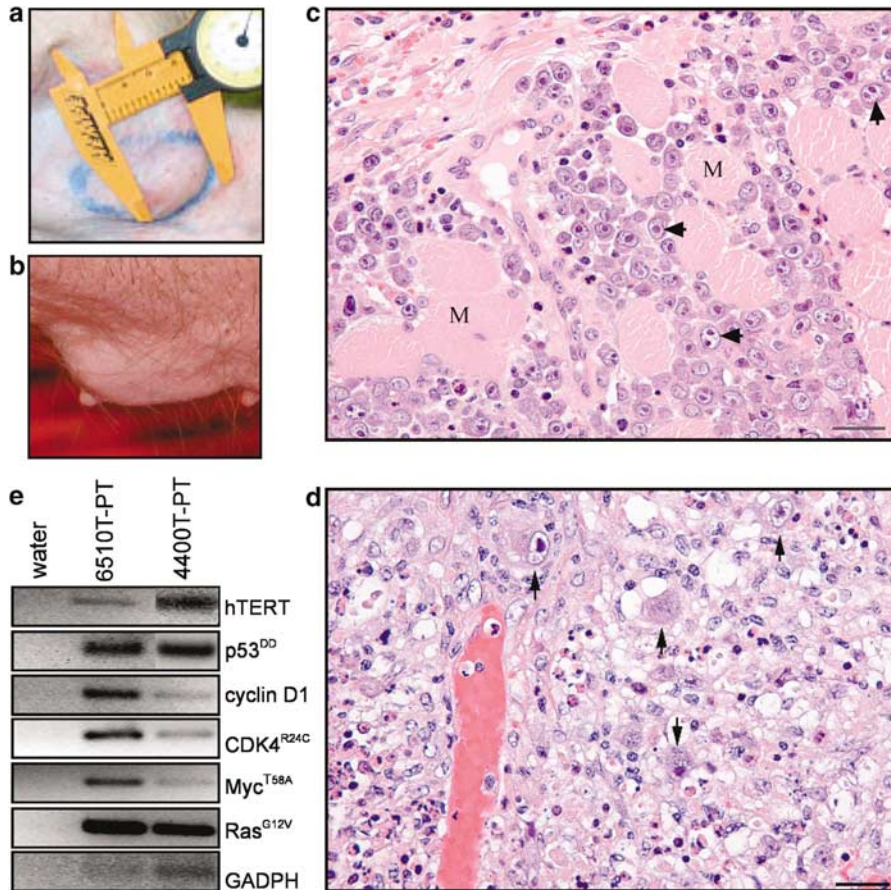


Figure 3 Genetically defined tumor growth in a pig. Fibroblasts from pigs #6510 or #4400 were serially infected with retroviruses encoding hTERT, p53^{DD}, cyclin D1, CDK4^{R24C}, c-Myc^{T58A} and H-Ras^{G12V}, generating the cell lines 6510T and 4400T, respectively. 6510T cells were then injected orthotopically behind both ears and 4400T into the mammary fat pad of their respective immunosuppressed isogenic host animal and (a and b) developed visible tumors in the pigs. When stained with H&E, (c) the 6510T tumors showed subcutaneous muscle (M) infiltration (arrows), which displayed markedly anaplastic and polymorphic phenotype with prominent nuclei and abundant chromatin (Bar = 20 μm). (d) Tumors formed upon injection of 4400T cells into the mammary fat pad. However, in this tumor a severe inflammatory response was seen because cyclosporin treatment was removed and the tumor was removed after tumor regression had begun. The tumor showed degradation and necrosis of the tumor cells (arrows) scattered among a severe inflammatory response (innate immune response) within the interstitial stroma of the mammary gland (Bar = 20 μm). (e) Both tumors were verified to express all six transgenes via RT-PCR. Water served as a negative control; GADPH served as loading control.

pBabe-hTERT + p53^{DD}, pBabe-cyclinD1 + CDK4^{R24C} and pBabe-c-Myc^{T58A} + H-Ras^{G12V} using standard approaches (Armbruster *et al.*, 2001; Hamad *et al.*, 2002), generating the polyclonal cell lines 6510T, 6511T, 6512T, 6513T, 214T, 5023T, 4400T, 4400BT, 2310T and 2311T. Other porcine cell lines assayed, including SK-RST (CRL-2842), PK(15) (CCL-33) and ST (CRL-1746), were obtained from American Type Culture Collection (ATCC) and characterized by them to be normal. These cell lines were then serially infected in a similar manner to the dermal fibroblast to generate the transformed cell lines SK-T, PK-T and ST-T. To generate cell lines expressing five of these six cDNAs, primary cultures of fibroblasts from pig 6510 and 214 were infected with different combinations of two of the three aforementioned plasmids, followed by infection with retroviruses pBabepuro-cyclinD1•HA, pBabehygro-CDK4^{R24C}•HA, pBabeblasto-c-Myc^{T58A}, pBabeneo-p53^{DD}, pBabepuro-flag•H-Ras^{G12V} or pBabepuro-flag•hTERT. Vector control cells were sequentially infected in parallel the other cultures three times with pBabepuro.

RT-PCR

RNA was isolated using the RNazol B reagent according to the manufactures protocol (Tel-Test Inc., Friendswood, TX, USA) from the described cell lines 48 h after the last infection and reverse transcribed using Omniscript reagents (Qiagen, Valencia, CA, USA) with oligo dT primers (Invitrogen, Carlsbad, CA, USA). Resultant cDNA was used to verify the absence or presence of expression of specific transgenes by PCR amplification with one primer specific to the transgene and another specific to a transcribed region of the pBabe plasmids as noted: 5'-TGGCTGTGCCACCAAGCATT and 5'-TTTCCACACCTGGTTGC (hTERT), 5'-GCTCACTCAGCTACCTGAA and 5'-ATGCCTTGCAAAATGGCG (p53^{DD}), 5'-AACATGGACCCCAAGGCC and 5'-TTCTGCCTGCTGGGGAG (cyclin D1), 5'-GGTGGTACCTGAGATGGA and 5'-TAGCTTGCCAAACCTACAGG (CDK4^{R24C}), 5'-ACGAGCACAAGCTCACC and 5'-TTTCCACACCTGTTGC (c-Myc^{T58A}) and 5'-GCACGCACTGTGGAATCT and 5'-TAGCTTGCCAAACCTACAGG (H-Ras^{G12V}). Because cDNAs were cloned into different sites in plasmids

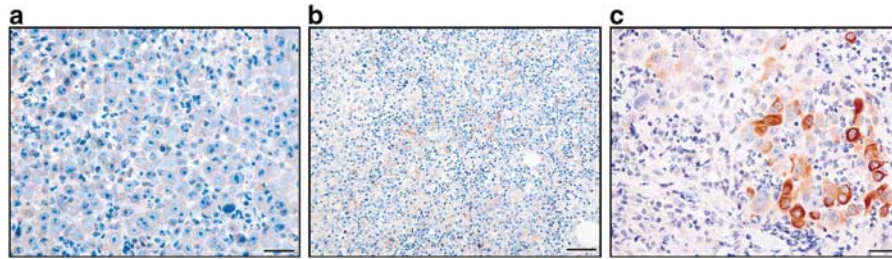


Figure 4 Immunohistochemical analysis of porcine tumors. Tumors derived from cell lines 6510T and 4400T were embedded into paraffin, sectioned and stained for vimentin, desmin and cytokeratin. (a) 6510T tumor cells immunohistochemically labeled (yellow to brown color) positive for vimentin intermediate filament proteins and were admixed with inflammatory cells (Bar = 20 μ m). (b) Less than 5% of 4400T tumor cells within the inflammatory milieu immunohistochemically labeled (yellow to brown color) positive for cytokeratin (Bar = 40 μ m). (c) Less than 5% of 6510T tumor cells immunohistochemically labeled (yellow to brown color) positive for cytokeratin intermediate filaments (Bar = 20 μ m).

encoding one versus two transgenes, the following primers were also used for PCR: 5'-GAGGTGCAGAGCGACTAC and 5'-GCTGTTCACTGCAATCCA (hTERT), 5'-GCTCACTCCAGCTACCTGAA and 5'-TTCTGCCTGCTGGGAG (p53^{PD}), 5'-AGGAGGAGGAAGAGGAGG and 5'-TTTCCACACCTGGTTGC (cyclin D1), 5'-GACTGGCTCGAGATGTAT and 5'-TACTTCTGCCTGCTGGGG (CDK4^{R24C}), 5'-ACGAGCACAAGCTCACC and 5'-TTTCCACACCTGGTTGC (c-Myc^{T58A}) and 5'-GCATCCCTACATCGAGA and 5'-TACTTCTGCCTGCTGGGG (H-Ras^{G12V}). Glyceraldehyde phosphodehydrogenase (GAPDH) was amplified as described previously (Armbruster *et al.*, 2001).

Soft agar

10³ cells per 3 cm plate (in triplicate) were suspended in soft agar as described (Cifone and Fidler, 1980) and colonies > 30 cells scored after 4 weeks. Assays were done independently twice.

Tumor growth

Under a protocol approved by the Duke University and the University of Illinois Institutional Animal Care and Use Committees, 1 \times 10⁷ of cells listed in Table 1 mixed with Matrigel (BD Biosciences, San Diego, CA, USA) were injected subcutaneously into the flanks of two, three or four severe-combined immunodeficient/beige mice per cell line, after which tumor volumes were determined at regular intervals as described previously (Hamad *et al.*, 2002) or 1 \times 10⁸ of 6510T, 4400T or 4400BT cells mixed with a similar number of isogenic control normal cells were injected orthotopically behind each ear or into the mammary fat pad of the donor pig

treated daily with cyclosporine (20 mg/kg), azothioprine (2 mg/kg) and prednisolone (4 mg/kg). For systemic tumor growth analysis, 2 \times 10⁷ of 2310T or 2311T cells were resuspended in 1 ml phosphate-buffered saline and then injected into the jugular vein of each immunosuppressed (see above drug regimen) isogenic host animal for systemic delivery of the cells. The pigs were then monitored for signs of sickness and euthanized after 4 weeks, as per Institutional Animal Care and Use Committee request, regardless of health status. A full necropsy was performed and lung tissue was then sent for pathology analysis. Tumor embedding, sectioning and H&E staining were done using standard approaches.

Immunohistochemistry

All immunohistochemical staining was conducted using the avidin-biotin method, diaminobenzidine chromogen and Mayer's hemotoxylin as the counter stain.

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