A humanized stromal bed is required for engraftment of isolated human primary squamous cell carcinoma cells in immunocompromised mice

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Abstract

Epithelial cancers are the most common malignancies and the greatest cause of cancer mortality worldwide. The incidence of keratinocyte-derived (non-melanoma) skin cancers (NMSCa) is increasing rapidly. Despite access to abundant tumor tissue and ease of observation, acceptance of NMSCa as model carcinomas has been hindered by the lack of a reliable xenograft model. Herein we describe conditions that allow routine xenoengraftment of primary human squamous cell carcinoma (SCCa) cells. Tumor development required creation of an appropriate stromal bed prior to xenografting tumor tissue onto the backs of athymic nude mice. We also demonstrate that the stromal bed must be "humanized" if primary human SCCa is to be propagated from cell suspensions. SCCa xenografts recapitulated the histological grade and phenotype of the original tumors with considerable fidelity, even after serial passage, irrespective of the histological grade of the primary human SCCa. To our knowledge this previously unreported model can be used for drug testing, as well as for studies that are relevant to the biology of primary human SCCa and other epithelial cancers.

Keywords

Skin cancer; squamous cell carcinoma; mouse models

Introduction

Cancers of epithelial tissues (skin, stomach, lung, breast, prostate and colon) approximately account for 85% of all cancers and 75% of all cancer-related deaths in the USA and worldwide (Altekruse *et al*., 2009; Ferlay *et al*., 2010*)*. Among carcinomas, although not

included in these cancer statistics because of their relative low mortality, the incidence of non melanoma skin cancer (NMSCa) (basal cell carcinoma and squamous cell carcinoma (SCCa)) is rising faster than all other cancers (Christenson *et al*., 2005; Diepgen *et al*., 2002; Green *et al*., 1996; Buettner *et al*., 1998; Holme *et al*., 2000; Benton *et al*., 2008). Currently, NMSCa are the most frequent of all cancers and affect 1 million Americans per year, 250,000 of whom have SCCa (http://www.cancer.org/docroot/CRI/content/CRI_2_2_1X_How_many_people_get_nonmelanoma_skin_cancer_51.asp?sitearea=). Despite the high incidence and ease of observation, the study of primary human SCCa as a model of epithelial cancer has been hindered by the lack of reproducible *in vivo* tumor tissue growth assays.

Although the genetic basis of human SCCa has not been completely characterized and likely involves a number of different alterations (Boukamp., 2005), approximately 50% of all SCCa harbor a UV light-induced p53 mutation in one or both alleles (Ziegler *et al*., 1994; Brash *et al*., 1991). Also, since overexpression of the RAS signaling pathway is observed in 75% of all SCCa, RAS likely plays a critical role in SCCa initiation and maintenance (Dajee *et al*., 2003). Previously it has been suggested that a minimal set of alterations, including RAS activation and NFκB pathway inactivation or upregulation of cyclin dependent kinase 4, are sufficient to transform normal human keratinocytes resulting in SCCa-like tumors in a subcutaneous xenograft model (Dajee *et al*., 2003; Ortiz-Urda *et al*., 2005).

Primary human skin (either intact or as reconstituted human skin equivalents) can be successfully grafted onto the backs of immunocompromised mice (Guerret *et al*., 2003; Pouliot *et al*., 2002). These grafts retain many features of normal human skin: structure (Bohnert *et al*., 1989; Kim *et al*., 1992), epidermal differentiation (Demarchez *et al*., 1986) and function (Kim *et al*., 1992; Demarchez *et al*., 1986). However, establishing primary human SCCa xenografts has proved to be difficult. Most successful xenografts of human SCCa involve cell lines. Rheinwald and Beckett reported two primary human cutaneous SCCa as well as 4 orally-derived SCCa from cells that had been passaged in tissue culture prior to successful grafting onto athymic nude mice (Rheinwald *et al*., 1981). Intriguingly, reproducible tumor growth required implantation of 3×10^6 cultured SCCa cells. Similarly, the Fusenig laboratory reported engraftment of two poorly differentiated primary human SCCa from tissue cultured passaged cells onto athymic nude mice (Boukamp *et al*., 1982; Tilgen *et al*., 1983). Independent of these early successes and despite the subsequent availability of additionally immunocompromised mouse strains that have allowed successful xenografts of other tumor types, xeno-engraftment of primary human SCCa remains challanging (Takizawa *et al*., 1997).

Orthotopic xenograft models are essential for investigation of tumor biology, notably in the identification of tumor initiating cell subpopulations, as well as for drug testing. The lack of standardized *in vivo* models that allow establishment and propagation of SCCa, and characterization of tumor initiating cells in SCCa has been a significant problem. To address this issue, we developed a reliable *in vivo* orthotopic xenograft assay that allows routine growth of tumors from directly implanted primary human SCCa tissue and cell suspensions.

Results

Orthotopic primary human SCCa xenografts rarely take

Normal skin can be xenografted onto immunocompromised mouse strains, either as intact tissue or alternatively reconstituted human skin equivalents (Guerret *et al*., 2003; Pouliot *et al*., 2002; Bohnert *et al*., 1989; Kim *et al*., 1992; Demarchez *et al*., 1986). To develop an *in vivo* assay for primary human SCCa, we first attempted tumor growth by xenografting intact tumor tissue ($0.5cm^3$) in which epithelial-stromal relationships are maintained onto various immunocompromised mice. Despite using nude, SCID-beige and NOD-SCID mice strains, onto which we subcutaneously implanted 22 different primary human SCCa's, reproducible tumor growth was not achieved (Row 1, Table 1). Although surprising, our observation is consistent with the literature (Rheinwald *et al*., 1981; Takizawa *et al*., 1997).

Creation of a stromal bed facilitates primary human SCCa xenograft growth *in vivo*

We hypothesized that since primary human SCCa are highly vascularised tumors, insufficient blood supply at the subcutaneous graft site might be responsible for graft failure. To facilitate xenograft engraftment and to increase vascularity, we induced a stromal reaction by first implanting either a subcutaneous glass disc (Roop *et al*,, 1986) or Gelfoam™ dressing (Zeamari *et al*., 2004) 2 weeks prior to the intact tumor tissue xenograft (Fig. 1a and b). We utilized athymic nude and SCID-beige mouse strains since these had allowed growth of a single clinically aggressive SCCa. Of 25 different primary human SCCa tissue xenografts, 22 demonstrated tumor growth as confirmed by serial tumor measurements and subsequent histology (Fig. 1c and Row 2, Table 1). All histological grades of primary human SCCa successfully engrafted and grew, including: 4 of 6 poorly, 7 of 7 moderately and 11 of 12 well-differentiated SCCa. All tumors maintained their original morphology *in vivo*, including large well-differentiated tumors (Fig. 1d). The growth rates of human SCCa xenografts were consistent with the relatively slow growth rate of *in situ* SCCa in patients (Fig. 1c). Intriguingly, xenograft growth was observed in athymic nude, but not in more immunocompromised SCID-beige, mice. None of the four different SCCa samples that grew as xenografts in athymic nude mice grew in SCID-beige mice, despite simultaneous engraftment using the identical experimental protocol. Although the number of SCID-beige mice is low, one would have expected at least an equal if not greater rate of graft takes in the more immunocompromised mice. Thus, primary human SCCa xenograft growth was not achievable in more severely immunocompromised mice strains, and was clearly dependent upon the prior creation of a stromal reaction.

To confirm that xenograft tumors contained primary human SCCa epithelial cells, we stained tumor xenograft sections with antibodies to human HLA antigens, murine MHC class 1 antigens (Fig. 2a), human cytokeratins (Fig. 2b) and human p53 (Fig. 2c). The p53 antibody clone used binds to the DNA binding domain and specifically recognizes the mutated protein. After 12 weeks, most tumor stroma was comprised of murine cells. Primary human SCCa xenografts retained human HLA, keratin and mutant p53 protein expression demonstrating the survival and growth of primary human SCCa keratinocytes. The xenograft tumors were also amenable to serial passage (Fig. 2d). We concluded that athymic

nude mice supported subcutaneous primary human SCCa xenografts, after creation of fibrovascular beds by implantation of either glass discs or Gelfoam™ dressings.

Primary human SCCa-derived cell suspensions require human fibroblasts to form a xenograft

We next attempted to adapt the *in vivo* model that we had created for intact tumor tissue to allow tumor growth *in vivo* from primary human SCCa-derived cell suspensions. First, we demonstrated that tumor xenograft growth was possible in 3 of 3 SCCa xenografts with fragments of tumor tissue (Fig. 3a–c, Row 3 Table 1). To derive single SCCa cell suspensions, we modified the enzymatic digestion protocol for normal skin dissociation (Fig. 3d,e), resulting in between 30 and 70% cell viability by FACS analysis (Fig 3f). However, when we attempted to initiate SCCa tumor growth by xenografting unsorted cell suspensions derived from human SCCa, we achieved growth in only 3 of 24 SCCa cell suspension xenografts after $> 10^6$ cells were resuspended in Matrigel™ and injected into the subcutaneous fibrovascular bed created in athymic nude mice (Row 4 Table 1). To improve the proximity of stromal and tumor cells, we next supplemented primary human SCCa cell suspensions with an additional 10^6 primary normal human or SCCa associated fibroblasts prior to implantation. With this approach, we achieved no tumor growth from 8 different primary human SCCa (Row 5 Table 1), however we did not attempt implantation with greater numbers of fibroblasts.

As success in xenografting primary human breast cancer cells required cleared murine mammary fat pads to be first "humanized" by the pre-implantation of human fibroblasts (Kuperwasser *et al*., 2004), we hypothesized that primary human SCCa xenografts from single cell suspensions might also require "humanization". Successful and reproducible xenografts of human SCCa cell suspensions were ultimately achieved when large numbers of normal human fibroblasts were used to "humanize" xenograft recipient sites. One million (10^6) primary normal human fibroblasts were first suspended in Matrigel™ and implanted with the glass disc or Gelfoam™ dressing. After 14 days, SCCa xenograft cell suspensions were co-injected with an additional 10^6 primary human normal fibroblasts suspended in Matrigel™ into the prepared xenograft sites (Fig. 4a). This approach yielded 28 successful xenograft tumors in 29 attempts (Table 1) using cell suspensions derived from 29 different primary human SCCa's: 16 well differentiated SCCa, 7 moderately differentiated SCCa and 6 poorly differentiated SCCa. The histological patterns of these xenograft tumors recapitulated the original primary human SCCa histologies irrespective of the histological classification of the tumors (Fig. 4b). For success, a minimum number of SCCa cells were required such that xenograft success was assured if 3×10^6 unsorted primary human SCCa cells were xenografted. Tumor growth was not reproducible with xenografts containing $< 3 \times 10^6$ unsorted primary human SCCa independent of the histological grade of the original tumor (Fig. 4c). Robust xenograft growth was observed in athymic nude mice and not the more immunocompromised NOD-SCID strain (Row 6 Table 1), in 0/4 attempts. Primary human SCCa xenograft tumors could also be serially passaged into new athymic mice as cell suspensions (Fig. 4d). Hence, we successfully created an orthotopic *in vivo* xenograft model using primary human SCCa cell suspensions that reproducibly recreated the original tumor phenotype, independent of the original histological grade.

Discussion

Herein we describe an *in vivo* xenograft model for primary human SCCa using intact tumor tissue and cell suspensions in which *in vivo* tumor growth is reliable and recreates the original tumor hierarchical architecture, irrespective of the SCCa histological grade. In contrast to previously described models, *in vitro* passage was not required and primary tumor tissue or cell suspensions could be directly implanted. The high frequency and relative ease with which primary human SCCa tissue can be accessed, combined with the simplicity of this model, will facilitate the study of primary human epithelial cancer growth and drug testing. Since, *in vivo* growth can be assured with implantation of primary human SCCa cell, this assay can also be used for the study of potential tumor initiating cell populations.

Because no standardized assay has been established for growing primary human cutaneous SCCa xenografts, we describe our extensive efforts to develop an efficient *in vivo* xenograft mouse model in some detail. In our model, creation of a fibrovascular stromal bed by pre-implantation of glass discs or Gelfoam™ dressings was critical, suggesting the relative importance of a well developed tumor niche environment. These approaches were adapted from previous models for murine SCCa (Roop *et al*,, 1986) and peritoneal metastases (Zeamari *et al*., 2004) respectively, in which enhanced efficiency of tumor take was demonstrated. Likewise, *in vivo* growth from primary human SCCa cell suspensions required co-implantation of 10^6 primary human fibroblasts prior to, and along with, human cells derived from SCCa. Previously, the "humanization" of murine stroma was found to be necessary for successful human breast cancer xenografts (Kuperwasser *et al*., 2004). The requirement for pre-implanted human stromal cells is not well understood, since there is abundant vascularization and murine stroma in place even in the absence of human cells.

We were surprised that the success rate for establishing human SCCa xenografts was consistently higher in athymic nude mice than in more immunocompromised mice, including SCID-beige and NOD-SCID mice, even though all mice developed a fibrovascular stroma. Athymic nude mice retain some residual immunity with functional NK cells, but when attempting to xenograft tumor pieces after the creation of a stromal bed the administration of NK neutralizing antibody did not hinder nor enhance tumor growth (n=3). Athymic nude mice also retain functional B-lymphocytes, suggesting this could facilitate tumor growth. Interestingly, the paradoxical role of both the adaptive and innate immune responses, particularly B-lymphocytes, in facilitating mouse SCCa growth has been previously noted (Clevers, 2004; De Visser *et al*., 2006; Karin *et al*., 2006) and SCCa growth is observed in inflamed human skin (e.g.: chronic wounds, burn injury, discoid lupus erythematosus, lichen sclerosus, lichen planus, hidradenitis supprativa and lupus vulgaris (Alam *et al*., 2001)). Thus, the reactive tissue milieu in the prepared graft site may have been more inflammatory in the athymic immunodeficient nude mice recipients and facilitated SCCa growth (de Visser *et al*., 2005). However, patients receiving immunosuppressants for the management of renal transplants are at greater risk for developing clinically aggressive SCCa once tumors have been established. The development of a SCCa *in vivo* xenograft model using athymic nude mice is consistent with what is known about the role of inflammation in the pathogenesis of SCCa and the promoting effect

of immunodeficiency. SCCa xenografts grew slowly and none of these tumors metastasized or were lethal within 12 weeks *in vivo*, consistent with the SCCa clinical phenotype in patients.

In conclusion, we have created a simple, reliable *in vivo* xenograft model for primary human SCCa. This assay will be widely applicable for drug testing, as well as for studies to elucidate the biology of primary human SCCa and other epithelial cancers.

Material and Methods

Immunofluorescence

Immunofluorescence was performed using standard techniques with the following primary antibodies: pancytokeratin (Dako, clone AE1/3), Mouse MHC class I (AbD Serotec, clone 2G5), Human HLA (AbD Serotec, clone YTH 862.2) and mutant p53 (Calbiochem, clone Pab240).

For cytospin analysis, normal human skin keratinocytes, RS4 lymphocyte cells (ATCC) or SCCa single cells were washed and resuspended in PBS at a concentration of 10^5 cells per ml. 100 μl aliquots of cells were added to each slide cytofunnel (Shandon, Thermo Scientific), spun at 800 rpm for 5 minutes in a cytocentrifuge (Shandon Cytospin 2 cytocentrifuge), fixed in acetone and labelled with a pancytokeratin antibody as above.

Cell preparation

Normal foreskins and human squamous cell carcinoma were obtained after IRB approval of experiments, written, informed patient consent and adherence to the Helsinki Guidelines. Tumor tissue was mechanically dissociated, and then incubated for 2 hours at 37°C in a 100 ml sterile conical flask containing medium 199 with 200 u/ml Dispase (BD Biosciences) and 200 u/ml Ultrapure Collagenase Type III (Sigma) with stirring on a magnetic stirring plate in a tissue culture CO_2 incubator. The semi-dissociated mixture was pipetted into sterile 50 ml tubes and, after centrifugation at 200g for 5 minutes, supernatants were discarded. The remaining tissue was then incubated at 37°C for 5 minute with 0.05% trypsin with EDTA. Tissue remnants were removed from cell suspensions by filtration through a 40 μm cell strainer.

Flow cytometry

Samples were assessed using a FACSCalibur flow cytometer (BD Biosciences) and mouse flurochrome-conjugated IgG subtype isotype controls (BD Pharmingen). A live cell gate was created using 7-Amino-Actinomycin D (BD Pharmingen) to label dead cells.

Transplantation of human SCCa cell suspensions

Athymic nude homozygous foxn1^{nu} (Jackson Lab), SCID-beige and NOD-SCID (Taconics) mice were housed and utilized under conditions approved by the Animal Care and Use Committee at the National Cancer Institute. Mice were anaesthetized and either Gelfoam™ dressing (Johnson & Johnson) or sterilized glass discs were implanted into the dorsal subcutaneous space, together with 10^6 primary human fibroblasts suspended in 100 μl of

Matrigel (BD Biosciences) for single cell suspension experiments, and wounds were closed with surgical staples (Mikron). After 14 days, mice were anaesthetized, glass discs were removed and SCCa cells that had been suspended in 100 μl of Matrigel was injected into the subcutaneous space or alternatively into the in-situ gelfoam™ dressing. After 12 weeks, mice were euthanized via CO_2 inhalation, and tumors were removed for analysis.

Acknowledgements

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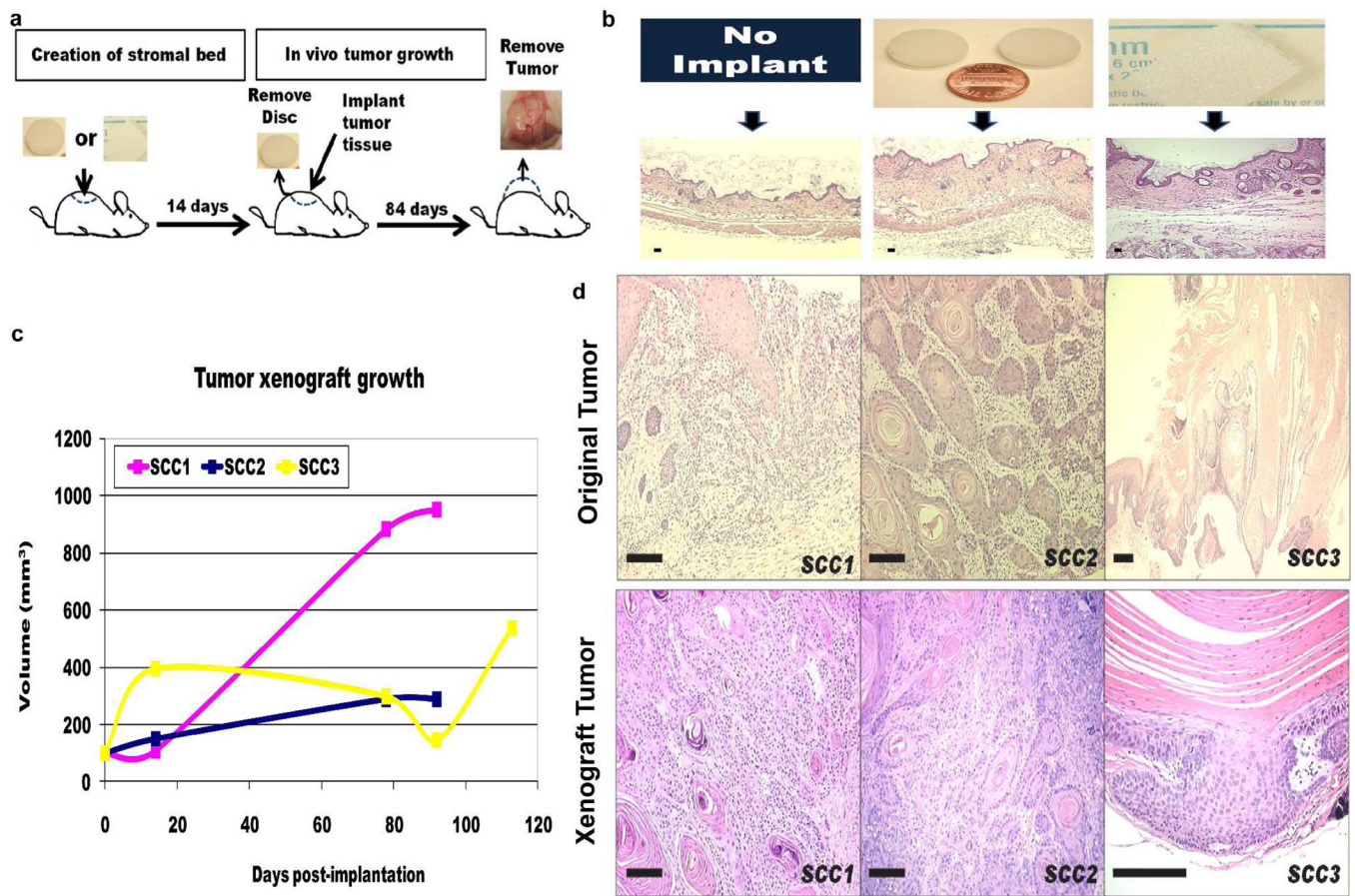
Figure 1. Creation of a SCCa *in vivo* xenograft model that can accurately recapitulate and propagate human SCCa from intact tumor tissue

a, Successful engraftment of primary human SCCa tissue required prior creation of a stromal bed, into which the tissue is implanted after 14 days. After 12 weeks, tumor growth was determined by histological sampling of all xenograft sites. **b,** H&E stained sections of athymic nude mouse skin demonstrate a fibrovascular proliferation following subcutaneous implantation of either a glass disk or Gelfoam™ dressing for 2 weeks. **c,** Tumor growth after implantation of similar size intact SCCa tissue. **d,** Xenograft tumors that developed from intact, primary human SCCa preserved the original tumor morphology. All scale bars are 100μm.
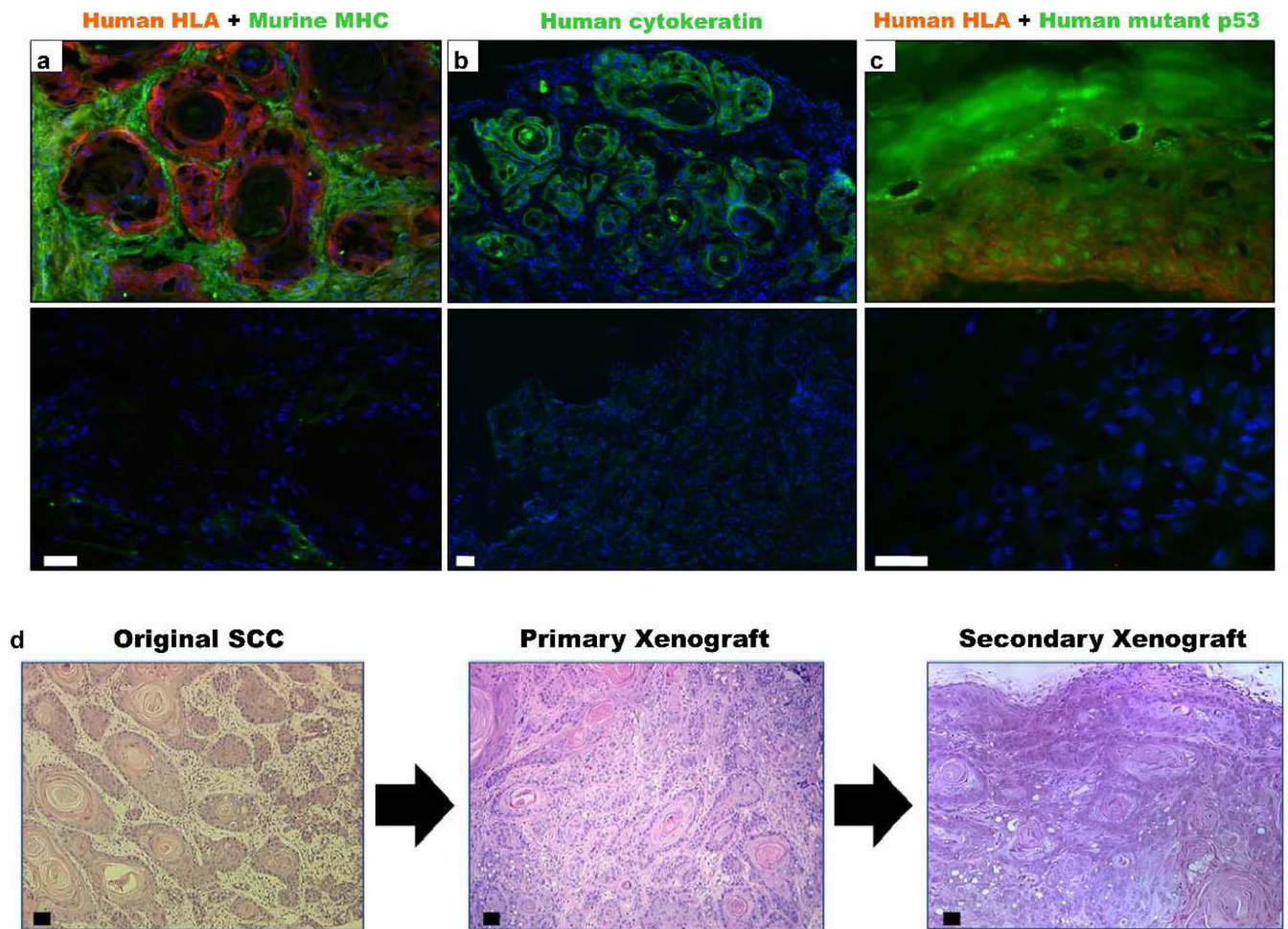
Figure 2. Xenografts express human markers and can be passage *in vivo*
Primary human SCCa-derived xenograft tumors expressed: **a,** human HLA antigen, **b,**
cytokeratin and **c,** nuclear accumulation of mutant p53. Respective isotype controls are
shown in panels underneath. Xenograft tumor stroma was predominantly replaced by murine
cells that expressed murine MHC class 1. Non- specific staining was observed within the
dead squamous accumulations. **d,** Recreation of the original tumor morphology upon *in vivo*
serial passage of the previously xenografted tumor. Histology after 12 weeks was similar to
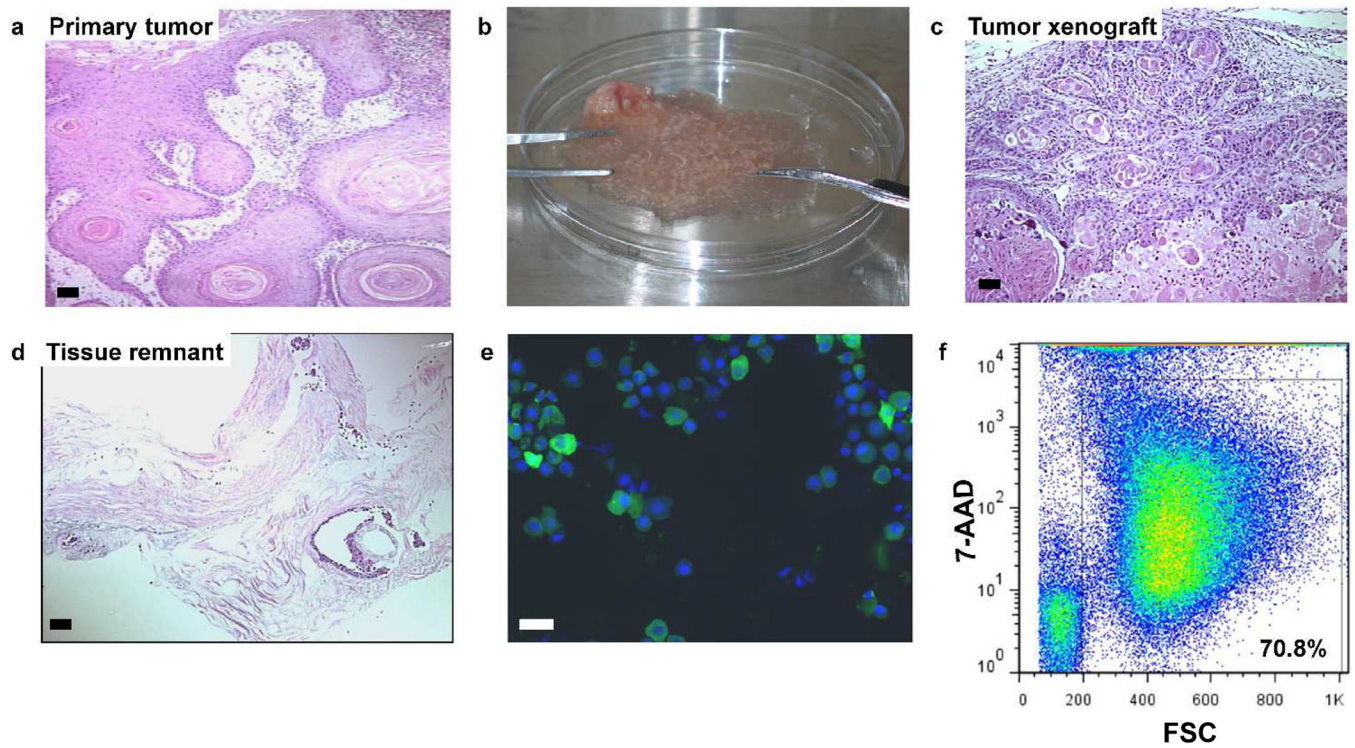the original and primary xenograft tumors. All scale bars are 100 μm.

Figure 3. SCCa mechanical dissociation and generation of a single cell suspension
a, Primary human SCCa was **b,** mechanically dissociated using surgical scissors, **c,** the minced tumor tissue was then implanted onto wound beds created by either a glass disc or gelfoam dressing that had been implanted for 14 days in the athymic nude mice. Shown is a typical tumor after 12 weeks. **d,** cell suspensions were created by additional enzymatic dissociation so that few cells remained in the remnant tissue. **e,** tumor cells could be labeled with a human pancytokeratin antibody and **f,** approximately 70% of the tumor dissociated cells were viable, as assessed by FACS. All scale bars are 100 μm.
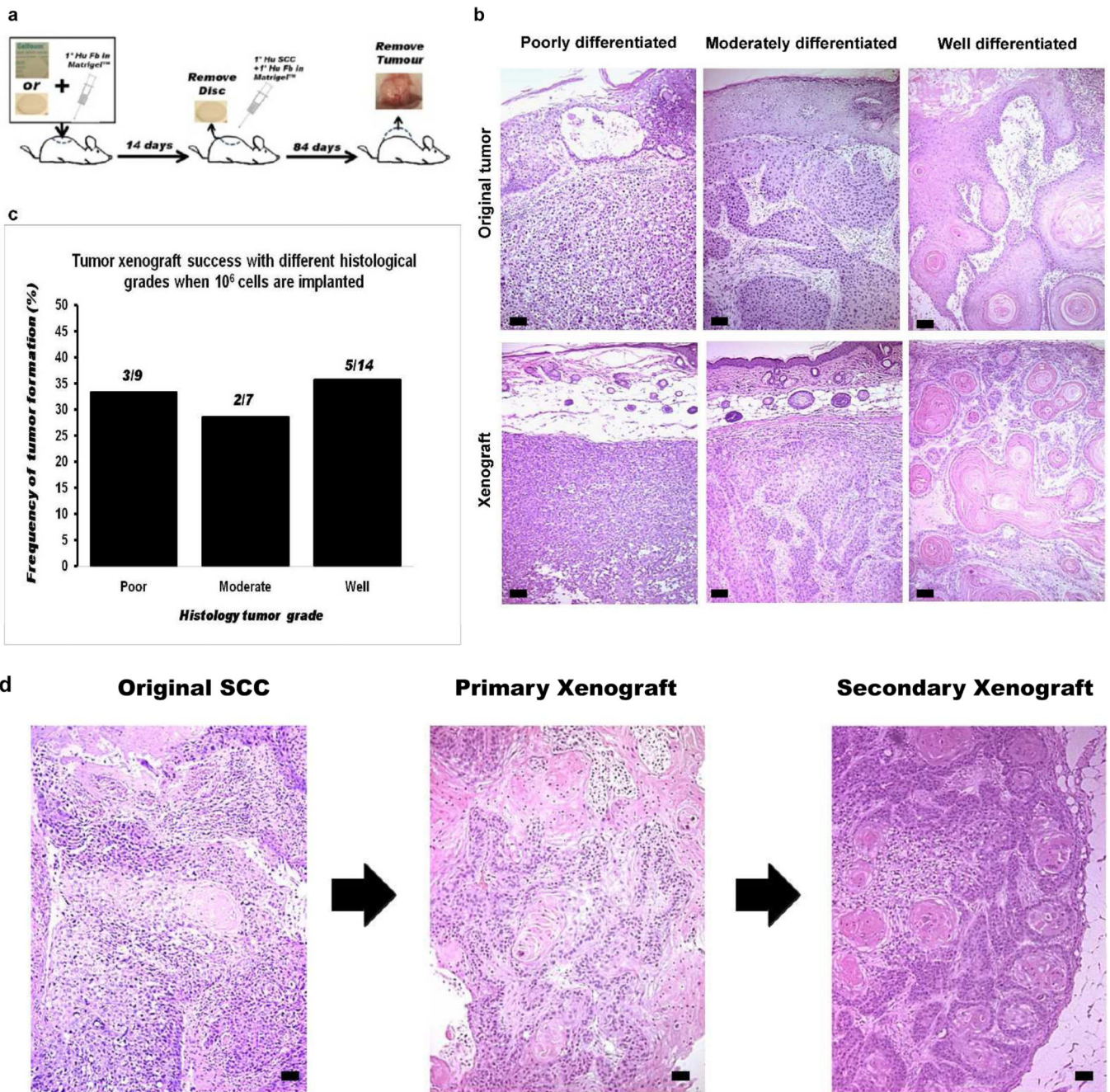
Figure 4. Xenografts can accurately recapitulate and propagate human SCCa from cell suspensions

a, Schematic **b,** SCCa xenografts from cell suspensions recreated the original tumor morphology **c,** with no difference in xenograft growth based upon the original tumor histological grade. Xenograft success was only dependent upon the number of tumor cells implanted and **d,** harvested tumor tissue could be dissociated and successfully implanted into naive athymic nude mice. All scale bars are 100 μm.

Table 1

Development of an *in vivo* SCCa model

SCCa tumor xenograft	Graft site preparation	Xenograft tumor frequency in different mouse strains		
		Nude	**SCID-beige**	**NOD-SCID**
Intact tumor tissue	None	1 of 22	1 of 5	0 of 5
Intact tumor tissue	Creation of a stromal bed	22 of 25	0 of 4	ND
Minced tumor tissue	Creation of a stromal bed	3 of 3	ND	ND
Single cell suspension	Creation of a stromal bed	3 of 24	ND	ND
Single cell suspension with 10^6 primary human fibroblasts	Creation of a stromal bed	0 of 8	ND	ND
Single cell suspension with 10^6 primary human fibroblasts	Creation of a "humanized" stromal bed	28 of 29	ND	0 of 4

ND = Experiment not performed