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Identification and characterization of tumor initiating cells in human primary cutaneous squamous cell carcinoma

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Abstract

Primary human squamous cell carcinoma (SCCa) are heterogeneous invasive tumors with proliferating outer layers and inner differentiating cell masses. To determine if tumor initiating cells (TIC) are present in SCCa, we utilized newly developed reliable *in vitro* and *in vivo* xenograft assays that propagate human SCCa, and demonstrated that a small subset of SCCa cells (~1%) expressing Prominin-1 (CD133) in the outer layers of SCCa were highly enriched for TIC (~1/400) compared to unsorted SCCa cells (TIC ~1/10^6). Xenografts of CD133+ SCCa recreated the original SCCa tumor histology and organizational hierarchy, while CD133- cells did not, and only CD133+ cells demonstrated the capacity for self-renewal in serial transplantation studies. We present a model of human SCCa in which tumor projections expand with outer leading edges that contain CD133+ TIC. Successful cancer treatment will likely require that the TIC identified in cancers be targeted therapeutically. The demonstration that TIC are present in SCCa and are enriched in a CD133-expressing subpopulation to our knowledge has not previously been reported.

Keywords

Skin cancer; squamous cell carcinoma; tumor initiating cells; cancer stem cells; mouse models

Introduction

The molecular pathogenesis of human cutaneous SCCa likely involves a number of different genetic and epigenetic alterations (Boukamp, 2005) including frequent mutations in p53 and upregulation of Ras activity. (Ziegler *et al*., 1994; Brash *et al*., 1991;. Dajee *et al.*, 2003).

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Histologically, human SCCa are heterogenous tumors that contain both proliferating and differentiating keratinocytes (Watanabe *et al*., 1995; Murphy *et al*., 1984) organized in a hierarchy that recapitulates normal epidermal development. Since the cellular hierarchy of normal epidermis is reconstituted by keratinocyte stem cells, it is possible that human SCCa are also initiated by cancer stem cells or tumor initiating cells (TIC) that possess long-term proliferative and self-renewal capacity and can reconstitute the tumor hierarchy and cellular heterogeneity in SCCa (Dick, 2009). In tumors that lack a clear organizational hierarchy, as a recent study suggests in the case of melanoma, TIC may not be present in a distinct subpopulation or may be present at very high frequencies (Dick, 2009; Quintana et al., 2008; Eaves, 2008). The isolation and characterization of potential TIC in cutaneous SCCa would facilitate the elucidation of the fundamental changes required to achieve both tumor formation and hierarchical organization.

One approach to identify TIC-enriched sub-populations in human SCCa could involve characterization of the anatomical heterogeneity of human SCCa using both biological (differentiation) and cell surface markers that identify keratinocyte stem cells in the hair follicle bulge region (CD200) and other populations in other hair follicle regions and epidermis (CD24, CD34, CD71, CD146) (Ohyama *et al*., 2006). Application of additional cell surface markers that identify TIC in other epithelial human cancers, including CD44 (hyaluronan receptor) for breast cancer (Al-Hajj *et al*., 2003), head and neck cancer (Prince *et al*., 2007), and colorectal cancer (Dalerba *et al*., 2007), and CD133 (prominin-1) for lung cancer (Eramo *et al*., 2008) and colon cancer (O'Brien *et al*., 2007; Ricci-Vitiani *et al*., 2007; Shmelkov *et al*., 2008) could then be added to narrow the search.

A sensitive reproducible *in vivo* orthotopic xenograft mouse model is needed to demonstrate that TIC are present in distinct sub-populations of human cutaneous SCCa cells. In a companion paper, we describe a recently developed model that is capable of initiating and maintaining the heterogeneous SCCa hierarchy when isolated tumor cells are implanted into immunocompromised mice (Patel *et al*., 2011). We now describe the isolation and characterization of a small subpopulation of tumor cells that exhibits long-term proliferative and self renewal capacities and gives rise to more differentiated tumor cells. We propose that these cells include SCCa TIC.

Results

Primary human squamous cell carcinomas are heterogeneous with characteristic patterns of epidermal proliferation and differentiation

Primary human cutaneous SCCa are comprised of multiple finger-like tumor projections (Figure 1a) characterized by inner cell masses of keratinocytes that do not proliferate and express involucrin (Figures 1b and 1c; Figure S1) and Ki-67+ proliferating keratinocytes that are located on the outer edges of tumor projections (Figures 1b and Figure S1). These results suggest that human SCCa keratinocytes differentiate after they leave the proliferating layer, similar to normal epidermis (Watanabe *et al*., 1995; Murphy *et al*., 1984). The presence of proliferating and differentiating keratinocytes within primary human SCCa's is consistent with an organizational hierarchy suggesting the presence of TIC that can reconstitute and maintain human SCCa.

Defining distinct keratinocyte subsets in primary human SCCa

We utilized a panel of CD markers (CD24, CD34, CD71, CD146, and CD200) that we had previously demonstrated could distinguish distinct keratinocyte populations within normal human hair follicles (Ohyama *et al*., 2006). CD71 (transferrin receptor), normally expressed by proliferating outer root sheath keratinocytes of hair follicles, was only expressed by proliferating outer cell layers of primary human SCCa (Figures 1c and 1d; Figure S2) while CD24 and CD146 were each expressed exclusively by differentiating keratinocytes in tumor inner cell masses (Figures S2). CD71 expression was confirmed by FACS analysis and was consistently detected on approximately 8% ± 6% (n=9) of keratinocytes in cell suspensions prepared from human SCCa (Table S1). Since human SCCa contained variable numbers of inflammatory cells (mean 16% ± 16%; n=45) (Tables S1 & S2), all FACS analysis included monoclonal antibodies reactive with CD45, the common leukocyte antigen, to identify these cells. CD34 was not detected on human SCCa keratinocytes by immunohistochemical analysis, but CD34 was identified on endothelial cells in the SCCa stroma (Figure S2). Of interest, although we previously demonstrated that CD200 could identify human keratinocyte stem cells located in the hair follicle bulge area, we did not detect CD200 expression on keratinocytes in human SCCa, either by immunohistochemical or FACS analysis (Figure S2).

A subset of primary human SCCa keratinocytes expresses CD133

CD44 and CD133 expressing TIC have been identified in other cancers (Al-Hajj *et al*., 2003; Prince *et al*., 2007; Dalerba *et al*., 2007; Eramo *et al*., 2008; O'Brien *et al*., 2007; Ricci-Vitiani *et al*., 2007; Shmelkov *et al*., 2008; Singh *et al*., 2004; Yi *et al*., 2007). Expression of CD44, used to enrich TIC populations in breast, head and neck, pancreas and colorectal cancers (Al-Hajj *et al*., 2003; Prince *et al*., 2007; Dalerba *et al*., 2007), was detected by immunofluorescence (data not shown) and FACS exclusively on CD45+ non-keratinocytes scattered throughout SCCa tumors (Figure 1d), consistent with previous reports of absent or down-regulated CD44 expression on SCCa keratinocytes (Janes *et al*., 2006).

In contrast, primary human SCCa tumor sections labeled for CD133 using antibody AC133 (epitope 1, glycosylation-dependent) demonstrated basal layer cell surface expression on relatively rare cells in proliferating CD71+ outer cell layers (Figure 1e). CD133+ cells also expressed cytokeratin 14 but did not express CD31 (PECAM-1), thereby excluding endothelial cells and identifying CD133+ cells as keratinocytes (Figures 1e, Figure S3). FACS analysis of 39 primary human SCCa demonstrated CD133+ expression by 0.9% ± 0.9% of SCCa cells (Figure 1d, f and Table S2). CD133+ SCCa keratinocytes were less heterogeneous when compared to equivalent numbers of total primary human SCCa single cells with regard to forward and side scatter (Figure S4). Similar percentages of CD133+ CD45− cells were present in different histologic grades of human SCCa by FACS analysis (Figure 1f). Thus, CD133+ keratinocytes comprised a relatively rare subset (approximately 1%) of keratinocytes in human SCCa and were located in the CD71+ outer proliferating layers of SCCa tumor projections.

CD133+ human SCCa keratinocytes are enriched for colony forming cells in an *in vitro* assay for human SCCa

Primary human SCCa cells formed colonies when grown in an adapted keratinocyte colony forming assay that incorporates a NIH3T3 feeder layer to support growth (Terunuma *et al*., 2007). During culture, primary human SCCa cells attached to NIH 3T3 cells in the feeder layers and not to tissue culture plate surfaces. After 14 days in culture, compact spheroidal colonies formed with > 100 cells tethered to NIH 3T3 feeder cells (Figures 2a). In contrast, normal human keratinocytes attached directly to tissue culture plates and formed adherent monolayer colonies (Terunuma *et al*., 2007) (Figure 2a). Increased numbers of tethered sphere colonies formed when increased numbers of SCCa tumor cells were cultured, demonstrating a dose response relationship (Figure 2b). The cultured tethered spheres could also be serially passaged with approximately 50 tethered sphere colonies formed from an initial inoculum of 10^5 human SCCa cells. Also, when colonies identified by inverted light microscopy were isolated using cloning cylinders and then serially passaged these resulted in equivalent numbers of secondary colonies. Thus the serial passage studies demonstrated that only 1–2 colony-forming cells were present on average in each tethered sphere. Importantly, these colonies contained tumor-derived keratinocytes (Figure 2c). To confirm that these tethered sphere colonies contained TIC, we also demonstrated that xenografts of tethered sphere colonies recreated human SCCa tumors when implanted in immunocompromised mice (Figure 2d).

To determine if TIC were enriched in the different SCCa keratinocyte subsets, we screened sorted CD71+ and CD133+ subpopulations for increased colony forming efficiency that might indicate enrichment of TIC. Highly purified CD133+ and CD71+ human SCCa keratinocyte subsets were isolated using magnetic bead (MACS™) separation or FACS-sorting with greater than 92% and 80% purity respectively based on FACS analysis (Figure 2e). Cytospin analysis of the CD133+ CD45− and CD133− CD45− purified subsets demonstrated keratin expression in all CD133+ CD45− cells and most CD133− CD45− cells, confirming their keratinocyte origin and purity (Figure S5; n=3), and also demonstrated the presence of the same p53 mutation detected in the parental SCCa tumor (Figure S6). We determined that the CD133+ CD45− SCCa keratinocytes were > 7-fold more efficient in colony formation than the CD133− CD45− sorted and unsorted total SCCa populations; $p<0.01$ (Figures 2f–h). By comparison, CD71+ CD45− SCCa cells were ~2-fold increased in spheroid colony formation efficacy compared to CD71− CD45− SCCa cells (Figure 2h).

Since the differences in colony formation efficiency might reflect different proliferation rates for the sorted populations, cell cycle analysis showed that the CD133+ CD45− population was relatively quiescent compared to the CD133− CD45− population (4.2% versus 13.1% cells in G2-M, respectively (Figures S7), and thus may represent a relatively quiescent population in the outer proliferating SCCa layer. These results suggest that CD133+ CD45− SCCa cells may be candidates for TIC.

TIC frequency in unsorted primary human SCCa

Successful and reproducible xenografts of human SCCa cell suspensions onto immunocompromised mice were ultimately achieved when large numbers of normal human fibroblasts were used to "humanize" the xenograft recipient sites (Patel *et al*., 2011). One million (10^6) primary normal human fibroblasts were first suspended in Matrigel™ and implanted subcutaneously with glass discs or Gelfoam™ dressing. After 14 days, SCCa xenograft cell suspensions were co-injected, along with an additional 10^6 primary human normal fibroblasts suspended in Matrigel™ into the xenograft sites (Figure 3a). Using this approach, successful xenografts were assured if $\geq 3\times10^6$ unsorted primary human SCCa cells were xenografted (28 successful xenograft tumors in 29 attempts) with SCCa xenografts recapitulating the original SCCa histology. The SCCa xenograft success rate demonstrated a clear dose response (n=82 xenografts), irrespective of the histological grade, with no SCCa growth detectable for xenografts initiated with $\leq 10^4$ unsorted primary human SCCa cells (Figure 3b). The estimated TIC frequency in unsorted SCCa cell suspensions is 1 TIC in 1.4×10^6 total unsorted SCCa cells (0.00007%) (Table S3), a TIC frequency within the range previously reported for other human and mouse carcinomas (Dick, 2009, Kennedy, 2007).

CD133+ primary human SCCa keratinocytes are enriched for TICs

We assessed TIC enrichment in CD133+ and CD71+ SCCa subpopulations because of their increased CFE *in vitro*. For CD133+ CD45− sorted SCCa keratinocytes from 28 different human SCCa specimens, reproducible xenograft tumor growth (n=41 xenografts) was seen following implantation of $10^2 – 10^5$ CD133+ CD45− keratinocytes. We estimate that TIC frequency is 1 TIC for every 432 CD133+ CD45− SCCa keratinocytes (0.23%) (Figure 4a; Table S4). Importantly, CD133+ CD45− SCCa xenografts, after 12 weeks of *in vivo* growth, recreated the original primary human SCCa histology (Figure S8). The measured sizes of the SCCa xenograft tumors, correlated with the numbers of CD133+ CD45− cells implanted (Figure S9). Of note, sorted CD133− CD45− SCCa keratinocytes did not yield SCCa xenograft tumors, even at numbers sufficient for reproducible growth with unsorted cells. 3×10^6 CD133− CD45− cells were implanted from 20 different SCCa in 33 failed xenograft attempts (Figure 4a, first column labeled CD133−) without growth.

When various numbers of CD71+ CD45− SCCa keratinocytes from 6 different SCCa specimens were xenografted into 11 mice, only one SCCa tumor was detected in a mouse that received 10^4 CD71+ CD45− keratinoctyes. No tumor growth was observed when 10^5 CD71− CD45− SCCa keratinocytes were xenografted (data not shown).

These results support our contention that TIC reside in the outer layers of human SCCa tumor projections, and that TIC can be greatly enriched using the cell surface marker CD133. Sorted CD133+ CD45− keratinocytes were able to initiate and recreate the histological heterogeneity of the original tumors, while CD133− CD45− cells keratinocytes failed to form tumors.

CD133+ human SCCa keratinocytes can self-renew

An essential property of TIC is their ability to self-renew (in analogy to tissue stem cells) and to reconstitute SCCa tumor heterogeneity during serial transfer into new recipient mice. To test this, we serially transferred CD133+ SCCa keratinoctyes derived from SCCa xenografts that were initially prepared from 8 different primary human SCCa specimens (Figure 4b). When cell suspensions from the second generation SCCa xenografts were analyzed by flow cytometry, approximately 1% of the SCCa keratinocytes were CD133+ (0.7% +/− 0.5%; n=11) (Table S5), strikingly similar to the percentage of CD133+ CD45− SCCa keratinocytes in the original SCCa tumors. Different histologic grades of second generation SCCa xenografts contained comparable percentages of CD133+ CD45+ cells (Figure S10). When serially xenografted into recipient mice, CD133+ SCCa keratinocytes obtained from the initial SCCa xenografts produced secondary SCCa xenograft tumors in a dose-dependent manner when 10^2 to 10^4 keratinocytes were implanted (Figure 4c) and had an estimated TIC frequency of 1 TIC per 863 CD133+ keratinocytes (Table S6). Of the 14 secondary CD133+ xenografts from the 8 different primary xenograft tumors, 10 demonstrated xenograft growth (Figure 4c) and all that grew recapitulated original tumor histologies (Figure S8) and maintained the original patterns of differentiation (involucrin expression), proliferation (Ki67 expression) and mutant p53 expression (Figure 5). CD133− keratinocytes from initial xenografts again failed to initiate tumors when serially implanted (Figure 4c, first column (CD133− cells)) thus arguing against *in vivo* selection. In conclusion, primary human SCCa contain TIC that reside in a small fraction of CD133+ CD45− SCCa keratinocytes located in the basal layer of the tumors and exhibit the stem cell property of self renewal.

Discussion

During human SCCa growth, tumor projections or “fingers” with proliferating outer layers of CD71+ keratinocytes invade into the underlying dermis. These proliferating leading edges surround central masses of more differentiated keratinocytes that are CD24+ and CD146+ and that express the late differentiation marker involucrin. Relatively rare small clusters of CD133+ TIC keratinocytes are also present within the proliferating CD71+ leading edge of SCCa tumor projections.

Our data provide strong experimental support for the concept that TIC in human SCCa are present and enriched in the CD133+ SCCa subpopulation: 1) the CD133+ subset can accurately recreate the histology and organizational hierarchy of the original SCCa tumor when transplanted in a xenograft mouse model, while CD133− sorted SCCa cells are consistently unable to recapitulate human SCCa; 2) the CD133+ subpopulation is capable of efficient serial passage while CD133− lack serial passage ability, demonstrating self-renewal, high proliferative capacity and long-term SCCa reconstitution in the CD133+ subset; 3) the percentage of CD133+ cells in the original tumors and in serial passaged xenografts was relatively invariant (approximately 1%) and the TIC frequency in these CD133+ subsets also was relatively constant (approximately 1/500). Additionally, within the CD133+ population, subsets that are even more highly enriched for TIC may be present and ultimately identifiable.

TIC have been proposed for a number of cancers (Dick, 2009). In the cancer stem cell model, tumor growth is determined by a relatively small sub-population of cells which are solely responsible for initiating and maintaining the tumor. In contrast to the classical model of tumor growth, the cancer stem cell model predicts that the majority of the tumor is composed of differentiated cells with limited proliferative capacity that are unable to regenerate the tumor. Consistent with this view, SCCa often contain differentiated keratinocytes resulting in the “horny pearls” used by pathologist to diagnose them. Similar to normal epidermal maintenance, TIC are believed to maintain tumor growth by virtue of their stem cell-like ability to self renew and produce differentiated progeny along predetermined lineages, and their long lived proliferative capacity. The cancer stem cell model predicts that effective cancer therapies must target this subpopulation of cells (TIC).

We do not know if TIC in human SCCa are derived from normal keratinocyte stem cells or more committed progenitor cells in human epidermis. CD133+ cells can be detected in normal human epidermis by FACS analysis, but the function of these cells in normal epidermis and whether these cells have a functional relationship to the CD133+ keratinocytes that are enriched for TIC in human SCCa is uncertain. Another unresolved question is what functional role, if any, the prominin-1 (CD133) membrane glycoprotein has in TIC biology. Although prominin-1 has previously been shown to interact with membrane cholesterol microdomains and to be involved in the processing and release of membrane particles from neural progenitors and epithelial cells, a direct biological link to stem cell behavior remains elusive (Dubreuil *et al.*, 2007).

We hypothesize that human cutaneous SCCa are derived from quiescent CD133+ malignant keratinocytes, so-called TIC, which differentiate and give rise to invasive tumors. These CD133+ keratinocytes merit further study and should help us understand the origin of TIC and the signaling pathways are important for their maintenance. Additionally, it is possible that similar molecular mechanisms of tumor initiation may exist for other epithelial squamous cell carcinomas, such as head and neck cancers, and the selective targeting of their TIC may be an effective therapeutic approach.

Material and Methods

Immunofluorescence

Immunofluorescence was performed using standard techniques with the following primary antibodies: Involucrin (Abcam, clone Sy5), Ki67 (Dako, clone mib-1), CD71 (BD Pharmingen, M-A712), Keratin 14 (gift from Dr Julie Segre), CD133 (Miltenyi Biotec, clone AC133) and CD31 (BD Pharmingen, clone WM59).

Immunohistochemistry

Immunohistochemistry was performed using standard antigen retrieval techniques with the following primary antibodies: CD24, CD34, CD71, CD146 (all BD Pharmingen), CD200 (AbD Serotec, clone ox-104), pancytokeratin (Dako, clone AE1/3), involucrin, Ki67 and p53 (BD Pharmingen, clone DO-7).

Cell preparation

As previously described (Patel, 2011), in brief human squamous cell carcinomas were obtained with IRB approval. Tumor tissue was mechanically dissociated, then incubated for 2 hours with Dispase (BD Biosciences) and Ultrapure Collagenase Type III (Sigma) at 37°C. Supernatants were removed after brief centrifugation, and the remaining tissue was then incubated at 37°C for 5 minutes with trypsin 0.05% with EDTA to create single cell suspensions. Tissue remnants were removed by filtration through a 40 μm cell strainer.

Flow cytometry

Samples were assessed using a FACSCalibur flow cytometer (BD Biosciences) and mouse fluorochrome-conjugated IgG subtype isotype controls (BD Pharmingen). Live cell gates were created using 7-Amino-Actinomycin D (BD Pharmingen) to label dead cells. Cells were labeled according to manufacturer's instructions with fluorochrome-conjugated antibodies, CD44-PE (BD Pharmingen), CD71-PE or APC (BD Pharmingen), CD45-FITC (BD Pharmingen), AC133-APC (Miltenyi-Biotec), and 293C3-APC (Miltenyi-Biotec). Data was analyzed using Flowjo software (Tree Star Inc).

Cell sorting

For magnetic separation, immediately after enzymatic dissociation, cells were sorted using the Dead Cell Removal Microbead kit (Miltenyi-Biotec), as per manufacturer's instructions. Viable cells were initially negatively sorted using CD45 microbeads and then AC133 microbeads were used to isolate CD133 positive and negative populations using a mini-MACS Separator (Miltenyi-Biotec) and MS columns (Miltenyi-Biotec) for *in vitro* (n=3) and *in vivo* (11 of 28 primary CD133+ CD45− and CD133− CD45− xenografts) experiments.

For flow sorting using a FACS Aria (Becton Dickinson), cells were labeled with CD45−FITC, AC133-APC or CD71-PE and the vital dye 7-Amino-Actinomycin D.

In vitro assay and tissue culture

Tumor samples were subjected to mechanical and enzymatic dissociation as described above under Cell Preparation. Primary human SCCa suspensions formed spheroidal colonies when grown in an adapted keratinocyte colony forming efficiency assay that incorporates a NIH3T3 feeder layer to support growth, and that we had previously used to detect normal human keratinocyte stem cells.

Transplantation of human SCCa cell suspensions

As described previously (Patel, 2011), athymic nude homozygous foxn1^{nu} (Jackson Lab), SCID-beige and NOD-SCID (Taconics) mice were maintained under conditions approved by the Animal Care and Use Committee. Mice were anaesthetized and either Gelfoam™ dressings (Johnson & Johnson) or glass discs were implanted into dorsal subcutaneous spaces 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 and glass discs were removed, and

then unsorted or magnetic or flow sorted CD133+ and CD133− purified populations together with 10^6 primary human fibroblasts resuspended in 100 μl of Matrigel were injected into spaces or alternatively into the gelfoam™ dressings *in-situ*. After 12 weeks, mice were euthanized by CO_2 inhalation and tumors were removed for analysis.

Statistical analysis

The frequencies of CD45+, CD71+ and CD133+ cells are described as mean +/− standard deviations. The differences between the mean number of colonies (Figures S2c,d) and fold changes in colony number over unsorted populations (Figure 2e) were calculated using a paired t-test. Also, a t-test was used to compare the colony forming efficiencies of CD133+ versus CD133− and CD71+ versus CD71− subpopulations. For *in vivo* limiting dilution assays, the frequencies of cancer initiating cells were calculated using L-Calc software (Stem cell technologies Inc.), with Chi square analysis to determine internal consistency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

TIC	Tumor initiating cells
SCCa	Squamous cell carcinoma

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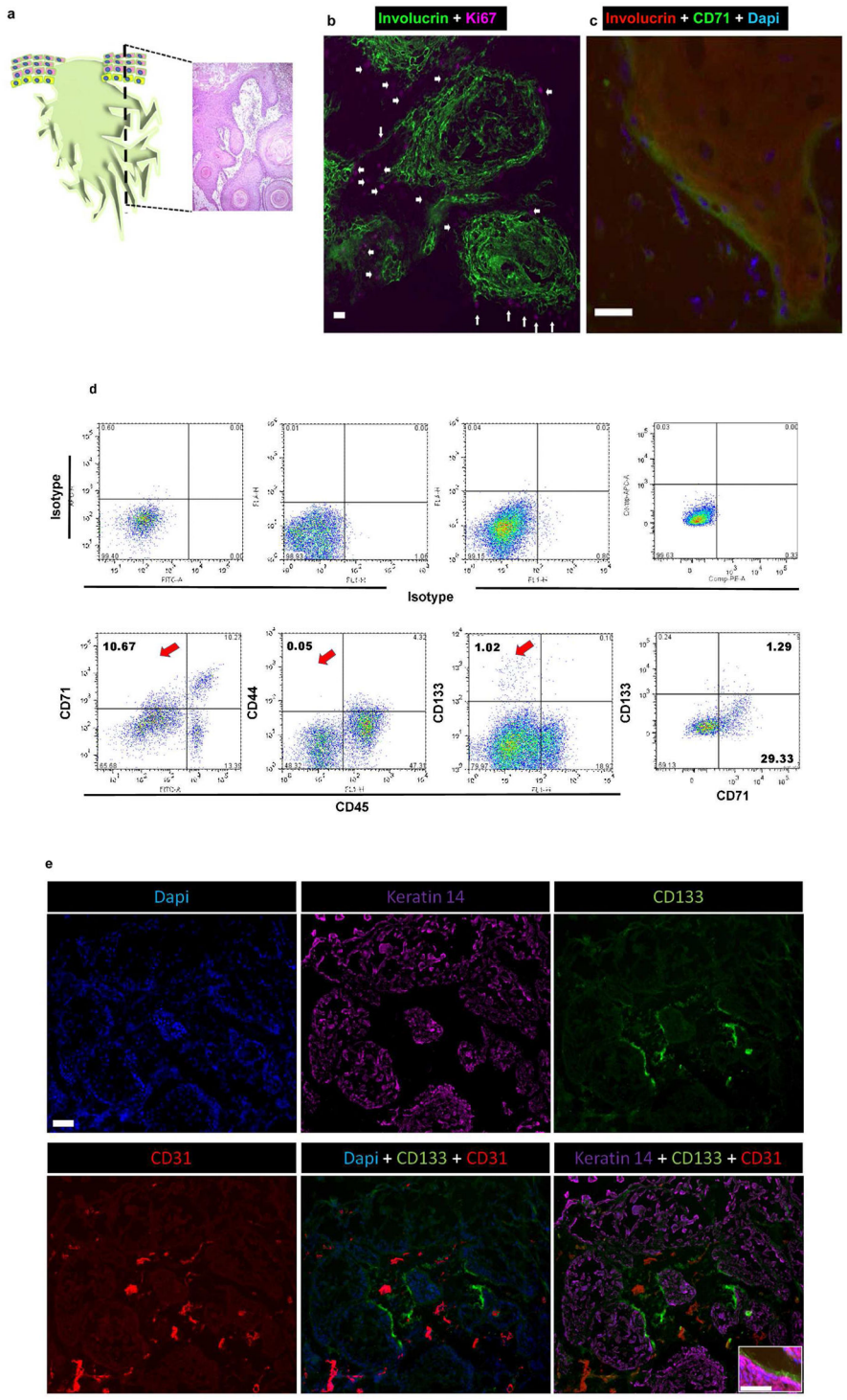


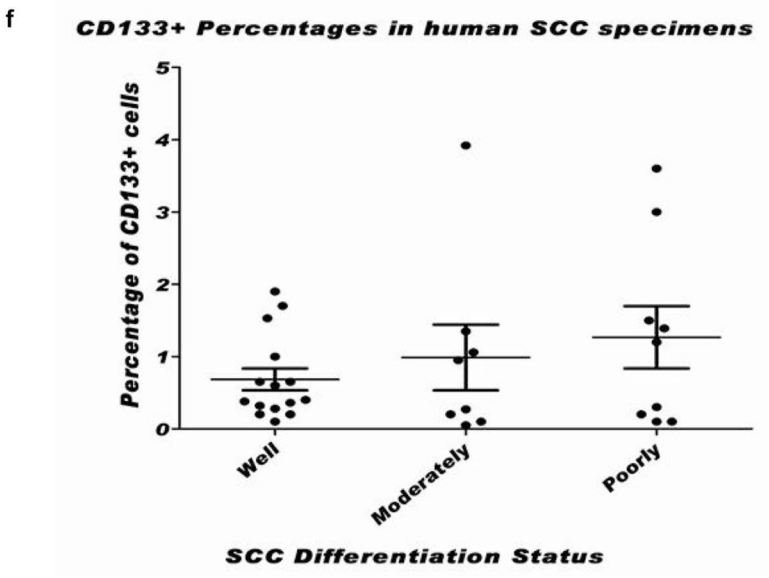
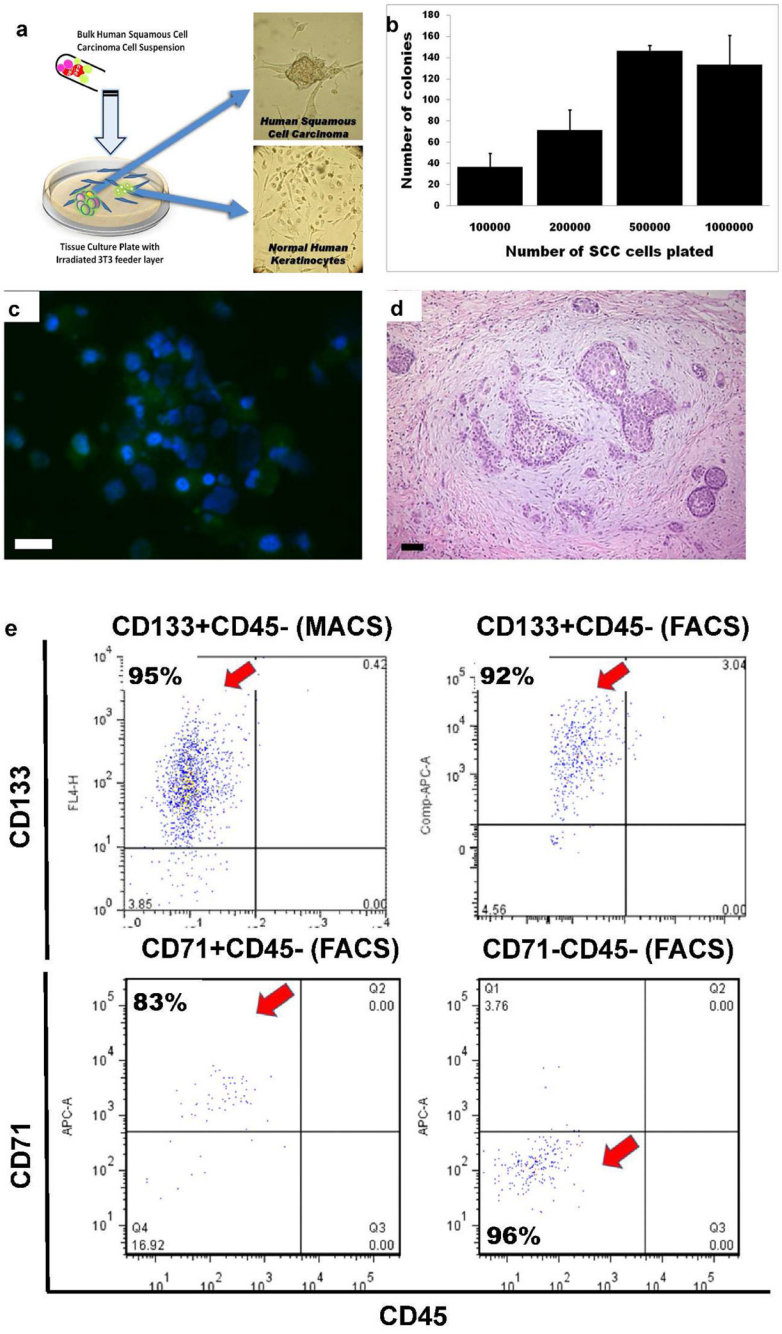


Figure 1. Characterization of SCCa cellular subsets

a, Schematic of a primary human SCCa and stained section. Immunofluorescence of a SCCa tumor section labeled for, **b,** involucrin (green) and KI67 (purple), and **c,** CD71 (green), involucrin (red) and counterstained with DAPI (blue). **d,** FACS of primary human SCCa's demonstrating cell surface marker expression together with isotype controls. The red arrows indicate the CD45 negative tumor subpopulation of interest. Also shown is a FACS plot of CD133 and CD71 of a CD45− subpopulation. **e,** Immunofluorescence of an SCCa section labeled with CD133 (green), CD31 (red), keratin 14 (purple) and DAPI (blue). All scale bars are 100 μm. **f,** CD133+ CD45− percentages from total cell suspensions derived from 32 different histological grades of primary human SCCa.



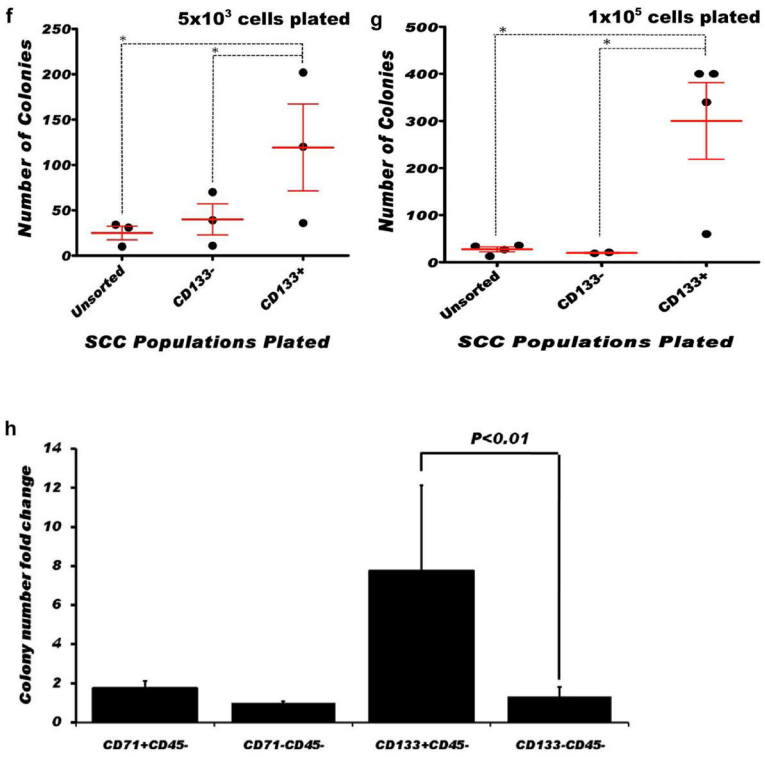


Figure 2. A CD133+ subpopulation in human SCCa with increased colony forming efficiency *in vitro*

a, A schematic showing how normal keratinocytes form adherent colonies while SCCa tumor keratinocytes form spheriodal colonies in our *in vitro* assay. **b** The number of colonies emerging from culture was dependent upon the number of cells plated. **c,** Spheroids were then dissociated and after cytospin onto a slide, were labeled with anti– pancytokeratin antibody, revealing the presence of keratinocytes within the spheroids. **d,** These cell culture colonies formed tumors, when xenografted onto the backs of prepared athymic nude mice. **e,** FACS for purity following magnetic bead separation (Macs™) or FACS of CD133+ CD45− (red arrow) primary human squamous cell carcinoma subpopulations (above panels). FACS for purity of the CD71+ CD45− (red arrow, left panel) and CD71− CD45− (red arrow, right panel) sorted subpopulation (lower panels). **f and g,** CD133 sorted cells demonstrate a statistically significant increased colony forming efficiencies as compared to equivalent numbers of unsorted and CD133− sorted cells (n=6, the two experiments utilized different primary tumor samples) ($p<0.01$). **h,** Fold change in colony forming efficiencies for CD133+, CD133−, CD71+ and CD71− subpopulations compared with unfractionated SCCa controls.

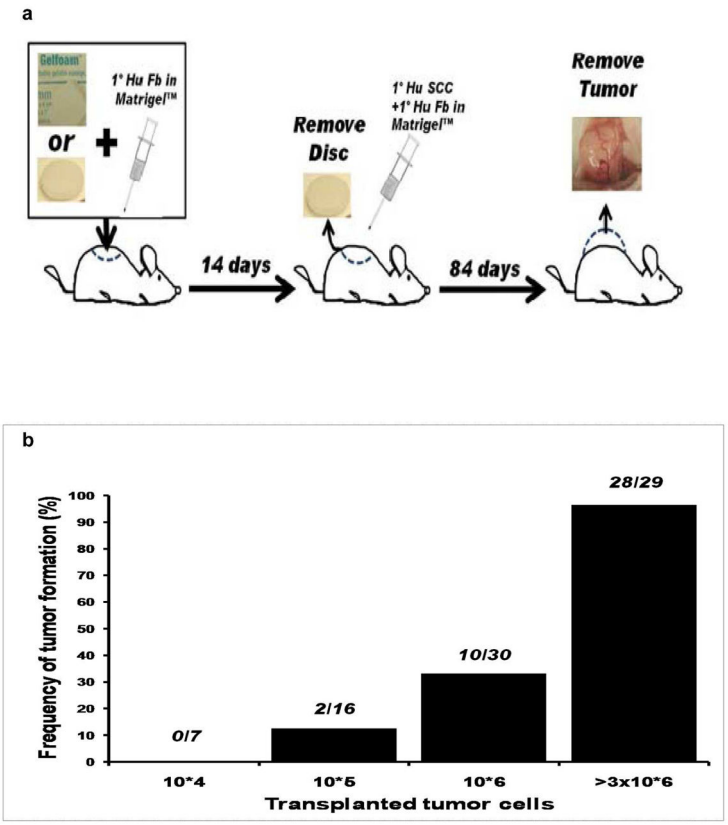


Figure 3. Creation of a SCCa in-vivo xenograft model that can accurately recapitulate and propagate human SCCa

a, Successful generation of primary human SCCa xenografts from cell suspensions required pre-implantation of either a glass disc or a Gelfoam™ dressing, combined with 10^6 primary human fibroblasts suspended in Matrigel™. After 14 days, SCCa tumor cells were combined with 10^6 primary human fibroblasts in Matrigel™ and implanted into either the Gelfoam dressing or the glass disc site. **b,** Xenograft tumor growth (n=82) following implantation of different doses of total unfractionated SCCa cells.

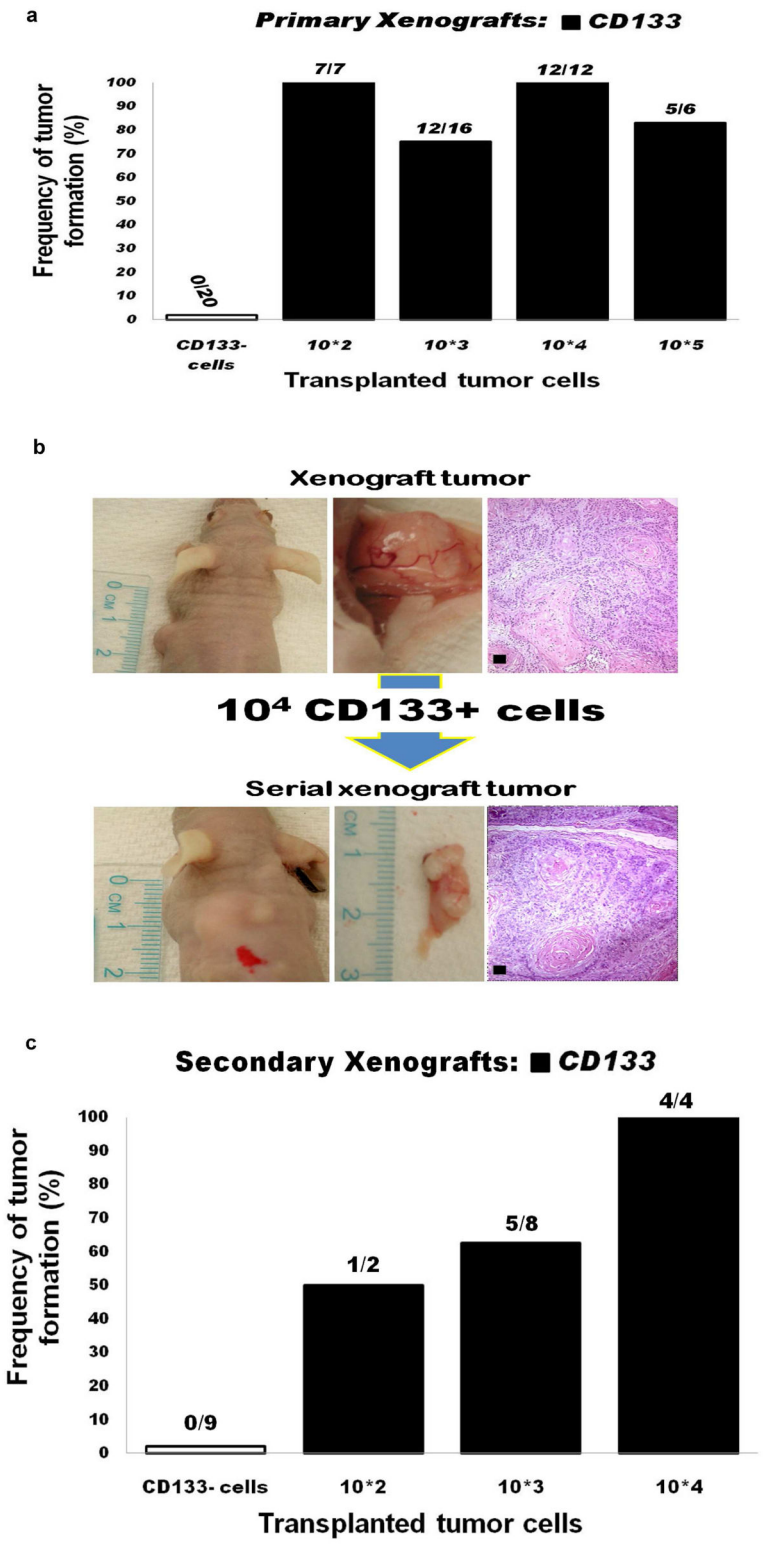


Figure 4. The CD133+ SCCa subpopulation is enriched for TIC

a, Xenograft tumor frequencies (n=42) after inoculation of CD133+ and CD133− sorted cells from 28 different primary human SCCa at varying doses, shown as a percentage and

total. No xenograft tumors developed from SCCa CD133− cell suspensions (n=20; far left column) at doses of: 3×10^6 (n=1), 2×10^6 (n=1), 4×10^5 (n=1), 2×10^5 (n=1), 10^5 (n=16). **b,** Eight human SCCa xenografts were removed and CD133+ and CD133− cells were isolated for serial passage (secondary xenografts) into athymic nude mice. **c,** serial passage secondary xenograft tumor frequency (n=14) for CD133+ cells isolated from primary SCCa xenografts. No secondary xenograft tumors developed from CD133− sorted cells (n=8; far left column), despite doses of: 10^6 (n=2), 2×10^5 (n=1), 10^5 (n=5).

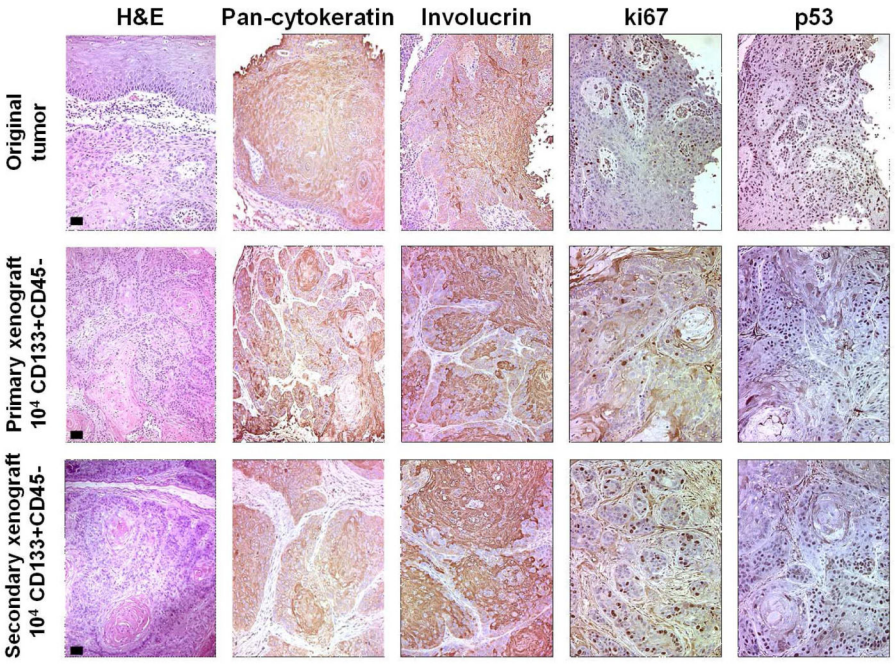


Figure 5. Primary and secondary xenografts generated from CD133+ CD45− human SCCa cells resemble the original SCCa tumors

Parent tumor histologies are compared to both primary and secondary xenografts derived from implantation of CD133+ SCCa cells. The initial passage represents a xenograft generated from the injection of 10^4 CD133+ CD45− primary human SCCa cells. The secondary xenograft was generated from the injection 10^4 CD133+ SCCa cells isolated from the primary CD133+ CD45− xenograft. The histology of the three tumors shows a well to moderately differentiated SCCa. The immunohistochemical markers AE1/3, involucrin, KI67 (MIB-1), and p53 reveal comparable staining patterns in both the primary and secondary CD133+ CD45− xenografts, as compared to the parent tumor. All scale bars are 100 μm.