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In vitro generation of human cells with cancer stem cell properties

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Abstract

Cancer stem cells (CSCs) have been implicated in the maintenance and progression of several types of cancer. The origin and cellular properties of human CSCs are poorly characterized. Here we show that CSC-like cells can be generated *in vitro* by oncogenic reprogramming of human somatic cells during neoplastic transformation. We find that *in vitro* transformation confers stem cell properties to primary differentiated fibroblasts, including the ability to self-renew and to differentiate along multiple lineages. Tumours induced by transformed fibroblasts are hierarchically-organized and the cells which act as CSCs to initiate and maintain tumour growth are marked by the stage-specific embryonic antigen SSEA-1. Heterogeneous lineages of cancer cells in the bulk of the tumour arise through differentiation of SSEA-1^{+} fibroblasts and differentiation is associated with loss of tumorigenic potential. These findings establish an experimental system to characterize cellular and molecular properties of human CSCs and demonstrate that somatic cells have the potential to de-differentiate and acquire properties of CSCs.

The cancer stem cell hypothesis postulates that cancers are hierarchically-organized and only a subset of cells, the cancer stem cells (CSCs), drive cancer growth[1,2]. Defining features of putative CSCs are tumour-initiation potential, self-renewal capacity and the ability to differentiate into heterogeneous, non-tumorigenic cancer cells[1,2]. Little is known about the cellular origin of CSCs. In some cancers, CSCs arise by transformation of adult stem cells[3–6]. In addition, committed progenitors can acquire self-renewal ability and function as CSCs[7,8]. Whether differentiated somatic cells can aberrantly reprogram and acquire CSCs features is unclear.

CSCs have been implicated in the growth of several human cancers[9–17], but the characterization of their biological properties has been hampered by the difficulty of isolating pure CSC populations and manipulating them *ex vivo*. The inability in CSC populations isolated from clinical samples to retrospectively identify the cell which suffered

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AUTHOR CONTRIBUTIONS

P.S. conceived the study and carried out the experimental work. P.S. and T.M. designed the experiments and wrote the manuscript.

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the first oncogenic hit and drove tumorigenesis has prevented direct comparison between a CSC and its non-neoplastic counterpart.

Human differentiated cells can be transformed *in vitro* by expression of telomerase (hTERT), the oncogenic H-RasV12 mutant and concomitant inhibition of p53 and pRB pathways by simian virus 40 (SV40) Large-T (LT) and Small-T (ST) antigens[18]. Such transformed cells nucleate tumours when injected into immunocompromized mice[18]. We demonstrate here that during *in vitro*-transformation a subpopulation of fibroblasts reprogram to a more primitive, multipotent cell type which possesses all hallmarks of CSCs and generates hierarchically-organized tumours.

RESULTS

In vitro-transformed fibroblasts acquire differentiation ability

We *in vitro*-transformed primary skin fibroblasts by stably expressing hTERT, H-RasV12 and SV40 Large-T (LT) and Small-T (ST) antigens[18] (Supplementary Fig. S1a) and examined tumours induced by the transformed cells in immunocompromized mice. In line with their mesenchymal origin, tumours lacked distinct differentiated structures, but were heterogeneous in their composition and contained cells with various morphologies (Fig. 1a, upper left panel). Immunohistochemical staining showed expression of markers of various lineages in tumours derived from transformed fibroblasts. Cells positive for endothelial (hCD34), muscle (myosin) and neural (tubulin-β-III) markers were detected in several tumours (Fig. 1a). Semi-quantitative RT-PCR analysis confirmed expression of markers of various lineages in tumours, while very low or undetectable levels were expressed in the transformed fibroblasts prior to injection (Fig. 1b). No individual tumour expressed all markers, indicating that tumours induced by transformed fibroblasts did not have features of teratomas. These observations suggest that tumorigenic fibroblasts derived by *in vitro* transformation acquire the ability to differentiate along multiple lineages *in vivo*.

To directly demonstrate that the transformation process confers differentiation ability to somatic fibroblasts, we induced their differentiation *in vitro* along two distinct lineages. In contrast to hTERT-immortalized control fibroblasts, transformed cells from two independent fibroblast cell lines efficiently differentiated into adipocytes and osteoblasts with similar efficiency as human mesenchymal stem cells (Fig. 1c–f). Taken together, these results indicate that during *in vitro* transformation differentiated fibroblasts reprogram into a less committed, more primitive cell type capable of differentiating along various lineages during tumour growth. This property is reminiscent of the ability of CSCs to generate intra-tumour heterogeneity by differentiating along various lineages. We therefore explored the possibility that cells with CSCs properties arise during transformation of differentiated fibroblasts.

SSEA-1 is a marker of tumorigenic in vitro-transformed cells

In hierarchically-organized tumours, not all cells contribute equally to tumour growth and only a distinct subset of cells, the CSCs, is tumorigenic[2,19]. To identify potential markers of putative CSCs generated by *in vitro* transformation we probed for surface markers enriched

in two cell lines of transformed fibroblasts compared to the corresponding non-tumorigenic hTERT-immortalized fibroblasts. Several known CSC markers, including CD44[12], CD133[13,14], ABCG2[20] or ABCB1[20], and the human embryonic stem cell (hES) markers SSEA-4, TRA-1-60 or TRA-1-81[21] were present in *in vitro*-transformed fibroblast cell lines, but were detected at similar levels in hTERT-immortalized control cells, disqualifying them as markers of tumorigenicity in our system (Fig. 2a). In contrast, the stage-specific embryonic antigen SSEA-1, a hES cell early differentiation marker[21], previously identified as a CSCs marker in human and mouse brain tumours[17,22,23], was detected in ~1% of transformed cells but was absent from primary and hTERT-immortalized cells (Fig. 2a,b, Supplementary Fig.S2a). Immunofluorescence microscopy confirmed the presence of SSEA-1 in ~1% of transformed cells while no positive cells were detected in the immortalized population (Fig. 2c). Similarly, ~ 9% of *in vitro*-transformed mammary epithelial cells[24] were immunopositive for SSEA-1 while only ~ 0.2% of SSEA-1$^+$ cells were detected in primary cells ($p < 0.001$) (Fig. 2d), indicating that acquisition of SSEA-1 upon *in vitro*-transformation is not a unique property of fibroblasts. SSEA-1$^+$ cells were also detected in clinical samples of fibrous tissue cancers with SSEA-1$^+$ cells present at varying abundances in 59% of the analyzed tumours (n = 69) (Fig. 2e).

To rule out the possibility that rare, pre-existing stem cells in the population of primary cells are the source of SSEA-1$^+$ cells, we infected primary fibroblasts with limiting amounts of viruses so that only ~1:25,000 cells were infected with all three transforming factors. Under these conditions, the probability of infecting a rare stem cell present in the cell population is low (Supplementary Fig. S2b). Four randomly selected colonies of infected cells all contained SSEA-1$^+$ cells and injection of two clones into immunocompromized mice resulted in tumour formation, indicating that SSEA-1$^+$ cells do not arise from transformation of rare, pre-existing stem cells (Fig. 2f, Supplementary Fig. S2b). We conclude that the presence of the hES cell early differentiation marker SSEA-1 in populations of differentiated cells correlates with acquisition of tumorigenicity upon transformation. These observations suggest SSEA-1 as a marker of putative CSCs generated by *in vitro* transformation.

Characterization of SSEA-1$^+$ transformed fibroblasts

A characteristic feature of stem cells is their ability to both self-renew and generate phenotypically distinct daughter cells[25,26]. SSEA-1$^+$ sorted cells grown in culture rapidly generated a heterogeneous progeny with the percentage of SSEA-1$^+$ cells in the population decreasing to that of the unsorted population in 6 days (Fig. 3a). In agreement, immunofluorescence microscopy revealed that ~21% of cell divisions resulted in one SSEA-1$^+$ and one SSEA-1$^-$ daughter cell, whereas ~79% of divisions yielded two SSEA-1$^+$ daughter cells (Fig. 3b,c, Supplementary Fig. S3), confirming that SSEA-1$^+$ cells can generate SSEA-1$^-$ progeny *in vitro*. Conversely, SSEA-1$^-$ fibroblasts can spontaneously convert into SSEA-1$^+$ cells. SSEA-1$^+$ cells appeared during culturing of sorted SSEA-1$^-$ cell populations 5 days after sorting and their abundance increased over time (Fig. 3a). The presence of SSEA-1$^+$ cells was not due to selection of contaminating SSEA-1$^+$ cells after sorting, because individual clonal populations originated from single SSEA-1$^-$ cells contained various fractions of SSEA-1$^+$ cells (8.4%, 2.3% and 1.9%), indicating that SSEA-1$^-$ fibroblasts stochastically convert into SSEA-1$^+$ cells (Fig. 3d,e).

We characterized the expression profiles of SSEA-1$^+$ and SSEA-1$^-$ fibroblasts by microarrays analysis. ~300 genes were differentially expressed in the two subsets of cells (Fig. 3f). Amongst the most upregulated genes in SSEA-1$^+$ fibroblasts were several involved in tumorigenesis and several cancer biomarkers, whereas many tumour suppressors were downregulated (Supplementary Table 1). Differences in the transcriptional profiles of SSEA-1$^+$ and SSEA-1$^-$ fibroblasts were not due to different expression levels of hTERT, H-RasV12 and SV40-T antigens (Supplementary Fig. S1b).

In vitro-generated SSEA-1$^+$ fibroblasts are tumour initiators

To test whether *in vitro*-generated SSEA-1$^+$ cells have tumour-initiating capacity as predicted for CSCs, we performed an *in vivo* lineage-tracking experiment to assess the relative contribution of SSEA-1$^+$ and SSEA-1$^-$ fibroblasts to tumour initiation in a model of competitive tumour development. SSEA-1$^+$ cells from a GFP-labelled transformed fibroblast cell line and SSEA-1$^-$ cells from an unlabeled line generated from the same parental line (see Methods) were co-xenografted and the percentage of GFP-labelled cells in the resulting tumours was evaluated (Fig. 4a). As a positive control, when 100% of unsorted GFP-labelled transformed cells were injected, the vast majority of tumour cells were GFP$^+$ (89.3% ± 2.3) (Fig. 4b). When GFP-labelled SSEA-1$^+$ cells were co-injected with competing unlabeled SSEA-1$^-$ cells (28% :72%), 4 out of 5 tumours showed a high percentage of GFP-positive cells (76.1% ± 7.7) comparable to the positive control, while one tumour showed a lower fraction (30%) (Fig. 4b). Similarly, tumours derived from a mixed population of GFP-labelled SSEA-1$^+$ fibroblasts and unlabeled non-tumorigenic hTERT-immortalized fibroblasts (28% :72%) contained mostly GFP$^+$ cells (87.7 ± 2.7%) (Fig. 4b). These results demonstrate that SSEA-1$^+$ fibroblasts are the cells primarily responsible for tumour development and contribute to most of the tumour mass, while SSEA-1$^-$ fibroblasts are mostly non-tumorigenic. Immunohistochemical staining of tumour sections confirmed these results (Fig. 4c). As a control, GFP-labelled SSEA-1$^-$ cells mixed with unlabeled SSEA-1$^-$ cells (29% :71%) resulted in growth of only 2 out 4 tumours, in which the relative abundance of GFP-labelled cells in the tumours was not increased compared to the injected cells (21.8 ± 21.5 vs. 29%, $p > 0.025$), as expected for labelled and unlabeled cells with equal tumorigenicity (Fig. 4b). In accordance with our observation that SSEA-1$^+$ fibroblasts can spontaneously originate from SSEA-1$^-$ cells, we found SSEA-1$^+$ cells in SSEA-1$^-$-derived tumours (Supplementary Fig. S4), suggesting that SSEA-1$^-$ fibroblasts may serve as precursors of tumorigenic SSEA-1$^+$ cells. Taken together, these results demonstrate that tumour formation is predominantly driven by SSEA-1$^+$ cells, consistent with their function as CSCs.

SSEA-1$^+$ fibroblasts differentiate into heterogeneous, non-fibroblastic cancer cells

CSCs generate the bulk of a tumour by differentiating into heterogeneous, non-tumorigenic cancer cells[2,19]. In line with differentiation of tumour-initiating SSEA-1$^+$ fibroblasts, tumours originating from either unsorted or SSEA-1$^+$ fibroblasts contained >90% of SSEA-1$^-$ cells (Fig. 5a,b). Furthermore, immunohistochemical staining of serial sections from tumours derived from GFP-labelled SSEA-1$^+$ cells detected cells positive for endothelial (hCD34), muscle (myosin) and neural (tubulin-β-III) markers in GFP-labelled

areas (Fig. 5c), indicating that GFP-labelled SSEA-1$^+$ fibroblasts had differentiated into multiple cell types during tumour growth.

To demonstrate that single SSEA-1$^+$ transformed fibroblasts are multipotent we examined tumours induced by clonally-derived populations from SSEA-1$^+$-sorted cells. Tumours induced by three individual clones expressed markers of multiple germ layers in various combinations (Supplementary Fig. S5a), indicating that single SSEA-1$^+$ fibroblasts have the potential to initiate heterogeneous tumours. Directed differentiation along multiple mesenchymal lineages *in vitro* confirmed multipotency of clonal populations derived from single SSEA-1$^+$ fibroblasts (Supplementary Fig. S5b,c).

In line with differentiation of transformed fibroblasts into other cell types during tumour growth, several mesenchymal markers were expressed at reduced levels in tumours. About ~80% of tumour cells expressed lower levels of CD166 compared to the injected fibroblasts, as assessed by FACS, and quantitative RT-PCR showed a ~5-fold decrease in *CDH2* and *FGF2* (Fig. 5d–f). Other markers such as CD29 and CD10 were unchanged, indicating that tumour cells do not lose all mesenchymal features (Fig. 5e). Furthermore, upon tumour dissociation only a minor percentage of cells (~ 0.003%–1%, dependent on the number of injected cells), displayed fibroblastic morphology and could be propagated *in vitro* in the same conditions as the injected fibroblasts (Fig. 5g). We refer to these cells as tumour fibroblasts. Tumour fibroblasts expressed high levels of CD166, *CDH2* and *FGF2* and were positive for SSEA-1 (Fig. 5d–f,h). Conversely, SSEA-1$^-$ cells from primary tumours were non-fibroblastic and expressed reduced levels of mesenchymal markers (Fig. 5d, Supplementary Table S2). This observation highlights that tumour SSEA-1$^-$ cells generated by differentiation of SSEA-1$^+$ fibroblasts during tumour growth are a distinct cell state from the SSEA-1$^-$ fibroblasts which arise during transformation. Taken together, these results demonstrate that SSEA-1$^+$ *in vitro*-transformed fibroblasts are multipotent and generate tumour heterogeneity by differentiating into non-fibroblastic cells, as expected for CSCs.

SSEA-1$^+$ fibroblasts maintain hierarchically-organized tumours

A critical feature of CSCs is that they generate hierarchically-organized tumours in which their progeny lose tumorigenic potential during differentiation and do not contribute to tumour maintenance. As a consequence, only a minor fraction of cells in a tumour are tumorigenic[2]. To assess whether *in vitro*-generated SSEA-1$^+$ fibroblasts give rise to hierarchically-organized tumours we estimated the number of tumorigenic cells in primary tumours. About 1: 1,875 tumour cells were able to grow in soft agar and ~1:5,000 cell were able to form spheres in suspension in low adherent conditions, a common feature of various CSCs[27] (Fig. 6a–c). Furthermore, limiting dilution transplantation experiments in NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice estimated a frequency of tumorigenic cells of 1:8,805 *in vivo* (Fig. 6d). Thus, consistent with hierarchical organization, tumours induced by *in vitro*-transformed fibroblasts contain only a small fraction of tumorigenic cells which maintain tumour growth.

To assess whether it is the multipotent SSEA-1$^+$ cells which sustain tumour growth rather than the differentiated SSEA-1$^-$ cells, we isolated SSEA-1$^+$ and SSEA-1$^-$ cells form primary tumours and assessed their tumorigenic potential. While ~1: 1,250 and ~1:5,000

SSEA-1$^+$ tumour cells were clonogenic in soft agar and sphere formation assays, respectively, SSEA-1$^-$ cells did not form colonies in either condition (Fig. 6b,c). In agreement, the number of tumorigenic cells *in vivo* in the two subsets of cells was significantly different and SSEA-1$^-$ cells induced tumours only at high dose (1:2,045 for SSEA-1$^+$cells and 1:46,766 for SSEA-1$^-$ cells, p = 1.46e–05) (Fig. 6d). Despite the difference to SSEA-1$^-$ cells, the relatively low frequency of tumorigenic cells in the subset of SSEA-1$^+$ cells indicated heterogeneity of the subpopulation. When we isolated the subset of fibroblastic SSEA-1$^+$ cells from tumours (~ 1% of the sorted population) we observed significant enrichment in the number of clonogenic and tumorigenic cells. About 1:3 and 1:16 cells were able to grow in soft agar and as spheres in suspension, respectively ($p < 0.001$), and 1:80 cells were tumorigenic *in vivo* (p= 6e–08) (Fig. 6a–d). These results demonstrate that the cells responsible for tumour maintenance are a subpopulation of SSEA-1$^+$ tumour cells which maintain a similar fibroblastic cellular nature as the cells that initiate the tumour, while their differentiated progeny is non-tumorigenic. We conclude that SSEA-1$^+$ fibroblasts generated by *in vitro* transformation, like CSCs, posses the ability to give rise and maintain hierarchically-organized tumours.

Hierarchical organization of tumours induced by *in vitro*-transformed cells was confirmed in allotransplantation experiments using murine cells. BALB/c primary skin fibroblasts were transformed by introducing H-Ras-V12 and SV-40 T-antigens[28] and injected in BALB/cAnNCr-*nu/nu* mice. Similarly to human tumours, primary murine tumours contained a low number of tumorigenic cells (~1:36,735) and tumour fibroblasts (~5% of the tumour cell population) were enriched for clonogenic (~1:28 vs. ~1:517 in the unsorted population, $p < 0.001$) and tumorigenic cells (~1:1,056 vs. ~1:36,735, p = 2.9e–06) (Supplementary Fig. S6a–c). As for human cells, SSEA-1$^+$ cells were absent from primary mouse fibroblasts and appeared in the transformed population (Supplementary Fig. S6d,e). Unlike human SSEA-1$^+$ fibroblasts, however, mouse SSEA-1$^+$ fibroblasts did not generate SSEA-1$^-$ progeny *in vitro* or *in vivo*, were not enriched in the tumour-maintaining fibroblastic population of primary tumours, and were not more clonogenic than SSEA-1$^-$ cells (Supplementary Fig. S6b,f–i). These observations indicate that both human and mouse tumorigenic cells induced by *in vitro*-transformation give rise to hierarchically-organized tumours, but are characterized by different markers, in line with the notion that SSEA-1 marks different cell states in human and mouse[21].

SSEA-1$^+$ CSC-like cells exhibit a distinct molecular signature

To characterize the molecular changes associated with cellular differentiation during tumour growth we compared the transcriptional profiles of human SSEA-1$^+$ tumour fibroblasts and their SSEA-1$^-$ progeny isolated from three primary tumours. The two subsets of tumour cells exhibited distinct transcription profiles in every tumour with up to 1100 genes differentially expressed (≥2-fold) (Fig. 6e, Supplementary Table 2). ~230 genes were differentially expressed in all tumours in SSEA-1$^+$ and SSEA-1$^-$ cells (≥2-fold, $p< 0.05$) (Fig. 6e, Supplementary Table 2). Importantly, 70% of these common genes were expressed at similar levels ($p>0.05$, P< 2-fold) in SSEA-1$^+$ tumour fibroblasts and in the SSEA-1$^+$ transformed fibroblasts that were injected to induce primary tumours (Fig. 6f). These results confirm that the tumour-maintaining SSEA-1$^+$ cells retain a fibroblastic identity whereas

their SSEA-1$^-$ progeny acquire different cellular phenotypes. In line with differentiation and loss of tumorigenic potential, upregulated genes in SSEA-1$^-$ cells included several regulators of differentiation and inhibitors of cellular proliferation, whereas genes promoting tumorigenesis, oncogenic miRNAs, and negative regulators of differentiation were upregulated in SSEA-1$^+$ tumour fibroblasts (Supplementary Table 2).

Self-renewal and evolution of SSEA-1$^+$ CSC-like cells in tumours

A key property of CSCs is their ability to self-renew in tumours. The capacity of SSEA-1$^+$ tumour fibroblasts to reconstitute secondary tumours and their molecular similarity with the SSEA-1$^+$ transformed fibroblasts which initiate primary tumours are indicative of their self-renewal capacity *in vivo* (Fig. 6d,f, Fig. 5d–f). In addition, SSEA-1$^+$ tumour fibroblasts from secondary tumours transplanted into new recipient mice formed tertiary tumours, indicating self-renewal over several passages (Fig. 7a). At every passage, the percentage of SSEA-1$^+$ cells in the tumours increased (~3.1%, ~7.9%, and ~25%) (Fig. 7a,b) and higher abundance of SSEA-1$^+$ cells correlated with higher number of clonogenic cells (~1:3759, ~1:1162, and ~1:156) as assed by colony formation in soft agar and by faster development of tumours (27d, 13d and 11d) (Fig. 7a,c). This result suggests evolution of SSEA-1$^+$ tumour fibroblasts cells over passages and selection of more aggressive cells, yet maintaining a hierarchical organization of tumours, a phenomenon described for several types of CSCs[29,30]

Taken together, these results demonstrate that *in vitro*-transformed SSEA-1$^+$ fibroblasts have all hallmarks of CSCs including multipotency, self-renewal and the ability to generate hierarchically-organized tumours by differentiating into phenotypically distinct, non-tumorigenic cells.

DISCUSSION

We show here that cells with CSC properties can be generated *in vitro* by oncogenic reprogramming of somatic cells. We find that during *in vitro* transformation, a distinct subpopulation of cells arises which is responsible for tumour initiation and maintenance. These cells are marked by the presence of SSEA-1, have self-renewal potential, are multipotent and generate hierarchically-organized tumours by differentiating into non-tumorigenic cells. SSEA-1$^+$ CSC-like cells are molecularly distinct from SSEA-1$^-$ cells in the population of transformed fibroblasts and from their differentiated progeny in tumours, in that they express a tumorigenic transcription program, while repressing numerous tumour suppressors.

The CSC model postulates that CSCs may arise either from stem cells or progenitors, or may be generated from differentiated somatic cells through multiple mutagenic events[1,19]. While there is evidence, particularly from studies on hematological cancers, that CSCs can derive from tissue stem and progenitor cells[3,4,7,8], whether somatic cells can do so has been unclear. Our findings demonstrate that somatic cells possess enough plasticity to reprogram and acquire CSC properties upon oncogenic insults *in vitro* and may act as cells of origin of cancer.

Oncogenic reprogramming of differentiated cells into CSC-like cells appears to be a stochastic process within the population of *in vitro*-transformed cells since, despite the presence of telomerase, H-RasV12 and SV40-T antigens in all fibroblasts, not all cells are equally tumorigenic and tumour formation is mainly driven by SSEA-1^+ fibroblasts. Our observation that SSEA-1^- transformed fibroblasts can stochastically convert into SSEA-1^+ cells and are able to induce inefficient tumour growth, suggests a model where telomere maintenance, activation of an oncogene, and inhibition of tumour suppressor pathways generate pre-malignant SSEA-1^- cells, which then develop, via additional mutations or epigenetic changes, into malignant SSEA-1^+ cells (Fig. 8).

The reprogramming of differentiated cells into CSC-like cells upon transformation is reminiscent of the de-differentiation of somatic cells into induced pluripotent stem (iPS) cells[31]. Interestingly, most reprogramming factors employed to induce iPSs have been implicated in tumorigenesis[32–36], suggesting that reprogramming and cellular transformation might occur through related pathways. Moreover, inhibition of p53 enhances reprogramming of fibroblasts into iPS cells, although they are genetically unstable[38]. It is conceivable that during transformation, normal pathways governing development and cell fate may be co-opted in the absence of functional tumour suppressor mechanisms. De-differentiation of somatic cells into malignant stem cells or into normal pluripotent stem cells may thus represent different versions of similar reprogramming events.

Our ability to generate CSC-like cells *in vitro* will be a useful tool for the molecular characterization of CSCs. While rare and heterogeneous CSC populations isolated from clinical samples cannot be easily characterized and manipulated, *in vitro*-generated CSC-like cells represent an experimentally amenable system which can be directly compared to its non-cancerous source. It will now be possible to interrogate pathways involved in acquisition and control of cellular self-renewal in a targeted fashion and to characterize genetic and epigenetic events associated with CSCs tumorigenicity with the goal of uncovering new mechanisms of tumorigenesis and possible therapeutic targets.

METHODS

Generation of cell lines

The Transformed-1 and -2 human cell lines were generated essentially as described[18] from a primary skin fibroblast cell line (CRL-1474 from ATCC) and a hTERT-immortalized fibroblast cell line (gift of R. Faragher[39]), respectively. Retroviral constructs expressing human telomerase, SV40 Early region (LT and ST antigens) and oncogenic HRas-V12 were introduced serially. Retroviral supernatants were produced by transfecting amphotropic Phoenix cells (Orbigen) with pBABE-puro-hTERT, pBABE-zeo-LargeT genomic or pBABE-neo-ras-V12 (gifts of P. Adams). Infected cells were selected using puromycin (1 μgml^{-1}, 2–3 days), zeocin (100 $ngml^{-1}$, 7–10 days) or neomycin (1 $mgml^{-1}$, 7 days), respectively. To transform cells under condition of low infection efficiency, the three retroviral constructs were introduced simultaneously into primary fibroblasts using low titer viruses so that ~ 1:25,000 primary cells contained all three constructs. Four clonal populations were isolated after selection with puromycin, zeocin and neomycin. Primary and transformed fibroblasts were grown in minimum essential medium (MEM) containing 15%

fetal bovine serum (FBS), 2 mM L-glutamine, 100 Uml^{-1} penicillin and 100 μgml^{-1} streptomycin. Primary skin fibroblasts isolated from 3 days old BALB/c mice (gift of L. Li, NCI) were transformed by serially introducing retroviral constructs expressing SV40 T antigens and HRAS-V12 as previously described[28]. Infected cells were selected with zeocin (0.4 $mgml^{-1}$, 7 days) or neomycin (0.4 $mgml^{-1}$, 7 days). Primary and transformed mouse fibroblasts were grown in D-MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 Uml^{-1} penicillin and 100 μgml^{-1} streptomycin. Primary mammary epithelial cells (Lonza) and *in vitro*-transformed mammary epithelial cells[24] were grown in HMEC bullet kit (Lonza). All cells were grown at 37 °C in 5% CO_2

Tumorigenicity assays

Six week-old male immunodeficient mice (BALB/cAnNCr-*nu/nu*, Animal Production Program (NCI-Frederick) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (The Jackson Laboratory)) were maintained in pathogen-free conditions. For generation of primary tumours, transformed fibroblasts (3×10^6, 3×10^5 or 3×10^3 per injection in 100 μl or 50 μl of Phosphate Buffered Saline (PBS)) were injected intradermally in BALB/cAnNCr-*nu/nu* mice. Tumour heterogeneity and differentiation of transformed fibroblasts were maximal when 3×10^3 cells were injected. To enhance engraftment efficiency, 2.97×10^5 hTERT-immortalized cells were used as carriercells when 3×10^3 transformed fibroblasts were injected. For generation of secondary tumours in limiting dilution experiments, primary tumours obtained from injections of 3×10^3 transformed fibroblasts were dissociated and graded numbers of tumour cells were resuspended in 50 μl of PBS containing 30% standard Matrigel (354234 BD Pharmingen). Cells were injected intradermally in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice that had beenlocally shaved with a depilatory cream. Dead cells were removed from the tumour preparations by cell sorting using 7-Amino-actinomycin D (7-AAD, BD Bioscience). Mice were monitored for up to 24 weeks after injection.

Tumour dissociation and fibroblasts isolation

Tumours were removed surgically when they measured 1–1.5 cm in diameter, minced, incubated in a solution containing 1:10 diluted Liberase 3 (Roche), corresponding to 2.8 Collagenase Wunsch units ml^{-1}, and 0.2 $mgml^{-1}$ DNAse (Roche) for 1 h at 37°C, filtered through a 70 μm cell strainer and washed with sterile PBS. Typically, ~70–80% dissociated cells were viable. Dissociated tumour cells were either sorted or plated to isolate tumour fibroblasts as previously described[40]. Briefly, dissociated tumour cells were plated in MEM medium containing 15 % FBS, 2 mM L-glutamine, 100 Uml^{-1} penicillin and 100 μgml^{-1} streptomycin for 18 h. Adherent cells were washed and grown for 3–7 days in the presence of 500 μgml^{-1} neomycin to eliminate mouse stromal cells, in the absence of specific growth factors necessary for non-fibroblastic adherent cell types. Vimentin immunostaining was used to confirm substantial purity (> 99%) of the isolated fibroblastic population. To isolate SSEA-1^+ tumour fibroblasts, adherent cells were selected as described above either from SSEA-1^+ -sorted tumour cells or from the unsorted population of tumour cells, as all tumour fibroblasts cells were positive for SSEA-1^+.

Flow cytometry analysis and cell sorting

Cultured cells detached from plates with Accutase solution (Sigma) and dissociated tumour cells were stained with anti-SSEA-1-Alexa 488 or – Alexa 647 (eBiosciences, 1:10), anti-SSEA-4 (1:50), anti-TRA-1-60 (1:50), anti-TRA-1-81(1:50) (Chemicon), anti-CD133-PE (Miltenyi Biotec, 1:10), anti-CD44-FITC (Caltag Laboratories, 1:20), anti-ABCG2-PerCP-Cy5.5 (Biolegend, 1:20), anti-ABCB1 (Kamiya, clone MRK16, 05 $\mu g \mu l^{-1}$) anti-CD166-PE (1:5), anti-CD10-PE (1:10) and CD-29-PE (1:10) (eBioscence) antibodies in Sort Buffer (15 mM HEPES buffer, 1% BSA, 2 mM EDTA, 100 $U ml^{-1}$ Dnase, 100 $U ml^{-1}$ penicillin and 100 $\mu g ml^{-1}$ streptomycin in PBS) for 45 min at 4 °C. For non-conjugated primary antibodies, cells were subsequently incubated with Alexa 647- conjugated secondary antibodies (Invitrogen 1:1600). Mouse IgMs-FITC were used as isotype control for the anti-SSEA-1 antibody. Stained cells were analyzed on the FACSCalibur flow cytometer (BD) using the CellQuest Pro software. For quantitation of GFP^+cells in tumours, cells were co-stained with biotin-conjugated anti-mouse H-$2k_d$ MHC class I (eBioscience, 1:200), followed by secondary detection with PerCP-Cy5.5-conjugated streptavidin (eBioscience, 1:200) to exclude mouse stromal and blood cells from the analysis.

For cell sorting, cultured transformed fibroblasts were stained with an anti-SSEA-1-Alexa 647 antibody and SSEA-1^+ and SSEA-1^- cells were isolated using the FACSAria II flow cytometer. Sorted fibroblasts were plated for 16h prior to injection. To sort viable, human SSEA-1^+ and SSEA-1^- cells from xenograft-derived tumours, dissociated tumour cells were co-stained with anti-SSEA-1-Alexa 647and biotin-conjugated anti-H-$2K_d$ MHC class I antibodies followed by secondary detection with Alexa 488-conjugated streptavidin (invitrogen, 1:2000), and 1.25 $\mu g ml^{-1}$ 7-AAD was added to the cells prior to sorting to identify dead cells. SSEA-1^+ and SSEA-1 cells in the 7-AAD^-,H-$K2_d^-$ subpopulation of cells were sorted.

Soft agar and non-adherent sphere formation assays

Soft agar assays were performed as described[18] by plating 5000 cells per well in 6-wells plates in 0.35% SeaPlaque Agar (Lonza) in MEM medium. For sphere formation, transformed cells were plated in Knockout DMEM medium (Invitrogen) supplemented with Knockout Serum Replacement (Invitrogen) in uncoated Petri dishes at clonogenic density (1000 cells ml^{-1}). Dead cells were removed from the tumour preparation by cell sorting using 7-AAD. hTERT-immortalized fibroblasts were used as negative controls for both assays.

Induction and quantitative analysis of transformed fibroblast differentiation

Adipogenesis and osteogenesis were induced and quantitatively analyzed as described[41].

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed by the Pathology and Histology Laboratory, NCI-Frederick. Primary antibodies used to stain xenograft-derived tumours and the tissue microarray (SO2084, Biomax US) were: anti-hCD34 (Vector 1:25), anti-tubulin-β-III (Chemicon 1:20), anti-myosin (NeoMarkers 1:500), anti-SSEA-1-CD15 (DAKO 1:25), anti-GFP (Abcam 1:5000). Positive and negative control tissues were used in all experiments.

To detect SSEA-1 in cultured cells by immunofluorescence microscopy cells were grown and stained on plastic plates as living cells, since fixation and growth on glass coverslips affected antigen detection. Immunodetection was performed by incubating cells in medium containing an anti-SSEA-1 antibody (Millipore, 1:50) for 15 min at 37 °C, followed by addition of a secondary antibody and Hoechst 33342 at a final concentration of 20 $\mu g ml^{-1}$ and 1.5 $\mu g ml^{-1}$, respectively, for additional 15 min at 37 °C. Cells were imaged on an Olympus IX70 inverted microscope using a 10X or 40x objective.

To detect asymmetrically divided cells, SSEA-1^+ sorted cells were plated at low density (~1 cell per field using a 10X objective) and incubated for 24–48h prior to immunodetection. Pairs of cells whose nuclei were closer than 20 μm were considered cells originated from the same cell. Alternatively, SSEA-1^+ sorted cells were treated with 10 μM blebbistatin (Sigma) for 24h as previously described[26], trypsinized and seeded on poly-lysine-coated coverslips for 10 min prior to immunodetection. Blebbistatin-treated cells were imaged on a Zeiss META confocal microscope using a 63x objective, acquiring 10–15 1-μm optical sections.

Lineage tracking

SSEA-1^+ and SSEA-1^- cells were isolated by cell sorting from an unlabeled and a GFP-labelled version of the Transformed-2 cell line. The GFP-labelled cell line expresses an inducible form of GFP-lamin A which does not affect the tumorigenicity of the cells (P. Scaffidi, unpublished results). Tumours were surgically removed when they measured ~1 cm in diameter. Half of the tumour was fixed in 4% PFA and embedded in paraffin for immunohistochemistry analysis and the other half was dissociated and analyzed by flow cytometry.

Microarrays and quantitative analysis of gene expression

RNA from tumours was extracted by disrupting tumours in Trizol reagent (Invitrogen) as described[42]. RNA from cultured cells was extracted using the RNAeasy kit (Qiagen). Semi-quantitative RT-PCR was performed as described[43] by performing 21–29 cycles of amplification, depending on the target, to ensure detection of products in the linear range of amplification. Amplification products were loaded on 1.5% agarose gels and visualized using Syber Green (Invitrogen).Quantitative RT-PCR was performed as described[41]. Primers used in this study are listed in Supplementary Table S3.

For microarray analysis, RNA was extracted from two biological replicates of SSEA-1^+ and SSEA-1^- transformed fibroblasts isolated by cell sorting and from SSEA-1^+ tumour fibroblasts and SSEA-1^- cells isolated from 3 primary tumours induced by 3,000 transformed fibroblasts. RNA labelling, hybridization on Affymetrix GeneChip Human Gene 1.0 ST arrays and data analysis were performed by the Laboratory of Molecular Technology, SAIC-Frederick, Partek software was used to normalize and analyze the data. Genes showing at least a 1.5-fold difference between SSEA-1^+ and SSEA-1^- transformed fibroblasts with p-value < 0.05 were considered differentially expressed. Paired T-test and Anova test were used to identify differentially expressed genes (p-value < 0.05, 2-fold difference) in SSEA-1^+ tumour fibroblasts and SSEA-1 tumour cells in all tumours and compare them with the values in the injected fibroblasts.

Accession number

The microarray data have been deposited in EMBL-European Bioinformatics Institute (accession number E-MEXP-2851).

Statistical analysis

Statistical significance of the differences in *in vitro* differentiation, quantitative RT-PCR experiments and *in vitro* clonogenicity assays was determined by one-tailed Student t-test. Statistical significance of the differences among populations measured by flow cytometry was determined by Kolmogorov-Smirnov (K-S) test. Statistical significance of the differences in the lineage tracking experiment was determined by χ^2 analysis. The experimental values (% GFP^+ cells in the tumours) were compared with the expected values under the hypothesis of equal tumorigenicity of GFP-labelled and unlabeled cells (% of GFP^+ in the injected cells). A χ^2 value > 6.63 indicates that the experimental values are different from the expected ones at the 99% confidence level and that GFP-labelled and unlabeled cells have differential tumorigenicity. Limiting dilution data were analyzed using the ELDA software application at http://bioinf.wehi.edu.au/software/elda/. The frequency of tumorigenic cells was estimated setting a 95% level of confidence and significance of the differences was determined by χ^2 analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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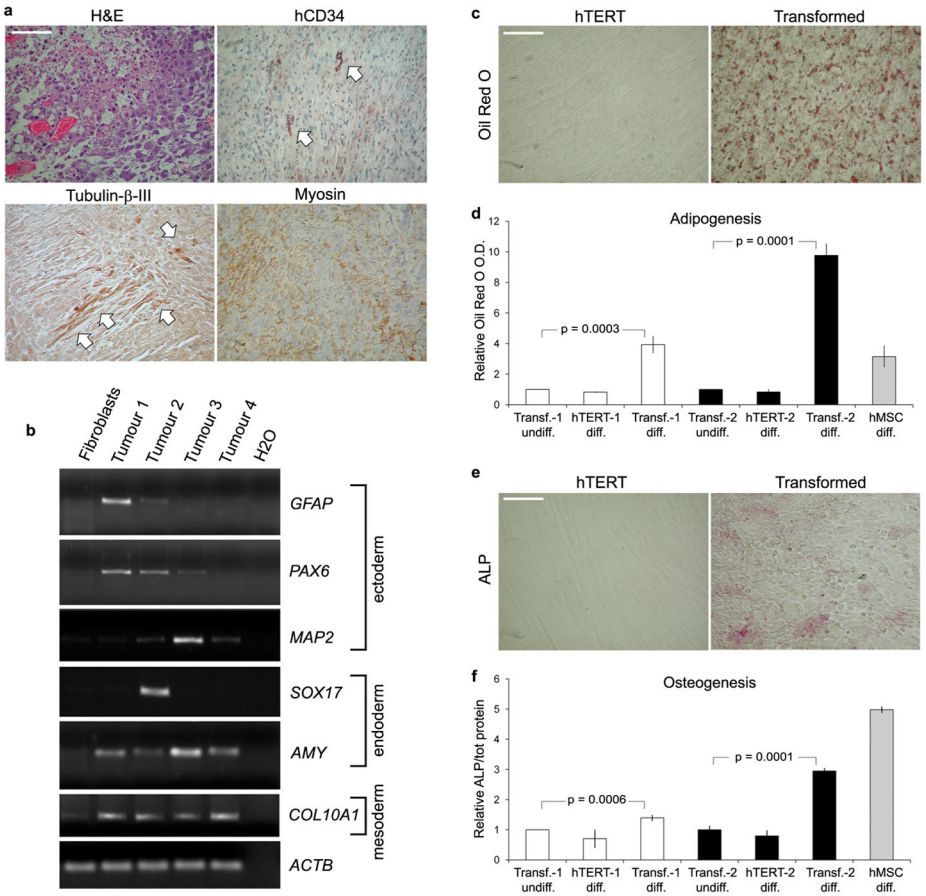

Figure 1. Differentiation of *in vitro*-transformed fibroblasts

(**a**) Hematoxylin and eosin staining (upper left panel) and immunohistochemical detection of the indicated proteins (brown) in paraffin sections of tumours generated by *in vitro*-transformed fibroblasts. Cells immunopositive for hCD34 and tubulin-β-III are indicated by arrows. Wide areas of myosin-positive cells were detected. Scale bar: 100 μm. (**b**) Semiquantitative RT-PCR analysis of expression levels of the indicated germ layer markers in transformed fibroblasts and 4 primary tumours induced by two independent transformed fibroblast cell lines (tumour 3 and 4 from Transformed-1 cell line and tumour 1 and 2 from Transformed-2 cell line). *ACTB* was used as control for RNA amount. Full scans of the gels, including molecular weight markers, are shown in Supplementary Fig. S7. (**c**, **e**) *In vitro* differentiation of Transformed-1 and Transformed-2 cell lines and the corresponding hTERT-immortalized control cells after (**c**) 5 d of adipogenic differentiation or (**e**) 7 d of osteogenic differentiation. Transformed cells were plated at densities of 1×10^4cells /cm^2 for adipogenesis and 3×10^3cells /cm^2 for osteogenesis. Scale bar: 100 μm. (**d,f**) Quantitative analysis of incorporated Oil red O after elution with isopropanol (**d**) or Alkaline Phosphatase (ALP) activity by luminescent analysis (**f**). Values represent means ± s. d. from three biological replicates. Statistical significance of the differences between uninduced and induced transformed cells is indicated.

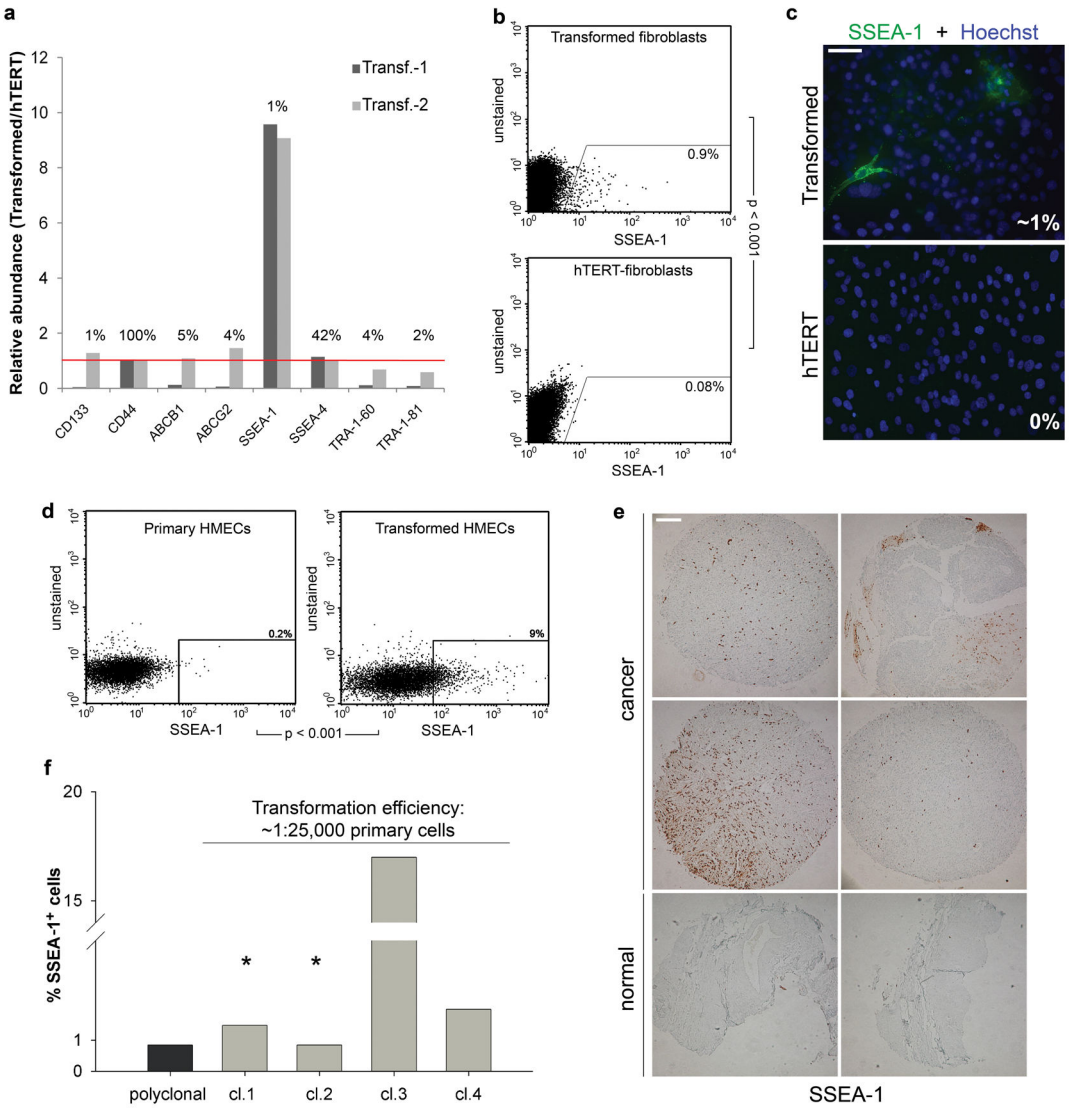

Figure 2. The presence of the stage-specific embryonic antigen SSEA-1 in populations of differentiated cells correlates with acquisition of tumorigenicity upon transformation

(**a**) Relative abundance of cells immunopositive for the indicated antigens in two independent cell lines of *in vitro*-transformed fibroblasts compared to the corresponding hTERT-immortalized control cell lines. The average percentage of positive cells in the two transformed cell lines for each antigen measured by flow cytometric analysis is indicated. The red line indicates a relative abundance of 1 and values above the line indicate enrichment of the immunopositive cells in transformed fibroblasts compared to hTERT-immortalized cells. (**b,c**) Immunodetection of SSEA-1 in the Transformed-2 cell line and the corresponding hTERT-immortalized control cell line by flow cytometry (**b**) and fluorescence microscopy (**c**). The percentage of SSEA-1$^+$ cells and the significance of the difference between the two populations determined by K-S test are indicated. The signal intensities and the percentage of SSEA-1$^+$ cells in the hTERT-immortalized cell lines were not different ($p > 0.05$) from the background measured in unstained cells or cells stained

with an isotype control antibody (Supplementary Fig.S2a). Scale bar: 40 μm. (**d**) Flow cytometric analysis of SSEA-1 in primary and *in vitro*-transformed mammary epithelial cells. The percentage of SSEA-1^+ cells is indicated. (**e**) Immunohistochemical detection of SSEA-1 in clinical samples from one inflammatory myofibroblastoma (top left panel) and three malignant fibrohistiocytomas. Representative images of varying abundance and distribution of SSEA-1^+ cells observed in the tissue microarray are shown. No SSEA-1^+ positive cells were detected in normal control tissues (bottom panels). Scale bar: 150 μm. (**f**) Abundance of SSEA-1^+ cells in a polyclonal population of primary fibroblasts transformed by serial introduction of transforming factors under conditions of high infection efficiency or in four clones obtained by simultaneous introduction of the transforming factors under conditions of low infection efficiency. Injection of the clones marked by an asterisk induced tumour formation in mice.

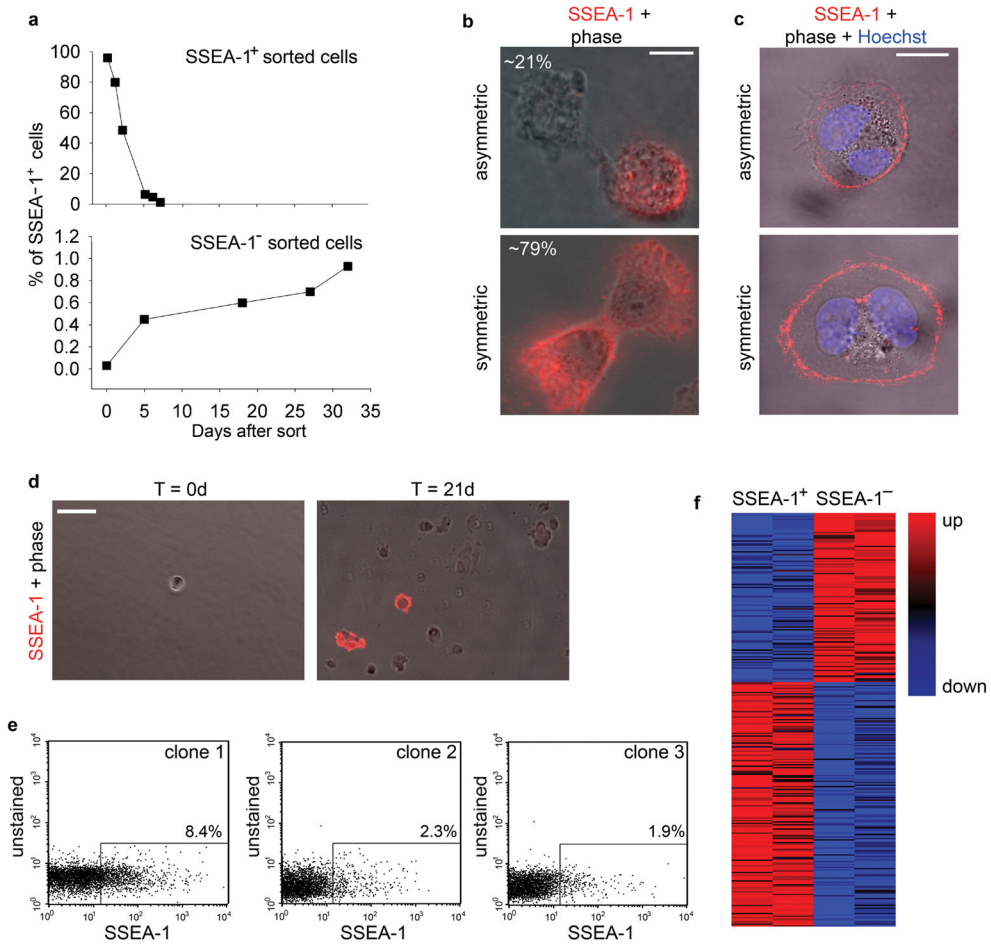

Figure 3. SSEA-1 identifies a biologically distinct subpopulation of transformedfibroblasts

(**a**) Abundance of SSEA-1$^+$ cells in populations of sorted SSEA-1$^+$ (top) and SSEA-1$^-$ fibroblasts (bottom) over time in culture in a representative time course experiment. About 1% of SSEA-1$^+$ cells were present in the unsorted population. (**b,c**) Immunodetection of SSEA-1 in untreated cells (**b**) or cells treated with 25mM blebbistatin for 24h (**c**) by fluorescence microscopy. Pairs of daughter cells were identified as described in Supplementary Fig. S3 and Methods. Scale bar: 20 μm. The percentage of cells undergone each type of cell division is indicated. (**d**) Immunodetection of SSEA-1 in an isolated single SSEA-1$^-$ -sorted fibroblast and in its daughter cells generated after 3 weeks of culture by fluorescence microscopy. Scale bar: 80 μm. (**e**) Flow cytometric analysis of SSEA-1 in three independent clonal populations resulting from single SSEA-1$^-$ fibroblasts after three weeks of culture. Variable percentages of SSEA-1$^+$ cells in each clone suggest that conversion to SSEA-1$^+$ fibroblasts occurs in a stochastic manner. (**f**) Heatmap representing relative expression levels of genes showing a difference of at least 1.5-fold between SSEA-1$^+$ and SSEA-1$^-$ fibroblasts with a p value < 0.05. Two biological replicates for each subpopulation were analyzed. Red and blue represents the highest and lowest value of each gene among all samples, respectively.

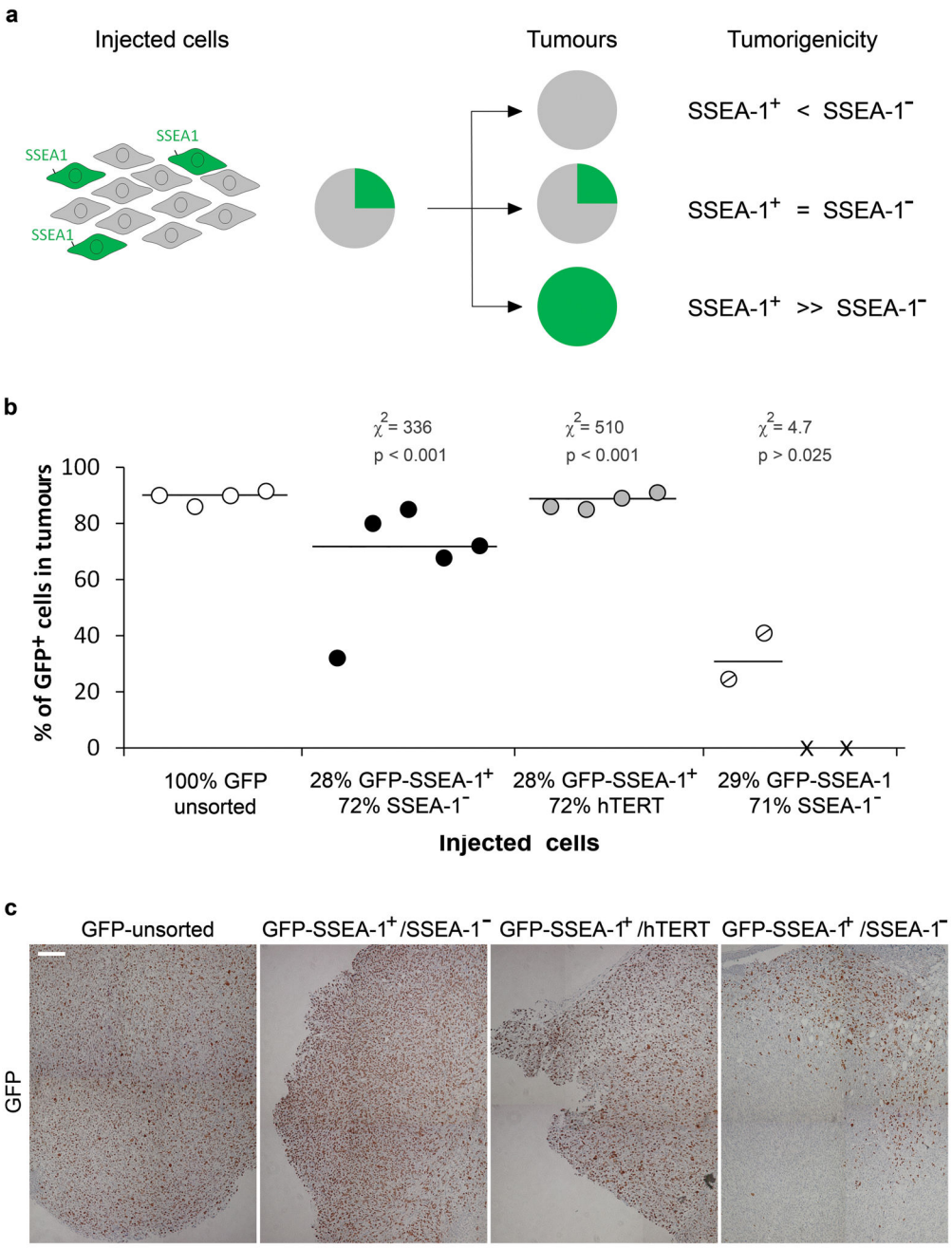

Figure 4. SSEA-1$^+$ fibroblasts are responsible for tumour initiation

(**a**) Experimental design to track cells responsible for tumour initiation among *in vitro*-transformed fibroblasts and possible outcomes. Higher percentage of GFP$^+$ cells in the tumours compared to that in the injected cells indicates greater tumorigenicity of labelled SSEA-1$^+$ cells than competing unlabeled SSEA-1$^-$ cells. (**b**) Percentages of GFP$^+$ cells in tumours induced by the indicated combinations of unlabeled and labelled cells measured by flow cytometric analysis. Dots represent individual tumours at 6 weeks after injection. Crosses are injections that failed to form tumours. Solid lines indicate median values of GFP$^+$ cells for each group. Statistical significance of the differences between the

experimental values and the expected values under the hypothesis of equal tumorigenicity of the injected unlabeled and labelled cells (see Methods) is indicated for each group. (**c**) Immunohistochemical detection of GFP (brown) in tumours resulting from injection of the indicated cells. Pictures of four adjacent fields were combined for each tumour. Scale bar: 150 μm.

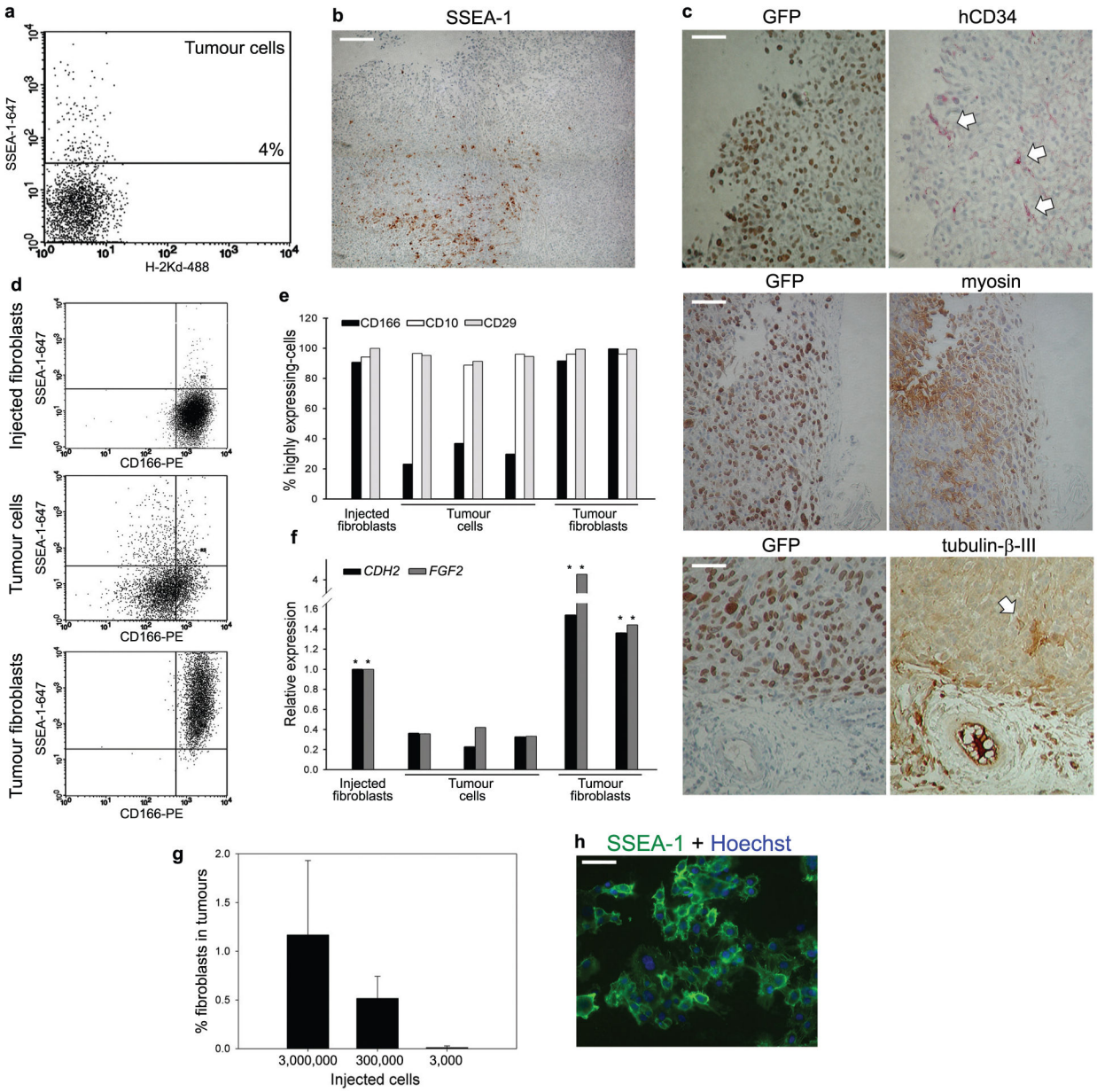

Figure 5. SSEA-1^{+} fibroblasts differentiate into non-fibroblastic cells during tumour growth

(**a,b**) Flow cytometric analysis (**a**) and immunohistochemical detection (brown) (**b**) of SSEA-1 in primary tumours. Human H-2Kd^{-} cells were gated and plotted. Pictures of four adjacent fields were combined. Scale bar: 150 μm. (**c**) Immunohistochemical detection (brown) of the indicated proteins in primary tumours resulting from injection of GFP-labeled SSEA-1^{+} fibroblasts mixed with unlabeled SSEA-1^{-} fibroblasts. Cells immunopositive for hCD34 and tubulin-β-III are indicated by arrows. Wide areas of myosin-positive cells were detected. Scale bars: 80 μm (upper and middle panels) and 50 μm (bottom panels). (**d**) Flow cytometric analysis of CD166 and SSEA-1 in transformed fibroblasts, a primary tumour, and fibroblasts isolated from the tumour. (**e**) Abundance of cells expressing high levels of the indicated antigens measured by flow cytometric analysis

in transformed fibroblasts, three representative primary tumours and two representative tumour fibroblast populations. The gate was set based on the values measured in the transformed fibroblasts. (**f**) Quantitative RT-PCR measuring expression levels of the mesenchymal markers *CDh2* and *FGF2* in the indicated samples. Values are normalized to the housekeeping gene *PPIA* and represent means from two replicates. Statistical significance of the differences compared to the tumour cells ($p < 0.001$) is indicated by an asterisk. (**g**) Abundance of fibroblasts in primary tumours resulting from injection of the indicated number of transformed fibroblasts. Values are relative to the number of viable tumour cells after dissociation and represent mean ± s. d. from three tumours. (**h**) Immunodetection of SSEA-1 in tumour fibroblasts isolated from primary tumours by fluorescence microscopy. Scale bar: 40 μm.

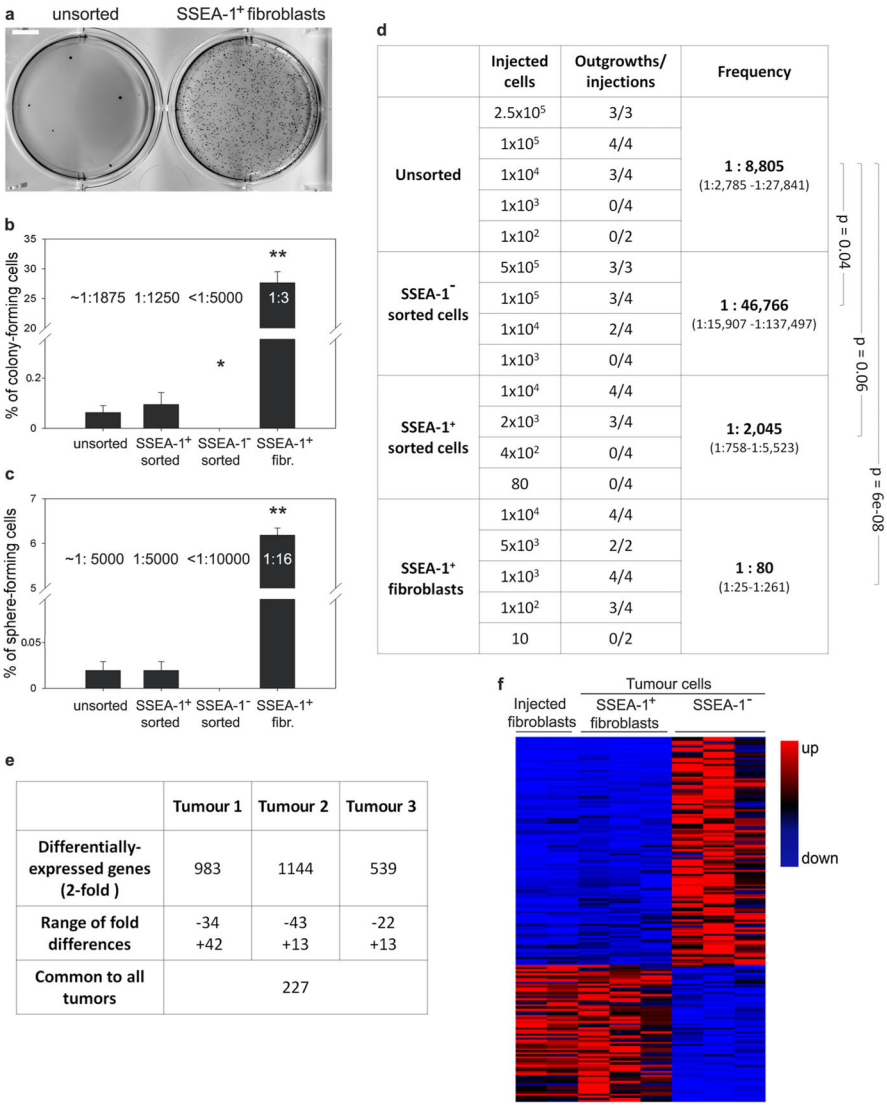

Figure 6. Hierarchical organization of tumours induced by *in vitro*-transformed fibroblasts

(**a**) Soft agar assay using unsorted cells form a primary tumour and SSEA-1$^+$ tumour fibroblasts. Scale bar: 400 mm. Similar results were obtained with cells from 5 other tumours. (**b**,**c**) Quantitative analysis of soft agar assay (**b**) and non-adherent sphere formation assay (**c**) using the indicated cells from a primary tumour resulting from injection of 3,000 transformed fibroblasts. The frequency of clonogenic cells is indicated. Values represent means ± s. d. from three replicates. Statistical significance of the differences compared to the unsorted population is indicated by one ($p < 0.005$) or two ($p < 0.001$) asterisks. (**d**) Limiting dilution transplantation assay into NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice using the indicated cells from two primary tumours resulting from injection of 3,000 transformed fibroblasts. The frequency of tumorigenic cells (estimate with upper-lower limits) was calculated by limiting dilution analysis as described in the Methods. Statistical significance of the differences compared to the unsorted population is indicated. (**e**) Summary of the differences in gene expression profiles between SSEA-1$^+$ tumour

fibroblasts and SSEA-1^{-} cells from three primary tumours. The number of genes differentially-expressed (≥2-fold) in each tumour and of those significantly different in all tumours (≥2-fold, $p < 0.05$) is indicated. (**f**) Heatmap representing relative expression levels of the subset (70%) of differentially-expressed genes in all tumours (SSEA-1^{+} tumour fibroblasts vs. SSEA-1^{-} cells, $p < 0.05$, ≥ 2-fold) which are expressed at similar levels in SSEA-1^{+} tumour fibroblasts and transformed fibroblasts ($p > 0.05$, < 2-fold). Values from two replicates of transformed fibroblasts and three tumours are represented. Red and blue represents the highest and lowest value of each gene among all samples, respectively.

a

	Injected cells	Outgrowths/ injections	% of SSEA-1$^+$ cells in tumours	Days to grow a ~0.1cm^3 tumour
Primary tumours	Transformed fibroblasts	4/4	3.1% ± 1.8	27 ± 4
Secondary tumours	SSEA-1$^+$ tumour fibroblasts	4/4	7.9% ± 1.6	13 ± 1
Tertiary tumours	SSEA-1$^+$ tumour fibroblasts	2/2	25.2% ± 2.2	11 ± 1

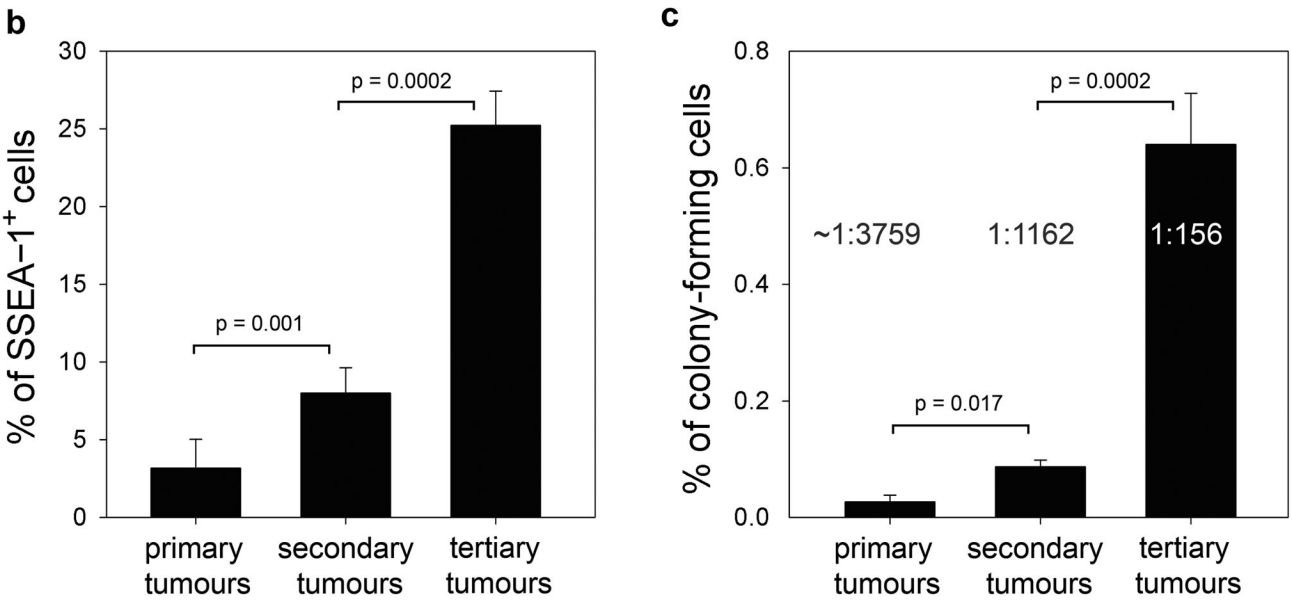

Figure 7. Self-renewal ability of SSEA-1$^+$ transformed fibroblasts

(**a**) Serial transplantation of SSEA-1$^+$ fibroblasts in BALB/cAnNCr-*nu/nu* mice. 3×10^6 cells were injected for all passages. (**b**) Percentages of SSEA-1$^+$ cells in the indicated samples measured by flow cytometric analysis. Values represent mean ± s. d. from 2 to 6 tumours. Statistical significance of the differences is indicated. (**c**) Quantitative analysis of soft agar assay using unsorted cells from primary, secondary and tertiary tumours. The frequency of clonogenic cells is indicated. Values represent mean ± s. d. from three replicates. Statistical significance of the differences is indicated.

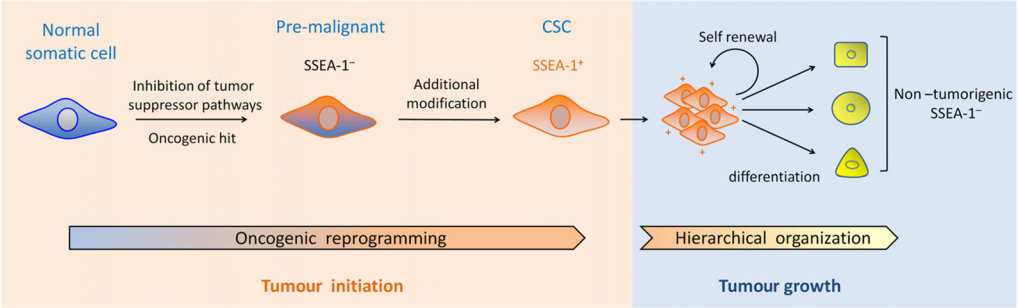

Figure 8. Model for reprogramming of somatic cells to CSCs

Inhibition of tumour suppressor cellular mechanisms and concomitant oncogenic activation convert somatic differentiated cells into pre-malignant SSEA-1$^-$ cells. This cellular intermediate is not tumorigenic. Additional, stochastic modifications lead to full reprogramming of somatic cells to SSEA-1$^+$ CSCs, conferring self-renewal and differentiation ability. These cellular properties allow reprogrammed somatic cells to initiate and maintain tumours. During tumour growth, SSEA-1$^+$ CSCs differentiate into phenotypically diverse, non-tumorigenic cancer cell, thereby generating heterogeneous and hierarchically-organized tumours.