Effects of estrogen on the proportion of stem cells in the breast

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Abstract

There is increasing evidence that breast cancers contain tumor-initiating cells with stem cell properties. The importance of estrogen in the development of the mammary gland and in breast cancer is well known, but the influence of estrogen on the stem cell population has not been assessed. We show that estrogen reduces the proportion of stem cells in the normal human mammary gland and in breast cancer cells. The embryonic stem cell genes *NANOG*, *OCT4* and *SOX2* are expressed in normal breast stem cells and at higher levels in breast tumor cells and their expression decreases upon differentiation. Overexpression of each stem cell gene reduces estrogen receptor expression, and increases the number of stem cells and their capacity for invasion, properties associated with tumorigenesis and poor prognosis. These results indicate that estrogen reduces the size of the human breast stem cell pool and may provide an explanation for the better prognosis of estrogen receptor-positive tumors.

Introduction

The adult mammary epithelium consists of a bilayered structure with a central lumen, an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. Estrogen is essential to normal mammary gland development, where it is responsible for epithelial cell proliferation and differentiation of healthy breast epithelium [1]. In addition to its fundamental role in the normal physiology of the mammary gland, exposure to estrogen is an important determinant of risk for breast cancer. The estrogen receptor (ER) belongs to the family of steroid and nuclear hormone receptors, transcription factors that bind to DNA and regulate transcription of target genes in response to ligand binding. Tamoxifen, an antagonist of ER, is widely used as hormonal therapy in the treatment of ER-positive breast cancer, although very frequently tamoxifen resistance develops [2]. Approximately three out of four cases of breast cancer express ER [2], which is highly predictive of the clinical response to hormonal therapy. ER-positive tumors have a better prognosis than other types of breast tumors in terms of overall survival, while ER-negative tumors have a more aggressive phenotype [3-5].

The tissue expansion and remodeling that occurs in the mammary gland during the multiple cycles of pregnancy, lactation and involution throughout a woman's lifetime suggest that there are stem cells and early progenitor cells in the adult mammary gland [6]. Adult stem cells are characterized by their self-renewal capacity, which allows the maintenance of the stem cell pool, and the ability to differentiate into the cell types of the tissue in which they reside, luminal and myoepithelial cells in the case of the mammary gland. Evidence has accumulated during the last several years supporting the existence of stem cells both in the mouse and human mammary gland [7]. Different strategies have been used to identify and isolate human breast stem/progenitor cells including FACS sorting for the expression of cell surface antigens. For example, EMA^+CALLA^+ and EMA^-CALLA^- cells can generate mixed colonies from a single cell [8]. In addition, an *in vitro* cell culture system has been described that allows the propagation of human mammary epithelial cells in an undifferentiated state based on their ability to proliferate in suspension as non-adherent mammospheres [9].

The *in vitro* propagation of breast cancer-initiating cells as mammospheres from breast cancer lesions and cell lines has also been reported [10,11]. Compelling data supporting the existence of breast cancer stem cells showed that cells isolated as Lin-$CD44^+CD24^{-/low}$ were capable of forming tumors in mice with higher efficiency than cells with alternate phenotypes [12]. Interestingly, Shipitsin and collaborators showed that the gene expression profile of $CD44^+$ cells resembles that of stem cells, and normal and tumor $CD44^+$ cells are more similar to each other than to $CD24^+$ cells from the same tissue [13]. Another fruitful approach identified aldehyde dehydrogenase activity ($ALDH^+$) as a shared common functional marker for both normal and malignant human mammary stem cells [14]. The identification of markers shared by both normal and malignant mammary stem cells lends support to the cancer stem cell hypothesis.

Recent studies suggest that cancer cells share molecular signatures that are similar to those of pluripotent embryonic stem (ES) cells [15]. The regulatory networks controlling the function of ES cells, including key regulators of ES cell maintenance, namely Nanog, Oct4 and Sox2 [16,17], are also found in some adult stem cell populations [18,19]. Moreover, genes associated with ES cell identity have been linked to tumor histology, supporting the possibility that these genes contribute to the stem cell-like phenotype of many tumors [20].

The proliferative role of estrogen in breast cancer cell lines and in ER-positive tumors is well established. Nevertheless, generally ER-positive tumors present a better prognosis. Presently, little is known about estrogen signaling in normal mammary epithelial cells and, particularly, in stem/progenitor cells [7]. Therefore, we wished to investigate the effects of estrogen on the stem/progenitor cell population, both in normal breast and in breast cancer. To this end, we used the expression levels of embryonic stem cells transcription factors Nanog, Oct4 and Sox2 to monitor the differentiation status of breast stem cells in the presence of either estrogen or tamoxifen and then examined the outcome of changing the expression levels of these three factors on the stem cell phenotype. We found that estrogen induces the breast stem cells to differentiate, which may provide an explanation for the better prognosis of ER-positive breast tumors.

Materials and methods

Isolation of human breast epithelial cells

Normal breast tissue (n = 14) was obtained from women (average age 39) undergoing reduction mammoplasty with no previous history of breast cancer. Tumor samples were obtained from core biopsies (n = 8) or from women who underwent therapeutic surgery (n = 3). For histological information, please refer to Online Resource (Table 1). In all cases the samples were reviewed by a consultant breast pathologist. All patients provided written informed consent, and the procedures were approved by the local Hospital Research Ethics Committee and the 'Ethics Committee of Clinical Investigation of Euskadi'. The breast tissue was immediately processed as previously described [8]. For further details please see Online Resource.

Mammosphere culture

Breast epithelial cells were plated in 75 cm^2 ultralow attachment flasks (Corning) at a density of 10.000 cells/cm^2 in primary culture and 5.000 cells/cm^2 in passages. Human breast cancer cell lines MCF-7 and T47D were plated at a density of 2.500 cells/cm^2 in initial cultures and 1.000 cells/cm^2 during passage. All cells were grown and processed as described in [9]. Experiments using primary epithelial cells treated with hormones were performed using samples from pre-menopausal women (n = 9). Please see Online Resources for further information (also for adherent cell culture).

Invasion assay

In vitro invasion and chemotaxis assays were performed in a 24-well BD Falcon™ HTS Multiwell Insert System containing an 8 μm pore size PET (PolyEthylene Terepthalate) membrane. For invasion assays, the top of the upper wells was coated with 2 μg of Matrigel Basement Membrane Matrix (BD) diluted in 50 μl of DMEM:F-12 medium and allowed to air-dry overnight. The following day the Matrigel was re-hydrated and 100,000 cells, previously starved in serum-free medium for 24 h were added to the upper well. The lower well was filled with 300 μl of chemoattractive medium containing 20% FBS and 300 μl of MCF-7 cell-conditioned medium (0.45 μm filtered). Medium alone was used as a negative control. After 72 h, cells on the upper surface of the membrane were removed by wiping with a cotton swab, and the cells remaining on the lower side of the membrane were fixed and stained with crystal violet. For chemotaxis assays the same procedure was followed but the upper wells were not coated with Matrigel. At least nine different fields from each well were counted to determine the

number of invading cells. The percentage of invasion was calculated by dividing the mean number of cells that invaded through the Matrigel with those that migrated through the control.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol (Invitrogen). When the cell number was lower than 0.5 x 10^6 cells the RNeasy Micro Kit (QIAGEN) was used. In all cases, RNA extraction was according to the instructions of the manufacturer. Real-time PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems), using the iTaq™ SYBR® Green Supermix with ROX (BioRad). cDNA was amplified using the following conditions: 95° C for 5 min, 40 cycles of amplification - 95ºC for 15 sec, 55-65ºC for 1 min (annealing temperatures, AT, depending on the primers) - and a dissociation stage. 36B4 was used as a reference transcript for normalization. cDNA from the embryonal carcinoma cell line NTera2/D1, which expresses the stem cell genes *NANOG*, *OCT4* and *SOX2*, was used as a positive control. The sequences of the primers used can be found as Online Resource.

Transcription assays and western blotting

The details for the transcription and western blotting assays, performed as in [21], can be found as Online Resource.

Lentivirus production

Lentivirus were produced by triple transfection of 293FT cells with the second generation packaging vector psPAX2 (Addgene plasmid 12260, deposited by Dr Didier Trono), the pMD.G vector (kind gift of Dr James Sutherland) encoding the envelope vesicular stomatitis virus glycoprotein (VSV-G), and either the pSin-EF2-Nanog-Pur, the pSin-EF2-Oct4-Pur or the pSin-EF2-Sox2-Pur vectors (Addgene plasmids 16578, 16579 and 16577, respectively, deposited by Dr James A Thomson). pSin-EF2-EGFP-Pur was used as control and obtained by replacing the Oct4 gene from the pSin-EF2-Oct4-Pur with an EGFP coding sequence. DNA plasmids were transfected and viral supernatant was collected every 24 hours for three days. The supernatant was filtered and concentrated 30-fold by ultracentrifugation. For lentiviral transductions, the concentrated supernatant containing polybrene (8 µg/ml final concentration, Sigma) was added to the cells at a 1:1 ratio. Cells were then incubated for 48 h prior to selection with 0.5-2 µg/ml of puromycin (Sigma).

Immunofluorescence

Cells grown on slides were fixed with 4% paraformaldehyde (Sigma), permeabilized with PBS 0.3% Triton-X-100 and blocked with 8% FBS in PBT (PBS containing 0.05% Tween-20). Cells were then incubated for 1 hour with one of the following primary antibodies: goat anti-NANOG (R&D Systems, AF1997), mouse anti-OCT3/4 (C-10, Santa Cruz, sc-5279) and goat anti-SOX2 (Y-17, Santa Cruz, sc-17320); and then with anti-goat alexa 568 (Molecular Probes, A11057) or anti-mouse alexa 647 (Molecular Probes, A31571) secondary antibodies. Finally, slides were mounted in Vectashield with DAPI (Vector) and visualized on the Leica confocal microscope.

Fluorescent activated cell sorting (FACS)

Human epithelial membrane antigen (EMA) and common acute lymphoblastic leukaemia antigen (CALLA) labeling was performed essentially as previously described [8]. For details please refer to Online Resource. The directly conjugated mouse PE anti-CD24 antibody (BD, clone ML5) and the directly conjugated mouse allophycocyanin (APC) anti-CD44 antibody (BD, G44-26) were used to label CD24 and CD44. Control samples were stained with isotype-matched control antibodies, PE conjugated non-specific mouse IgG2a,κ antibody (BD) and APC conjugated non-specific mouse IgG2b,κ antibody (BD) for CD24 and CD44, respectively. The viability dye 7-aminoactinomycin D (7AAD, BD) was added for dead cell exclusion.

Aldefluor assay

To measure cells with ALDH activity, the Aldefluor assay was carried out according to manufacturer's (Stemcell Technologies) guidelines. Briefly, dissociated single cells were suspended in Aldefluor assay buffer containing an ALDH substrate, bodipyaminoacetaldehyde (BAAA) at 1.5 mM, and incubated for 45 min at 37ºC. To distinguish between ALDH-positive and -negative cells, a fraction of cells was incubated under identical conditions in the presence of a 2-fold molar excess of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB).

Results

Estrogen reduces the pool of stem/progenitor cells in the normal mammary gland

We wished to investigate whether the pluripotent factors Nanog, Oct4 and Sox2 were differentially expressed in the human mammary gland. To this end we assessed the expression of *NANOG*, *OCT4* and *SOX2* mRNAs in freshly isolated organoids from reduction mammoplasties, in breast epithelial cells derived from the organoids and grown as adherent cells in the presence of serum, and in mammospheres originating from single breast epithelial cells. We observed that, with respect to the expression in freshly isolated organoids, the expression of all three pluripotency genes was lower in the differentiated (adherent) cells and it was significantly higher in mammospheres (Fig. 1a). Furthermore, when mammospheres were plated under differentiating conditions (with serum) there was a strong reduction in the expression of *NANOG*, *OCT4* and *SOX2* (Fig. 1b). In order to investigate the potential roles of estrogen and tamoxifen in cell-fate determination, we assessed the expression of *NANOG*, *OCT4* and *SOX2* in mammosphere cultures under different hormonal conditions. Progesterone receptor (PR, a known ER target) was induced in response to estrogen treatment of freshly isolated breast epithelial cells, indicating the presence of transcriptionally active ER (Fig. 1c). Naturally, the induction of PR by estrogen in the primary cells was more modest than that observed in ER-positive breast cancer cell lines since the expression of ER is lower [22] and steroid hormone receptors in primary cells display lower transcriptional activity than in cancer cells [23]. Nevertheless, estrogen treatment significantly reduced the expression of *NANOG*, *OCT4* and *SOX2* (Fig. 1d).

To confirm that culture of primary mammary epithelial cells as mammospheres enriches for stem/progenitor cells, the percentage of EMA^+CALLA^+ was assessed in cells grown in suspension at very low density. As we previously reported, freshly isolated EMA^+CALLA^+ (DP) cells from the mammary gland represent 0.5-1.4% of the total cell population [8]. This population of stem/progenitor cells was increased up to 5-fold in suspension cultures (Fig. 2a). Interestingly, estrogen treatment reduced the percentage of DP cells present in the mammospheres, while tamoxifen increased it with respect to the ethanol control (Fig. 2b). Furthermore, the expression levels of *NANOG*, *OCT4* and *SOX2* were strongly increased in the progenitor DP population when compared with the differentiated EMA^+ and $CALLA^+$ cell populations (Fig. 2c). These observations suggest that the stem cells proliferate more in the absence of estrogen or that estrogen induces differentiation of the progenitor cells and as a result depletes the pool of stem/progenitor cells.

Expression of NANOG, OCT4 and SOX2 in normal and tumor samples

The results above suggest that the pluripotency genes *NANOG*, *OCT4* and *SOX2* might be markers of stem/progenitor cells in the normal mammary gland. Next, we wished to assess whether this was also the case for breast tumors. To this end we compared *NANOG* and *SOX2* expression in tumor samples and adjacent normal tissue. *NANOG* and *SOX2* were more highly expressed in pathological samples (8/11 cases, Table 1 in Online Resource for Sox2 values) than in the normal counterpart samples (Fig. 3a). Furthermore, although material was too scarce to assess expression of the three markers, we could observe Sox2 protein expression in the tumor but not in the normal sample from the same patient (Fig. 3a, right). To monitor the effects of estrogen on the subpopulation of cancer stem/progenitor cells we examined the expression of *NANOG* and *SOX2* in breast tumor samples from which individual cells had been isolated and grown in suspension for 6 days in the presence of ethanol, estrogen or tamoxifen. Quantification by real-time PCR showed that estrogen treatment strongly reduced the expression of *NANOG* and *SOX2* in mammospheres, while tamoxifen increased their expression (Fig. 3b). We also determined the changes in *NANOG*, *OCT4* and *SOX2* expression in the $CD44^{+}CD24^{-/low}$ population of breast cancer stem/progenitor cells. Interestingly, the expression of all three genes was significantly increased in the stem/progenitor $CD44^{+}CD24^{-/low}$ subpopulation with respect to the expression in the rest of the tumor cell population (Fig. 3c). These results reinforce the value of these transcription factors as breast stem/progenitor cell markers both in normal and tumor samples.

Effects of estrogen and tamoxifen on mammosphere formation by MCF-7 breast cancer cells

To investigate further the potential role of Nanog, Oct4 and Sox2 in the regulation of breast stem cells, we used the ER-positive breast cancer cell line MCF-7. We plated MCF-7 cells on ultralow attachment plates at low density in the presence of estrogen, tamoxifen or ethanol. The number of mammospheres formed was highest in the presence of tamoxifen whereas estrogen treatment resulted in the formation of fewer, larger mammospheres (Fig. 4a). To ensure that the mammospheres formed were clonal in origin, tertiary mammospheres grown in the presence of hormones were dissociated and single cells were FACS sorted into ultralow attachment 96-well plates at a density of one cell per well and allowed to form mammospheres in the presence of the corresponding hormones. Estrogen reduced the frequency of mammosphere formation 6-fold compared with control cultures, while there was an increase induced by tamoxifen that was small but significant (Fig. 4b). Mammospheres formed in the presence of estrogen were larger than those with the other treatments, suggesting increased proliferation of the progeny of mammosphere-initiating cells, consistent with the known proliferative effect of estrogen on differentiated MCF-7 cells (Fig. 4c). In conclusion, estrogen inhibits the self-renewal capacity of stem/progenitor cells within MCF-7 mammospheres, as measured by their capacity to be serially passaged.

NANOG, OCT4 and SOX2 expression reflects the differentiation state of MCF-7 cells

Next, we determined whether the expression of the pluripotency factors also mirrored the changes in the differentiation state of MCF-7 cells. We observed by quantitative PCR that the expression of *NANOG* and *SOX2* was approximately 10-fold higher in suspension cultures than in adherent cultures, while the increase in *OCT4* expression was small but significant (Fig. 5a). The high expression of these factors was maintained in suspension cultures serially passaged as many as 20 times (data not shown). Estrogen reduced the expression of *NANOG*, *OCT4* and *SOX2* in mammospheres, while tamoxifen increased it (Fig. 5b). Furthermore, when we plated tertiary mammospheres on normal tissue culture plates in the presence of serum (differentiation conditions), the expression of *NANOG*, *OCT4* and *SOX2* was strongly reduced (Fig. 5b). These observations suggest that the expression of *NANOG*, *OCT4* and *SOX2* is indicative of the self-renewal capacity of breast stem/progenitor cells and that estrogen induces these cells to mature and proliferate. The same results were observed using another breast cancer cell line that expresses ER, T47D (Online Resource 1). Therefore, these observations are not limited to MCF-7 cells, but rather a more general phenomenon in ER-positive breast cancer cell lines.

Overexpression of Nanog, Oct4 and Sox2 increases the stem/progenitor cell population

In order to investigate the relevance of Nanog, Oct4 and Sox2 in the maintenance of breast stem cell properties, we overexpressed each of these transcription factors in MCF-7 cells using lentivirus. The efficient expression of the three factors was confirmed by immunofluorescence (Fig. 6a). It has been shown that Nanog, Oct4 and Sox2 form autoregulatory and feedforward loops [24] and, in agreement with this, we found that the overexpression of one of these factors increased the expression of each of the others (Fig. 6b). To monitor the effects of these factors on MCF-7 self-renewal, we tested the capacity of the cell lines to form mammospheres from cells plated at very low density. Nanog and Sox2 overexpressing cells formed mammospheres with higher efficiency than control cells and the mammospheres formed were smaller than those formed by control cells and had irregular shapes (Fig. 6c and 6d), suggesting that their ability to self-renew was increased but their ability to proliferate and/or differentiate was compromised. This was not the case for Oct4, possibly because Oct4 protein expression was only detectable in approximately 20% of the cell population (Fig. 6a).

We, and others, have previously reported the lack or low expression of ERα in breast stem/progenitor cells [8,25,26]. Here, we observed that MCF-7 cells overexpressing Nanog or Sox2 had reduced levels of ER expression, and this was even more evident when the cells were grown as mammospheres (Fig. 7a). Furthermore, expression of endogenous PR was also lower in cells overexpressing each of these factors (Fig. 7b). In addition, transient transfection assays showed that estrogen activation of ER-dependent transcription was reduced in cells expressing each of the

pluripotency genes, most notably in Sox2-expressing cells (Fig. 7c), indicating that ER, although present at lower levels, is still transcriptionally active.

We next determined whether ectopic expression of Nanog, Oct4 and Sox2 affected the stem/progenitor cells as defined by different phenotypes. Increased expression of *NANOG*, *OCT4* and *SOX2* considerably increased the presence of the $CD44^{+}CD24^{-/low}$ cell subpopulation (Fig. 8a), as well as the DP ($EMA^{+}CALLA^{+}$) (Fig. 8b) and $ALDH^{+}$ (Fig. 8d) cell populations, most notably when cells were grown as mammospheres (Online Resource 2). In addition, treatment of the overexpressing cells with estrogen strongly reduced the percentage of stem/progenitor cells, while tamoxifen significantly increased it (Fig. 8c). Moreover, the overexpression of *NANOG*, *OCT4* and *SOX2* was associated with a remarkable increase in cellular invasion (Fig. 8e). These results indicate that Nanog, Oct4 and Sox2 are involved in the maintenance of the stem/progenitor cell population in breast cancer cells and that estrogen reduces the proportion of stem/progenitor cells.

Discussion

Here we demonstrate that stem cells isolated from both normal human breast and breast tumor cells display increased expression of the embryonic stem cell genes *NANOG*, *OCT4* and *SOX2*. Moreover, we show that ectopic expression of any one of these factors, but in particular Nanog and Sox2, in breast cancer cells increases the pool of stem cells, the ability of the cells to form mammospheres and their capacity for invasion. The importance of estrogen and its receptor as negative regulators of the stem cell population is demonstrated by the fact that ER is expressed at low levels in stem cells and that estrogen inhibits expression of stem cell genes and mammosphere formation.

Our results suggest that ES, normal breast and breast cancer cells use the same genes to maintain a stem cell population. However, the expression of *NANOG*, *OCT4* and *SOX2* in tumors is higher than in normal cells. Several studies indicate that Nanog, Oct4 and Sox2 function as core transcription factors in the ES cell gene regulatory network [16,24], and this is underlined by the essential role that these factors play in the induction of pluripotent stem cells from differentiated cells [27-29]. It has been proposed that the function of this network is to prevent the stabilization of differentiation signals that exist in the environment or that might be intrinsic to stem cells [30]. We report here that one such signal comes from estrogen, which downregulates *NANOG*, *OCT4* and *SOX2* expression. Thus, by inhibiting the effects of estrogen, tamoxifen might prevent tumor stem cell differentiation leading to acquired resistance and the formation of more aggressive tumors in some cases. However, it is clear that ER-positive tumors do not represent a single entity and further molecular characterization would be helpful [31].

Based on our results, it seems reasonable to hypothesize that ER-positive tumors will be found to express lower levels of *NANOG*, *OCT4* and/or *SOX2* and that this will be associated with a more favorable prognosis. We did not observe a clear association between the ER status and the expression levels of *NANOG*, *OCT4* and *SOX2*, but this might be because of the small sample size and the unusual proportion of ER-positive and HER2-positive tumors in the sample analyzed. Analysis of a substantially larger number of samples is warranted to determine the potential of stem cell genes as markers of aggressiveness. In fact, high-grade ER-negative breast tumors have been reported to express an ES-like gene signature and activation of targets of Nanog, Oct4 and Sox2 is associated with aggressive tumor behavior [20]. Furthermore, there is precedence for expression of stem cell genes in tumors. Human gliomas, for example, express some embryonic stem cell-specific genes [32], Sox2 is preferentially expressed in breast tumors with a basal-like phenotype [33] and a core embryonic transcriptional profile that includes *OCT4* and *NANOG* was identified in stage 3 breast carcinomas [34].

We observed that overexpression of any one of the stem cell factors (Nanog, Oct4 and Sox2) increased the capacity of MCF-7 cells to form mammospheres, as well as the size of the stem cell pool and invasiveness. These results are consistent with other reports on the roles of Nanog and Sox2

in breast tumorigenesis [35,36]. This suggests that high expression of *NANOG*, *OCT4* and *SOX2* is associated with a breast cancer stem cell phenotype and that targeting any one of these factors might provide an improved therapy for poorly differentiated tumors. Our results also suggest that mutations or epigenetic changes that lead to higher expression of either Nanog, Oct4 and/or Sox2 could contribute to the development of tumors with a cancer stem cell phenotype.

Previous studies demonstrated that breast cancer patients whose tumors displayed a $CD44^{+}CD24^{-/low}$ phenotype have a poor prognosis, with shorter metastasis-free and overall survival [37,13]. These results suggest that the presence and frequency of $CD44^{+}CD24^{-/low}$ tumorigenic breast cancer cell populations have prognostic relevance [38]. In addition, several groups have shown that the $CD44^{+}CD24^{-/low}$ subpopulation of breast cancer cells expresses higher levels of pro-invasive genes and plays a role in the invasive step of metastasis [37,39,40]. Indeed, we observed higher expression of *NANOG*, *OCT4* and *SOX2* in the stem cell populations $CD44^{+}CD24^{-/low}$ and $EMA^{+}CALLA^{+}$ than in the rest of the sample population, and cells overexpressing these factors displayed an increase in the stem cell populations and were more invasive. The correlation between number/proportion of cells displaying stem-like features within the tumor and a worse prognosis has also been reported for ALDH1 expression in inflammatory breast cancer [41]. Furthermore, the intrinsic resistance of tumorigenic breast cancer cells to certain forms of therapy is reflected by the increase in the percentage of $CD44^{+}CD24^{-/low}$ cells and in the formation of mammospheres from tumor samples evaluated after chemotherapy [42]. Similarly, Pece and collaborators observed that poorly differentiated breast tumors contain more cancer stem cells than well-differentiated tumors [43]. Characterization of the pathways that regulate cancer stem cell differentiation might help to design better future therapies.

Our findings do not address the question of the origin of breast cancer stem cells [44], but they suggest that certain types of tumors might arise through transformation of either ER-positive progenitors or ER-negative stem/early progenitors that may asymmetrically divide to self-renew and give rise to undifferentiated ER-positive progenitor cells. In response to estrogen, ER-positive progenitors might then secrete paracrine factors, such as amphiregulin [45,46], that influence the proliferation and/or differentiation of ER-negative cells.

The relevance of estrogen in the development of the mammary gland and in breast cancer has been known for many years, but its influence on the stem cell population is not fully understood (reviewed in [7]). Our results suggest that estrogen reduces the pool of breast stem cells both in normal and breast cancer tissues. The number of mammospheres formed after serial passage at clonal density is believed to reflect stem cell self-renewal, whereas the size of the mammospheres formed reflects the rate of proliferation of more mature progenitor cells [9]. We observed that estrogen treatment reduces the number of mammospheres but increases their size. This suggests that estrogen not only reduces the pool of self-renewing stem cells, presumably by promoting their differentiation, but also promotes the proliferation of more differentiated progenitors and tumor cells, as expected considering the

known effects of estrogen on proliferation. In addition, tamoxifen antagonizes the mitogenic signals of estrogen in the tumor cells leading to smaller mammospheres. These results might seem paradoxical given that exposure to estrogen is considered to increase the risk of breast cancer (reviewed in [47]). Although the complexity of estrogen signaling has already been highlighted [48]. Ironically, one of the strongest and most extensively documented protective factors for breast cancer is early age of first pregnancy [47]. The protective effect of early childbirth has been attributed to mammary epithelial stem cell differentiation and a decrease in mammary stem cell numbers (reviewed in [49,50]). Thus, given that estrogen is required for mammary gland differentiation during puberty [51], it is possible that estrogen can have the beneficial effect of reducing the size of the mammary stem cell pool. Furthermore, clinical benefit has been observed using estrogen to treat women with metastatic breast cancer resistant to classical hormone therapy [52] and also in high doses to treat hormonally sensitive tumors [48], and higher circulating estrogen levels in postmenopausal breast cancer patients are associated with a less aggressive tumor phenotype [53]. The most striking evidence is that ER is a powerful predictive marker in breast cancer management and ER-positive tumors are more differentiated, and have a better outcome than basal or HER2-positive tumors. Our results agree with all these well-reported clinical observations. In order to self-renew, tumor cells should find ways to bypass the differentiation-promoting effect of estrogen, for example through loss of expression of ER. Consistent with this model, deletion of the breast cancer susceptibility gene BRCA1, which expands the stem cell population, reduces the expression of ER [54]. Moreover, women with ER-negative tumors are more likely to develop a second primary tumor and the second tumor is more likely also to be ER-negative [55]. In fact, it has been recently shown that poorly differentiated cancers contain more cancer stem cells than well-differentiated tumors [43], and the presence of cancer stem cells was associated with high histological grade, ER-negativity and poor overall survival [56].

The protective effects of short-term stimulation with hormone, either by early pregnancy or with the hormones estrogen and progesterone, have also been extensively studied in animal models. For example, short-term hormone exposure can prevent mammary tumorigenesis in two genetically engineered mouse models [57]. Recently, it has been reported that estrogen and progesterone together increased stem cell numbers in mice, but no difference was observed using estrogen alone [58,59]. These results are difficult to reconcile with the long-term protection against breast cancer offered by early pregnancy and with reports showing no difference in stem cell numbers between parous and virgin animals [60] or a decrease in the number of stem cells after an early pregnancy [61], which was considered an explanation for pregnancy-induced protection against breast cancer. Clearly, this is still a controversial area and further studies will be needed to decipher fully the effects of exposure to steroid hormones and breast cancer risk.

To conclude, our results implicate Nanog, Oct4 and Sox2 in the maintenance of human mammary stem cells. Finally, this work provides an explanation for the better prognosis and less aggressive phenotype of ER-positive tumors.

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Figure legends

Fig. 1 Mammospheres derived from breast epithelial cells express high levels of *NANOG*, *OCT4* and *SOX2* that are reduced by estrogen. **a** Real-time PCR analysis of *NANOG*, *OCT4* and *SOX2* expression in the normal mammary gland (n = 12). RNA was isolated from freshly isolated organoids (org) and from normal mammary epithelial cells grown under different conditions: adherent (adh) or as secondary mammospheres (ms). Expression in organoids was set at 1. The median is indicated by the horizontal bar, the mean is indicated by a solid square and the outliers by open circles. ** $p < 0.01$ (Mann-Whitney U test). **b** Expression of *NANOG*, *OCT4* and *SOX2* in mammospheres grown under differentiating conditions (dif) compared to the expression in mammospheres (ms, set at 1). **c** Levels of progesterone receptor (PR) expression in mammospheres exposed to ethanol (OH, black bar, set at 1), estrogen (E2, dashed bar) or tamoxifen (Tam, gray bar). **d** Expression of *NANOG*, *OCT4* and *SOX2* in mammospheres in the presence of the indicated hormones

Fig. 2 Breast stem/progenitor cells express higher levels of *NANOG*, *OCT4* and *SOX2* than the differentiated cells. **a** FACS analysis of DP (EMA^+CALLA^+) cells isolated from dissociated organoids (org, top) or from mammospheres (ms, bottom). **b** Fold change of the percentage of DP cells in mammospheres grown during 7 days in the presence of estrogen (E2, dashed bar) or tamoxifen (Tam, gray bar) compared to the control ethanol (OH, black bar), set at 1. **c** *NANOG*, *OCT4* and *SOX2* expression assessed by real-time PCR in DP and differentiated EMA^+ and $CALLA^+$ cells. Expression in EMA^+ cells was set at 1. All experiments were performed using at least three different breast samples, * $p < 0.05$; ** $p < 0.01$

Fig. 3 *NANOG*, *OCT4* and *SOX2* are highly expressed in breast cancer stem cells. **a** The expression of *NANOG* and *SOX2* in tumor samples (T) was compared to the expression in normal samples (N) from the same patients set at 1 by real-time PCR, and represented as fold induction. * $p < 0.05$, ** $p < 0.01$ (Mann-Whitney U test). On the right, Sox2 nuclear expression levels were examined by immunofluorescence in tumor and normal samples from the same patient. **b** Dissociated tumor cells were allowed to grow in suspension in the presence of ethanol (OH, black bar), estrogen (E2, dashed bar) or tamoxifen (Tam, gray bar). *NANOG* and *SOX2* RNA was quantified and represented as fold induction with the ethanol set at 1. **c** The $CD44^+CD24^{-/low}$ tumor cell population was isolated by FACS sorting and the expression of *NANOG*, *OCT4* and *SOX2* compared to the expression in the total tumor population (T) set at 1. All experiments were performed using at least three different tumor samples, * $p < 0.05$

Fig. 4 Estrogen treatment reduces the mammosphere formation capacity of MCF-7 cells. **a** MCF-7 cells were plated at low density in the presence of the carrier ethanol (OH), estrogen (E2) or

tamoxifen (Tam) at day 1 (1d, left). After 7 days in suspension culture (7d, middle) the mammospheres were trypsinized into single cell suspension and the cells (P1 0d) were allowed to form new mammospheres, and considered as passage 1. The process was repeated again every 7 days for several passages. Scale bar = 100 μm. (B) MCF-7 cells from passage 3 were FACS sorted at single cell level into 96-well plates in the presence of ethanol (black bar), estrogen (dashed bar) or tamoxifen (gray bar). The number of mammospheres formed was determined and represented as percentage. The experiment was done three times in triplicate. **c** The graph represents the sizes of the mammospheres formed in **b** as the percentage of mammospheres within a specific diameter: smaller than 50 μm, between 50 and 100 μm, between 100 and 200 μm and larger than 200 μm

Fig. 5 Expression of *NANOG*, *OCT4* and *SOX2* is increased in MCF-7 mammospheres and reduced by estrogen treatment. **a** Expression of *NANOG*, *OCT4* and *SOX2* in mammospheres (ms) compared to their expression set at 1, in adherent cultures (adh) ** $p < 0.01$. **b** Expression of *NANOG*, *OCT4* and *SOX2* in tertiary mammospheres (ms) and in mammospheres that were allowed to differentiate (dif) under adherent conditions with serum and in the presence of ethanol (OH, black bar), estrogen (E2, dashed bar) or tamoxifen (Tam, gray bar)

Fig. 6 Generation of MCF-7 stable cell lines (pooled) overexpressing Nanog, Oct4 and Sox2. **a** Immunostaining of MCF-7 cells transduced with lentivirus designed to express Nanog, Oct4, Sox2 or GFP. Scale bar = 40 μm (GFP cells) or 20 μm (Nanog, Oct4 and Sox2 cells). **b** Real-time PCR analysis of the expression of *NANOG*, *OCT4* and *SOX2* in the stable cell lines. GFP stable cell line expression was set at 1. **c** Cells from each cell line were FACS sorted at 50 cells/cm^2 into 6-well plates and allowed to grow in suspension. Top: representative photographs of the mammospheres formed by each stable cell line. Bottom: representative photographs of the mammospheres after staining with crystal violet. Scale bar = 100 μm. **d** Top: counting of the mammospheres formed in **c**. Bottom: percentage of mammospheres within specific size ranges: smaller than 50 μm, between 50 and 100 μm, between 100 and 200 μm and larger than 200 μm. The experiment was done three times in triplicate, * $p < 0.05$; ** $p < 0.01$

Fig. 7 Overexpression of Nanog, Oct4 and Sox reduces ERα expression and transcriptional activity. **a** ERα and GAPDH immunoblots of lysates of MCF-7 overexpressing cells grown as adherent (adh) or as mammospheres (ms). **b** PR and GAPDH immunoblots of lysates of MCF-7 overexpressing cells grown as adherent cells in the presence of ethanol (OH) or estrogen (E2) for 24 h. Cells had been hormone depleted for 72 h prior to treatment. Both isoforms, A and B, of progesterone receptor are indicated (PR-A and PR-B). **c** ERα transcriptional activity of the different MCF-7 cell lines in the presence of ethanol (OH) or estrogen (E2). β-galactosidase activity was used to control for

transfection efficiency. The graph shows the mean of three independent experiments done in triplicate. White (OH) and stippled (E2) columns cells were transfected with the control TK-luciferase reporter; Black (OH) and dashed (E2) columns cells were transfected with the ERE TK-luciferase reporter, ** $p < 0.01$

Fig. 8 Overexpression of Nanog, Oct4 and Sox2 increases the stem/progenitor cell population. **a** Isolation of $CD44^{+}CD24^{-/low}$ cells, **a**, or $EMA^{+}CALLA^{+}$ cells, **b**, for cells grown as adherent cultures (adh) or as mammospheres (ms). Non-transduced MCF-7 cells were used as control. **c** Fold change of the percentage of DP cells in cells grown as adherent cultures in the presence of ethanol (OH), set at 1, estrogen (E2) or tamoxifen (Tam) for 48 h. As before, cells were hormone depleted for 72 h prior to treatment. **d** ALDH1 enzymatic activity assessed by the ALDEFLUOR assay and flow cytometry of cells grown in adherence. **e** Representative photographs of chemotaxis assays (top) and Matrigel invasion assays (bottom) performed using the indicated cells. Each experiment was repeated three times and the results are represented as the percentage of invasion (right). Scale bar = 100 μm, * $p < 0.05$; ** $p < 0.01$

Figure 1

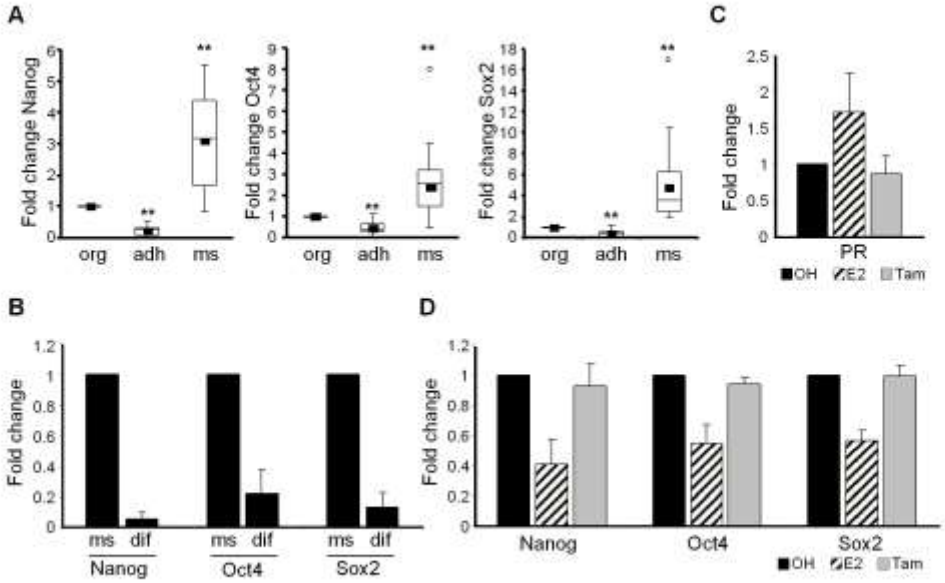
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Figure 2

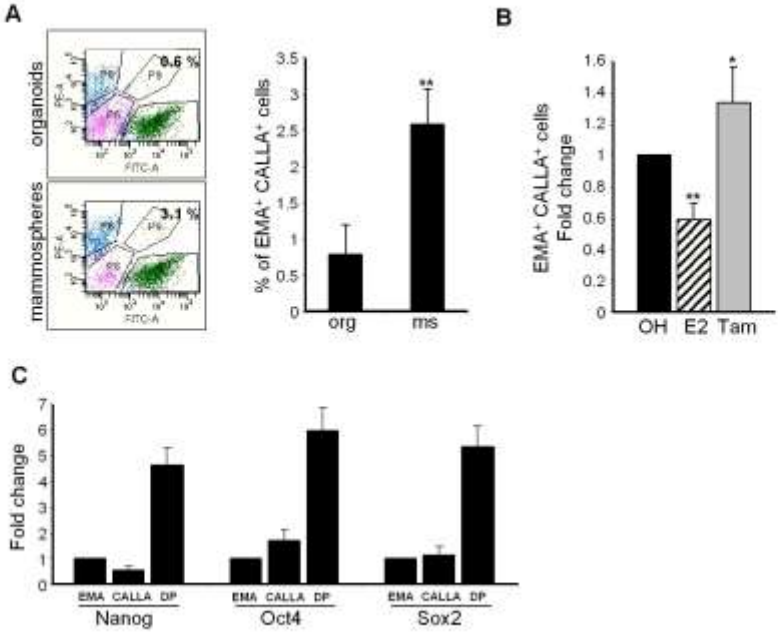
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Figure 3

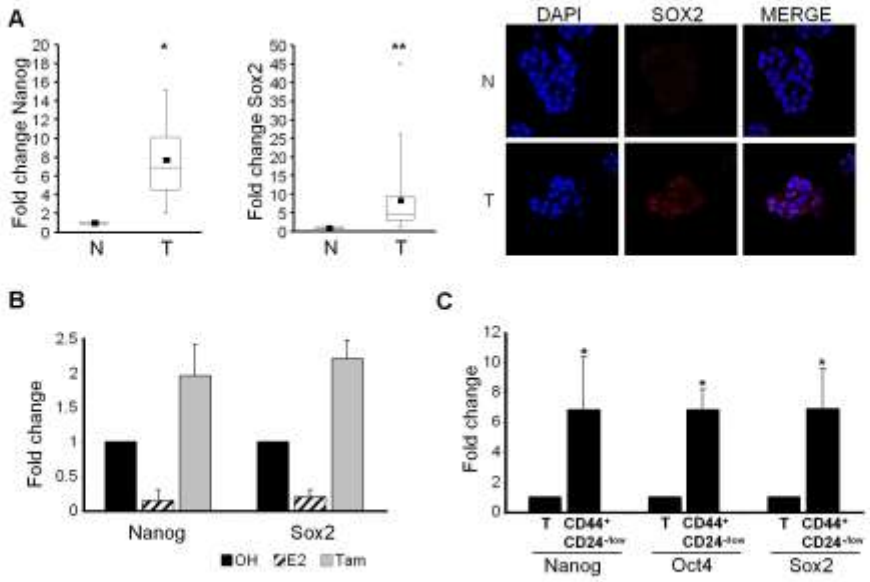
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Figure 4

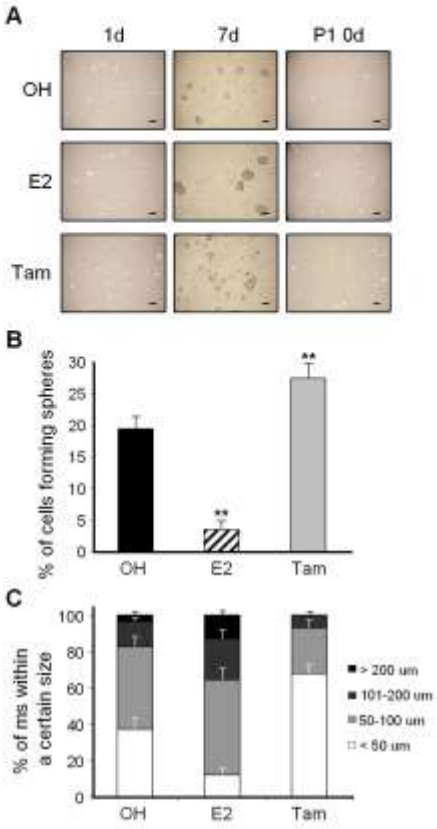
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Figure 5

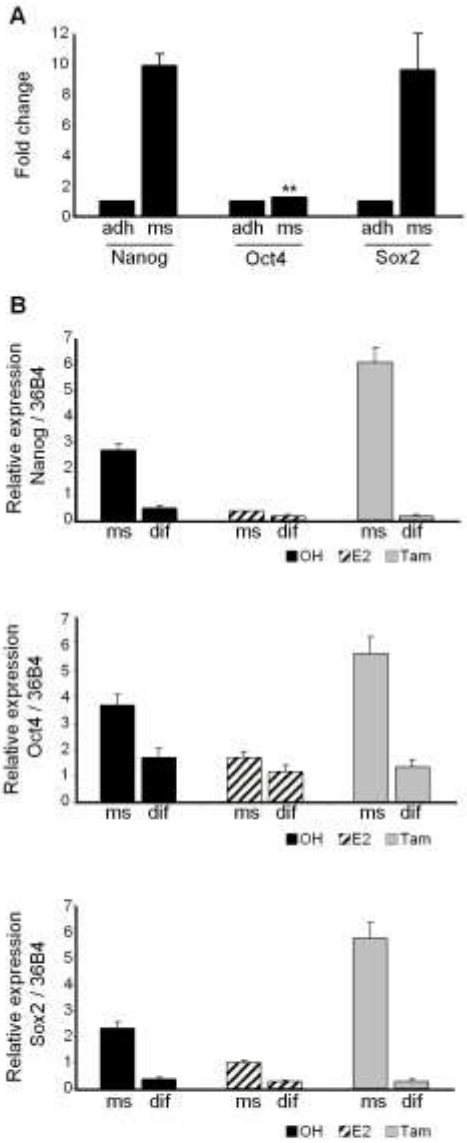
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Figure 6

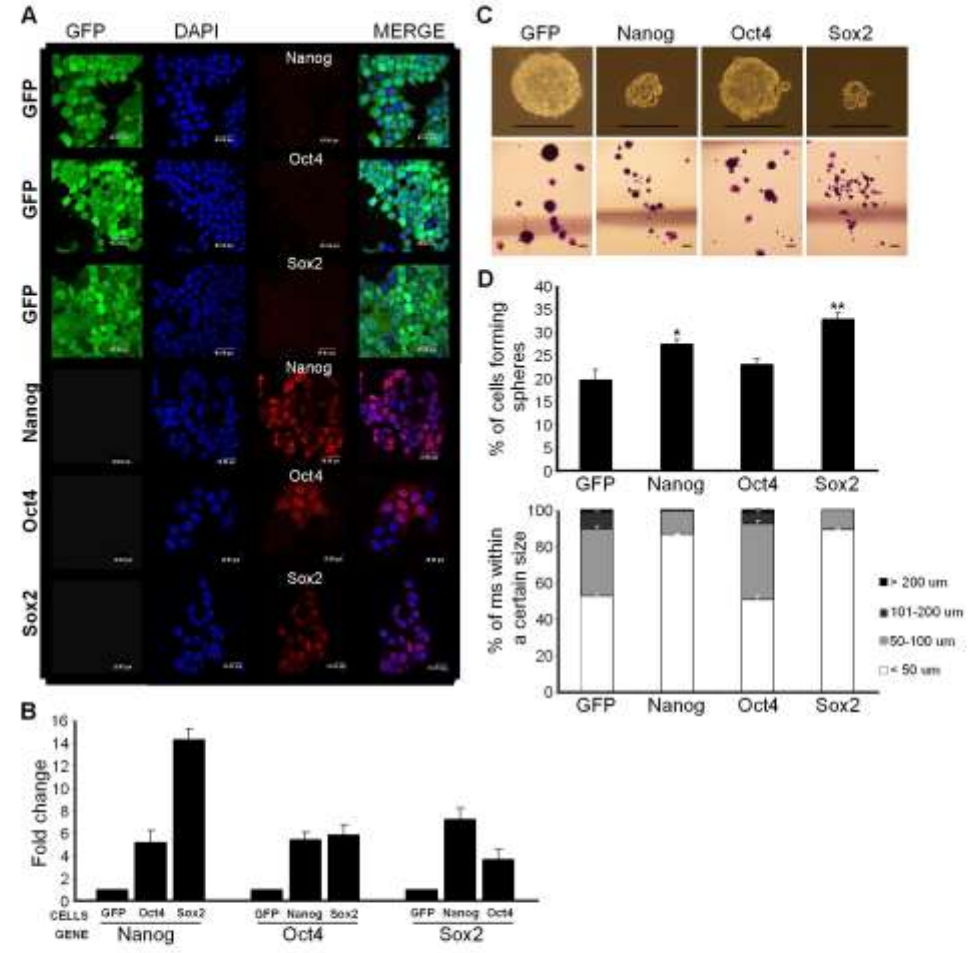
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Figure 7

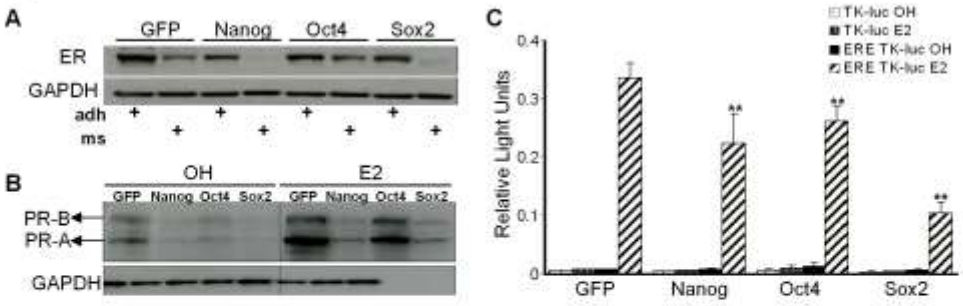
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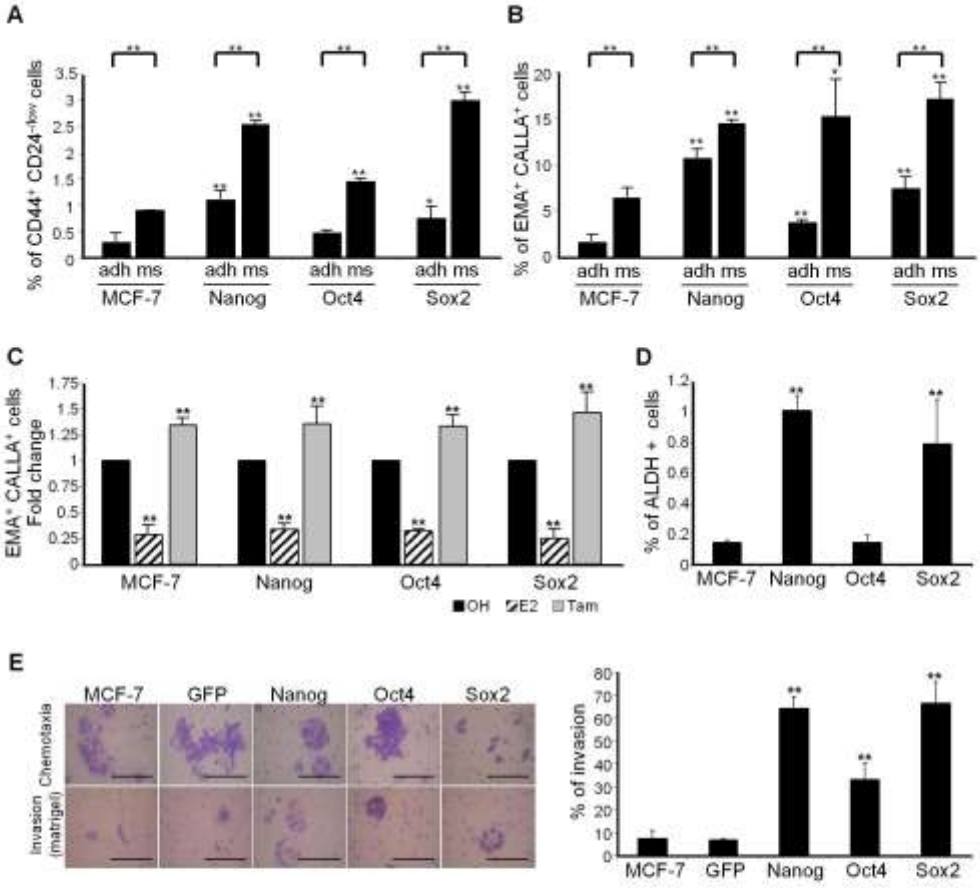
Figure 8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