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Sox10 regulates stem/progenitor and mesenchymal cell states in mammary epithelial cells

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Summary

To discover mechanisms that mediate plasticity in mammary cells, we characterized signaling networks that are present in the mammary stem cells responsible for fetal and adult mammary development. These analyses identified a signaling axis between FGF signaling and the transcription factor Sox10. Here we show that Sox10 is specifically expressed in mammary cells exhibiting the highest levels of stem/progenitor activity. This includes fetal and adult mammary cells *in vivo* and mammary organoids *in vitro*. Sox10 is functionally relevant, as its deletion reduces stem/progenitor competence, while its overexpression increases stem/progenitor activity. Intriguingly, we also show that Sox10 overexpression causes mammary cells to undergo a mesenchymal transition. Consistent with these findings, Sox10 is preferentially expressed in stem- and mesenchymal-like breast cancers. These results demonstrate a signaling mechanism through which stem and mesenchymal states are acquired in mammary cells, and suggest therapeutic avenues in breast cancers for which targeted therapies are currently unavailable.

Graphical Abstract

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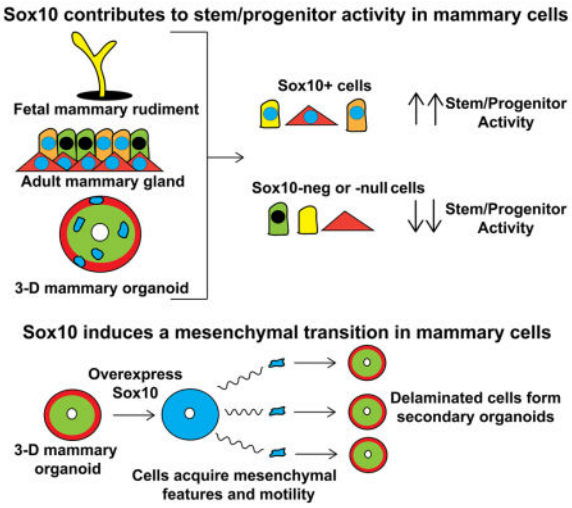
Author Contributions

CD and GMW designed the study. BTS, CJ, CD, JCH, CMP performed bioinformatic analyses. BTS, CT performed transplants. CD acquired all other data. CD wrote the manuscript; all authors facilitated revisions. GMW supervised the study.

Additional experimental procedures are described in the Supplement.



Keywords

Sox10; mammary stem cells; EMT; basal-like breast cancer; cancer stem cells

Introduction

The capacity to reprogram differentiated cells *in* and *ex vivo* indicates that the differentiated state is not as fixed as once thought (Takahashi and Yamanaka, 2006; Tata et al., 2013). This plasticity has important implications for cancer, where the dysregulation of stem and mesenchymal states appears to be critical in disease initiation and progression. Phenotypic lability may endow some types of cancer cells, often termed “cancer stem cells” (CSC), with a greater capacity to propagate the disease when assayed in a transplant setting (Al-Hajj et al., 2003; Bonnet and Dick, 1997). In contrast to CSCs, which typically exhibit mesenchymal characteristics, transcriptome analyses have revealed another class of tumorigenic cancer cells whose gene expression profiles resemble those of cells with known stem or progenitor cell functions. Tumors with these distinct “stem-like” cancer cells tend to appear less differentiated and behave more aggressively, while eliminating such cells can attenuate tumor progression (Chen et al., 2012; Eppert et al., 2011; Merlos-Suarez et al., 2011; Schepers et al., 2012). Stem-like cancer cells may arise either by cell of origin, in which the tumor originates in a stem/progenitor cell and retains those properties through tumorigenesis, or through reprogramming of differentiated cells into a stem-like state (Barker et al., 2009; Schwitalla et al., 2013). Because a significant fraction of triple-negative breast cancers contain stem-like cancer cells, we have focused on elucidating the molecular mechanisms that specify the mammary stem cell (MaSC) state, assuming that such knowledge will deepen our understanding of how such breast cancers initiate and progress.

The mammary gland contains at least two populations of cells with stem or progenitor qualities (Shackleton et al., 2006; Stingl et al., 2006). Luminal progenitors comprise a heterogeneous population of cells in the luminal fraction of the gland that possess clonogenic properties *in vitro* (Shehata et al., 2012). This population may contain the cell-

of-origin for stem-like basal-like breast cancers (Lim et al., 2009). Transplantation studies also demonstrate that the basal fraction of the gland contains cells capable of generating an entire mammary gland. These MaSCs are inferred to possess extensive proliferative, invasive, and multi-lineage differentiation potential, as a single MaSC can regenerate a functional gland (Shackleton et al., 2006).

Several fundamental aspects of MaSC biology remain to be elucidated. There is no consensus on the number of MaSCs within the gland, which has hindered analyses of the origin of breast tumors (Tomasetti and Vogelstein, 2015). There is also conflicting data about the topographical location of MaSCs in the gland, and the developmental timeframe during which these cells retain multi-lineage potential (Rios et al., 2014; Van Keymeulen et al., 2011). Both of these problems might be resolved by availability of markers enabling prospective MaSC identification. The mechanisms by which mammary cells enter and exit from the MaSC state also remain to be defined, and resolving this problem may present solutions to those concerning MaSC identification. One recent advance on this topic involves the demonstration that Sox9 and Slug act together to convert mammary epithelial cells into cells with MaSC-like properties (Guo et al., 2012). However, the degree to which this mechanism is utilized in the gland is not clear because the distribution and function of Sox9 or Sox9/Slug cells in unperturbed *in vivo* contexts remain to be defined. Moreover, mice that are deficient for *Slug* do form a complete native mammary gland, which suggests that Slug is not an essential determinant of the MaSC state (Nassour et al., 2012). Clearly, a better understanding of the transcriptional programs and extrinsic signaling mechanisms that regulate the MaSC state are required.

To investigate the biology of MaSCs and MaSC-like cells in cancer, our research has focused on the stem cells present during fetal mammary development. During mid-late embryogenesis, mammary cells are highly proliferative and invasive, and likely experience conditions such as hypoxia and growth-oriented metabolism that resemble those encountered by tumor cells (Masson and Ratcliffe, 2014). Fetal MaSCs (fMaSCs) may therefore most resemble the MaSC-like cancer cells in breast tumors. Indeed, we previously showed that fMaSCs exhibit both the organoid forming and mammary repopulating properties found in luminal progenitors and adult MaSCs, respectively (Spike et al., 2012). Transcriptome profiling of fMaSCs and adult MaSCs revealed that the fMaSC signature gene list is uniquely enriched in basal-like breast tumors, indicating the presence of fMaSC-like cells in such tumors. This shared biology suggests that fetal mammary development and fMaSCs can be utilized to identify molecular mechanisms that govern important functions in breast cancer.

Here we describe how analysis of fMaSCs revealed an important function for Sox10 in mammary cells. Sox family transcription factors have well defined roles in regulating cell fate decisions in different tissues, and at different stages of development (Sarkar and Hochedlinger, 2013). Sox factors generally induce preferential differentiation down one cell lineage path over another, often by antagonizing the activity of other lineage-specifying factors. This phenomenon has best been described with Sox2, and the elucidation of roles for Sox2 in multiple different cell fate decisions, each of which occurs in concert with other transcription factors (Sarkar and Hochedlinger, 2013). However, when Sox expression or

activity is balanced or kept at lower levels in the cell by other key factors, differentiation is forestalled and stem and progenitor functions arise (Kopp et al., 2008). This is consistent with an emerging model of stem cell specification through the balance of lineage specifiers (Loh and Lim, 2011). Sox factors can thus be mediators and markers of both differentiation and stemness, depending on expression level and cellular context.

Here, we report that Sox10 plays important regulatory roles in promoting both stem- and EMT-like properties in mammary stem cells. Critically, these stem and mesenchymal states are acquired independently of one another; this clear distinction prevents potential conflation of stem cell and mesenchymal properties, and demonstrates how these distinct states can be related by a single factor such as Sox10. We further present evidence that these functions may be conserved in certain types of aggressive breast cancers, and demonstrate the importance of FGF10 in a paracrine signaling mechanism that regulates Sox10.

Results

Sox10 is an fMaSC- and tumor-associated transcription factor regulated by FGF signaling

To identify molecular mechanisms that specify stem/progenitor cell functions in mammary cells, we analyzed transcriptome profiles of fMaSCs and their surrounding fetal stroma (fStr) (Spike et al., 2012). We prioritized both transcription factors that are differentially expressed in the fMaSC-enriched population and inferred signaling axes between fMaSCs and fStr that could regulate their expression. These analyses identified Sox10 as one of the most prominent transcription factors associated with the fMaSC population (Fig. 1A). This was of immediate interest, as Sox family transcription factors play important roles in pluripotent or tissue-specific stem cell states (Sarkar and Hochedlinger, 2013). Further, Sox10 in particular has been shown to be a critical transcription factor in reprogramming differentiated cells into multipotent stem/progenitor states (Hornig et al., 2013; Kim et al., 2014; Najm et al., 2013; Yang et al., 2013).

These analyses also revealed high relative expression of FGF7 and FGF10 in the fStr, and expression of multiple FGFR family members in the fMaSC population (Fig. 1A). FGF signaling plays a critical role in fetal mammary development, and we previously showed that fMaSCs could utilize FGF signaling to promote multipotent growth *in vitro* (Lu et al., 2008; Mailleux et al., 2002; Spike et al., 2012). Furthermore, FGF signaling has been shown to regulate the expression and function of different Sox family transcription factors in multiple developing tissues through a feedback loop of unknown mechanism (Chen et al., 2014; Seymour et al., 2012). These observations led us to hypothesize that an FGF signaling axis may regulate Sox10 expression in mammary stem/progenitor cells.

To address this, we grew fMaSCs in 3-D culture conditions in the presence of the pan-FGFR inhibitor, JNJ-42756493 (FGFRi). With vehicle-only, fMaSCs form organoids when either EGF or bFGF (FGF2) are added to the media, but fail to form organoids if neither growth factor is present (Fig. 1B, Supp. Fig. 1). Addition of FGFRi blocked organoid formation if FGF is the only available growth factor. However, organoid formation is rescued upon adding EGF to media containing FGFRi (Fig. 1B). As the number of dead cells does not increase in FGFRi-treated organoids (data not shown), these data demonstrate that fMaSC-

derived organoids can utilize FGF signaling, and indicate that FGFRi blocks FGF signaling without eliciting overt cytotoxicity.

To determine if FGF signaling regulates Sox10 expression in mammary cells, we measured Sox10 expression levels in fMaSC-derived organoids plated with vehicle or increasing concentrations of FGFRi. Organoid exposure to FGFRi resulted in significant dose-dependent decreases in Sox10 mRNA expression levels (Fig. 1C). Similarly, by using a *Sox10*-H2BVenus BAC transgenic mouse line (in which H2B-Venus is expressed under Sox10 transcriptional regulatory elements) to quantify the Sox10+ cells through Venus fluorescence, we found that FGFRi exposure significantly reduced the number of Sox10+ mammary organoid cells (Fig. 1D). This effect was observed in a serum-based medium or in a serum-free medium (SFM) containing defined growth factors (Fig. 1D, Supp. Fig. 1). Organoids that were generated from adult luminal progenitors also showed a reduction in Sox10+ cells following FGFRi exposure (Fig. 1D). fMaSCs grown in the presence of SFM with EGF + FGF10 developed into organoids with increased numbers of Sox10+ cells compared to fMaSCs grown only in SFM with EGF (Fig. 1E). This effect was not seen in fMaSCs grown with SFM containing EGF + FGF2, indicating a specific role for FGF10 signaling through its cognate receptor, FGFR2b. No significant differences in Sox10 levels were observed in fMaSCs grown +/− EGF (Supp. Fig 2). These data indicate that FGF signaling specifically regulates Sox10 expression levels in mammary cells.

To determine whether elevated Sox10 expression was a feature common to fMaSC and their associated human cancer counterparts, we next analyzed the expression of Sox10 across a panel of tumor samples representing two distinct breast cancer datasets. This analysis revealed that basal-like and claudin-low breast cancers tend to express significantly higher levels of Sox10 than the other subtypes of the disease (Fig. 1F), in accordance with two recent studies of Sox10 in breast cancer (Cimino-Mathews et al., 2013; Ivanov et al., 2013). These two subtypes comprise the bulk of triple negative breast cancers and both are frequently metastatic and aggressive. However, they differ in that basal-like breast cancers are weakly differentiated and the most fMaSC-like of the breast cancer subtypes, while claudin-low breast cancers possess the most EMT-like morphology and transcriptome amongst the breast cancer subtypes (Prat et al., 2010; Spike et al., 2012). These findings suggest that Sox10 expression may correlate with distinct stem and mesenchymal properties in human breast cancers.

Collectively these data identify Sox10 as an FGF responsive, mammary stem cell associated transcription factor with likely roles in normal and transformed mammary cells.

Sox10 is a fetal mammary stem cell marker that improves fMaSC purification

To elucidate the role of Sox10 in mammary cells, the *Sox10*-H2BVenus BAC transgenic mouse line was used to visualize Sox10+ cells. Consistent with the fMaSC transcriptome data, Sox10 was robustly expressed in all five fetal mammary rudiment pairs (Fig. 2A–C). The rudiments at these stages appear to be very primitive, as there is amorphous structure at E16, while at E18 the lumen has not yet formed and there is no clear segregation of the luminal marker keratin-8 (K8) and the basal marker keratin-14 (K14) (Fig. 2D).

Sox10+ fetal mammary cells were recovered using flow cytometry for more detailed molecular characterization. As cells in the rudiment can be distinguished from surrounding stromal cells by the epithelial cell adhesion marker (EpCAM), fetal Sox10+ mammary cells were isolated as Sox10+;EpCAM+. Consistent with Fig. 2C, nearly all cells appear to be Sox10+ within the rudiment by FACS analysis (Fig. 2E). It is possible that the stability of the H2B-Venus fusion protein may yield cells that no longer express Sox10 but still retain the Venus fluorescence and thus overrepresent Sox10 expression. To address this, a *Sox10*$^{flox\text{-}GFP}$ mouse line in which a less stable GFP reporter is expressed from native Sox10 transcripts was also analyzed, and confirmed that the majority of fetal mammary cells are Sox10+ (Supp. Fig. 3). Consistent with the *Sox10*-H2BVenus wholemount images, most single *Sox10*$^{flox\text{-}GFP}$ cells also co-expressed K8 and K14, suggesting that they may be bipotent progenitors or stem cells (Fig. 2F).

Stem/progenitor cell function in these Sox10+ fetal cells was next analyzed using *in vitro* and *in vivo* stem/progenitor cell assays. Single fMaSCs grown in 3-D culture conditions will clonally expand to generate bi-lineage organoids that resemble the architecture of the mammary gland with inner K8+ luminal cells and external K14+ basal cells (Spike et al., 2012). When E18 Sox10+ fetal cells were plated as single cells into 3-D culture conditions, they robustly formed bi-lineage organoids (Fig. 2G, 2H, Supp. Fig. 3). This demonstrates that the Sox10+ E18 population contains bipotent cells that generate both luminal- and basal-like cells. By contrast, the more rare Sox10^{neg} fetal mammary cells formed spheres at significantly reduced efficiency. As an *in vivo* metric of stem cell function, E18 Sox10+ fetal cells were also transplanted into cleared fat pads of immune-compromised mice. As few as five Sox10+ fetal cells were sufficient to generate a full mammary gland, further indicating that Sox10 positivity strongly correlates with fMaSC activity (Fig. 2I, Supp. Fig. 3). Collectively, the data demonstrate that Sox10 expression labels cells in the fetal mammary rudiment that possess bipotent stem/progenitor features.

Notably, the organoid forming efficiency for fetal cells recovered with the *Sox10*-Venus and EpCAM markers represents a >3x improvement over the original CD24 and CD49f fMaSC marker strategy we previously employed. We isolated and RNA-sequenced E17 Sox10+;EpCAM+ fMaSCs and their surrounding fetal stromal cells (Supp. Table 1). In parallel, we RNA-sequenced E17 fMaSCs isolated by sorting for CD24^{hi};CD49f^{+} cells to assess the purification afforded by Sox10 and EpCAM. Comparison of these transcriptome profiles revealed that numerous stromal-associated genes were removed from the E17 fMaSC profile by using Sox10 expression to purify fMaSCs (Fig. 2J). Taken together, our data show that using Sox10 as a marker produces an fMaSC population significantly purer than obtained previously.

Sox10 labels cells with stem/progenitor features in adult mammary tissues

We next analyzed Sox10 expression in the adult mammary gland. Immunofluorescence against positional markers such as EpCAM (high in luminal cells, low in basal cells) indicated that Sox10 expression was more restricted in the adult gland compared to the fetal mammary rudiment (Fig. 3A). To quantify the expression of Sox10 by cell type, *Sox10*-H2BVenus and *Sox10*$^{flox\text{-}GFP}$ adult glands were FACS sorted into basal and luminal

fractions using EpCAM/CD49f and the percentage of Sox10+ cells in each fraction was then determined. These analyses revealed that nearly all basal cells express Sox10, while ~50% of luminal cells express Sox10 (Fig. 3B, Supp. Fig. 4).

Mammary stem/progenitor cell assays were performed on these Sox10+ basal and luminal cells to better understand their function in the gland. Sox10+ and Sox10^{neg} luminal cells were isolated by FACS and plated into 3-D culture conditions. While Sox10+ luminal cells demonstrated sphere forming potential with luminal characteristics (18.0 +/− 2.1%), Sox10^{neg} luminal cells did not form spheres (0.3 +/− 0.3%, Fig. 3C, Supp. Fig. 4). This suggests that Sox10+ luminal cells demarcate the colony-forming luminal progenitor cells in the luminal fraction of the mammary gland. Consistent with this, Sox10+ cells do not express progesterone receptor, a mature luminal cell marker, which is instead exclusively expressed in Sox10^{neg} luminal cells (Fig. 3D). In the basal cell fraction, both Sox10+ and less common Sox10^{neg} basal cells were transplanted into cleared fat pads to determine MaSC function in an *in vivo* context. Sox10+ basal cells exhibited robust repopulation potential, whereas no successful transplantation was observed with Sox10^{neg} basal cells (Fig. 3E, F). Sox10+ luminal cells also failed to exhibit successful transplantation, further indicating that these are lineage restricted progenitor cells.

These data indicate that populations with known mammary stem/progenitor cell properties —fMaSCs in the fetal rudiment, repopulating MaSCs in the adult basal fraction, and luminal progenitors in the luminal layer of the mammary gland, all appear to express Sox10.

Sox10 labels cultured mammary cells with stem/progenitor characteristics *in vitro*

The correlation of Sox10 expression with mammary stem/progenitor populations *in vivo* led us to next investigate if Sox10 also labels cells with these properties in organoids grown from fMaSCs *in vitro*. To address this, *Sox10*-H2BVenus fMaSCs were grown into bi-lineage organoids in 3-D culture conditions. Intriguingly, these structures exhibited mosaic Sox10 expression in which Sox10+ and Sox10^{neg} cells were clearly evident (Fig. 4A). To determine if these cells differ in stem/progenitor functionality, these populations were isolated and replated into identical organoid-forming conditions to generate secondary organoids in a classic surrogate assay of self-renewal for stem cells. Notably, Sox10+ cells from primary organoids had significantly greater potential to form secondary organoids than Sox10^{neg} cells (Fig. 4B). Further, the secondary structures from Sox10+ cells were larger and yielded clear bi-lineage differentiation with both luminal and basal cell types present (Fig. 4C). The rare secondary outgrowths derived from Sox10^{neg} cells were by contrast smaller and appeared to lack the bi-lineage structure observed in primary and Sox10+ secondary organoids (Fig. 4C). These secondary organoids appeared to show more luminal-restricted Sox10 expression compared to primary organoids, which may reflect the restriction in stem/progenitor competence that occurs in this differentiation medium, and may mimic native mammary cell hierarchy. These data indicate that in addition to mammary cells *in vivo*, Sox10 labels populations with enhanced stem/progenitor functions in cultured mammary organoids *in vitro*.

Sox10 functionally contributes to stem/progenitor activity in mammary cells

We next determined if Sox10 actively contributes to fMaSC function by performing stem/progenitor assays on cells in which Sox10 expression was ablated by deletion. We infected *Sox10*$^{flox/flox}$ and *Sox10*$^{wild\text{-}type}$ fMaSCs with Cre-expressing lentivirus to delete Sox10 from the *Sox10*^{flox} cells. While Cre-infected *Sox10*$^{wild\text{-}type}$ fMaSCs generated typical organoids with luminal and basal architecture resembling the mammary gland, the Cre-infected *Sox10*$^{flox/flox}$ fMaSCs generated fewer organoids, and the structures that did form were typically smaller and failed to develop the morphological features of multi-lineage organoids (Fig. 4D, Supp. Fig. 5).

We also performed transplantation assays with Cre-infected *Sox10*$^{flox/flox}$ fMaSCs or *Sox10*$^{flox/flox}$ adult basal cells to determine if cells were capable of generating full outgrowths following Sox10 deletion. No full outgrowths following transplantation were observed in the Sox10^{null} MaSCs, whereas equivalent numbers of control cells exhibited successful transplantation (Fig. 4E, Supp. Fig. 5). Together these data indicate that Sox10 is required for full stem/progenitor cell functionality.

To determine if overexpression of Sox10 can increase stem/progenitor function in mammary cells, the Tet-on system was used to drive expression of human Sox10 in fMaSCs. fMaSCs isolated from a mouse strain that ubiquitously expresses the m2rtTA reverse tetracycline transactivator were infected with either LV-TRE-hSox10-2A-NLSVenus (doxycyline (dox) induces expression of Sox10 and Venus) or LV-TRE-NLSVenus (dox induces expression only of Venus) and allowed to form primary organoids. No apparent increase in primary organoid formation was observed with Sox10-overexpression (Sox10^{OE}). These primary organoids were then dissociated to single cells, replated into identical culture conditions, and scored for their ability to generate secondary organoids as a metric for increased persistence of stem/progenitor function. While fMaSCs that did not overexpress Sox10 showed low ability to form secondary organoids in differentiation medium (Fig. 5A), Sox10^{OE} fMaSCs now demonstrated robust secondary organoid formation (Fig. 5A, B). These data indicate that ectopic expression of Sox10 is able to increase or sustain stem/progenitor competence in cultured fetal mammary cells.

Ectopic Sox10 expression drives an EMT-like response in fMaSC-derived organoids

While measuring the stem/progenitor function of Sox10^{OE} cells, we discovered that primary organoids with Sox10^{OE} cells demonstrated a novel morphology in which the primary organoid was surrounded by individual cells (Fig. 5C). Video microscopy showed that the satellite cells originate from the delamination and extrusion of Sox10^{OE} cells from the primary organoid (Fig. 5D, Videos). We found that Sox10^{OE} (Venus+) cells no longer expressed keratin markers, suggesting that the mobility of the cells might result from Sox10^{OE}-induced EMT (Fig. 5E, Supp. Fig. 6). Sox10^{OE} cells also presented with additional EMT markers, including downregulated expression of E-cadherin and upregulated expression of vimentin (Fig. 5F, Supp. Fig. 6). No such changes were observed in organoids not exposed to Dox. These data demonstrate that Sox10 can directly mediate an EMT-like response when forcibly expressed at high levels in fMaSC-derived organoids.

We next determined if the EMT state could be reversed in Sox10^{OE} mammary cells and if they retained or could regain bipotential stem/progenitor function. Sox10^{OE} mammary cells were isolated from primary organoid cultures and replated into 3-D culture conditions with or without dox. The Sox10^{OE} mammary cells that were plated into dox, and thus maintained high Sox10 expression, often persisted as single cells and did not organize into secondary organoids (Fig. 6A). However, when these same cells were plated into dox-free media, and Sox10 levels were reduced to baseline (Supp. Fig. 7), the cells now favored the formation of bi-lineage secondary organoids (Fig. 6A).

The same phenomenon was observed when Sox10^{OE} organoids that had undergone EMT and cell delamination were subjected to a protocol that removed dox from the media and lowered Sox10 expression to basal levels. While organoids continuously exposed to dox and high Sox10 levels showed mostly persistent single cell satellite structures, the satellite cells in the dox-withdrawn organoids now initiated the formation of localized secondary organoids (Fig. 6B). These secondary organoids exhibited the same bi-lineage features of primary fMaSC organoids, indicating that these single Sox10^{OE} cells have the potential to produce both luminal- and basal-like cells (Fig. 6C). Notably, this robust secondary organoid formation occurred in the same strong differentiation media in which cells with retained stem/progenitor qualities are rare (Fig. 4B), indicating the downstream effects of Sox10 serve to counterbalance these pro-differentiation factors.

These data reveal that at high levels of expression, Sox10 induces a mesenchymal transition that enables cell migration away from primary organoids. These cells are then capable of undergoing a mesenchymal-epithelial transition (MET) that mediates the formation of secondary organoids, which appears to be favored when Sox10 expression levels are reduced.

FGF signaling is required for Sox10-induced cell motility

We next attempted to identify mechanisms through which Sox10 evokes stem/progenitor and EMT/motility functions in mammary cells. The feedback loop between Sox transcription factors and FGF signaling that appears to involve Sox10 and FGF10 in mammary cells (Fig. 1) suggests that these Sox10-mediated cell functions could involve FGF signaling. To test this, fMaSCs were manipulated to overexpress Sox10 as before, but this time in the presence of FGFRi. As expected, fMaSCs that were given vehicle formed primary organoids and the overexpression of Sox10 elicited an EMT-like delamination of cells (Fig. 7A). However, this cell delamination was significantly attenuated in organoids that were exposed to the FGFRi, as indicated by the absence of satellite cells surrounding the primary organoid (Fig. 7A, B). Sox10^{OE} organoids that were grown in media without FGF also failed to extrude satellite cells, confirming that it is inhibition of FGF signaling by the FGFRi that mediates this effect (Fig. 7C). These data suggest that the potentiation of FGF signaling can be one effector of Sox10 that mediates cell delamination, and that a pan-FGFRi blocks Sox10-induced motility in fMaSC-derived mammary organoids.

Transcriptome analyses of Sox10^{OE} cells indicate potential mediators of stem and EMT functions

To more comprehensively profile the state changes elicited by Sox10 and to identify other potential direct or indirect targets of Sox10 that could mediate the stem/progenitor and EMT-like functions of Sox10, we performed transcriptome profiling of Sox10^{OE} cells through RNA sequencing (Supp. Table 2). In parallel, we also isolated and RNA-sequenced control organoid cells that did not overexpress Sox10 for comparison. To assess the quality of the sequencing data, we determined if previously described targets of Sox10 were upregulated in response to Sox10 overexpression. Published targets such as Mitf, Mia, and ErbB3 all showed elevated expression in Sox10^{OE} cells (Bondurand et al., 2000; Graf et al., 2014; Prasad et al., 2011) (Fig. 7D). We also analyzed targets of FGF signaling, given our data linking Sox10 and FGF signaling. Among the targets induced by Sox10, we found that the FGF positive signaling regulator Etv5 was upregulated, while the FGF negative regulator Dusp6 was downregulated (Fig. 7D). This is consistent with the positive FGF-Sox10 loop indicated by our data, in which FGF acts to induce Sox10, while activated Sox10 then reinforces FGF signaling. These data validate that the differential expression of molecules between Sox10^{OE} and control cells can be used to identify targets of Sox10 or signaling network changes initiated by Sox10.

We next identified genes that were significantly differentially expressed in response to Sox10^{OE}. Gene ontology analysis with these gene lists indicated significant reprogramming of cellular function that is consistent with the observed phenotypic changes in Sox10^{OE} cells (Fig. 7D, Supp. Table 2). For example, Sox10^{OE} cells delaminate from the primary organoid where they tend to remain quiescent, and indeed this analysis finds genes associated with migration are upregulated with Sox10^{OE}, while genes associated with proliferation and adhesion are downregulated with Sox10^{OE}. Similarly, Sox10^{OE} cells in organoids lose differentiation marker expression and gain stem/progenitor function during this process, and indeed genes associated with differentiation are downregulated with Sox10^{OE}. These transcription data thus provide a hypothesis generating resource to determine how Sox10 elicits important state changes in normal or transformed mammary cells.

Notably, ErbB2 and the estrogen and progesterone hormone receptors all showed reduced expression levels following Sox10 overexpression. Sox10 is preferentially expressed in triple negative breast cancers that lack these three receptors (Fig. 1F). These data suggest that Sox10 may be one mechanism of functionally specifying this triple negative state.

Discussion

Our studies have used diverse strategies to reveal important roles for Sox10 in stem and progenitor functions within mammary cells. This is first indicated by the significant correlation between Sox10 expression and two aggressive subtypes of breast cancer that have previously been described as stem-like (basal-like) or EMT-like (claudin-low). We then present data that Sox10 consistently labels cells with stem/progenitor qualities in multiple contexts that include fetal, adult, and 3-D cultured mammary tissues. Sox10 may be a cell state regulatory node in mammary cells, as deleting Sox10 decreased stem/progenitor functions, while its ectopic activation both expanded stem/progenitor activity and induced

EMT. This suggests that relative expression levels of Sox10 can mediate either stem-like or EMT-like responses depending on context.

The link between Sox10 and both stem- and EMT-like cell functions is reminiscent of the published links between CSCs and EMT (Oskarsson et al., 2014). Importantly, it has been unclear to what extent CSCs are stem-like, given that their mesenchymal properties and transcriptome profiles often do not resemble those of bone fide stem cells. The enhanced motility of mesenchymalized cells may endow them with greater capacity to aggregate and form polyclonal “tumorspheres” in suspension cultures, or to invade and form tumors more efficiently in xenograft assays. These properties are clearly independent of stemness measured by transcription profiling, and should not be used as surrogates for stem cell function. These concerns have led to the rebranding of CSCs as “tumor-” or “xenograft-initiating cells”, which suggests the distinction between the stem-like cells in tumors identified transcriptionally, and the more EMT-like CSCs.

The data described here present clear evidence that the stem cell and mesenchymal states are related and can be interconverted in stem-like cells. We find that a single factor, Sox10, is able to contribute to cells entering each of these two states, and critically we show that it does so independently of the other state. Sox10+ cells that have not undergone EMT show increased levels of stemness in multiple contexts, while EMT occurs independent of stem cell activity. The separation of these states removes the aforementioned concerns about conflating stemness with properties of mesenchymal cells, and demonstrates that a single molecule such as Sox10 can link these two distinct states. Importantly, this affirms the link between stem-like and mesenchymal states, and defines a molecular mechanism by which these state conversions can take place.

These data also yield predictions about how mammary cells acquire stem cell-like properties in normal and cancerous states, and how these mechanisms may contribute to metastatic disease. The capacity of Sox10 to promote both stem-like and EMT-like behaviors, suggests that Sox10 could be a factor that mediates these two functions that are hypothesized to be directly responsible for tumor initiation and progression. Most notably, we have modeled the sequential stages of metastatic behavior using only Sox10 in 3-D mammary cell culture, as we find that: 1) $Sox10^{+}$ cells preferably form primary organoids, 2) $Sox10^{OE}$ activates EMT to elicit delamination and migration of cells away from the primary organoid, and 3) reduction of Sox10 levels in these cells reverses the EMT and initiates the establishment of separate organoids at secondary sites. It is easy to visualize how this could similarly play out in Sox10+ tumors, in which microenvironmental or genomic changes could induce fluctuations in Sox10 expression levels that cycle cells through these stem-like and EMT states to mediate metastasis.

Our findings also have implications for how stem/progenitor cell states may be specified in mammary cells. As discussed in the introduction, the balanced activation of specific lineage determining factors is a mechanism capable of mediating stem-like functions in cells. This model fits with observations of Sox family transcription factors, where Sox molecules have antagonistic relationships with other factors at cell fate decision points. By applying this model to Sox10 and mammary cells, our data indicate that Sox10 may specify the basal

lineage in mammary cells. This is apparent in the expression data, where Sox10 preferentially labels the basal cell fraction in the adult mammary gland, and the functional data, as $Sox10^{OE}$ can elicit EMT in mammary cells, and basal cells can be considered "partial EMT" based on their morphology. Furthermore, this model predicts that Sox10 should promote stem-like qualities when in balance with other factors. This is supported by our data linking Sox10 expression and function to stem-like properties, and our data demonstrating that lower levels of Sox10 expression increase efficiency of bi-lineage sphere formation and self-renewal. These data thus support a model in which cell fate decisions and stemness in mammary cells are regulated by a balance of lineage specifiers, of which Sox10 is one critical player that favors a basal lineage. However, there are pieces of our data that do not neatly fit this model, such as that $Sox10^{neg}$ cells produce mostly basal-like organoids and $Sox10^{OE}$ elicits cells that appear less differentiated. This suggests that a function of Sox10 may be to provide cell state plasticity, instead of, or in addition to, a role in lineage specification.

As described in the introduction, there is not a consensus on the localization and frequency for MaSCs. Our data and the balanced lineage specifier model suggest that a significant reservoir of Sox10-expressing poised basal cells exists, and that these cells could adopt activated stem/progenitor cell properties by the acquisition of antagonistic factors that bring Sox10 levels into an equilibrium that favors a stem cell state. This is consistent with work that indicates the majority of single basal cells have the potential to generate full mammary glands (Prater et al., 2014). Evaluating this model will require a better understanding of how Sox10 works in concert with other, presumably pro-luminal factors, such as Elf5, Gata3, and Notch signaling, among others. Similarly, it will be key to evaluate the relationship of Sox10 with basal lineage regulators such as p63 and Slug, and the stem-cell marker Lgr5 (Oakes et al., 2014).

Finally, two of our most striking results are that the use of an FGFR inhibitor profoundly affects the expression of Sox10 and the delamination phenotype induced through $Sox10^{OE}$. Notably, the deletion of *FGFR1* and *FGFR2* results in the loss of the transplantation competent population of mammary stem cells and compromises ductal remodeling, which mirror the roles for Sox10 in stem cell competence and cell motility shown here (Pond et al., 2013). Extrinsic signaling mechanisms in the stem cell niche that regulate the frequency and output of stem cells are potential targets for cancer prevention or treatment. Thus it will be key to determine if blocking FGF signaling also antagonizes the expression or downstream effects of Sox10 (or other Sox family transcription factors) *in vivo* in normal mammary tissue or tumors. Together these data imply a central role for FGF signaling and Sox10 in normal mammary function, and indicate that tight control is required to prevent it from eliciting malignant functions.

Experimental Procedures

Mammary cell preparation

Single cell preparations of fetal mammary cells were obtained by pooling freshly dissected fetal mammary rudiments from euthanized embryos into dissociation media: Epicult-B Basal medium (Stem Cell Technologies) supplemented with 5% FBS, pen/strep, fungizone,

hydrocortisone, collagenase and hyaluronidase. Rudiments were then dissociated to single cells by sequentially incubating them in dissociation medium for 1.5 hours at 37°C with gentle agitation, exposing them to ammonium chloride for 4 minutes on ice to remove erthyrocytes, and triturating them with dispase and DNase. Final suspensions were passed through a 40 um filter to remove aggregated cells, and stored in Hank's Balanced Salt Solution with 2% FBS for flow cytometry. Single cell preparations of adult mammary cells were prepared by dissecting out and mincing the #4 mammary glands from 6–12 week old virgin female mice. Glands were then dissociated by agitating them for 3–6 hours at 37°C in the same dissociation media. Cells were further processed as with the fetal cells, except that trypsin and accutase (Life Technologies) were also utilized prior to dispase treatment to facilitate disaggregation. Final suspensions were passed through a 40 um filter to remove cell clusters, and stored in Hank's Balanced Salt Solution with 2% FBS for flow cytometry.

Immunostaining and confocal analyses

Mammary tissues were immunostained through direct or indirect immunofluorescence. Confocal microscopy was performed with equipment from the Waitt Advanced Biophotonics Center at the Salk Institute, including Zeiss 780 inverted laser scanning confocal microscopes. Details of tissue preparation and staining protocol are included in the Supplement.

3-D organoid culture

To generate organoids, single mammary cells were plated at 50–650 cells per well in 96-well ultra low-adhesion plates (Costar) with Matrigel. Cells were plated in either restricted serum-free media (Epicult-B media with B-supplement (Stem Cell Technologies) containing heparin and pen/strep, and defined growth factors such as EGF, FGF2, and/or FGF10), or in serum-based MCF10A media (DMEM/F12 with 5% horse serum, hydrocortisone, cholera toxin, insulin, and ciproflaxin, supplemented with B27 supplement and EGF). Description of the plating protocol and analysis of these cells is in the Supplement.

4-D Organoid culture and imaging

m2rtTA fMaSCs were infected with LV-TRE-hSox10-2A-NLSVenus and plated onto glass bottom 35 mm dishes with a Matrigel bed in restricted serum-free media. After 72 hours, organoids were given fresh media and dox to induce Sox10/Venus expression. 8–24 hours later, cells were imaged at 10 minute intervals with a Zeiss CSU Spinning Disk Confocal Microscope in a climate-controlled environment of 5% CO_2 and 37° C. Images were assembled into movies using Imaris imaging software.

RNA sequencing and Bioinformatic analyses

RNA isolation, sequencing, and analysis are described in detail in the Supplement. The RNA-sequencing data are available at the gene expression omnibus under accession GEO: GSE71300.

Statistical analyses

A two-tailed student's t-test was used to quantify significance. P values were represented as follows: * - p<0.05, ** - p<.005, *** - p<.0001. The error bar in all figures is the standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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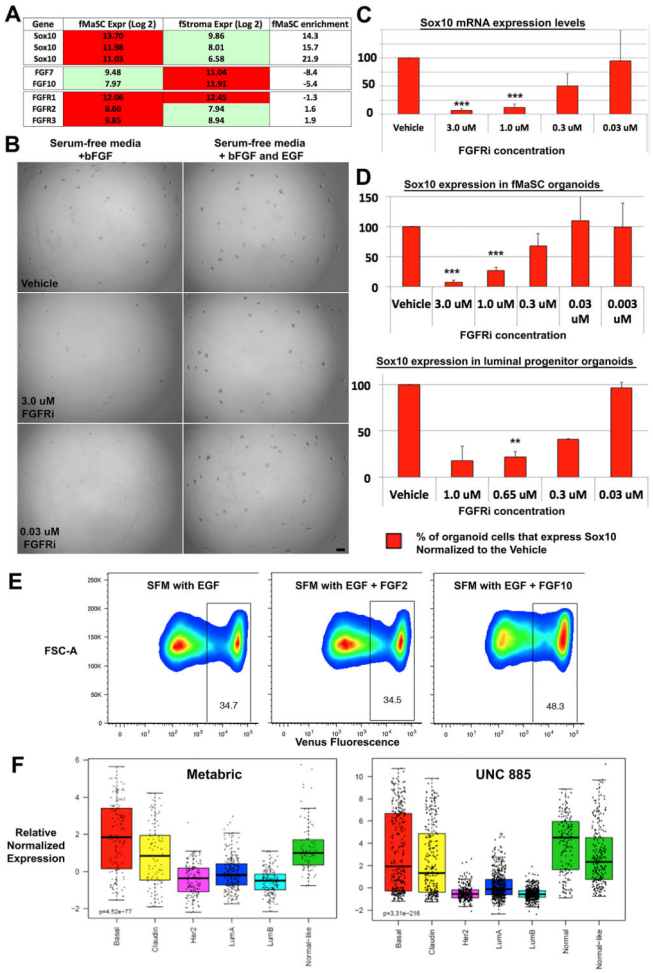


Figure 1.

A) Log2 microarray expression values for Sox10 and FGF signaling molecules in E18 fMaSCs and fStroma. **B)** E18 fMaSCs grown in 3-D culture conditions for 5–7 days with the indicated media. Scale bar 150 um. **C)** Sox10 mRNA levels expression levels in fMaSC-derived organoids grown with FGFRi for 7 days. **D)** FACS-based quantification of Venus+ cells in 7-day old FGFRi-treated organoids grown from *Sox10*-H2BVenus fMaSCs or adult mammary luminal progenitors. In C–E, the Y-axis represents the # of Venus+ cells as a % of the total # of cells in the primary organoids, normalized to the vehicle. **E)** FACS-based quantification of Venus+ cells in 8-day old organoids grown from E18 *Sox10*-H2BVenus fMaSCs in defined growth factors. X-axis is Venus fluorescence, # in box is % gated Sox10+ cells. **F)** Whisker plots for Sox10 expression from the Metabric and UNC885 breast tumor databases across multiple subtypes. Each dot is a Sox10 expression value from a particular tumor.

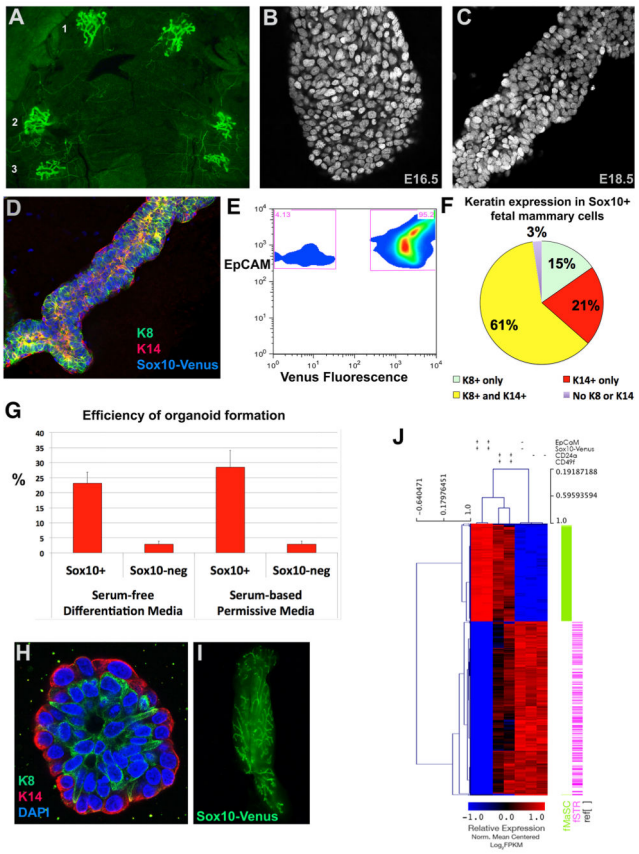
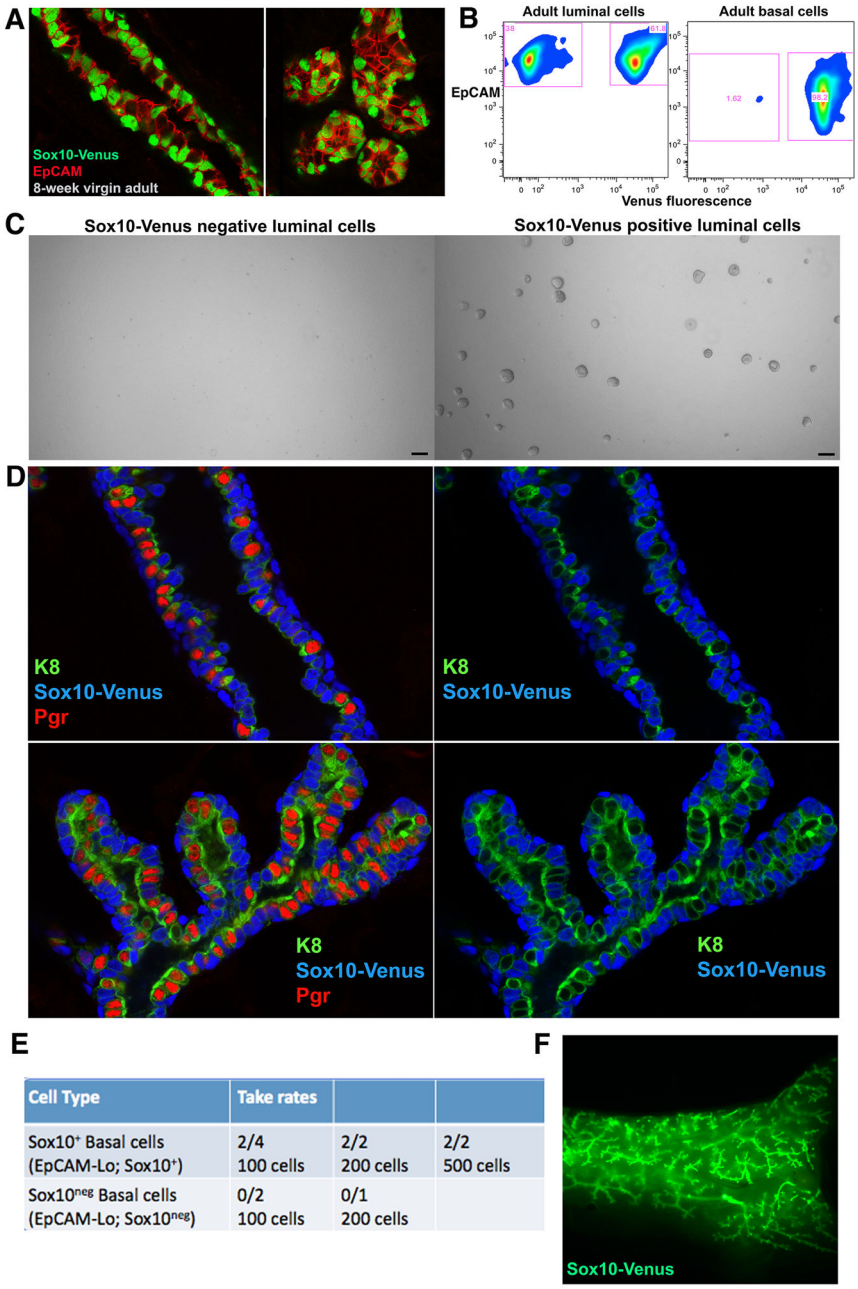


Figure 2.

A) Wholemount view of the 1–3 mammary rudiment pairs in an E18 *Sox10*-H2BVenus embryo. **B–C)** Venus fluorescence in E16 & E18 *Sox10*-H2BVenus mammary rudiments wholemounts. **D)** Wholemount mammary rudiment from E18 *Sox10*-H2BVenus embryo immunostained with luminal (K8) and basal (K14) markers. **E)** FACS of E18 *Sox10*-H2BVenus fetal mammary cells (pre-gated for EpCAM+ cells). **F)** Keratin immunostain of single E18 *Sox10*$^{\text{flox-GFP}}$ EpCAM+ fetal mammary cells. **G)** Efficiency of organoid formation from E18 *Sox10*-H2BVenus female mammary rudiments in two different media. Y-axis is # of organoids per 100 cells plated. **H)** A bi-lineage organoid derived from fMaSCs. **I)** A reconstituted mammary gland following transplantation of Sox10+ fetal cells visualized by *Sox10*-H2BVenus reporter. **J)** *Sox10*-H2BVenus-derived fMaSCs (columns 1 and 2), CD24/CD49f-derived fMaSCs (columns 3 and 4), and fStroma (columns 5–7) were RNA-sequenced and clustered (SAM: FDR<0.01%) using previously indicated differentially expressed genes between fMaSC (pink) and fStroma (green).



Cell Type	Take rates		
Sox10⁺ Basal cells (EpCAM-Lo; Sox10⁺)	2/4 100 cells	2/2 200 cells	2/2 500 cells
Sox10^{neg} Basal cells (EpCAM-Lo; Sox10^{neg})	0/2 100 cells	0/1 200 cells	

Figure 3.

A) Immunostain for EpCAM in an adult *Sox10*-H2BVenus mammary gland. **B)** FACS of Venus fluorescence (X-axis) in adult *Sox10*-H2BVenus luminal and basal populations (Y-axis is EpCAM). Displayed are luminal cells that were pre-gated as EpCAM^{hi};CD49f$^{low\text{-}med}$, and basal cells as EpCAM$^{low\text{-}med}$;CD49f^{hi}. **C)** Venus(−) or Venus(+) luminal cells from an adult *Sox10*-H2BVenus mammary gland cultured in 3-D for 6 days. Scale bar 65 um. **D)** Wholemount immunofluorescence for K8 and progesterone receptor (Pgr) from adult *Sox10*-H2BVenus mammary glands; right image lacks Pgr for easier visualization. **E)** Transplantation take rates for Venus(−) and Venus(+) basal cells from an adult *Sox10*-H2BVenus mammary gland. **F)** A reconstituted mammary gland

following transplantation of Sox10+ adult basal cells visualized by the *Sox10*-H2BVenus reporter.

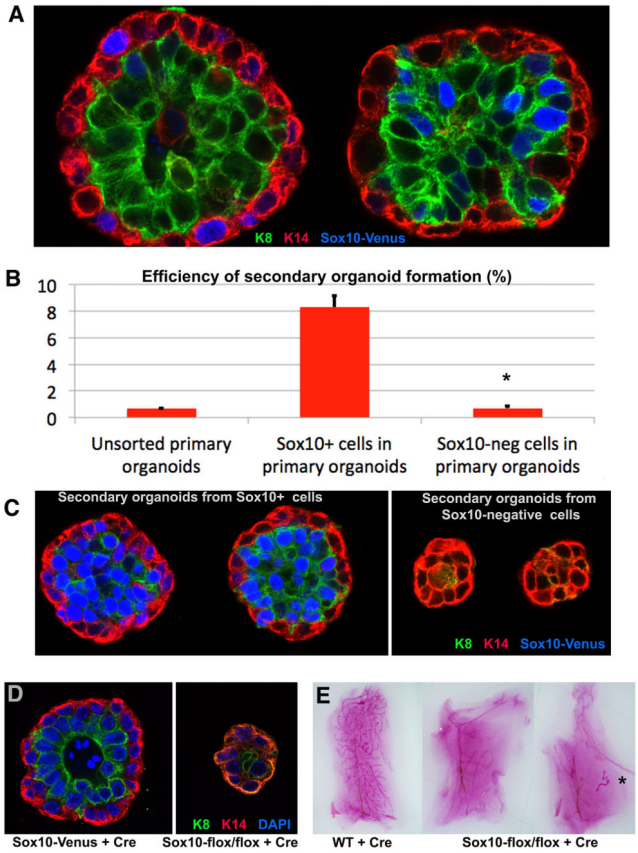


Figure 4.

A) Organoids from *Sox10*-H2BVenus fMaSCs contain Venus(+) and Venus(−) cells. **B–C)** Efficiency of secondary organoid formation for Venus(+) and Venus(−) cells taken from primary *Sox10*-H2BVenus fMaSC organoids grown in SFM. Y-axis is # of secondary organoids per 100 cells plated. **D)** Representative organoid formation following 3-D culture of Cre-infected *Sox10*$^{\text{wild-type}}$ or *Sox10*$^{\text{flox/flox}}$ fMaSCs. **E)** Carmine staining of transplanted Cre-infected *Sox10*$^{\text{wild-type}}$ or *Sox10*$^{\text{flox/flox}}$ fMaSCs into cleared fat pads. Transplants were considered takes if greater than half the fat pad was reconstituted; *marks a partial aborted outgrowth.

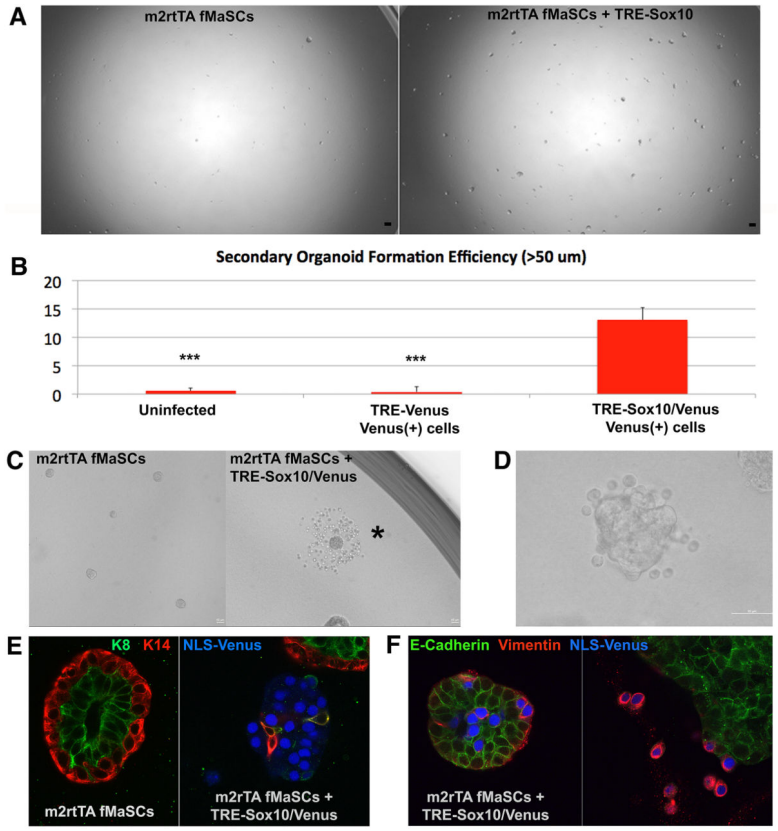


Figure 5.

A) Primary (1°) organoids from control (uninfected) or Sox10^{OE} m2rtTA fMaSCs were dissociated and replated into 3-D culture to form secondary (2°) organoids. Shown is 2° organoid growth after 7 days. Scale bar 75 um. **B)** Quantification of 2° organoid forming potential for Sox10^{OE} cells compared to uninfected or Venus-only infected cells. Y-axis is # of >50 um 2° organoids per 100 cells plated. **C)** Sox10^{OE} fMaSCs present with satellite single cell structures surrounding the 1° organoid (*). Scale bar 40 um. **D)** Active delamination of cells from a Sox10^{OE} organoid. **E)** Immunostains of control or Sox10^{OE} fMaSC organoids demonstrate the loss of keratin expression (red or green) in Sox10^{OE} cells (blue). Scale bar 50 um. **F)** Immunostains of Sox10^{OE} fMaSC organoids reveal upregulation of vimentin and loss of E-cadherin in Sox10^{OE} cells (blue).

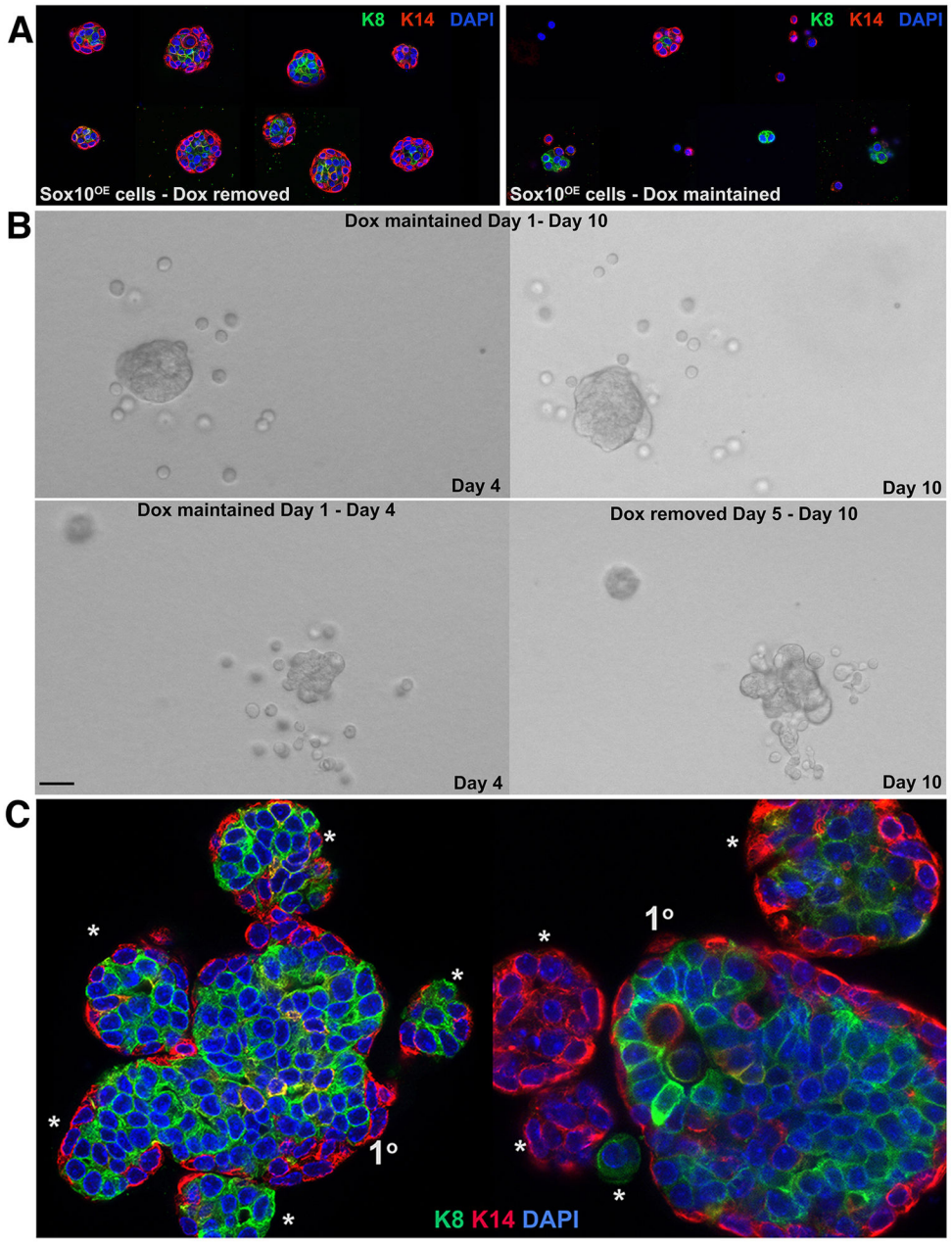
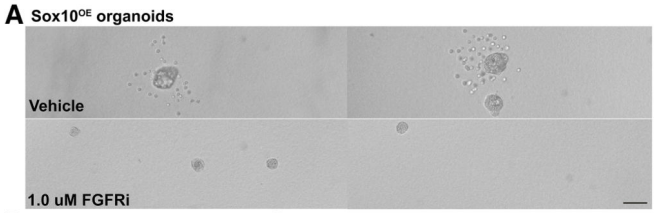
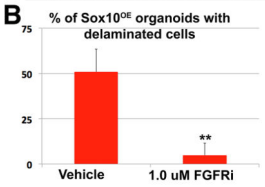
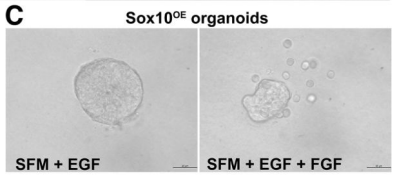


Figure 6.

A) Sox10^{OE} cells were isolated from 7-day old fMaSC-derived primary (1°) organoids and replated in 3-D culture +/− dox. Secondary outgrowths from these cells were immunostained for keratin markers after 7 days. **B)** Sox10^{OE} satellite cells form secondary (2°) organoids surrounding the 1° organoid at greater efficiency if dox is removed from the media after 4 days. Left/right are the same organoids over 10 days of culture. Scale bar 20 um. **C)** Sox10^{OE} cells were allowed to form 1° organoids in 3-D culture for 7 days, then dox was washed out of the media to ease Sox10 expression. 3–4 days after washout, the delaminated satellite cells initiated 2° organoid formation (*) around the 1° organoid.







D

	GO Terms	Genes	p-value
GO Terms associated with genes upregulated by Sox10-overexpression	Transport	221	4.90E-09
	Regulation of neuron projection development	32	1.20E-06
	Intracellular signal transduction	121	2.52E-06
	Cell projection morphogenesis	48	7.31E-06
	Regulation of cell communication	128	1.12E-05
GO Terms associated with genes downregulated by Sox10-overexpression	Cell adhesion	127	7.16E-25
	Cell differentiation	256	2.17E-23
	Regulation of cell proliferation	143	7.77E-22
	Regulation of programmed cell death	140	3.57E-19
	Tissue development	173	4.74E-31
	Organ development	256	1.71E-30

		Control	Sox10OE	Rank Change
Reported Sox10 or FGF signaling targets	Mitf	2.0	14.3	5272
	ErbB3	34.4	100.7	1978
	Mia	112.3	180.6	294
	Etv5	62.0	112.6	651
	Dusp6	459.2	200.5	-185
Genes associated with epithelial/mesenchymal or motile cell states	Postn	128.2	29.0	-6302
	Icam1	59.0	9.6	-6384
	Pvrl1	20.1	7.8	-3712
	Krt14	820.5	98.6	-1398
	Vim	293.3	1057.3	195
	Fyn	16.2	40.5	3187
	Enpp2	2.2	139.3	10873
	Limk1	3.9	15.1	4380
Genes associated with stem or differentiated cell states	FoxA1	63.9	24.9	-2843
	Esr1	4.2	0.3	-2961
	Pgr	5.7	2.0	-1952
	Myb	48.9	12.6	-4958
	Hmga1	74.5	20.0	-4071
	Runx1	62.5	8.7	-6829
	Id2	146.1	409.2	318
	Lbh	49.3	165.6	1359
	Dnmt3b	1.2	4.9	2630
	Tert	2.7	6.6	2067
	Kit	8.7	36.6	5429
	Tnfrsf19	0.0	161.6	13685

Figure 7.

A) Sox10^{OE} organoids were grown in 3-D culture in the presence of vehicle or 1.0 uM FGFRi. 100 um scale bar. **B)** Fraction of Sox10^{OE} organoids with extruded satellite cells after 6 days (Y-axis) in the presence of vehicle or 1.0 uM FGFRi. **C)** Sox10^{OE} organoids were grown in 3-D culture in SFM with EGF alone or EGF, FGF2, and FGF10. Scale bar 40 um. **D)** Gene ontology terms associated with significantly down- or up-regulated genes following Sox10^{OE} (top) and example notable genes with altered expression by Sox10^{OE} (bottom).