Loss of Sox10 in a HER2+ model prevents tumor initiation and induces luminal-to-basal reprograming in cancer cells.

Brennan Garland[1,3], Samuel Delisle[1,3], John Abou-Hamad, Christiano de Souza[3], Riana Zuccarini[1,3], Rebecca Auer[2,3,4] and Luc A. Sabourin[1,3]

1. Dept. of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, K1H8M5

2. Dept. of Biochemistry, Microbiology And Immunology, University of Ottawa, Ottawa, ON, K1H8M5

3. Dept. of Cancer Therapeutics, The Ottawa Hospital Research Institute, Ottawa, ON, K1H 8L6,

4. Dept. of Surgery, University of Ottawa, Ottawa, ON, K1H 8M5

Corresponding author: Luc A. Sabourin, PhD.

Associate Professor, University of Ottawa

Senior Scientist, Cancer Therapeutics,

Ottawa Hospital Research Institute

501 Smyth Rd, Ottawa, ON, Canada, K1H 8L6

T: 613-737-7700 ext. 79963

lsabourin@ohri.ca

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Abstract

The SRY-HMG-Box transcription factor SOX10 plays a critical role in neural crest development, but its function in epithelial tumorigenesis remains unclear. Here, we identify SOX10 as a key regulator of tumor-initiating activity in Neu-driven mammary cancers. Genetic ablation of Sox10 in the luminal compartment of MMTV-Neu (NIC) mice resulted in delayed but normal mammary gland development. Sox10 deletion conferred a dose-dependent delay in tumor onset, with a complete loss of tumor initiation in Sox10-deficient luminal cells. CRISPR/Cas9-mediated Sox10 inactivation in Neu-transformed tumor cells led to reduced 3D invasion and diminished self-renewal in mammosphere assays. Established Sox10-null cell lines exhibited markedly impaired growth in orthotopic transplant models and failed to colonize lung tissue following tail vein injection, suggesting a loss of tumor-initiating capacity. Transcriptomic profiling revealed that Sox10-deficiency in Neu+ tumor cells induces a luminal-to-basal/stem-like shift and the downregulation of several genes associated with genetic networks regulating stemness. Collectively, these findings demonstrate that Sox10 is required for a permissive cell progenitor state for Neu-driven tumor initiation and that it is critical to sustain the invasive and self-renewing traits that drive tumor progression and metastasis.

Significance

The Sox10 transcription factor is critical for mammary stem cell function and plasticity. Animal studies suggest that different subtypes of breast cancers arise from luminal epithelial cells and hypothesized the implication of luminal stem cells in breast cancer initiation. We show that Sox10 deletion in the luminal compartment of adult female mice abrogates tumor initiation. Sox10 inactivation impairs tumor cell growth, dissemination and reprograms luminal Neu+ tumor cells to a basal phenotype. Our data supports a model whereby luminal progenitors are required for tumor initiation, self-renewal and growth at distant sites. The findings provide a new therapeutic opportunity in the targeting of Sox10 networks to reduce the cancer stem cell content of mammary tumors.

Introduction

The HER2/ErbB2/Neu receptor tyrosine-kinase (RTK) is overexpressed in approximately 25-30% of human breast cancers (1, 2). Currently, the most effective form of treatment for HER2+ breast cancer remains chemotherapy in combination with trastuzumab (Herceptin). However, acquired or *de novo* resistance is observed in up to 70% of HER2+ metastatic breast cancers (3, 4). Murine models of HER2-positive breast cancer have confirmed the oncogenic role of HER2 in breast tumor initiation and progression (5, 6), yet identification of the cell population susceptible to HER2-mediated transformation remains largely undefined. This highlights the need to investigate regulators of lineage plasticity as a potential prerequisite for malignant reprogramming.

Sox10 (Sry-HMG Box 10) is a critical transcriptional regulator of cell fate determination and several developmental processes during embryogenesis (7, 8). Recently, it has been found that Sox10 expression is conserved in mammary epithelial cell populations, specifically in those with the highest degree of stem/progenitor cell activity (9). Consistent with this, conditional deletion of Sox10 in mice has been shown to disrupt normal mammary gland development, further highlighting its functional role in the maintenance of the mammary epithelial lineage (10).

Currently, the role of Sox10 in malignant progression remains elusive and controversial with different observations in various types of cancers (11, 12). In the context of breast cancer, we and others have shown that Sox10 is a marker for select basal-like carcinomas such as triple-negative breast cancers (13-17). Recenly, Sox10 has been shown to be highly expressed in luminal progenitors of the human mammary glands and is over-represented in TNBC brain metastases (18).

Using murine models of luminal-, and basal-like mammary tumorigenesis, Sox10-expressing tumours exhibited a de-differentiated state and acquired a more mesenchymal-like phenotype (19). Mechanistically, Sox10 was found to be directly bound to promoter regions of genes regulating epithelial-mesenchymal transition (EMT), stem/progenitor cell activity, and neural crest identity (19). Functional studies using C3-TAg mice, a model of basal-like breast cancer, revealed that Sox10 haploinsufficiency significantly prolongs survival, directly implicating it in disease progression (19). Supporting this, using a

murine model of HER2+ breast cancer, we have shown that increased numbers of Sox10+ cells correlates with a more rapid tumor initiation (20).

Our lab has recently shown that TCGA datasets revealed that approximately 15% of HER2+ breast cancers are Sox10^{hi} (21). Consistent with this, others have demonstrated substantial variability in proportions of the Sox10+ populations (2% - 79%) among HER2+ tumour samples (20, 22-25). Interestingly, Sox10^{hi} cohorts were associated with high EMT-like signatures, worse grade, and a stem-like phenotype, suggesting that Sox10+/HER2+ tumors represent a more aggressive de-differentiated subtype (20, 22, 25).

Despite these observations, whether the Sox10+ progenitor population is critical for HER2-driven tumorigenesis or represents a transitional state of cellular plasticity remains unresolved. Here, we investigated the functional and genetic consequences of conditional Sox10 deletion in MMTV-NIC mice (26). We find that luminal deletion of Sox10 delays mammary gland development but completely abrogate hyperplasia development and tumor initiation in MMTV-NIC mice. CRISPR-mediated deletion of Sox10 in established Neu+ cell lines impairs tumor growth in orthotopic assays and prevents tumor expansion in lung colonization studies. Tumor cells devoid of Sox10 show a luminal to basal/stem-like shift in gene signature. These results suggest that Sox10+ luminal progenitors are required for Neu-induced tumorigenesis and that the loss of Sox10 can reprogram cancer cells to a basal-like state.

Results

Sox10 inactivation delays mammary gland development

Previous studies have shown that Sox10 is required for full development of the mammary gland (10). However, the MMTV-Cre strains used have been shown to display intrinsic developmental defects due to transgene positional effects and leaky expression in the myoepithelial/basal layer (27, 28). As the MMTV-NIC and MMTV-Cre lines used here have been shown to be exclusively expressed in the luminal compartment (29, 30), we first assessed mammary gland development in this background.

Sox10$^{fl/fl}$ mice (31) were crossed with MMTV-Cre mice (29) and mammary gland development was assessed at 4 and 11 weeks of age. As previously reported (29), LacZ staining marked Cre expression in rudimentary mammary glands as early as P14 (Suppl Fig.1A). At 4-weeks of age, Sox10$^{fl/fl}$:MMTV-Cre females showed a slight delay in mammary tree invasion, end bud numbers and ductal branch point morphogenesis (Fig. 1A-C). However, by about 11-weeks of age, no obvious defects were observed in post-pubertal virgin females and mammary glands were indistinguishable from that of the wildtype group (Fig.1A-C). Furthermore, no defects in epithelial organization were observed as indicated by similar proportions of luminal (CK8) and basal cells (CK14) (Fig. 1D). While basal cells remained Sox10+, immunohistochemistry showed lower expression of Sox10 and approximately a 60% reduction in Sox10+ luminal cells in 11-week-old in Sox10$^{fl/fl}$:MMTV-Cre females (Fig. 1E, F). Inefficient but progressive deletion of this Sox10 allele throughout the lifespan of the animals has also been observed previously (31). Supporting this, in 16 week-old MMTV-NIC mice, other than the basal layer, virtually all luminal cells were devoid of Sox10 (Suppl Fig.1B) In contrast to a previous report (10), it is likely that delayed inactivation of Sox10 in luminal progenitors in our model resulted in the development of mature mammary glands. This allowed us to assess tumor development in homozygote Sox10$^{fl/fl}$ mice in the MMTV-NIC background.

Sox10 deletion in MMTV-NIC mice inhibits tumor initiation

To assess the role of Sox10 in HER2-mediated mammary tumourigenesis, Sox10$^{fl/fl}$ mice were crossed with transgenic MMTV-Neu-IRES-Cre (NIC) mice (26). The MMTV-promoter used here drives luminal-specific expression of a bicistronic transcript containing both a constitutively active Neu/ErbB2 (NeuNDL2-5) and Cre recombinase (29). Supporting the MMTV-Cre:Sox10$^{fl/fl}$ observations, analyses of MMTV-NIC:Sox10$^{fl/fl}$ mice also revealed no overt mammary glands defects (not shown). Kaplan-Meier analyses showed a significant increase in disease-free survival (average 76-days) for MMTV-NIC:Sox10$^{fl/+}$ heterozygote mice when compared to wild-type MMTV-NIC:Sox10$^{+/+}$ (Fig. 2A). Surprisingly, MMTV-NIC:Sox10$^{fl/fl}$ mice remained disease free for the duration of the study, suggesting a complete absence of tumor initiation. Western blot analysis of tumour lysates from MMTV-NIC:Sox10$^{fl/+}$ mice revealed a ~50% reduction in Sox10 expression along with the luminal marker CK8 (Fig. 2B). Palpable tumours were readily detectable in MMTV-NIC:Sox10$^{+/+}$ about 80 days sooner than in MMTV-NIC:Sox10$^{fl/+}$ mice, suggesting delayed tumor initiation and that Sox10 haploinsufficiency markedly affect tumor initiation (Fig.2C). However, onset to endpoint proceeded at the same rate in both group, suggesting that tumor growth was unaffected (Fig. 2D). Supporting our onset to endpoint data, no quantitative differences were observed for the proliferative and apoptotic markers, Ki-67 and cleaved caspase-3, respectively (Suppl Fig.2B,C). To further investigate the observed delay in tumour onset, mammary glands were collected at 16-weeks of age, prior to tumor detection and assessed for hyperplastic lesions. Sox10 staining in 16 week-old MMTV-NIC:Sox10$^{fl/fl}$ revealed a marked reduction in Sox10+ luminal cells with numerous Sox10+ basal cells (Suppl Fig.1B), suggesting efficient Sox10 excision. Hematoxylin staining of 16-week-old mammary gland sections revealed the presence of numerous hyperplastic lesions in wildtype MMTV-NIC:Sox10$^{+/+}$ mice (Fig.2E,F; Suppl Fig.2D). However, no hyperplasia were observed in age matched MMTV-NIC:Sox10$^{fl/+}$ mice (Fig.2E,F; Suppl Fig.2F). Approximately 8 weeks was required for heterozygote mice to reach a level of hyperplastic

burden similar to that of wildtype mice (Fig.2E, F). Supporting an absence of tumor formation, no hyperplasia were ever detected in MMTV-NIC:Sox10$^{fl/fl}$ mice, up to 420 days of age (Suppl Fig.2F).

The Sox9 transcription factor has been shown to be critical for luminal lineage commitment and tumor progression in a TNBC mouse model (32-34). Therefore, we assessed Sox9 expression in 16 week-old mammary glands. For all MMTV-NIC:Sox10 genotypes, strong Sox9 expression was observed in both the basal and luminal layers (Suppl Fig.2G), suggesting that the loss of Sox10 does not affect Sox9 expression. Importantly, despite Sox9 expression, tumor initiation was impaired suggesting that Sox9 cannot compensate for Sox10 in this process. Together, these data suggest a critical role for Sox10 in the initiation of Neu-induced mammary tumourigenesis.

Sox10-deficient Neu^{+} cell lines have reduced stemness and tumorigenic potential

Genetic studies revealed that Sox10 is expressed in mammary progenitor cells (9, 18, 33, 35-37). Because of the critical role of Sox10 in mammary stem/progenitor cell function (9, 19), we hypothesized that the delay in tumor initiation *in vivo* was due to reduced tumor stem cell activity following the loss of Sox10.

Immunohistochemical analyses of endpoint tumors revealed that all tumors from wildtype and Sox10$^{fl/+}$:NIC heterozygotes expressed Sox10. This might reflect heterogeneity and the variable TIC content of those tumors. However, as previously reported for human HER2 tumors (20, 22, 23, 25), the number of Sox10+ nuclei was found to vary greatly between tumors (~15-80%; Suppl Fig.2A). Similarly, western blot analysis of MMTV-NIC- and MMTV-NDL-derived tumor cell lines showed markedly different levels of Neu and Sox10 expression (Suppl Fig.3A). To further characterize the observed *in vivo* phenotype, we derived additional low passage Sox10-expressing tumor cell lines from endpoint tumors. Two independent tumor cell lines expressing Sox10 (BG1 and BG2; Suppl Fig.3A) were successfully established from MMTV-NeuNDL tumour-bearing mice (5, 38). Immunostaining showed approximately 60-90% *Sox10*+ nuclei for BG1 and BG2 cells (Fig.3A). To inactivate Sox10, two guide RNAs (sgRNA/RFP) targeting amino acids 168-175 (sgRNA1) or 67-74 (sgRNA2) were transduced into cell

lines expressing Cas9 and RFP+ cells were flow sorted. Sox10 knockout was then assessed by immunofluorescence, western blotting and RT-qPCR. In both lines, transduction with sgRNA-2 resulted in markedly reduced levels of Sox10 protein with less than 5% of *Sox10*+ nuclei (Fig. 3A, B; Suppl Fig.3B). Therefore, the sgRNA-2 pools were selected for further studies. Both Neu+/Sox10^{-} BG1 and BG2 cell lines exhibited growth rates similar to that of the NTC control in culture (Suppl Fig.3C), suggesting that Sox10 is not required for tumor cell proliferation or cell viability.

Deletion of Sox10 did not have any effects on cell migration as tested in transwell chambers (Suppl Fig.3D, E). However, in a 3D Matrigel invasion assay, Sox10-deficient cells showed a modest but significant reduction in invasive potential (Fig. 3C; Suppl Fig.3). While a small decrease in primary mammosphere formation was observed, Sox10-deficient cells showed a marked reduction in secondary sphere formation, suggesting impaired self-renewal capacity (Fig. 3D). Both Sox10-deficient lines showed similar results (not shown). Although no effect on proliferation was observed, orthotopic injection of BG2-Sox10^{+} and Sox10^{-} tumor cells into the mammary fat pad of immunodeficient mice revealed a marked delay in tumor growth in the Sox10-deficient group (Fig. 3E, F). Interestingly, Sox10 immunohistochemistry on tumors at endpoint showed that the proportion of Sox10+ cells was similar between Sox10+ and Sox10^{-} cell lines (Supplemental Fig.3G), suggesting that the tumors arising upon injection of Sox10^{-} cells are due to residual Sox10-expressing subpopulations. As Sox10 deletion does not affect cell proliferation *in vitro*, these data suggest that Sox10 loss impairs the tumor stem cell content and self-renewal, reducing tumorigenic potential.

Sox10 is required for orthotopic growth and lung colonization in vivo

Western blot analyses show a wide range of Sox10 expression in NIC- and Neu-NDL-derived tumor lines with some showing undetectable levels (Suppl Fig.3A). This suggests that Sox10 expression may be lost upon prolonged passaging in culture. Supporting this, we have observed *Sox10* loss over time in BG2 cells (see Suppl Fig.3A). However, BG1 cells displayed stable Sox10 expression over multiple passages and were selected for additional *in vivo* studies. To avoid the potential expansion of residual Sox10^{+} cells

upon transplantation, the BG1sgRNA-2 pools were cloned and expanded. Two Sox10-deficient clones were randomly selected for further analyses (see Suppl Fig.3H)

To compare the tumorigenic potential and cancer stem cell (CSC) activity, ten-fold dilutions of Sox10^{+} and Sox10^{-} cells were injected orthotopically in NSG mice. Tumor volume measurements over time showed no measurable growth in all animals injected with Sox10-deficient tumor cells (Fig.4A). Supporting this, a drastic reduction in tumor weight was observed in all animals bearing Sox10^{-} cells (Suppl Fig.4A). Other than rare small pockets of Sox10+ cells, immunohistochemical analysis of residual tissue confirmed the absence of *Sox10* expression (Fig. 4B). Together, these data suggest that the CSC activity is markedly impaired in Sox10-deficient cells.

To test whether Sox10-null cells could seed at distant sites, control and Sox10^{-} clones were injected into the tail vein of immunodeficient mice. Supporting the orthotopic results, control cells displayed a heavy metastatic burden with numerous lesions in the lungs whereas the Sox10-null cells failed to produce readily detectable metastases (Fig.4C&D; Suppl Fig.4C). Interestingly, staining of lung sections from mice injected with Sox10^{-} cells shows areas of Cas9-positive cells, indicating the presence of Sox10-null BG1 cells (Fig.4D). This suggests that Sox10-deficient tumor cells can extravasate into the lungs but cannot efficiently expand. As for mammary glands in adult mice, Sox10-deficient Neu+ tumor cells express Sox9 (Suppl Fig.3H), suggesting that Sox9 expression is not sufficient to promote tumor growth in vivo in the absence of Sox10. Together, these findings support the requirement for Sox10 for both CSC maintenance and the expansion of metastatic niches in Neu-driven mammary cancer.

Sox10 depletion in Neu^{+} transformed cells induces basal reprogramming

To gain further insights into Sox10-dependent genetic programs in our model, we compared bulk RNA-sequencing of BG1 Sox10-knockout clones (sgRNA2-1, sgRNA2-2) to non-targeting control (NTC) cells. Pearson-correlation matrix (Suppl Fig. 5A) showed clustering of both Sox10 knockout clones compared to the NTC. Comparison of the significantly differentially expressed genes (DEGs) in each knockout clone revealed >50% overlap of downregulated and upregulated genes between the two

clones with over 1500 common genes in both sets (Suppl Fig. 5B, C). Interestingly, multiple luminal lineage markers as well as luminal progenitor markers were found to be significantly downregulated in the RNA-Seq data (EpCAM, CK8, CK18, Foxa1, Ngfr, Etv1 and Elf5) (Fig.5A) and validated by q-PCR (Fig.5B) from both Sox10 knockout clones. Classical basal markers such as keratins 5 and 14, Acta2, and Trp63 (Fig.5C) and genes associated with multipotent mammary stem cell (MaSC) identity were also upregulated (Lgr6, Id4, Snai2, Foxc1, and Wnt6; Fig.5A), suggesting re-programming following the loss of Sox10.

Using the Reactome database and overrepresentation analysis on our list of common DEGs, we found that cytoskeletal remodelling, integrin interactions and ECM production were all likely affected by Sox10 knockout (Suppl Fig. 5D). Typically, these processes are associated with basal/MaSC-like cells, supporting a basal/MaSC-like or EMT-like shift and loss of luminal identity in Sox10-deficient tumor cells. Gene set enrichment analysis (GSEA) on the common DEGs using the C2:CGP (Curated - Chemical and Genetic Perturbations) gene set collection identified multiple gene signatures associated with reduced luminal and increased mesenchymal/MaSC identity (Fig. 5D). Comparison to previous genetic profiling of murine and human breast cancers, (39-41) revealed that Sox10-deficient Neu+ cells display a strong positive enrichment (NES = 1.84) for genes up-regulated in MaSCs (Suppl Fig.6A) but strong negative enrichment (NES = 2.16) for down-regulated genes (Suppl Fig.6B). Supporting this, Sox10 knockout negatively enriched (NES = -1.95) for genes up-regulated in luminal-like breast cancer cell lines (Suppl Fig.6C, D). Although differences are observed between the various gene lists, GSEA analyses suggest that Sox10-knockout induces reprogramming resulting in a mesenchymal/stem-like state. This is consistent with PROGENy pathway activity inference showing elevated NFkB and hypoxia signalling, both of which have been associated with mammary stem/progenitor populations (42, 43). In addition, Sox10 deletion seems to reduce MAPK and JAK-STAT signalling, important pathways for maintaining luminal progenitor identity (44-46). Overall, genetic profiling of Sox10-deficient Neu+ tumor cells reveals a phenotype shift towards a more basal/stem-like state.

Discussion

Previous studies have demonstrated the expression of Sox10 in murine and human mammary progenitors (9, 10, 18, 19). Supporting a role for Sox10 in mammary stem cell function, its conditional deletion in the basal and luminal compartments impairs mammary gland development (10). Variable proportions of Sox10+ cells have been reported for both TNBC and HER2+ breast cancers (20, 22, 23, 25), suggesting that it is expressed in subpopulations of cancer cells. Using a conditional Sox10 allele, we tested the effect of Sox10 luminal deletion on tumor progression in a HER2+ mouse model. Our data show that luminal Sox10 deletion abrogates Neu-induced tumor initiation. Consistent with previous data using a basal-like model (19), Sox10 heterozygote females showed significantly delayed tumor initiation. These similarities across distinct models suggest that Sox10 dosage plays a rate-limiting role in mammary tumorigenesis.

Interestingly, using different MMTV-Cre/NIC transgenic lines (26, 29), we only observed a delay in mammary gland development. In a Neu+ oncogenic background, our studies reveal an essential role for Sox10 in Neu-driven mammary tumorigenesis. Using both *in vivo* and *in vitro* approaches, we demonstrate that Sox10 is required for tumor initiation, the maintenance of tumor-initiating potential and lung colonization. Inactivation of Sox10 in tumor cells results in the loss of luminal markers and the induction of a basal/stem-like gene signature, suggesting genetic reprogramming. Interestingly, in a MMTV-PyMT model, the seeding of distant metastases was shown to be associated with tumor cells of luminal origin (16). Here, the acquisition of a basal-like phenotype may also impair lung colonization.

In a large T antigen mouse model, Sox9 upregulation can drive luminal-to-basal plasticity and its deletion prevents the progression of hyperplasia to breast carcinomas (32). However, we found that Sox9 expression was unaffected in luminal cells following Sox10 deletion *in vivo*. This suggests that Sox9 is unable to compensate for Sox10 in driving tumor initiation. In Sox10-deficient Neu+ tumor cells, endogenous Sox9 was also unable to rescue tumor growth in orthotopic transplants. Collectively, these findings suggest that Sox10 expression is required to establish a “transformation-competent” state in mammary progenitors. This supports prior reports on the role of Sox10 in transcriptional programs

associated with multipotency and plasticity (47-50). In the absence of Sox10, oncogenic drivers such as HER2/Neu, fail to initiate tumors, highlighting its gatekeeping role.

In contrast to the previously reported developmental arrest (10), we only observed a delay in mammary gland development. This is likely due to MMTV-Cre strain differences and genetic background. Whereas the MMTV promoter used here is restricted to the luminal layer in an FVB/n background, the MMTV-Cre lines used elsewhere show both luminal and basal expression (27, 28), ablating all Sox10+ progenitors. Here, luminal deletion of Sox10 may allow delayed reprogramming of the basal cells and the development of functional mammary glands. Alternatively, delayed Cre-mediated deletion of Sox10 (31) could also account for the observed developmental phenotype. Nevertheless, luminal deletion throughout the lifespan of the animals likely prevented tumor onset in the NIC model.

Importantly, the complete absence of mammary tumorigenesis was not due to impaired mammary gland development, as NIC:Sox10$^{fl/fl}$ glands formed normally and retained luminal architecture. RNA-seq analysis of Sox10-deficient tumor cells revealed a downregulation of luminal markers. One possibility is that Sox10 deletion induces a loss of luminal progenitor “identity” that is necessary for MMTV-driven Neu transformation. Sox10-deficient tumor cells also upregulated a subset of basal/stem-like markers, suggesting that Sox10 may also recruit or activate genes implicated in active repression of basal gene expression. One possibility is that Sox10-deficient transformed cells may adopt a lineage-intermediate state with partial luminal identity without full acquisition of a basal/stem-like lineage phenotype. This supports the notion that Sox10 can drive lineage plasticity and is permissive for tumor initiation by sustaining a flexible luminal progenitor transcriptional landscape. The loss of Sox10 may result in a hybrid/transitional state unable to support tumorigenesis.

Previous studies in MMTV-Neu and MMTV-PyMT mice supported the general notion that mammary tumors originate from stem/progenitor cells (51, 52). Similarly, lineage tracing studies showed that basal-like breast tumors etiologically arise from luminal progenitors rather than from basal stem cells (53-55), suggesting luminal-to-basal reprogramming. In a MMTV-PyMT model, Sox10 may be required for this

luminal to basal plasticity (16). In agreement with those findings, our data support a model whereby breast tumor initiation in a HER2 model requires Sox10+ luminal progenitors.

Transcriptomic analyses of Sox10-deficient tumor lines revealed downregulation of gene programs associated with stemness and luminal identity (Fig.5). Although our analysis identified similarities with previous genetic profiling of $Sox10^{hi/lo}$ tumor cells (19) (Etv1, Sox8, Tfap2c, Dlx1), significant differences were also observed (LMO4, Snai2, Wnt10a, Zeb2). Such Sox10-associated differences were also observed between tumor models and human TCGA data (19). As hypothesized previously (50, 56), this supports the notion that the "reprogramming" functions of Sox10 may be dependent on the oncogenic signal or trigger. This could affect the complement of Sox10-associated co-factors and downstream gene expression profile. This transcriptional flexibility would either maintain luminal identity or allow re-programming toward basal-like states. This would also support a critical role for Sox10 across the spectrum of breast cancer subtypes.

Although genetic profiling suggests a re-programming to a basal/stem-like state in Sox10-null tumor cells, orthotopic growth is markedly impaired, suggesting a depletion of TIC activity. Our data show that Sox10 loss may disrupt expression of canonical and non-canonical Wnt signaling pathways as well as downregulate several stemness factors (eg. Notch3, Etv1, Elf5, Msx1). Interestingly, Sox10 inactivation also upregulates *Sfrp1*, a Wnt suppressor upregulated by the deletion of Ezh2 (57). Conversely, loss of Sox10 downregulates *Etv1*, shown to be required for the maintenance of the TIC phenotype (58). It is then likely that Sox10-regulated luminal identity and self-renewal networks involve distinct regulators, resulting in a shift to a basal/stem-like state with a loss of stemness. These findings position Sox10 as a key transcriptional regulator maintaining a hybrid epithelial/mesenchymal state; a feature recognized as a hallmark of plastic, tumorigenic cells (50).

Although our Sox10-deficient tumor cell lines downregulate luminal markers, they retain Neu expression *in vitro*. However, it is possible that Sox10-/- progenitors lose their luminal identity prior to transformation *in vivo*. This would lead to an absence of tumor initiation. In established tumors and transformed cell lines, Neu expression may become decoupled from the luminal state. Nevertheless, the

presence of Sox10+ luminal progenitors appears to be necessary for Neu-mediated transformation and maintenance of CSC populations.

Interestingly, we found that Sox10 expression is progressively lost in most NDL-derived tumor cell lines with extended passaging in 2D cultures. Similarly, no Sox10 expression has been reported in the PY230 cell line (19) and several human TNBC (18). Our early passage BG1 and BG2 lines display ~60-90% Sox10+ cells, suggesting heterogeneity within cell lines. Interestingly, only BG1 cells retained their Sox10+ subpopulation upon passaging, suggesting that most cell lines lose their Sox10+ content over time. This may result in partially differentiated populations with reduced self-renewal potential. In contrast to a human TNBC line (18), CRISPR/Cas-mediated Sox10 gene inactivation in two of our cell lines did not silence Neu expression nor did it induce cell death or growth inhibition, suggesting that it is not required for *in vitro* survival or proliferation. However, orthotopic transplants showed that Sox10 is essential for maintaining stemness. These findings highlight the importance of using early-passage lines or the use of *in vivo* systems for functional studies, as long-term cultures may evolve or enriched for different subpopulations.

Our data supports a critical role for Sox10+ luminal progenitors in Neu-induced tumor initiation and niche expansion at distant sites. Whether acting to enforce lineage identity or sustain self-renewal programs, Sox10 appears to be a regulator of tumor competency and progression across distinct breast cancer subtypes. In luminal tumors, Sox10 may be required to sustain a progenitor-like state permissive to HER2-driven transformation. In basal-like contexts, it may support lineage reprogramming from luminal origins. This dual role suggests that therapeutic targeting of Sox10 or its downstream effectors could impact not only tumor initiation, but also the maintenance of CSCs and the evolutionary trajectory of tumors as they acquire more aggressive phenotypes.

Materials and Methods

Animals

All animal experiments were pre-approved and conducted in accordance with University of Ottawa Animal Care and Veterinary Service guidelines. Rosa26R-LacZ, MMTV-NDL, MMTV-NIC, and MMTV-Cre transgenic mice were generously provided by Dr. William Muller (McGill University), while $Sox10^{fl/fl}$ mice were a kind gift from Dr. Michael Wegner (Friedrich-Alexander-Universität, Germany). Founder NSG mice were purchased from Jackson Laboratories and bred in-house. For survival studies, NIC females were monitored biweekly for tumour development starting at 12 weeks of age. Tumour onset was defined by a total tumour burden of 5 mm^3, and progression was tracked until endpoint of 17 mm^3, at which point mice were euthanized and tumours collected. For hyperplasia analysis, NIC females were sacrificed at 16-, and 24-weeks of age. Fourth inguinal mammary glands were harvested, fixed in 10% buffered formalin for 72 hours, washed in PBS (3 × 5 min), and stored in 70% ethanol at 4°C until processing. Embedding, sectioning (4 µm), and mounting were performed by the University of Ottawa Pathology Core Facility. For whole-mount analysis, glands were dry-mounted on glass slides, defatted in 100% acetone for 7 days, stained in hematoxylin overnight, and destained in 70% EtOH:1% HCl for 48–72 hours. Glands were then dehydrated in 100% ethanol followed by xylene.

For orthotopic injection studies, cell lines were resuspended in a 1:1 PBS:Matrigel mixture and injected into the third or fourth mammary gland of 12-week-old female NCG mice. Tumour development was monitored biweekly by palpation until endpoint was reached. For intravenous injections, 1×10^6 cells in PBS were injected into the left or right lateral tail vein of 12-week-old NCG mice. Mice were monitored twice weekly for changes in body weight, respiration rate, and behavioral changes such as hunched posture and lack of grooming until the end of the 28-day study period. Other than immune compromised hosts, all mice were maintained on an FVB/n background. Mice were weaned at P21, and genotyping was performed using DNA extracted from ear notches with the DNeasy Blood & Tissue Kit (Qiagen, Cat. No: 69504). PCR was performed using REDTaq DNA Polymerase (VWR, Cat. No: 76620-472). Primer sequences and annealing conditions are listed in Supplementary Table 1.

Western Blotting

Protein lysates were prepared from either cultured cell lines or freshly resected tumor tissue. Samples were washed in ice-cold PBS, then homogenized and lysed in RIPA buffer supplemented with protease/phosphatase inhibitors and processed as described previously (21). Protein concentration was determined using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, cat: 5000006). Equivalent amounts of total protein (30ug) were resolved on polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes and probed with the indicated antibodies.

Primary cell line isolation and CRISPR-Cas9 Gene Inactivation

At endpoint, tumors were mechanically dissociated, incubated overnight at 37°C in Gentle Collagenase/Hyaluronidase digestion medium (1:10 in DMEM; StemCell, cat: 7919). And blood cells were then lysed in 0.8% NH_4Cl. Cells were resuspended in 1 mg/mL DNase I (Sigma, cat: 10104159001) and filtered through a 40 µm strainer. Lineage-negative (Lin^-) cells were isolated using the Mouse Mammary Stem Cell Enrichment Kit (Stemcell Technologies, cat: 19757), according to the manufacturer's protocol. Cells were cultured in a 1:1 mix of supplemented DMEM (Corning, cat# 17-207-CV) and Ham's F-12 (ThermoFisher, cat# 11765070) as previously described (21, 59).

For CRISPR knockout, guide RNAs targeting the Sox10 ORF in exons 2 and 3, respectively, were designed using Benchling (Benchling.com) and cloned into BsmBI-digested pLKO H2B-mRFP-2A-puro (gift from Dr. Daniel Schramek, University of Toronto). Target cells were first transduced with pLenti-Cas9-Blast lentivirus and selected for blasticidin resistance and Cas9 expression. Cells were subsequently transduced with the pLKO H2B-mRFP-2A-puro plasmid containing Sox10-targeting guides, and RFP^+ cells were flow sorted.

Immunohistochemistry and fluorescence staining

Tissue samples were fixed in 10% buffered formalin, washed in PBS, and stored in 70% ethanol at 4°C until processing. Embedding, sectioning (4 µm), and mounting were performed by the University of Ottawa Pathology Core Facility. Sections were deparaffinized before undergoing antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) and quenched using 3% hydrogen peroxide (Sigma, cat: H1009). Sections were permeabilized, blocked and incubated overnight at 4°C with primary antibodies (see Supplementary Table 1). Detection was achieved with SignalStain® Boost IHC Detection Reagent (NEB, cat: 8125S/8114S) and Vector DAB Peroxidase Substrate Kit (MJS BioLynx, cat: VECTSK4100), followed by hematoxylin/eosin counterstaining. Slides were imaged using a ZEISS™ Axioscan 7 microscope and analyzed with Zen 2.3 software.

For fluorescence immunostaining, cells grown on coverslips were fixed, permeabilized incubated with primary antibodies (59) (Supplementary Table 1). Antigens were visualized using Alexa Fluor-conjugated secondary antibodies and counterstained with DAPI (1:5000, ThermoFisher, cat: D1306). Mounted coverslips were imaged using a ZEISS™ Axio Imager 2 microscope with Zen 2.3 software.

Real-time Quantitative PCR

Cell line and tissue RNA isolation and subsequent cDNA preparation was completed using TRIzol (ThermoFisher, cat: 15596026) and Superscript III (ThermoFisher, cat: 18080093) as per manufactures' protocols. RT-qPCR was performed using an Applied Biosystems 7500 Real-Time Fast PCR thermocycler. Relative mRNA expression was calculated using the ΔΔCT method and normalizing to total levels of ribosomal 18S.

Proliferation Assay and Boyden Chamber Assay

Growth curves were obtained as described using $2.5x10^4$ cells plated in 6-well plate on Day 0. Cell counts were performed at 24, 48, 72, and 96 hours using trypan blue exclusion Vi-Cell cell counter (Beckman Coulter). For transwell assays, serum starved cells ($5x10^4$) were seeded in the upper chamber of Collagen coated Corning™ Transwell™ inserts (8 µm pore size) containing 1% FBS DMEM/F12.

Migrated cells were counted using DAPI staining at 4 hours post-seeding (60). Absolute cell numbers were counted using a ZEISS™ Axioscan™ 7 microscope and analyzed with Zen™ 2.3 software.

Mammosphere forming Assay and 3D Invasion Assay

Mammosphere assays were performed as described (21)using 2×10^3 cells in primary and secondary assays into low-attachment 24-well plates (Sigma-Aldrich, cat# CLS3473). Spheres were grown in DMEM/F12 supplemented with Mammary Epithelial Growth Supplement (ThermoFisher, cat# S0155) and B-27 (ThermoFisher, cat# 17504044), and cultured for 7 days. Spheres ≥ 50 µm in diameter were enumerated. 3D invasion assays were performed as described (61) using cell spheres plated on a 1:1 Matrigel:Collagen type I mixture and cultured for 7 days. Spheres were imaged every 24-hours using an EVOS™ M5000 (Invitrogen, cat: AMF5000SV).

RNA-Sequencing Processing and Quantification

Triplicate RNA samples were subjected to RNA-seq at the Genome Quebec Facility. Mouse RNA-seq reads were quality filtered using the rfastp R package and filtered paired-end reads were aligned to the mm10 reference genome using subjunc (Rsubread v2.14.2) (62, 63). Differentially expressed genes were identified with DESeq2 (v1.40.2) (64). A DESeqDataSet was created from the raw count matrix with group information (NTC, sgRNA2.1, sgRNA2.2) and genes with adjusted $p < 0.05$ (Benjamini–Hochberg) and $|\log2FC| > 1$ were considered significant. Rlog transformed counts were used to compute a Pearson correlation matrix; sample distances were defined as 1 minus the correlation value. The distance matrix was displayed as a heatmap using the pheatmap package (v1.0.12) (65) with Euclidean distance used for hierarchical clustering. Pathway activity scores were computed with PROGENy (v1.18.2) (66) using the top 100 downstream target genes per pathway to calculate scores. Z-score-normalized samples were visualized using a heatmap with unsupervised hierarchical clustering.

Shared DEGs (adjusted p < 0.05, |log2FC| > 1) from both Sox10 knockout clones were analysed using the enrichPathway() function from the ReactomePA package (v1.42.0) (67). The ten most significant (adjusted p < 0.05) pathways were selected for visualization. GSEA was performed using the clusterProfiler package (v4.6.2) (68)with mouse gene sets from the MSigDB C2: Chemical and Genetic Perturbations (CGP) collection obtained through msigdbr (v7.5.1) (69-71). The top 10 positively and negatively enriched gene sets were visualized (gseaplot2) and ordered by normalized enrichment score (NES). Rlog-transformed gene expression values were used to generate a heatmap for a curated list of lineage-related genes. Expression values were row-scaled (z-score) and hierarchical clustering was performed using Euclidean distance.

Fluorescence activated cell sorting (FACS)

FACS was carried out as previously described (72) Briefly, cells were collected and washed with 1X DPBS, stained with Zombie NIR viability dye (Biolegend, cat: 423106) for 20 min and subsequently rinsed with 1X DPBS with 1% BSA. Cells were then stained with FC block (Biolegend, cat:101320) for 20 min and sorted for RFP+ populations. FACS sorting was completed using a Cytek Aurora spectral flow cytometery.

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Data sharing plan

This paper did not generate any original code. RNA-Seq data will be deposited and publicly available as of the date of publication.

Declaration of interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Luminal *Sox10*-deletion induces delayed mammary gland development. (A) Representative images of hematoxylin stained MMTV-Cre:Sox10$^{+/+}$ and MMTV-Cre:Sox10$^{fl/fl}$ mammary gland whole-mounts at 28-, and 80-days of age. Quantification of terminal end buds (B) and ductal branch points (C) in stained MMTV-Cre:Sox10$^{+/+}$; Sox10$^{+/fl}$ and Sox10$^{fl/fl}$. Data shown as mean ± SEM. n ≥ 10 mice/group. (D) Immunohistochemistry on MMTV-Cre:Sox10$^{+/+}$ and MMTV-Cre:Sox10$^{fl/fl}$ mammary glands at 11 weeks of age showing similar proportions of luminal (CK8) and basal (CK14) cells. (E) MMTV-Cre:Sox10$^{+/+}$ and MMTV-Cre:Sox10$^{fl/fl}$ glands were stained for Sox10 expression at 11 weeks of age and the efficiency of luminal Sox10 deletion was quantitated in panel (F). **,****$p < 0.05$, and 0.0005, respectively. Scale bar 50μ.

Figure 2. Luminal *Sox10*-deletion impairs Neu-induced tumor initiation. (A) Kaplan-Meier disease-free survival curves in MMTV-NIC:Sox10$^{+/+,fl/+,fl/fl}$ mice. Data shown as mean ± SEM. n ≥ 8 mice/group (**B)** Endpoint tumour lysates were assessed for the expression of Sox10, Neu, and CK8 using Western blot analysis. Time for tumour onset (0.5 cm^3) (**C**) and progression to endpoint (0.5 cm^3) (**D**) were measured for all MMTV-NIC genotypes. n≥ 15 mice/group. Quantification of total mammary gland coverage (E) and average size of hyperplastic lesions (F) in 16-, and 24-week-old MMTV-NIC mice from all genotypes. n≥ 6 mice/group. *,**,****$p < 0.05$,0.01, and 0.0005, respectively.

Figure 3. *Sox10*-deficient NeuNDL cell lines have reduced stemness and tumorigenic potential. (A) Immunofluorescence staining for Sox10 and (B) proportion of Sox10+ nuclei following CRISPR-Cas9 mediated *Sox10* ablation in NeuNDL BG2 cells. (**C**) 3D organoid collagen:matrigel invasion was measured in the BG2-sgRNA2 pools and NTC control over 6 days in serum free conditions. (D) Primary and secondary mammosphere efficiencies were measured in BG2-sgRNA-1, sgRNA-2 and NTC pools after 7-days in ultra-low attachment plates and imaging. (E) 10^6 BG2-sgRNA-2 or NTC cells were

subcutaneously injected into the flanks of NCG mice and tumour volumes were recorded bi-weekly until endpoint of 1.7cm^3 was reached by the first tumour in the cohort. Data shown as mean ± SEM. n = 4 mice/group. (**F**) Examples of resected tumors from cells injected in (E). *,***,****p < 0.05, 0,01 and 0.0005, respectively. Scale bar 50µ.

Figure 4. Sox10-deficent Neu+ cells show reduced TIC content and impaired lung colonization ability. (A) NTC, BG1sgRNA-2.1 and BG1sgRNA-2.2 NDL clones were injected orthotopically at 10^6, 10^5 or 10^4 cells into NCG mice and tumour progression was monitored bi-weekly for 32 days. Data is shown as mean ± SEM. n ≥ 4 mice/group (B) Tumors or residual tissue at the injection sites were removed and stained for Sox10. Scale bar 50µ. (C) Wet lung weight from tail vein colonization assays following injection of 10^6 NTC, BG1sgRNA-2.1 or BG1sgRNA-2.2 Sox10$^{+/-}$ NDL cells. n≥ 5 mice/group (D) Hematoxylin-eosin-stained lung tissues showing extensive colonization by BG2-NTC control cells. Bottom 6 panels: Immunohistochemistry for Cas9 shows widespread presence of BG1-NTC cells but few small foci of BG1sgRNA-2 cells. ***, ****p < 0.01 and <0.001, respectively. Scale bar 50µ.

Figure 5. Sox10 deletion in Neu+ tumor cells promotes a luminal to basal/stem-like reprogramming. (A) Triplicate Rlog-transformed gene expression values were used to generate a heatmap for a curated list of luminal and basal markers in BG1sgRNA2-1 and sgRNA2-2 cells. **(B)** Quatitative PCR (q-PCR) validation of selected luminal/basal markers in NTC, BG1sgRNA2-1 and sgRNA2-2 cells. (C) Gene Set Enrichment Analysis (GSEA) of genes ranked by the average Wald statistic of both Sox10 knockout clones (sgRNA2-1 and sgRNA2-2). Gene sets were derived from the MSigDB C2: Curated Gene Sets – Chemical and Genetic Perturbations (CGP) collection for *Mus musculus*. The dot plot displays the top positively (activated in Sox10-knockout) and negatively (supressed in Sox10-knockout) enriched gene sets by normalized enrichment score (NES) with adjusted $p < 0.05$. Dot size reflects the number of contributing genes, and color indicates adjusted *p*-value.

Supplemental Figure 1. Early Cre expression and Sox10 deletion in MMTV-Cre mice. (A) MMTV-Cre:ROSA26R mice show LacZ expression as early as 2 weeks in newborn mice. (B) Efficient Sox10 deletion in 16-week old Sox10-floxed MMTV-NIC mice showing little to no Sox10+ luminal cells.

Supplemental Figure 2. MMTV-NIC:Sox10-null luminal cells express Sox9 but do not develop hyperplasias. Endpoint tumor sections were assessed for the proportion of Sox10+ (A), Ki67+ (B) and active Caspase3 (C). Representative FFPE sections of 16-week-old NIC:Sox10$^{+/+}$ (D) and NIC:Sox10$^{+/fl}$ (E) mammary glands stained with hematoxylin to assess hyperplastic lesion coverage and numbers as presented in Figure 2. (F) Representative sections H&E sections showing no hyperplastic lesion in a 420 day-old MMTV-NIC:Sox10-null female. A wildtype female is shown for comparison. (G) Sox9 immunohistochemistry in 16 week-old NIC:Sox10$^{+/+}$ and NIC:Sox10$^{fl/fl}$ mice showing abundant luminal Sox9+ cells in Sox10-deficient mice.

Supplemental Figure 3. Sox10 inactivation in Neu+ cells does not impair proliferation or motility *in vitro*. (A) Western blot comparison of Sox10 levels in various established MMTV-NDL and MMTV-NIC cell lines. CK8; cytokeratin 8. (B) Western blot analysis of Sox10 knock down in cloned BG1 clones relative to the Sox10 knock-down pool (blk) and NTC control. (C) Cell growth was measured over 4 days using manual cell count following the plating of $5x10^4$ BG2-sgRNA-2 cells. (D & E) Collagen haptotactic migration assay on BG2-sgRNA-2 cells as assessed Boyden chamber. For both BG1- (not shown) and BG2-sgRNA-2, no proliferation or motility defects were observed. (F) Representative image of a 3D collage:matrigel invasion assay showing the “invasive perimeter” used in Figure 3C quantitation. (G) Tumors excised at endpoint following injection of BG2-NTC or sgRNA-2 pools were sectioned and stained for Sox10 expression. Tumors arising from BG2sgRNA-2 showed numerous Sox10+ nuclei, suggesting the expansion of non-targeted subpopulations. (H) Western blot analysis of Sox10 and Sox9 in BG1 clones and control.

Supplemental Figure 4. Sox10 inactivation in Neu+ cells impairs tumor cell expansion and lung colonization. Following the injection of 10^6, 10^5 or 10^4 BG1-NTC, BG1sgRNA2-.1 or BG1sgRNA2.2 cells into NCG mice, tumors were excised (A) and weight was recorded (B) at endpoint. (C) Representative images of wet lungs isolated from colonization assays using 10^6 BG1 cells. Lung weight was recorded and reported in Figure 4C. ***, ****; $p<0.01$ and <0.005, respectively

Supplemental Figure 5. (A) Euclidean distance matrix of rlog-transformed RNA-seq data showing sample clustering based on 1 minus Pearson correlation, comparing Sox10 knockout clones (sgSox10_2, sgSox10_6) and non-targeting control (NTC). **(B)** Venn diagrams illustrating overlap of significantly downregulated (blue) and upregulated (red) genes (adjusted $p < 0.05$, $|\log FC| > 1$) identified in independent Sox10 knockout clones relative to NTC. **(C)** Volcano plot of differentially expressed genes common to both Sox10 knockout clones. **(D)** PROGENy pathway activity scores inferred from rlog-transformed gene expression of pathway target genes, calculated per replicate. Scores were z-score normalized across samples (row-wise), and hierarchical clustering was applied to both pathways and samples. (E) Overrepresentation analysis of shared differentially expressed genes (adjusted $p < 0.05$, $|\log FC| > 1$) using the Reactome database. The top 10 enriched pathways were selected based on adjusted p-value and are displayed ordered by gene ratio. Dot size represents the number of enriched genes per pathway, and dot color indicates adjusted p-value.

Supplemental Figure 6. (A, B) Enrichment plots for the LIM_MAMMARY_STEM_CELL gene sets identified in Figure 5C. The UP gene set (genes elevated in mammary stem cells) is positively enriched (NES = 1.84) in Sox10 knockout clones (A). The DN gene set (genes downregulated in mammary stem cells) is negatively enriched (NES = -2.16) in Sox10 knockout clones (B). (C and D) Enrichment plots for the CHARAFE_BREAST_CANCER_LUMINAL_VS_ MESENCHYMAL gene sets identified in Figure

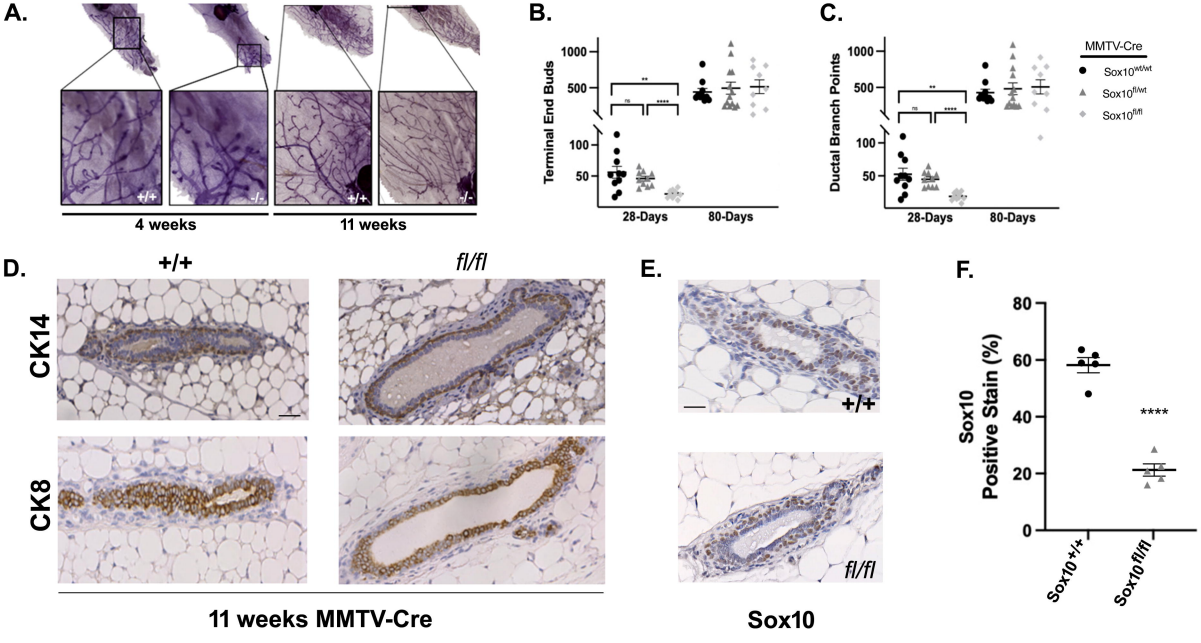
5C. The DN gene set (genes elevated in mesenchymal compared to luminal breast cancer) is positively enriched (NES = 1.91) in Sox10 knockout clones (C). The UP gene set (genes elevated in luminal compared to mesenchymal breast cancer) is negatively enriched (NES = -1.95) in Sox10 knockout clones (D).

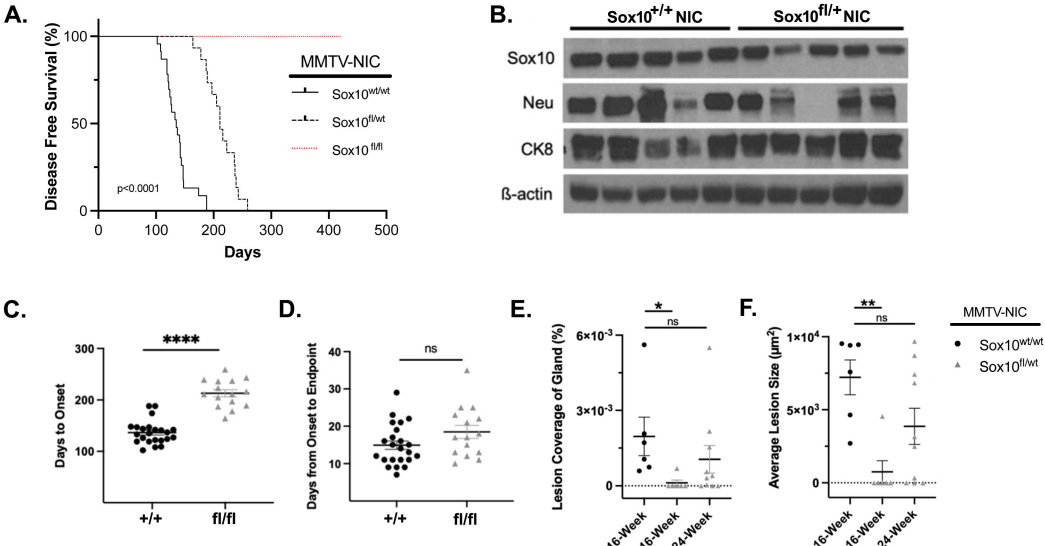
	Supplier	Identifier
Antibody		
Sox10	Abcam	Cat#ab180862
		WB - 1:1000 *IHC - 1:400*
Cleaved Caspase 3	Cell Signalling Tech	Cat#9661
		IHC - 1:400
Ki67	Santa Cruz	Cat#sc-23900 *IHC - 1:150*
Cre	Cell Signalling Tech	Cat#ab53280
		IHC - 1:50
Keratin 8	Abcam	Cat#15036S
		WB - 1:1000 *IHC - 1:800*
Keratin 14	Cell Signalling Tech	Cat#ab7800
		IHC - 1:100
Neu	Sigma	Cat#OP15 *WB - 1:2000*
		IHC - 1:100
Actin	Sigma	Cat#A5316
		WB - 1:20000
Cas9	Cell Signalling Tech	Cat#14697S *IHC - 1:100*
Sox9	Cell Signalling Tech	Cat#82630S
		WB - 1:1000
		IHC - 1:200
Goat anti mouse igG HRP conjugate	BioRad	Cat#1706516
Goat anti rabbit igG HRP conjugate	BioRad	Cat#1706515
Goat anti-Rabbit IgG (H+L) Alexa Fluor™ 488		*WB - 1:5000*
	BioRad	Cat#1706515
Goat anti-Mouse IgG (H+L) Alexa Fluor™ 594		WB - 1:5000
Oligonucleotides	Invitrogen	Cat#A-11008 IF: 1:1000
qPCR Primers	Invitrogen	Cat#A-11005
Krt8 Forward: TCCATCAGGGTGACTCAGAAA	This manuscript	IF: 1:1000
Krt8 Reverse: CCAGCTTCAAGGGGCTCAA	This manuscript	

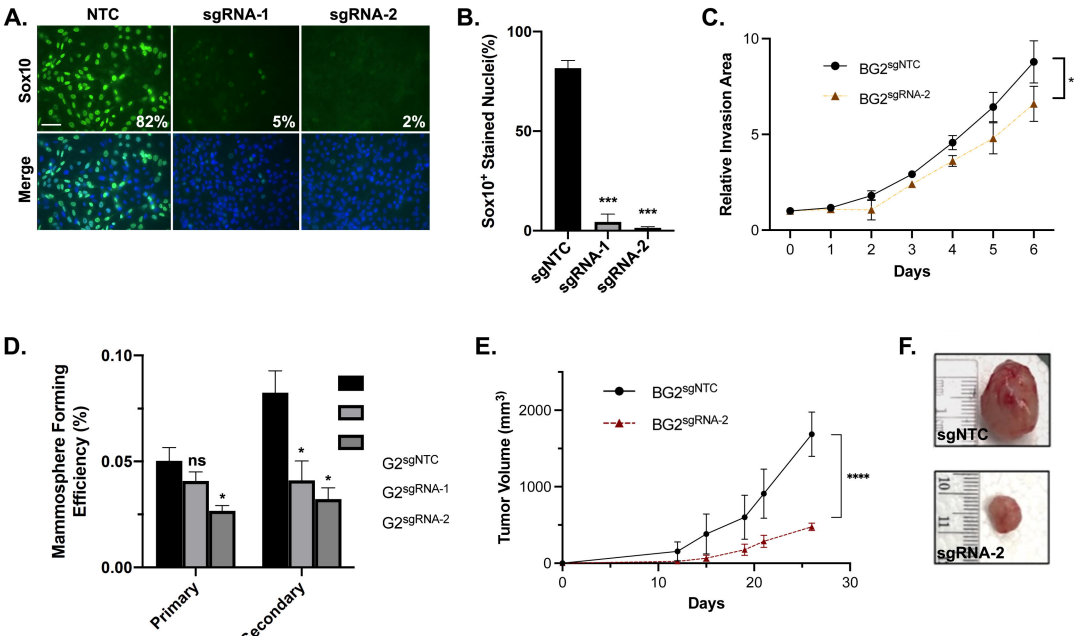
Krt18 Forward: TCAAGATCATCGAAGACCTGAGG	This manuscript	N/A
Krt18 Reverse: GCGCATGGCTAGTTCTGTC	This manuscript	N/A
Elf5 Forward: ATGTTGGACTCCGTAACCCAT	This manuscript	N/A
Elf5 Reverse: GCAGGGTAGTAGTCTTCATTGCT	This manuscript	N/A
Foxa1 Forward: ATGAGAGCAACGACTGGAACA	This manuscript	N/A
Foxa1 Reverse: TCATGGAGTTCATAGAGCCCA	This manuscript	N/A
Etv1 Forward: GCAAGTGCCTTACGTGGTCA	This manuscript	N/A
Etv1 Reverse: GCTTCAGCAAGCCATGTTTCTT	This manuscript	N/A
Krt5 Forward: TCTGCCATCACCCCATCTGT	This manuscript	N/A
Krt5 Reverse: CCTCCGCCAGAACTGTAGGA	This manuscript	N/A
Krt14 Forward: AGCGGCAAGAGTGAGATTTCT	This manuscript	N/A
Krt14 Reverse: CCTCCAGGTTATTCTCCAGGG	This manuscript	N/A
Acta2 Forward: GTCCCAGACATCAGGGAGTAA	This manuscript	N/A
Acta2 Reverse: TCGGATACTTCAGCGTCAGGA	This manuscript	N/A
Trp63 Forward: TACTGCCCCGACCCTTACAT	This manuscript	N/A
Trp63 Reverse: GCTGAGGAACTCGCTTGTCTG	This manuscript	N/A
Sox10 Forward: AGGTTGCTGAACGAAAGTGAC	This manuscript	N/A
Sox10 Reverse: CCGAGGTTGGTACTTGTAGTCC	This manuscript	N/A
18S Forward: AGTCCCTGCCCTTTGTACACA	This manuscript	N/A
18S Reverse: GATCCGAGGCCTCACTAAAC	This manuscript	N/A
Genotyping Primers		N/A
Sox10 Forward: CAGGTGGGCGGGGCTCTT *Sox10 Reverse 1: GTGAGCCTGGATAGCAGCAG*	This manuscript This manuscript	N/A
Sox10 Reverse 2: TCCCAGGCTAGCCCTAGTG	This manuscript	
Cre Forward: TATTCGGATCATCAGCTACACCAGAG	This manuscript	N/A N/A
Cre Reverse: GGGATTGCTTATAACACCCTGTTACG	This manuscript	N/A
Neu Forward: GTTTCCTGCAGCAGCCTACGC	This manuscript	N/A
Neu Reverse: TTCCGGAACCCACATCAGGCC	This manuscript	N/A

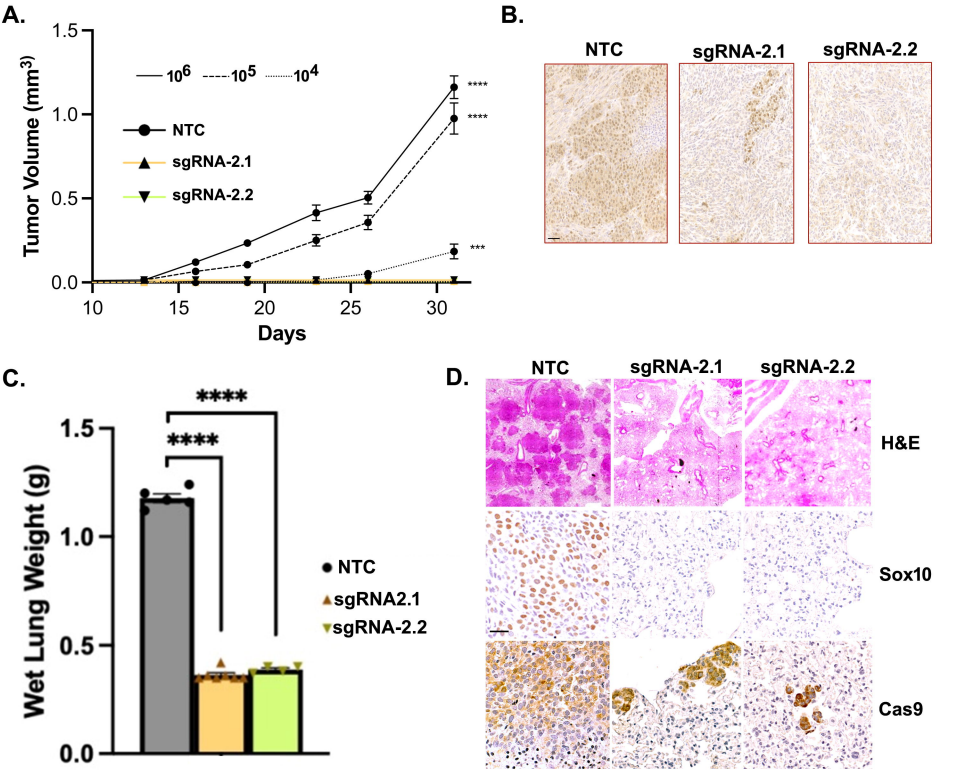
RosaR26R Forward: AATCCATCTTGTTCAATGGCCGATC	This manuscript	N/A
RosaR26R Reverse: CCGGATTGATGGTAGTGGTC	This manuscript	N/A
CRISPR sgRNA Guides		N/A
sgNTC: CACCGATCGGTATGTTTAGGGTT	This manuscript	N/A
sgNTC: AAACAACCCTAAACATAACCGATC	This manuscript	
sgSox10-1: CACCGGTTGGTACTTGTAGTCCGGA *sgSox10-1: AAACTCCGGACTACAAGTACCAACC*	This manuscript This manuscript	N/A
sgSox10-2: CACCGGTTCCCCGTGTGCATCCGCG *sgSox10-2: AAACCGCGGATGCACACGGGGAACC*	This manuscript This manuscript	N/A
Recombinant DNA		
pCMV-dR8.2 dvpr		
pCMV-VSV-G pLenti Cas9-Blast	 Addgene	N/A Cat#8455
pLKO H2B-mRFP-2A puro	Addgene Dr. D.Schramek; Toronto	Cat#8454 N/A
	Dr. D.Schramek; Toronto	N/A

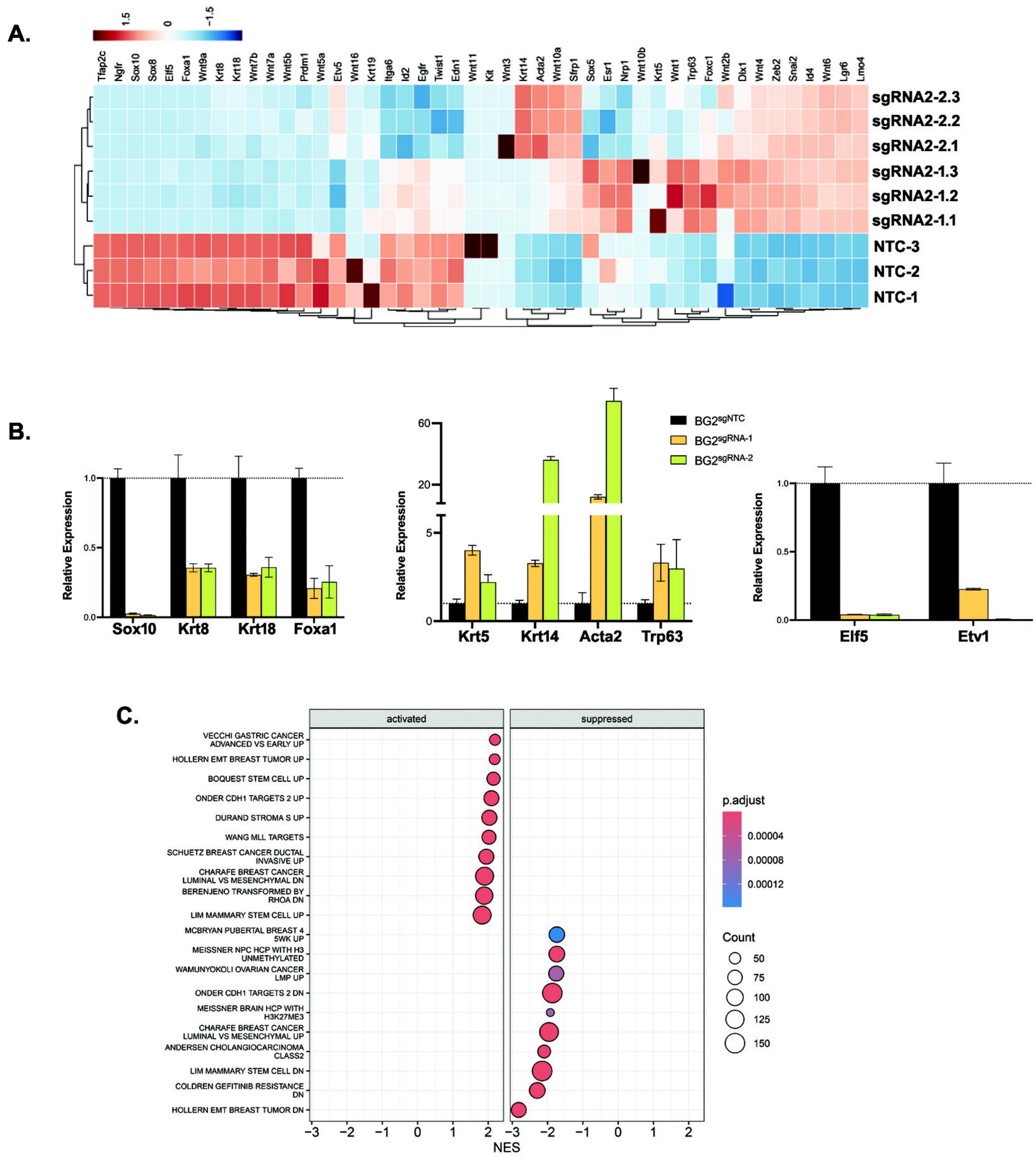
Supplemental Table 1.


A.
+/+
-/-
+/+
-/-
4 weeks
11 weeks
B.
Terminal End Buds
1000
500
100
50
**
ns
****
28-Days
80-Days
C.
Ductal Branch Points
1000
500
100
50
**
ns
****
28-Days
80-Days
MMTV-Cre
Sox10wt/wt
Sox10fl/wt
Sox10fl/fl
D.
+/+
fl/fl
CK14
CK8
11 weeks MMTV-Cre
E.
+/+
fl/fl
Sox10
F.
Sox10 Positive Stain (%)
80
60
40
20
0
****
Sox10+/+
Sox10fl/fl


A.
Disease Free Survival (%)
100
50
0
p<0.0001
0
100
200
300
400
500
Days
MMTV-NIC
Sox10wt/wt
Sox10fl/wt
Sox10 fl/fl
B.
Sox10+/+ NIC
Sox10fl/+ NIC
Sox10
Neu
CK8
ß-actin
C.
Days to Onset
300
200
100
****
+/+
fl/fl
D.
Days from Onset to Endpoint
40
30
20
10
ns
+/+
fl/fl
E.
Lesion Coverage of Gland (%)
6×10-3
3×10-3
0
*
ns
16-Week
16-Week
24-Week
F.
Average Lesion Size (µm2)
1×104
5×103
0
**
ns
16-Week
16-Week
24-Week
MMTV-NIC
Sox10wt/wt
Sox10fl/wt


A.
NTC
sgRNA-1
sgRNA-2
Sox10
Merge
82%
5%
2%
B.
Sox10+ Stained Nuclei(%)
100
50
0
***
***
sgNTC
sgRNA-1
sgRNA-2
C.
Relative Invasion Area
10
5
0
BG2sgNTC
BG2sgRNA-2
*
0
1
2
3
4
5
6
Days
D.
Mammosphere Forming Efficiency (%)
0.10
0.05
0.00
ns
*
*
*
Primary
Secondary
G2sgNTC
G2sgRNA-1
G2sgRNA-2
E.
Tumor Volume (mm3)
2000
1000
0
BG2sgNTC
BG2sgRNA-2
****
0
10
20
30
Days
F.
sgNTC
sgRNA-2


A.
Tumor Volume (mm3)
1.5
1.0
0.5
0.0
10
15
20
25
30
Days
10^6
10^5
10^4
NTC
sgRNA-2.1
sgRNA-2.2
****
****
***
B.
NTC
sgRNA-2.1
sgRNA-2.2
C.
Wet Lung Weight (g)
1.5
1.0
0.5
0.0
****
****
NTC
sgRNA2.1
sgRNA-2.2
D.
NTC
sgRNA-2.1
sgRNA-2.2
H&E
Sox10
Cas9


A.
1.5
0
−1.5
Tfap2c
Ngfr
Sox10
Sox8
Elf5
Foxa1
Wnt9a
Krt8
Krt18
Wnt7b
Wnt7a
Wnt5b
Prdm1
Wnt5a
Etv5
Wnt16
Krt19
Itga6
Id2
Egfr
Twist1
Edn1
Wnt11
Kit
Wnt3
Krt14
Acta2
Wnt10a
Sfrp1
Sox5
Esr1
Nrp1
Wnt10b
Krt5
Wnt1
Trp63
Foxc1
Wnt2b
Dlx1
Wnt4
Zeb2
Snai2
Id4
Wnt6
Lgr6
Lmo4
sgRNA2-2.3
sgRNA2-2.2
sgRNA2-2.1
sgRNA2-1.3
sgRNA2-1.2
sgRNA2-1.1
NTC-3
NTC-2
NTC-1
B.
Relative Expression
1.0
0.5
0.0
Sox10
Krt8
Krt18
Foxa1
60
20
5
0
Krt5
Krt14
Acta2
Trp63
BG2sgNTC
BG2sgRNA-1
BG2sgRNA-2
Elf5
Etv1
C.
activated
suppressed
VECCHI GASTRIC CANCER ADVANCED VS EARLY UP
HOLLERN EMT BREAST TUMOR UP
BOQUEST STEM CELL UP
ONDER CDH1 TARGETS 2 UP
DURAND STROMA S UP
WANG MLL TARGETS
SCHUETZ BREAST CANCER DUCTAL INVASIVE UP
CHARAFE BREAST CANCER LUMINAL VS MESENCHYMAL DN
BERENJENO TRANSFORMED BY RHOA DN
LIM MAMMARY STEM CELL UP
MCBRYAN PUBERTAL BREAST 4 5WK UP
MEISSNER NPC HCP WITH H3 UNMETHYLATED
WAMUNYOKOLI OVARIAN CANCER LMP UP
ONDER CDH1 TARGETS 2 DN
MEISSNER BRAIN HCP WITH H3K27ME3
CHARAFE BREAST CANCER LUMINAL VS MESENCHYMAL UP
ANDERSEN CHOLANGIOCARCINOMA CLASS2
LIM MAMMARY STEM CELL DN
COLDREN GEFITINIB RESISTANCE DN
HOLLERN EMT BREAST TUMOR DN
−3
−2
−1
0
1
2
NES
p.adjust
0.00004
0.00008
0.00012
Count
50
75
100
125
150