TFAP2C Governs the Luminal Epithelial Phenotype in Mammary Development and Carcinogenesis

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Abstract

Molecular subtypes of breast cancer are characterized by distinct patterns of gene expression that are predictive of outcome and response to therapy. The luminal breast cancer subtypes are defined by the expression of ER-alpha (ERα)-associated genes, many of which are directly responsive to the Transcription Factor Activator Protein 2C (TFAP2C). TFAP2C participates in a gene regulatory network controlling cell growth and differentiation during ectodermal development and regulating ESR1/ERα and other luminal cell-associated genes in breast cancer. TFAP2C has been established as a prognostic factor in human breast cancer, however, its role in the establishment and maintenance of the luminal cell phenotype during carcinogenesis and mammary gland development have remained elusive. Herein, we demonstrate a critical role for TFAP2C in maintaining the luminal phenotype in human breast cancer and in influencing the luminal cell phenotype during normal mammary development. Knockdown of TFAP2C in luminal breast carcinoma cells induced EMT with morphological and phenotypic changes characterized by a loss of luminal-associated gene expression and a concomitant gain of basal-associated gene expression. Conditional knockout of the mouse homolog of TFAP2C, Tcfap2c, in mouse mammary epithelium driven by MMTV-Cre promoted aberrant growth of the mammary tree leading to a reduction in the CD24hi/CD49fmid luminal cell population and concomitant gain of the CD24mid/CD49fhi basal cell population at maturity. Our results establish TFAP2C as a key transcriptional regulator for maintaining the luminal phenotype in human breast carcinoma. Furthermore, Tcfap2c influences development of the luminal cell type during mammary development. The data suggest that TFAP2C plays an important role in regulated luminal specific genes and may be a viable therapeutic target in breast cancer.

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Keywords
TFAP2C; breast cancer; mammary development; luminal; gene expression; EMT

INTRODUCTION

TFAP2C (AP-2C, AP-2γ) has been implicated in breast cancer development and progression; however, the role of TFAP2C in mammary epithelial differentiation and carcinogenesis requires more detailed analysis. Within the adult human mammary gland, TFAP2C expression can be found in epithelial and myoepithelial compartments. Overexpression of TFAP2C occurs in breast cancer, however, there are contradictory data regarding whether elevated expression of TFAP2C is associated with a worse clinical outcome. TFAP2C has been implicated in the regulation of genes that play a critical role in breast cancer biology including estrogen receptor-alpha (ERα) and HER2/c-erbB2. TFAP2C targets many of the genes that characterize the luminal ERα-positive breast cancer phenotype and transcriptional regulation by TFAP2C may further involve coordinate regulation with ERα and FOXA1. These findings indicate that TFAP2C may be critical for development of the luminal breast cancer phenotype. However, more definitive studies are required to delineate the role of TFAP2C in controlling the luminal breast cancer subtype.

TFAP2C is a developmentally regulated gene that controls early stages of epidermal differentiation in the ectoderm. Overexpression of the mouse homolog of TFAP2C, Tcfap2c, in a MMTV/neu transgenic mouse model resulted in a reduction in the number of tumors and a prolongation of latency but appeared to enhance proliferation and promoted tumor progression. Overexpression of Tcfap2c can lead to non-physiologic effects and gene knockout may provide clarity to the role of human TFAP2C in mammary development and oncogenesis. Total genetic knockout of the Tcfap2c gene resulted in embryonic lethality due to a placental defect. Jager, et al. successfully knocked-out Tcfap2c using Sox2cre in the whole animal and reported effects on mammary duct branching, development and mouse mammary epithelial cells (MMEC) proliferation. The effects did not appear to be the result of alterations in hormone expression. However, survival of the animals occurred at a low frequency raising questions about rare, selective effects and the potential that the effects were secondary to knockout of Tcfap2c in cells other than MMEC.

The purpose of the current study was to elucidate the role of TFAP2C in luminal breast cancer and mammary development. To better characterize the effects of TFAP2C in models of luminal breast cancer, we evaluated the effects on gene expression and cell morphology caused by knockdown of TFAP2C expression in both transient and stable knockdown systems. To clarify the role of TFAP2C in mammary development, we studied the phenotypic effects caused by conditional knockout of the mouse homolog of TFAP2C, Tcfap2c, utilizing MMTV-Cre for gene knockout in mouse mammary epithelial cells (MMEC). To the extent possible, we sought to characterize common physiologic effects in the human breast cancer and mouse mammary systems with the goal of better understanding...
the molecular effects of TFAP2C gene regulation in normal and cancer states of the mammary gland.

RESULTS

TFAP2C Targets the Luminal Breast Cancer Gene Expression Cluster

Previous studies indicated an important role of TFAP2C in regulating the expression of ERα and other genes involved in hormone response and ChIP-SEQ suggested that TFAP2C targets many of the genes associated with the luminal breast cancer phenotype. As seen in Figure 1A, ChIP-SEQ analysis of binding sites for TFAP2C demonstrated peaks associated with the regulatory regions of ERα-associated genes including ESR1, GATA3, FBP1, FOXA1, MYB, RET, KRT8 and MUC1. The first intron of CD44 was also noted to have a major site for TFAP2C binding, potentially indicating a role for TFAP2C-mediated repression of this key phenotypic marker.

Based on the published molecular profiling of breast cell lines of different phenotypes, we compared the ChIP-SEQ data with the genes characterizing various subclasses of gene profiles, including the Basal, Luminal Cytokeratins, ERα-associated and Luminal Differentiation gene sets. The genes were characterized as either a TFAP2C target gene, not a TFAP2C target or indeterminate if reads could not be uniquely assigned to the gene under analysis. A summary of the analysis is shown in Figure 1B, and shows that nearly all genes designated as Luminal Differentiation and ERα-associated were found to be directly targeted by TFAP2C. Approximately 60% of the Luminal Cytokeratins were found to be TFAP2C targets. By contrast only 10% of the genes in the Basal category were targeted by TFAP2C. Since the ChIP-SEQ data experiments were performed in MCF-7 cells, it is not clear to what extent epigenetic alterations or other molecular distinctions may alter how TFAP2C targets the genome, which might be different in basal cell lines. Nevertheless, the data demonstrate a non-random association between genomic binding pattern of TFAP2C and the set of genes associated with the luminal breast cancer subtype.

To assess the functional effects of binding, the expression of TFAP2C was knocked down in two luminal breast cancer cell lines—MCF-7 and T47-D—and the expression of several luminal target genes was assessed by RT-PCR and western blot. MCF-7 and T47-D cells express TFAP2C protein and are considered to be functional models for hormone-responsive Luminal A (ERα-positive, ERBB-2 non-amplified) breast cancer. As seen in Figure 2, knockdown of TFAP2C repressed expression of the luminal target genes in both cell lines, as noted by a reduction in RNA and protein. The CD44 gene, which is commonly down-regulated in luminal and up-regulated in basal cell types, demonstrated the opposite effect in MCF-7 and T47-D cells with increased expression with knockdown of TFAP2C. These data indicate that TFAP2C plays a critical role in regulating the expression of genes characteristic of the luminal breast cancer subtype. Furthermore, the data suggest that TFAP2C may be involved in active repression of genes associate with the basal phenotype.
Stable Knockdown of TFAP2C Induced a Luminal to Basal Transition

Transient knockdown of TFAP2C expression does not allow a complete evaluation of effects, some of which might involve epigenetic alterations that would require extended periods of growth to observe. Furthermore, the kinetics of alteration of gene expression can vary with the specific gene, and the completeness of knockdown can vary between transfection experiments. We sought to characterize the effect of stable TFAP2C knockdown on the phenotype and pattern of gene expression in luminal breast cancer cells. To further explore the role of TFAP2C in luminal cancer cells, we generated stable cell clones with knockdown of TFAP2C using lentiviral-mediated shRNA delivery (Figure 3). Stable MCF-7 clones with non-targeting shRNA vectors (sKD-NT) and those with TFAP2C-targeting shRNA vectors (sKD-C, clone 45 and 46) were isolated following extended puromycin selection. sKD-C cells demonstrated a significant knockdown of TFAP2C both at the RNA and protein level, with a concomitant up-regulation of the homologous family member TFAP2A compared to sKD-NT cells (Figure 3A, D), further confirming specificity of knockdown for the TFAP2C family member. sKD-NT cells retained a morphological appearance similar to parental MCF-7, while sKD-C cells developed altered morphology with multiple pseudopodia as noted by brightfield microscopy (Figure 3B).

Based on the gain of a mesenchymal-like morphology, we examined changes in the pattern of gene expression comparing Luminal Differentiation vs. Mesenchymal markers. As seen in Figure 3C, the pattern of gene expression as determined by microarray demonstrated that the luminal differentiation markers, as a group, were down-regulated in sKD-C cells compared to sKD-NT cells. Changes in several of the key markers were confirmed by western blot (Figure 3D). Stable knockdown of TFAP2C resulted in up-regulation of VIN/vimentin and CDH2/N-cadherin and repression of CDH1/E-cadherin as noted by both microarray (Figure 3C) and western blot (Figure 3D). CD44 expression, which is up-regulated in basal breast cancer and during epithelial-mesenchymal transition (EMT), was found to be up-regulated in sKD-C cells by microarray, western blot and RT-PCR (Figure 3C–E). Since CD24 was repressed (as noted on the microarray) and CD44 up-regulated with stable knockdown of TFAP2C, the data suggest a potential effect on the cancer stem cell (CSC) population. In order to determine whether the changes were occurring in the same cells, flow cytometry for CD44 and CD24 was performed in sKD-C cells compared to sKD-NT. We detected a doubling of the cell population expressing the cancer stem cell markers CD44^{hi}/CD24^{low} in the sKD-C cells compared to the sKD-NT cells (Figure 3F). These data show that the loss of TFAP2C in the Luminal A MCF-7 cell line promoted a mesenchymal change consistent with EMT, with an increase in the percentage of cells expressing cancer stem cell markers.

The data suggest that loss of TFAP2C was associated with a transition from a luminal to a basal breast phenotype. Hence, we analyzed the expression of genes characteristic of the luminal and basal subtypes. Using the taxonomic patterns identified by Kao et al., the pattern of gene expression was grouped as the Luminal or Basal Gene Cluster. sKD-C cells demonstrated a near-global loss of luminal-type gene expression, simultaneously up-regulating a large number of basal-type genes compared to the sKD-NT control line (Figure...
4A). To confirm these results, we performed RT-PCR and western blot analysis from multiple genes in both groups. Among the luminal-type genes, sKD-C cells demonstrated loss of GATA3, FOXA1, FBP1, MYB, RET, ESR1, KRT8, and MUC1 expression at both the RNA and protein level compared to sKD-NT cells (Figure 4A–C). Identical results were found for a separate cell clone derived by transfection of a different TFAP2C shRNA from a distinct part of the gene (clone 46). Furthermore, from the basal-type genes, MMP14, KIRREL, CALD1, GSTP1, and SFRP1 were all confirmed to have elevated expression in the sKD-C cells (both clones 45 and 46) compared to sKD-NT (Figure 4D–F). Taken together, these results confirm the pattern identified in the microarray analysis and definitively illustrate a migration from a luminal to a basal pattern of gene expression in MCF-7 cells following stable loss of TFAP2C.

Conditional KO of Tcfap2c

To clarify the role of TFAP2C in luminal development, we utilized a mouse model with conditional knock out of the mouse homolog of TFAP2C, Tcfap2c. We examined a conditional knockout utilizing MMTV-Cre to specifically eliminate the expression of Tcfap2c in mouse mammary epithelial cells (MMEC) (Supplemental Figure S1). To address the cumulative effect of TCFAP2C loss on mammary development, we evaluated whole mounts from 6- and 12-week old animals (Figures 5A, S2). At 6-weeks, KO ducts had delayed migration through the mammary fat pad and a decrease in the percentage invasion of the TEB into the mammary fat pad (48% vs. 67%, p=0.05) (Figure 5B). KO glands also appeared qualitatively different, with more end-bud like structures visible (Figure 5A, inset). Using a defined square area of 10^6 px2 immediately distal to and centered on the lymph node (LN) in digital images of equal magnification, the KO glands had both fewer branch points (31 vs. 46, p=0.04) and more end buds (67 vs. 50, p=0.02) than control glands (Figure 5C). These differences were not present in glands from 12-week old KO mice, though qualitative differences in the bud structures remained identifiable (Figure S2). Tcfap2cL/L MMTVCre+ and Tcfap2cwt/wt MMTVCre+ animals did not exhibit any noticeable quantitative or qualitative phenotype at 6 weeks (Figure S3), indicating that the effects described were attributable to the role of Tcfap2c. Both KO and control animals were capable of supporting litters (data not shown), suggesting that the developmental defect in KO animals was not sufficient to disrupt mammary function.

Immunochemistry performed on 6-week old Tcfap2cL/L MMTVCre+ animals (KO) demonstrated loss of TCFAP2C protein in the luminal MMEC within mammary ducts (Figure 6A). TCFAP2C protein was expressed in the myoepithelial cell layer in KO and control animals. Immunochemistry for cytokeratin-5 (CK5) and cytokeratin-8 (CK8) staining was used to identify myoepithelial and luminal subpopulations within mammary epithelium, respectively (Figure 6A). Qualitatively, luminal and basal cytokeratin staining patterns were similar between KO and control groups. A larger percentage of the ducts in 6-week KO mammary glands had stratified epithelium though not statistically different due to variation within the control population (data not shown). Interestingly, we noted CK5-positive cells within luminal structures (Figure 6B) that was distinctly different than the control animals. In addition, increased CK5 staining was noted in TEB structures in KO animals. To quantify these differences, the numbers of CK5-positive cells in the luminal cell
area was counted and confirmed a statistically significant increase in the KO compared to control animals (Figure 6C). The percentage of CK5-positive cells present in TEB structures were determined and demonstrated a statistically significant increased in the percentage of CK5-positive cells in KO animals (Figure 6D).

Examining the data in the context of the effects of TFAP2C knockdown in luminal breast cancer cells, we postulated that some of the effects of Tcfap2c KO in MMEC might be due to alterations in the luminal vs. myoepithelial (or basal) cell compartments. For example, KO of Tcfap2c was noted to have an increase in CK5-positive myoepithelial cells within the luminal spaces, suggesting that loss of Tcfap2c might be interfering with proper development of the luminal epithelium. Such an effect might also account for the differences noted on whole mount. Flow cytometry using defined markers of luminal and myoepithelial cells was performed to evaluate the luminal and basal populations within the epithelial compartment and gating was performed to remove the stromal elements to examine the Lin−MEC population. FACS analysis was performed from both pooled and individually isolated mammary glands (Figure 7). In cells isolated from KO glands compared to control, we identified a reduction in the CD24\textsuperscript{hi}CD49\textsuperscript{f} luminal population (average -11.9% reduction, p=0.002) and a concomitant increase in the CD24\textsuperscript{mid}CD49\textsuperscript{hi} basal population (average increase 14.9%, p=0.014). Additionally, a decrease in the normalized luminal:basal ratio was identified, with KO glands having on average a luminal:basal ratio of 33.4% that seen in the control group (p=0.0003). Taken together, the data strongly suggest that knockout of TCFAP2C resulted in an alteration in luminal development and a shift from the luminal to the basal compartment.

**DISCUSSION**

The clinical breast cancer subtypes are defined by characteristic patterns of gene expression, which can predict outcome and response to therapy. Hence, defining the mechanisms that establish the patterns of gene expression of the different breast cancer subtypes will likely form the basis for novel treatment strategies. The current study advances our understanding of the transcriptional mechanisms that establish the luminal breast cancer subtype and provides additional evidence that similar transcriptional mechanisms influence the development of the luminal epithelial cell during mammary development. The data demonstrate the functional effects of Tcfap2c in establishment of the luminal mammary compartment in MMEC and the critical role of TFAP2C in maintenance of the luminal differentiated phenotype in hormone responsive breast cancer. Maintenance of the luminal, hormone response breast cancer phenotype is an active process that requires TFAP2C as well as other transcription factors, since loss of TFAP2C induced a luminal to basal transition in the pattern of gene expression. Qualitatively similar results were obtained with transient knockdown using siRNA in two different cell lines and with stable knockdown in MCF-7 cell clones with shRNA cassettes targeting different regions of TFAP2C mRNA. Although the effects in the normal mammary gland were not as profound as in cell culture, the timing of Tcfap2c knockout and the relatively incomplete nature of the process likely relates to MMTV-Cre expression and thereby limits the extent of the effect of Tcfap2c elimination. However, finding parallel molecular changes in MMEC and human breast
cancer cell lines indicates that similar transcriptional mechanisms controlled by TFAP2C are operating in luminal mammary development and luminal breast cancer.

Transcriptional processes that induce a luminal to basal transition have gained significant interest of late. The transcription factor ELF5 has been shown to repress ERα and a set of ERα-associated genes with promotion of basal breast cancer characteristics. Similar to the effects of TFAP2C, PDEF has recently been shown to induce luminal differentiation in basal epithelial cells. In p18/Ink4c deficient mice, heterozygous germline mutation of Brca1 reduced development of luminal progenitors, inhibited the expression of luminal markers and induced tumors with basal-like characteristics. It has been reported that loss of ErbB3 from the MMEC compartment was associated with impaired Akt and MAPK signaling and induced a shift in gene expression from a luminal to basal pattern. Furthermore, the changes in luminal gene expression were partially rescued by activation of Akt and MAPK signaling, indicating that the regulation of luminal genes was sensitive to Akt and MAPK activation. Using a tumor model based on expression of MMTV-PyVmT, mammary tumors arising in ErbB3-deficient animals demonstrated increased expression of the basal marker, CK5. Knockdown of FOXA1 in luminal breast cancer cell lines induced a partial shift from a luminal to basal expression signature and these findings are consistent with other studies indicating that TFAP2C, FOXA1, and ERα co-target many of the genes of the luminal expression cluster. Interestingly, FOXM1 has an opposing effect and was found to repress luminal differentiation of the mammary gland. Similar to our results, FOXM1 plays an important role in controlling the differentiation of luminal epithelial progenitors; KO of FOXM1 disrupted development of the lobuloalveolar unit early in pregnancy, but later in pregnancy these differences are less evident and the mice lactate normally. Given the role of FOXM1 in regulating breast cancer phenotype, the data herein related to TFAP2C confirm an association between gene regulation during mammary development and regulation of the luminal-associated genes in breast cancer. Furthermore, the findings support a critical interaction between AP-2 and forkhead transcription factors in controlling patterns of gene expression in mammary development and breast cancer phenotype. Based on these findings, it is clear that maintenance of the luminal phenotype and its associated pattern of gene expression is an active process dependent upon the activity of a number of transcriptional regulators. A deeper understanding of the transcriptional mechanisms that maintain the breast cancer phenotypes offers the potential of altering the clinical characteristics of primary breast cancers, which will inform novel treatment strategies for hormone insensitive luminal breast cancers or potentially the aggressive basal cancer phenotype.

Breast cancer stem cells (CSC) were identified to reside within the CD44+/hi/CD24−/low population of tumor cells. We have shown that knockdown of TFAP2C in luminal breast cell lines led to induction of CD44 and resulted in an increase in the CD44+/hi/CD24−/low population. Similarly, KO of Tcfap2c in MMEC increased the percentage of basal cells characterized as the CD24mid/CD49fhi cell population within the MMEC. We have not examined the mammary stem cell (MaSC) compartment directly, which requires more detailed analysis utilizing markers for MaSC. However, the data presented herein leads to the conclusion that, within luminal breast cancer, TFAP2C represses CD44 expression and...
thereby reduces the population of cells expressing cancer stem cell markers. Further evidence that TFAP2C inhibits expression of CD44 is based on recent findings that forced over-expression of TFAP2C in a basal breast cancer cell line that does not express endogenous AP-2 proteins repressed CD44 expression. Basal or triple-negative breast cancers have a higher percentage of cells that express CSC markers than other breast cancer subtypes and the percentage of cells with CSC markers increases after chemotherapy indicating the relative resistance of this tumor cell population. Therefore, it is possible that inducing TFAP2C activity in basal breast cancer and thereby eliminating the CSC population may be an effective treatment either alone or in combination with conventional chemotherapy.

In summary, our finding provides compelling evidence that TFAP2C has a significant role in maintaining the luminal gene expression pattern characteristic of luminal breast cancer and influences the development of the luminal cell type in normal mammary glands. Loss of TFAP2C in luminal breast cancer lines induces a luminal to basal cell transition and was associated with the development of a mesenchymal expression pattern with loss of CDH1/E-cadherin and an increase in VIN/vimentin and CDH2/N-cadherin expression. Further study will be needed to more fully characterize whether the process of luminal to basal transition (or epithelial-mesenchymal transition, EMT) that occurs with loss of a key transcription factor such as TFAP2C induces a cell type that mirrors the clinical basal or triple-negative breast cancer subtype. However, the findings herein suggest the possibility that altering the transcriptional activity of AP-2 factors may induce changes in the clinically relevant phenotype of breast cancer, thus offering the potential of a novel treatment approach.

MATERIALS AND METHODS

Cell Lines

MCF-7 and T47-D cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained as previously described.

ChIP-SEQ

ChIP-SEQ tracks utilized in these studies were based on previously described studies. ChIP-SEQ data is available in GEO database under accession number GSE44257.

Generation of Stable cell Lines

Stable knockdown of TFAP2C expression in the MCF-7 cells was achieved using two different MISSION shRNA Lentiviral Particles based on pLKO.1-puro vector containing shRNA targeting TFAP2C Cat#TRCN0000019745 (resulting in clone 45), Cat#TRCN0000019746 (resulting in clone 46) and Non-Mammalian shRNA Control Cat#SHC002 (Sigma-Aldrich, St. Louis, MO, USA). All experiments were performed with early passage (<20 cell passages). MCF-7 origin of stable cell lines was confirmed by determining STR profile and subsequent comparison with ANSI/ATCC ASN-0002-2011 database (Authentication of Human Cell Lines: Standardization of STR Profiling).
**RNA isolation, cDNA synthesis and Real-Time-PCR**

Total RNA was isolated from cell lines using Rneasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized from 1 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Gene expression was monitored by quantitative real-time PCR. TaqMan primers and detection probes for genes were: TFAP2A Hs01029413_m1, TFAP2C Hs00231476_m1, GATA3 Hs00231122_m1, FOXA1 Hs00270129_m1, FBP1 Hs00983323_m1, MYB Hs00920556_m1, RET Hs01120030_m1, ESR1 Hs00174860_m1, KRT8 Hs01595539_g1, MUC1 Hs00159357_m1, MMP14 Hs01037009_g1, KIRREL Hs00217307_m1, CALD1 Hs00921982_m1, GSTP1 Hs02512067_s1, SFRP1 Hs00610060_m1, CD44 Hs01075861_m1, GAPDH Hs02758991_g1 (Applied Biosystems, Foster City, CA, USA). Expression values were normalized to the mean of 18S rRNA 4319413E-0803037 (Applied Biosystems, Foster City, CA, USA) as an endogenous control.

**Western blots**

Protein was isolated into RIPA buffer supplemented with Halt Protease Inhibitor (cat 78430, Thermo Scientific, Rockford, IL, USA). Western blots were done using the following primary antibodies at their recommended dilutions according manufacturer’s instructions: TFAP2-α 3154-1, TFAP2-γ 2384-1, FBP1 3288-1, ERα 1115-1, CALD1 1089-1, SFRP1 5467-1, KRT8 2032-1, GSTP1 6689-1, MMP14 2010-1, MUC1 2900-1, MYB 3195-1, RET 3454-1 (Epitomics, Burlingame, CA, USA); KIRREL (NEPH1) F-16, GATA3 HG3-31, FOXA1 (HNF-3α) Q-6, GPDH 6C5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Microarray**

All required RNA sample preparations, the subsequent hybridization and performing microarray analysis were done at the University of Iowa DNA Facility according the manufacturer’s recommended protocols. Arrays were performed in biologic triplicates with technical duplicates (hence six rows for each clone). Briefly, 200 ng total RNA was converted to amplified biotin-cRNA using the Ambion TotalPrep RNA Amplification Kit Cat# AMI1791 (Ambion, Austin, TX, USA). Biotin-cRNA was mixed with Illumina hybridization buffer and placed onto Illumina HumanHT-12v4 BeadChips Part No. BD-103-0204 (Illumina, San Diego, CA, USA). Hybridization was preformed at 58°C for 17h with rocking in an Illumina Hybridization Oven Cat# SE-901-1001 (Illumina, San Diego, CA, USA). Arrays were washed, blocked, and stained with streptavidin-Cy3 Cat# PA43001 (Amersham/GE Healthcare, Piscataway, NJ, USA) according to the Illumina Whole-Genome Gene Expression Direct Hybridization Assay protocol. Beadchips were scanned with the Illumina iScan System ID #N054 (Illumina, San Diego, CA, USA) and data were collected and analyzed using the GenomeStudio software v2011.1 (Illumina, San Diego, CA, USA). Microarray data is available in NCBI’s GEO database under accession number GSE44203.

**Flow Cytometry**

Samples were prepared with staining for CD44 using Mouse Anti-Human CD44 (Pgp-1) Cat# GWB-1F90D6 (GenWay Biotech, San Diego, CA, USA) and for CD24 using Anti-
Human CD24 PE Clone: eBioSN3 Cat#12-0247-42 (eBioscience, San Diego, CA, USA) according to manufacture protocol. All samples were control stained for live cells with FxCycle Violet Stain Cat# F10347 (Life Technologies, Grand Island, NY, USA). Flow cytometry was performed on a FACS (fluorescence-activated cell sorting) DiVa (Becton Dickinson, Franklin Lakes, NJ, USA) machine. Cells were first gated around the Fx Violet, followed by side and forward scatter profiles to eliminate adherent cells. Gating thresholds were established for CD44 and CD24 positivity in order to analyze for the CD44^{hi}/CD24^{-low} putative cancer stem cell populations.

**Mice and Characterization of TFAP2C Mammary Knockout**

Mice harboring the exon 6 LoxP-flanked Tcfap2c (Tcfap2c^{L/L}) allele were obtained from Dr. Trevor Williams, and mice harboring the MMTV-Cre allele were purchased from Jackson Laboratories (003553, Bar Harbor, ME, USA). All animals used for studies were from F4 progeny breeds to limit genetic variability. Genotyping was performed by Transnetyx, Inc (Cordova, TN, USA) on tissue harvested from tail-snip biopsy. All animals were cared for in accordance with guidelines established by the University of Iowa Institutional Animal Care and Use Committee. Whole mounts were accomplished as previously described according to Plante et al., using mammary gland 4. Immunohistochemical analysis was accomplished on FFPE samples from mammary gland 3. Data from whole mount testing was assessed using Student’s 2-tailed T-test.

**Flow Cytometry of Mouse Mammary Glands**

Mammary gland 4 was isolated from 10–12 week old female animals and prepared for flow cytometry as previously described. For pooled gland analysis, we used the EasySep Mammary Stem Cell Enrichment Kit (cat 19757, Stem Cell Technologies, Vancouver, Canada) for the enrichment of mammary epithelial cells by eliminating CD31-, CD45-, and Ter119-positive (lin+) cells before cytometric analysis according to manufacturer’s protocols. For single gland analysis, a negative gating strategy was used to eliminate Lin^{+} cells. Flow cytometry was accomplished using a BDFACSCanto II instrument (BD, Franklin Lakes, NJ, USA). Antibodies for flow cytometry were all from BD Biosciences (San Jose, CA, USA) and were as follows: CD49f-AlexaFluor 647 (clone GoH3, cat. 562494), CD24-FITC (clone M1/69, cat. 561777), TER-119-PECy7 (clone TER-119, cat. 557853), CD45-PECy7 (clone 30-F11, cat. 552848), CD31-PECy7 (clone 390, cat. 561410), CD16/CD32-FcBlocker (clone 2.4G2, cat. 553142). Cells were incubated with a cocktail of all antibodies at appropriate concentrations. TER-119/CD45/CD31-positive cells were gated out of analysis for CD24 and CD49f. Calculations and gating was accomplished in FlowJo (TreeStar, Inc). Data was tested for normality and comparisons were made using 2-tailed Student’s T tests.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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Figure 1. ChIP-SEQ Demonstrates TFAP2C Binds to Luminal Target Genes
A. Examples of ChIP-SEQ data from MCF-7 cells for select luminal target genes $GATA3$, $FBP1$, $FOXA1$, $MYB$, $RET$, $ESR1$, $KRT8$ and $MUC1$ showing that TFAP2C binds to the regulatory regions of the genes. Data also show that TFAP2C binds to the first intron of the $CD44$ gene. The y-axis represents normalized coverage (reads per million mapped). Full ChIP-SEQ dataset available under accession number GSE44257. B. Summary of analysis showing preference of TFAP2C binding to Luminal Differentiation and ERα-associated genes compared to Basal gene expression cluster.
Figure 2. Knockdown of TFAP2C in Luminal Cell Lines
TFAP2C was knocked down in MCF-7 and T47-D cells using siRNA compared to non-targeting (NT) siRNA. **A.** Relative RNA expression for genes shown normalized to expression with NT siRNA (line at 1.0) and demonstrated significant reduction in TFAP2C and luminal target genes and increase in CD44 expression. **B.** Western blot for protein expression shows reduced expression of TFAP2C and luminal genes with increase in CD44 expression. **C.** Relative RNA expression as in panel A performed with T47-D cells. **D.** Western blot for protein expression as in panel C performed with T47-D.
Figure 3. Stable knockdown of TFAP2C induced EMT
MCF-7 cell clone 45 derived by stable expression of shRNA targeting TFAP2C (sKD-C) compared to cell clone derived with non-targeting shRNA (sKD-NT). The clone used for all data in this figure was sKD-C-clone 45. A. Expression pattern of TFAP2A and TFAP2C by RT-PCR in stable cell clones. B. Cell morphology with using the brightfield microscopy 400x magnification. C. Section of expression array comparing sKD-NT vs. sKD-C cells demonstrates down-regulation of Luminal Differentiation Markers and up-regulation of Mesenchymal Markers. D. Western blot showing protein expression of AP-2 proteins and examples of luminal differentiation and mesenchymal markers. E. Relative expression of CD44 gene by RT-PCR for TFAP2C shows increased CD44 RNA in sKD-C cells, confirming array in A and protein in D. F. Distribution of CD44/CD24 subpopulations in stable cell clones of MCF7 by flow cytometry demonstrate an increase in the CD44^{hi}/CD24^{low} population.
Figure 4. Expression profile of sKD-NT and sKD-C stable cell clones

A. Heatmap of selected genes (p <0.01) for luminal associated genes for sKD-C, clone 45 compared to sKD-NT clone. B. Expression of RNA for luminal-marker genes normalized to expression in sKD-NT cells (line at 1.0) for sKD-C, clone 45 and clone 46. C. Western blots showing repression of protein expression of luminal target genes in sKD-C, clone 45 and clone 46 compared to sKD-NT cells. D. Heatmap of selected basal genes (p <0.01) for sKD-C clone 45. E. Expression of RNA for select basal genes normalized to expression in sKD-NT cells (line at 1.0) for sKD-C, clone 45 and clone 46. F. Western blot showing protein expression for selected basal genes for sKD-C, clone 45 and clone 46, compared to sKD-NT cells. Expression of TFAP2C confirmed to be abrogated in sKD-C, clone 46 by western blot (note: expression of TFAP2C in clone 45 presented in figure 3).
Figure 5. Whole Mounts of Mammary Gland-Specific Tcfap2c Knockout Mouse

A. Representative whole mounts of mammary gland 4–6-week old virgin animals. Inset demonstrates qualitative differences between overall gland structures, including more end buds and fewer branch points in KO glands.

B. Quantitation of whole mounts revealed reduced distal migration of TEBs with reduced fat pad invasion in KO (−/−) animals compared to control (wt/wt), n=6 glands per group, statistics from two-tailed Student’s T Test.

C. Quantitation of branch points and end buds, revealed an increase in end bud structures and a decrease in branch points in KO (−/−) compared to control (wt/wt) glands. N=6 glands per group, statistics from two-tailed Student’s T Test.
Figure 6. Immunohistochemical Analysis of Tcfap2c Knockout Mouse

A. Immunohistochemical analysis of mammary gland 3 from virgin control and KO mice at 6 weeks of age. KO (Tcfap2c<sup>−/−</sup>) glands demonstrated loss of TCFAP2C reactivity in luminal cell populations, but not in myoepithelial basal cell populations. CK5 and CK8 staining of the same representative duct in control and KO animals demonstrate CK5 staining of the same representative duct in control and KO animals demonstrate CK5 staining of myoepithelial layer and CK8 staining of luminal cells. Last panel notes CK5 staining in terminal end bud (TEB) structures with increased CK5-positive cells in KO animals. B. Examples of CK5 staining in luminal cells in single cell lined mammary ductal structures in KO animals. C. Quantification of CK5 staining demonstrated a statistical increase in KO animals. D. Quantification of CK5 staining in TEB structures demonstrates statistical increase in KO animals; n= 3 mice per group and data from 10 TEB/mouse; p=0.01, student’s t-test.
Figure 7. Flow Cytometric Analysis of Tcfap2c Null Mammary Glands
A/B. Representative images from individual flow cytometry runs from different litters. A. CD31--, CD45-, and Ter119-negative (Lin−) cells were derived from mammary gland 4 pooled from five control and five KO 10–12 week-old adult animals. B. Example of data from single mammary gland isolated from control and KO animal. For FACS analysis, cells were gated for CD31-, CD45-, and Ter119-negative (Lin−) cells. C–E. Quantitation of flow cytometric analysis based on all experiments including pooled and single mammary gland data. Total numbers of FACS flow data combined were five control (wt/wt) and five KO (−/−) samples (one FACS run from five pooled glands and four FACS runs from individual mammary glands). Cells from KO glands had a statistically significant reduction in
identifiable luminal cells (C), a statistically significant increase in identifiable basal cells (D), and a statistically significant reduction in the luminal/basal ratio (E); statistical analysis using Student’s T-test.