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EMT programs promote basal mammary stem cell and tumor-initiating cell stemness by inducing primary ciliogenesis and Hedgehog signaling

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Tissue regeneration relies on adult stem cells (SCs) that possess the ability to self-renew and produce differentiating progeny. In an analogous manner, the development of certain carcinomas depends on a small subset of tumor cells, called “tumor-initiating cells” (TICs), with SC-like properties. Mammary SCs (MaSCs) reside in the basal compartment of the mammary epithelium, and their neoplastic counterparts, mammary TICs (MaTICs), are thought to serve as the TICs for the claudin-low subtype of breast cancer. MaSCs and MaTICs both use epithelial–mesenchymal transition (EMT) programs to acquire SC properties, but the mechanism(s) connecting EMT programs to stemness remain unclear. Here we show that this depends on primary cilia, which are nonmotile, cell-surface structures that serve as platforms for receiving cues and enable activation of various signaling pathways. We show that MaSC and MaTIC EMT programs induce primary cilia formation and Hedgehog (Hh) signaling, which has previously been implicated in both MaSC and MaTIC function. Moreover, ablation of these primary cilia is sufficient to repress Hh signaling, the stemness of MaSCs, and the tumor-forming potential of MaTICs. Together, our findings establish primary ciliogenesis and consequent Hh signaling as a key mechanism by which MaSC and MaTIC EMT programs promote stemness and thereby support mammary tissue outgrowth and tumors of basal origin.

Significance

Breast cancer is one of the most common cancers and causes of cancer-related death worldwide. Tumor recurrence following therapy is attributed to a subset of tumor-initiating cells (TICs) with stem cell (SC) properties. Similar to normal adult SCs that drive tissue regeneration, TICs regenerate tumors after treatment and thereby enable dissemination throughout the body. Thus, a better appreciation of the mechanisms that induce and maintain SCs in normal and neoplastic tissues is critical for understanding normal tissue regeneration and improving breast cancer therapy. Here we show that key developmental epithelial–mesenchymal transition programs promote stemness of mammary SCs by inducing primary ciliogenesis and Hedgehog signaling. These results provide insights into intraepithelial and intratumoral heterogeneity that have an impact on certain breast cancers.


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Unrelated studies show that epithelial–mesenchymal transition (EMT) programs support the stemness of MaSCs (6, 7, 25–27). The EMT transcription factor (EMT-TF) Slug is expressed within populations of basal cells that are enriched for MaSCs (6, 7, 25–27). Moreover, the self-renewal capacity of these cells in organoid and transplantation/reconstitution assays is enhanced or suppressed by Slug overexpression or knockdown, respectively (7, 27). Indeed, Slug inhibition appears to promote luminal epithelial differentiation (26, 28). Consistent with these roles, Slug-knockout mice show a delay in mammary gland development (25). Despite these advances, it remains unclear how EMT programs enable the acquisition of stemness in cells of the mammary gland. When considering this question, it is important to note that EMT programs do not function as a simple binary switch from epithelial to mesenchymal states but instead generate a spectrum of phenotypic E–M states between these two extremes, only a subset of which is thought to enable stemness (29).

Breast cancers have been divided into various molecular subtypes, which are thought to arise from various cell lineages within the mammary epithelial differentiation hierarchy (1). Claudin-low tumors are thought to arise from the MaSCs of the basal compartment, and they display many of the defining characteristics of these cells. Claudin-low tumors are associated with activation of the EMT program (30), and the tumorigenic capacity of their mammary TICs (MaTICs) relies on EMT-TF programs in orthotopic mouse tumor models (6, 7, 26, 31). Additionally, Hh pathway components are up-regulated in poorly differentiated MaTICs of claudin-low and other breast cancer subtypes, and activation of Hh signaling correlates both with MaTIC expansion (12, 32–36) and with the formation of mammary tumors that express markers of the EMT program (37, 38). Most importantly, the self-renewal capacity of MaTICs is either activated or suppressed by activation or inhibition of Hh signaling, respectively, independent of breast cancer subtype (32–36). Collectively, these findings argue that EMT and Hh programs both play key roles in the formation of MaSC and MaTICs. However, the relationship and epistasis of EMT and Hh programs in either population have been obscure. In this study, we show that primary ciliogenesis plays a critical role in linking these two processes.

The primary cilium is a microtubule-based structure that is transiently assembled on the cell surface by the centrosome during the G0/G1 stages of the cell cycle (39). The function of the primary cilium was widely neglected until the discovery that ciliogenesis is essential for normal development (40), and the primary cilium was widely neglected until the discovery that primary ciliogenesis is essential for normal development (40), including development of the mammary gland (41). During embryogenesis, primary cilia coordinate the activation of various core signaling pathways (42, 43). Hh signaling is one of the known cilia-dependent pathways, in part because the GLI-TFs that function as downstream Hh effectors are processed within the primary cilium to yield either cleaved transcriptional repressors or full-length activators (14, 44). In this study, we establish that EMT programs result in the induction of primary ciliogenesis, which in turn enables Hh signaling and consequent acquisition of stem cell (SC) function.

Results

We initiated this study by hypothesizing that EMT programs act to promote the stemness of the MaSCs and MaTICs of claudin-low breast tumors by inducing primary ciliogenesis, thereby enabling active Hh signaling. This hypothesis was based on the prior findings that primary cilia are observed almost exclusively in the basal compartment of the adult mammary epithelium (41) and that epithelium-specific Shh expression induced the formation of hyperplastic ciliated lesions arising from the basal cell lineage (20). To test our hypothesis, we first asked whether primary cilia were observed with any frequency in MaSC-enriched cell populations. Since the Slug EMT-TF is expressed in MaSC-enriched basal cells, albeit in cells that may reside in different states along the E–M axis, we used a Slug–internal ribosome entry site (IRES)-YFP knockin mouse line in which the locus encoding Slug was engineered to express both Slug and a YFP reporter (7). Mammary gland sections from 8- to 10-wk-old virgin females were stained for YFP (indicative of Slug expression), α-smooth muscle actin (αSMA, a basal cell marker), and two primary cilium markers, Arl13b and acetylated tubulin. Remarkably, primary cilia were detected on 51% of the YFP+ cells in the basal compartment of mature ducts but were largely absent from YFP+ cells (P ≤ 0.001) (Fig. 14 and Fig. S1 A and B). We also examined remaining terminal end buds, in which the cap cells express Slug (25), and observed primary cilia in the YFP+ cells (Fig. SIC). Consistent with published data (41), we also detected primary cilia in the supporting stroma of both mature ducts and terminal end buds and found that a subset of these ciliated cells were YFP+ (Fig. S1 B and C). Since the stroma is known to express both Slug and, at higher levels, the related EMT-TF Snail (27), we stained for their common downstream target, the EMT-TF Zeb1 (Fig. S1B). We found that Zeb1 was consistently expressed in primary cilia-bearing cells of both the basal layer (lower Zeb1) and stroma (higher Zeb1) of the mature duct. We also assessed primary cilia in primary cilia in nontransgenic mice using FACS to isolate stromal–cell–free populations that were enriched for either MaSC-basal (CD24+CD49fhi) or luminal (CD24lowCD49fai) cells, the latter serving as a basal MaSC-deficient control (Fig. 1 B and C). Once again, primary cilia were detected on the majority of MaSC-enriched basal cells but on few of the luminal controls (P ≤ 0.001) (Fig. 1C). Hence, primary cilia were associated with the Slug EMT–TF–expressing, SC-enriched mammary epithelial cell population.

We then asked whether EMT programs actively promote primary ciliogenesis using HMLE cells. These are human mammary epithelial cells harvested from mammoplasty and immortalized by ectopic expression of the hTERT and SV40 Large T proteins (45). HMLE cells are more basal-like, as judged by gene-expression analyses (46). Rare mesenchymal variants exist within the HMLE population (6), but the great majority display epithelial characteristics (6), and thus we refer to HMLE cells as “E-like.” Given the basal-like nature of HMLE cells, we wanted to determine whether primary cilia and Slug expression exist in this population. Thus, we subjected the parental HMLE cells to FACS, employing the same markers used to identify primary MaSC-enriched basal cells, and screened the isolated CD24+CD49fhi population for Slug and Arl13b expression (Fig. S2 A and B). We observed primary cilia on 30.88 ± 5.97% of the Slug+ cells but rarely on the Sluglo cells (P ≤ 0.01). These findings established that primary cilia exist on a subset of HMLE cells and reinforced our conclusion of a correlation between EMT-TF expression and primary ciliogenesis.

In previous work, we generated HMLE variants in which ectopic expression of the Snail, Twist, or Zeb1 EMT-TFs or knockdown of E-cadherin was used to drive EMT and thereby generate more mesenchymal cells (6, 31). These variants display epithelial characteristics (6), and thus we refer to them as “E-like” (Fig. 1D and Fig. S2 C and D). Since primary cilia are assembled specifically during the G0/G1 cell-cycle phases, we serum-starved HMLE lines in either the E-like or M-like state and assessed primary cilia representation by staining for Arl13b. Remarkably, the E-like variants all exhibited dramatic increases in both the number and length of primary cilia relative to their E-like counterparts, indicating that EMT induction promotes primary ciliogenesis (Fig. 1D).
EMT programs induce primary ciliogenesis. (A) Normal mammary gland sections from Slug-IREs-YFP animals (8–10 wk old, n = 3) were stained for the indicated proteins (Inset: 1.4× magnification), and the percentage of ciliated cells was quantified (mean ± SEM). Representative results (from three independent experiments) are shown. (B and C) Luminal and MaSC-enriched basal cells from nontransgenic adult females (8–10 wk old, n = 3) were isolated by FACS using the indicated cell-surface markers (B) and were plated and examined for morphology by brightfield microscopy or for ciliated cells by immunofluorescence for the indicated proteins (mean ± SEM) (C). Representative results from three independent experiments are shown. (D) Morphology and percent ciliated cells were determined as described above for E-like, control (CTL), sh (short hairpin)CTL, and M-like (Snail, Twist, Zeb1, shEcad) HMLE cells. Representative results from three independent experiments are shown. (E) Bilateral orthotopic implantations were conducted with shCTL or shEcad HMLE variants; representative mice are shown. Tumor burden per mouse (mean ± SEM) was determined 8 wk postimplantation with two sites of implantation per mouse and four mice per cell type. (F) Sections from the resulting shEcad HMLE tumors were stained with H&E or for large T antigen and Arl13B to identify the tumor cells and cilia, respectively. Representative images are shown. [Scale bars: 100 μm for brightfield images (except in F, where the scale bar: 15 μm) and 15 μm for immunofluorescence images.]

To establish whether cell-cycle phasing contributed to the increased frequency of primary cilia in the EMT-induced cells, we also assessed their representation in the presence of added mitogenic growth factors. Once again, primary cilia were far more prevalent on M-like cells than on their E-like counterparts (Fig. S2D), even though these cell populations contained G0/G1 subpopulations of comparable sizes (Fig. S2F). Analysis of adult mammary gland sections also revealed no difference in proliferative index that could account for the differential representation of cilia on MaSC-enriched basal cells compared with luminal cells (Fig. S2F). We conclude that EMT programs actively induce primary cilia assembly and that this process could not be ascribed simply to promotion of entry into the G0/G1 phases of the cell cycle.

HMLE cells have been transformed through H-RASG12V expression to generate a tumorigenic population, termed “HMLE,” which becomes more M-like upon E-cadherin knockdown (Fig. S2C) (31, 45). Of note, this switch causes acquisition of tumor-initiating capacity (31), as evidenced by increased ability to form tumors that model claudin-low human breast cancers following orthotopic transplantation into the mammary fat pad (46) (Fig. 1E). Importantly, we found that E-cadherin knockdown in cultured HMLE (creating M-like cells) also greatly promoted primary ciliogenesis (Fig. 1D and Fig. S2G). Moreover, primary cilia were clearly apparent on a subset (30.4 ± 1.6%) of in vivo mammary tumor cells arising from the orthotopic transplant of the M-like (shEcad expressing) HMLE cells (Fig. 1F). Taken together, these data show that EMT programs operating within both MaSC-enriched basal cells and their neoplastic counterparts are associated with induction of primary ciliogenesis.

We proceeded to determine whether EMT-TF induction of primary ciliogenesis resulted in Hh pathway activation. As part of the Hh signaling program, active GLI-TFs promote their own transcription, and thus GLI1 mRNA levels are frequently used as a surrogate marker for Hh pathway activation (12). Accordingly, we isolated by FACS, populations of MaSC-enriched basal and stromal cells (both heterogeneous for EMT-TF expression) and luminal cells (EMT-TF deficient) (Fig. 1B) and conducted real-time qPCR for Gli1 and other markers (Fig. 2A). Gli1 mRNA was detected at higher levels in the MaSC-enriched basal cells (P < 0.01) and at highest levels in stromal cells (P < 0.001), relative to the luminal controls. Notably, the levels of Gli2 correlated well with those of Zeb1, which encodes the key EMT-TF in these three populations (Fig. 2A). Gli2 also showed modestly, but significantly (P < 0.05), higher expression in MaSC-enriched basal cells than in luminal cells (Fig. S3A). Moreover, analysis of two existing gene-expression datasets also showed significantly

Fig. 1. EMT programs induce primary ciliogenesis. (A) Normal mammary gland sections from Slug-IREs-YFP animals (8–10 wk old, n = 3) were stained for the indicated proteins (Inset: 1.4× magnification), and the percentage of ciliated cells was quantified (mean ± SEM). Representative results (from three independent experiments) are shown. (B and C) Luminal and MaSC-enriched basal cells from nontransgenic adult females (8–10 wk old, n = 3) were isolated by FACS using the indicated cell-surface markers (B) and were plated and examined for morphology by brightfield microscopy or for ciliated cells by immunofluorescence for the indicated proteins (mean ± SEM) (C). Representative results from three independent experiments are shown. (D) Morphology and percent ciliated cells were determined as described above for E-like, control (CTL), sh (short hairpin)CTL, and M-like (Snail, Twist, Zeb1, shEcad) HMLE cells. Representative results from three independent experiments are shown. (E) Bilateral orthotopic implantations were conducted with shCTL or shEcad HMLE variants; representative mice are shown. Tumor burden per mouse (mean ± SEM) was determined 8 wk postimplantation with two sites of implantation per mouse and four mice per cell type. (F) Sections from the resulting shEcad HMLE tumors were stained with H&E or for large T antigen and Arl13B to identify the tumor cells and cilia, respectively. Representative images are shown. [Scale bars: 100 μm for brightfield images (except in F, where the scale bar: 15 μm) and 15 μm for immunofluorescence images.]
higher expression of Gli1, Gli2, Snai2 (Slug), and the mesenchymal marker Vim (vimentin) in murine MaSC-enriched basal cells than in luminal or luminal progenitor cells (Fig. S3 B and C).

To determine whether shifts between epithelial vs. mesenchymal states can modulate Hh pathway signaling, we extended our analyses to the paired HMLE and HMLER cell lines. Here we observed significant up-regulation of GLI1 and GLI2 mRNAs and proteins in the M-like (post-EMT induction) variants compared with the E-like (pre-EMT) controls (Fig. 2B and Fig. S3D). Thus, EMT programs can induce Hh pathway activation in both normal and neoplastic mammary epithelial cells. Gli-TFs can be activated via both Smo-dependent and -independent events. To probe for a potential role of Smo, we treated M-like HMLE or HMLER cells with the Smo inhibitor erismodegib (Fig. S3E). The drug had opposing and dose-dependent effects on Gli1 (down-regulated) and Gli2 (up-regulated) mRNA levels. These data show that Smo is able to modulate Gli-TF regulation in M-like HMLE or HMLER cells, but the differential response of Gli1 and Gli2 suggests that both canonical and noncanonical Gli pathways play roles in their activation in M-like cells.

Previous studies in nonmammary tissues had shown that GLI2 and GLI3 accumulate in the primary cilium as part of the process by which Hh signaling mediates Gli-TF activation (14). Accordingly, we used immunofluorescence to determine the localization of GLI2 and GLI3 within both MaSC-enriched basal cells and M-like (shEcad-expressing) populations of HMLER cells. In both cases, these Gli-TFs were detected within the primary cilia (Fig. 2C). Notably, in the M-like HMLER cells, GLI2 and GLI3 were particularly enriched at the cilia tip, a known indicator of potent Hh pathway activation (44). Thus, in the context of both normal and tumorigenic mammary SC populations, EMT programs activate primary ciliogenesis, which then enable engagement of Hh signaling.

We wished to determine whether primary cilia are actually required for induction of Hh signaling. To address this question, we used CRISPR/Cas9 to mutate the genes encoding two essential ciliogenesis regulators, KIF3A and IFT20, in M-like HMLER cells (Fig. S4). This yielded cell populations with partial reduction of either KIF3A or IFT20 protein levels and thus partial loss of primary cilia, due to the cell-to-cell variability in the inactivation of KIF3A and IFT20 (Fig. S4 B and C). We then generated single-cell clones to identify two mutant clones for each gene that completely lacked either KIF3A or IFT20 protein expression due to frameshift mutations (Fig. 3A and Fig. S4D). As anticipated, these clones, but not the sg (small-guide)CTL control cells, lacked primary cilia (Fig. 3 B and C). Notably, the sgKIF3A and sgIFT20 mutant clones all retained their M-like morphologies (Fig. 3B) and displayed no alteration in their proliferative capacity in monolayer (Fig. S4E). Most importantly, we found that all four cilia-deficient clones had significantly lower levels of GLI1 and GLI2 mRNA than did the sgCTL control cells (Fig. 3D). As a parallel approach, we also treated M-like HMLER cells with the ciliogenesis inhibitor ciliobrevin A
(Fig. 3E and Fig. S4F). Ciliobrevin A (20 μM) reduced the frequency of cilia from 45.95 ± 6.37% to 9.62 ± 4.42%, and this was accompanied by a significant down-regulation (P ≤ 0.001) of GLI1 and GLI2 mRNA (Fig. 3 E and F and Fig. S4F). We also confirmed that GLI1 and/or GLI2 protein levels were reduced by ciliogenesis inhibition (Fig. S3D). Collectively, these data show that primary cilia are dispensable for maintenance of the M-like state and proliferative capacity in monolayers, but they enable induction of Hh signaling within SC-enriched populations. Interestingly, residual levels of GLI transcripts were detected in both the knockout and ciliobrevin A-treated cells, suggesting the existence of cilium-independent Hh/GLI-TF signaling, as previously reported (47).

We also wished to learn whether primary cilia and/or induction of Hh signaling play causal roles in the acquisition of stemness. Initially, we addressed this question by assaying the ability of normal basal MaSCs to form organoids using a 3D-Matrigel assay, which has been shown to recapitulate the regenerative capacity of MaSCs faithfully in transplant assays (7). We began by generating organoids from MaSC-enriched basal cells isolated from nontransgenic mice and determining that a subset of the cells displayed primary cilia, which were coincident with Slug expression (Fig. 4A). We then showed that Hh signaling plays a key role in stemness, consistent with prior reports (12, 13). Specifically, we treated isolated MaSC-enriched basal cells with the GLI1/2 inhibitor GANT61 (48) and showed that this significantly reduced their ability to form organoids (P ≤ 0.001) (Fig. 4B). Having validated the contribution of Hh signaling, we also asked whether primary cilia are required for organoid formation. For this, we isolated MaSC-enriched basal cells by FACS and transduced these with lentiviruses expressing the puromycin-resistance gene CAS9 together with CTL, KIF3A, or IFT20 small-guide RNAs to inactivate these genes. Because MaSCs are primary cells, we could not use single-cell cloning to isolate clonal populations of knockout cells. Instead, we cultured the cells briefly in puromycin to enrich for populations of vector-transduced cells, which were heterogeneous in their inactivation of KIF3A or IFT20. We introduced these directly into organoid assays and observed that the sgKIF3A- and sgIFT20-containing populations both yielded approximately half the number of organoids formed by the sgCTL control population (P ≤ 0.001) (Fig. 4C). Importantly, all the organoids arising from the sgKIF3A-containing populations maintained primary cilia, indicating that these arose from nontargeted cells (Fig. S4G). Hence, we concluded that Hh signaling plays a critical role in enabling stemness of basal MaSCs and that primary cilia are essential for this stemness.

Finally, we asked whether primary cilia also support the stemness of MaTICs using the single-cell KIF3A and IFT20 CRISPR/Cas9 mutant clones that we had generated from the M-like HMLER (shEcad) line. We assayed the tumorigenic capacity of these cells compared with that of the sgCTL HMLER cells using mammary fat pad implantation to do so (Fig. 4D). Transplantation of sgCTL HMLER cells successfully yielded tumors (Fig. 4D) with a frequency similar to that of the parental M-like HMLER cells (Fig. 1E). In stark contrast, although there was no alteration in their proliferative capacity in monolayer (Fig. S4E), the primary cilium-deficient HMLER cells exhibited either greatly reduced (sgKIF3A Cl.1) or completely abolished (sgKIF3A Cl.2, sgIFT20 Cl.1, and sgIFT20 Cl.2) ability to generate tumors (Fig. 4D). Therefore, primary cilia play essential roles in the tumor-initiating capacity of MaTICs.

Discussion

Previous studies have revealed that intraepithelial Hh signaling enables expansion of normal and malignant stem/progenitor cells and promotes tumorigenesis (12, 13, 20, 25–27). Similarly, several EMT-TFs are known to promote the stemness of both MaSCs and MaTICs (6, 7, 25–27). Our data emphasize a clear connection between EMT programs and Hh signaling in basal MaSCs and elucidate an epistatic relationship between these processes. Specifically, we show that EMT programs activate primary cilogenesis, which then enables Hh signaling. Most importantly, we demonstrate that ablation of primary ciliogenesis abrogates both the stemness of normal MaSCs in organoid assay and the tumor-forming capacity of MaTICs. Collectively, these data establish an ordered pathway of EMT programs → primary ciliogenesis → Hh signaling → stemness, and reveal this as a key mechanism enabling normal and transformed mammary stem cells to maintain their SC properties.

Our data do not exclude the possibility that primary cilia act to promote additional signaling pathways in MaSCs and/or MaTICs; indeed, we think this is entirely likely. Nonetheless, our results clearly highlight the importance of Hh signaling, which, based on the divergent response to Smo inhibitor, seems likely to result from both canonical and noncanonical Hh pathways. There are already extensive existing data supporting both epithelial and stromal roles for Hh signaling in mammo-

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**Fig. 4.** Primary ciliogenesis promotes stemness of MaSCs and MaTICs. (A) Organoids from MaSC-enriched basal cells were cut and stained for the indicated proteins (Inset: 2x magnification). (Scale bars: brightfield image, 100 μm; and immunofluorescence images, 35 μm). (B and C) Organoid-forming capacity was determined for sorted MaSC-enriched basal cells after treatment with vehicle (CTL) or GLI1/2 inhibitor (GANT-61, 20 μM) (B) or transduction with the indicated single-guide (sg) RNAs (C). Organoid numbers were quantified 7 d after plating and normalized to the controls (n = 3, mean ± SEM). (Scale bars in B and C: 500 μm). Representative results from three independent experiments are shown. (D) Bilateral orthotopic implantations were conducted with the indicated HMLER variants, and tumor burden per mouse was determined as described in the legend of Fig. 1E.
estingly, a recent report also
stages of differentiation (1).

KIF3A and IFT20 guide RNAs were selected from the
γ and γ′

Hh signaling pathway that we have
observed in the luminal compar-
As we and others (41) have shown, ciliated cells are rarely
uncovered in MaSC-enriched populations.

It is important to consider the relevance of our findings to
different breast subtypes. Our data reveal a critical role
for primary cilia in both MaSC-enriched basal cells and the
MaTICs for HMLER M-like cells, which yield tumors that display the
hallmarks of basally derived claudin-low subtype.

We note that other breast cancer subtypes, including basal-
like, HER2+, and luminal A and B, are thought to arise from the
luminal lineage at different stages of differentiation (1).

As we and others (41) have shown, ciliated cells are rarely
observed in the luminal compartment. We hypothesize that
the requirement of primary cilia will differ across various
breast cancer subtypes, reflecting the presence or absence of
cilia in the corresponding cells of origin. Consistent with this
model, a number of studies have assessed the representation of
primary cilia in mammary hyperplastic lesions and tumors (20, 50–54) and have arrived at differing conclusions about their
frequency, ranging from rare (52–54) to elevated (20)
levels. Not all of these studies allow conclusions about cilia
number in relation to subtype, however, when this information is apparent, the data show that cilia are present at high levels in
Shh-dependent hyperplasia arising from the basal layer (20),
the setting that is relevant to our study, but are rare in tumors that are derived from the luminal layer (54).

Notably, at the time of submission of this manuscript, a study by Has-
sounah et al. (55) concluded that ciliogenesis acts to suppress
breast cancer. While Hassounah et al. apply their findings to
breast cancer generally, their data come from the study of
MMTV-PyMT-driven tumors, which are luminaly derived,
and thus do not impact the conclusion that basal-derived tu-
mors are cilia-dependent. In support of this conclusion, a re-
cent study suggested that primary cilia may specifically promote
estrogen receptor alpha (ERα)-negative breast cancer meta-
tasis (56). Notably, there is strong reason to believe that
subtype-specific dependence on primary cilia in breast cancer is
relevant for other tumor types. For example, specific subsets of
skin cancer (basal cell carcinomas) and brain cancer (me-
dulloblastomas, SHH subtype) are believed to employ primary
cilia, while other subtypes have low levels of cilia (57–59).

Additional experiments will be required to establish whether
low cilia representation in these other subtypes equates to
cilia independence.

Even among basal-derived breast tumors, we anticipate some
degree of intratumoral heterogeneity representing the representa-
tion of ciliated cells. First, since basal body maturation and pri-
mary ciliogenesis are tightly regulated during the cell cycle,
instructions. Supernatants containing lentiviruses were collected 48 and 72 h posttransfection. Primary mammary epithelial cells or HMLE cells growing in a monolayer were transduced with the supernatants-containing viruses in the presence of 8 µg/mL Polybrene (Sigma-Aldrich). Transduced primary mammary epithelial cells and HMLE cells were selected with 2 µg/mL puromycin (GIBCO) or 6 µg/mL blasticidin (GIBCO). HMLE clones were selected by FACS 1 mo after infection. Mutations were validated by PCR amplification and Sanger sequencing of the targeted loci. Oligonucleotides used for CRISPR mutations and sequencing are listed in Table S2. Information.

Organoid Assay and Orthotopic Tumor Cell Implantation. Matrigel organoid culture was performed as described previously (7). Briefly, freshly isolated mammary epithelial cells or transduced cells were cultured in complete Epicult-B medium (STEMCELL Technology) containing 5% Matrigel (Corning). Organoids were counted 7–14 d after seeding. For orthotopic cell implantations, tumor cells were resuspended in a 1:1 mixture of complete MEGM medium (Lonza) with Matrigel.

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