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Establishing estrogen-responsive mouse mammary organoids from single Lgr5⁺ cells^{☆,☆☆,★}

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Abstract

Recent evidence suggests that mammary cells expressing R-spondin receptor and Wnt pathway regulator Lgr5, regarded as a stem cell marker in multiple tissues, might represent mammary stem cells (MaSCs). Whether Lgr5 marks a multipotent subpopulation of Lin-CD24^{low/med}CD49^{high} MaSCs remains controversial. To some extent the differing results reflect different assays used to assess properties of stemness, including lineage tracing *in vivo*, mammosphere culture, and mammary fat pad transplantation assays. To address this issue directly, we isolated Lgr5⁺ cells from mammary glands of *Lgr5-lacZ* mice and established organoids based on principles adapted from studies of Wnt-driven Lgr5⁺ cell populations in other organs. Mammary organoids were grown from single Lgr5⁺ mammary cells in Matrigel, the substratum of choice for intestinal organoids, and in a growth factor cocktail containing EGF, Wnt3a and R-spondin, designed to optimally activate the endogenous Wnt signaling program of stem cells. Colonies derived from single Lgr5⁺ cells manifest at least four distinct cell populations: Lgr5⁺ and Lgr5⁻ basal cells and

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Appendix A. Supplementary data

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c-Kit⁺ and c-Kit⁻ luminal cells that spontaneously organize into a ductal structure with basal cells around the periphery and luminal cells lining an interior cavity, reminiscent of normal mammary duct structure. Lgr5⁺ cell-derived organoids were sustainable during prolonged passaging. In contrast, although Lgr5⁻ cells expand into primary colonies, colony-forming efficiency immediately dissipated upon passaging. Furthermore, reproductive hormones induce epithelial cell proliferation resulting in marked increases in lumen diameter accompanied by squamous transdifferentiation. We propose this estrogen-responsive, self-organizing duct-like structure derived from single murine Lgr5⁺ mammary cells represents a “mini-breast” organoid.

Keywords

Lgr5⁺; Stem cells; Mammary gland; Organoid

1. Introduction

The existence of mouse mammary stem cells (MaSCs) was first posited based on the capacity of fragments of mammary gland to regenerate whole glands upon transplantation into cleared fat pads [1]. Subsequent serial transplantation and limiting dilution assays of mammary epithelial cells pointed to the existence of a rare subset of mouse MaSCs [2]. Mouse MaSC-enriched populations are characterized by surface marker expression as Lin⁻CD24⁺CD29^{high} [3] or Lin⁻CD24^{low/med}CD49^{high} [4]. The leucine-rich-repeat-containing G-protein-coupled receptor 5 (Lgr5) is regarded as a stem cell marker in multiple mammalian tissues including small intestine, colon, stomach, skin, and hair follicle [5–8]. Lgr5 is a receptor for R-spondin ligands, which act as agonists of Wnt-mediated signaling *via* the β -catenin/TCF pathway [7,8]. Recent evidence suggests that Lgr5 might also represent a mammary stem cell marker [9–11]. However, whether Lgr5 marks a multipotent subpopulation of Lin⁻CD24^{low/med}CD49^{high} MaSCs remains controversial. *In situ* lineage-tracing studies and mammary gland reconstitution assays have shown that Lgr5 progeny are restricted to the luminal lineage prior to day 12 after birth but thereafter switch, becoming committed to the basal compartment [9]. Visvader and co-workers, however, found that Lgr5⁺ cells exhibit bipotential capability [10]. Furthermore, comparison of the repopulating capabilities of Lgr5⁺ and Lgr5⁻ cells in fat pad transplantation assays has yielded disparate results. Werb and colleagues showed that single Lgr5⁺, but not Lgr5⁻, cells generate whole mammary glands with 24% efficiency [11], while another group reported that both Lgr5⁺ and Lgr5⁻ cells display regenerative capability upon transplantation assay [10]. Further complicating the understanding of whether Lgr5⁺ cells represent legitimate mammary stem cells, Wang et al. have reported that Lgr5⁻ but not Lgr5⁺ cells form colonies in 3D culture [12]. Thus a coherent picture of the role of Lgr5⁺ mammary cells as mammary stem cells cannot be drawn based on the current literature.

Here we address the role of Lgr5⁺ cells as potential mammary stem cells *ex vivo* based on principles established in other organ systems to evaluate the Wnt/R-spondin pathway. From single Lgr5⁺ mammary cells we were able to grow colonies that contain all major ductal cell types, display the property of self-renewal, and self-organize into duct-like structures with basal cells at the periphery, luminal cells in the interior, and a hollowed out central space.

Furthermore, reproductive hormones induce colony proliferation, marked increases in lumen diameter, and squamous transdifferentiation. We propose this estrogen-responsive, self-organizing duct derived from a single murine *Lgr5*⁺ mammary cell represents a “mini-breast” organoid.

2. Materials and methods

2.1. Mice

Lgr5-lacZ (*Lgr5*^{tm1Ah}) knock-in mice (a kind gift from Hans Clevers, University Medical Center Utrecht, Netherlands) in which *lacZ* reporter gene expression is driven by endogenous *Lgr5* regulatory sequences [5] and *Lgr5-EGFP-IRES-creERT2* knock-in mice (Jackson Laboratories) in which an *EGFP-IRES-creERT2* cassette is integrated at the first ATG codon [5] were bred and maintained in the MSKCC animal facility. Heterozygous female *Lgr5-lacZ* mice were crossed with C57BL/6 (Jackson Laboratories) males and offspring were genotyped as described [5]. All animal studies were approved by the MSKCC Institutional Animal Care and Use Committee.

2.2. Beta-galactosidase staining

Inguinal mammary glands were resected from female mice [11], fixed in 4% paraformaldehyde (PFA) at 4°C for 30 min, washed 3 times in rinse buffer (2 mM MgCl₂, 0.01% Na deoxycholate, 0.02% NP-40 in PBS) and incubated overnight at 37°C in 40 mg/ml 4-chloro-5-bromo-3-indoyl-β-D-galactopyranoside (X-gal), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆·H₂O, 2 mM MgCl₂ in PBS [13]. Mammary glands were washed in rinse buffer, dehydrated using graded alcohols and rinsed with xylene, and then tissue was mounted with xylene-based mounting medium. Tissue sections (5 μm) were counterstained with Fast Red.

2.3. Primary cell preparation

#3 and #4 mouse mammary glands from 8 to 12 week old virgin female mice were finely minced and then incubated in culture medium [RPMI 1640 with 25 mM HEPES, 5% fetal bovine serum (FBS), 1% penicillin-streptomycin, 300 U/ml collagenase IV (Sigma C7657)] for 3 h at 37 °C. After erythrocyte cell lysis using Red Blood Cell-Lysing Buffer (Sigma R7757), a single cell suspension was obtained by incubation with 0.25% trypsin-EDTA at 37 °C for 5 min, followed by 0.1 mg/ml DNase I (Sigma D5025) for 5 min with gentle pipetting, and filtration through a 40 μm cell strainer [14]. The EASY Sep Kit (StemCell Technologies Cat. 19758) was used to enrich mammary epithelial cells by removal of non-epithelial cell types using antibodies to CD45, CD31, TER119 and BP-1 according to manufacturer's instructions.

2.4. Fluorescence-activated cell sorting (FACS)-based detection of *Lgr5-lacZ*⁺ cells

FACS-gal analysis was performed using the fluorogenic β-galactosidase substrate fluorescein di-β-D-galactopyranoside (FDG) [15]. The FluoReporter® *lacZ* Flow Cytometry Kit (Invitrogen F-1930) was used according to manufacturer's instructions. Briefly, single cell suspensions were prepared from mammary glands by mincing and collagenase digestion. Cells were thereafter suspended in PBS containing 2% FBS at a final concentration of 10⁷ cells/ml. 100 μl cell suspension was loaded with 100 μl of pre-warmed

(37°C) 2 mM FDG working solution by mixing rapidly, and incubated at 37°C for 1 min. The reaction was terminated by adding 1.8 ml ice-cold PBS containing 2% FBS. Cells were maintained on ice for 20 min prior to flow cytometry assay.

2.5. Cell labeling and flow cytometry assays

The following antibodies were used for cell labeling: TER119-PE-Cy7, CD24-PE and CD49f-APC (eBioscience). Antibody incubations were performed at 4°C for 30 min in PBS containing 4% FBS, and stained cells were washed twice with PBS and stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies). FACS was performed with a FACSaria flow cytometer (Becton Dickinson). Purity of sorted populations was routinely >95%.

2.6. Preparation of Wnt3a and Rspodin1 conditioned medium

For preparation of Wnt3a conditioned medium [16], L-Wnt3a cells (ATCC CRL-2647) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS for 4 days, and conditioned medium (CM) [16] was collected and sterilely filtered. Wnt activity in CM was measured using the Wnt-responsive mammary epithelial reporter cell line (C57MG) [17] infected with WntRgreen transcriptional reporter virus (C57MG/WGR) [18]. In these cells, the GFP reporter is activated by Wnt3a-stimulated β-catenin/TCF signaling and the percentage of activated cells relates linearly to Wnt activity.

For preparation of R-spondin1 CM, 293T-HA-Rspo1-Fc cells (a kind gift from Dr. Calvin J Guo, Stanford University School of Medicine, CA) were grown to confluence, then incubated in Advanced DMEM/F12 medium (Gibco Life Technology) for 1 week prior to harvesting CM. R-spondin1 CM activity was tested using HEK 293T cells stably transfected with a SUPER TOPFLASH Wnt reporter (a kind gift of Dr. James Chen, Stanford University School of Medicine, CA) as per [19].

2.7. In vitro colony formation assay

FACS-sorted cells were resuspended at a density of 3.3×10^4 cells/ml in chilled 100% growth factor-reduced Matrigel (BD Bioscience). The gel was allowed to polymerize and then covered with medium containing 100 ng/ml EGF, 50% Wnt3a CM and 20% R-spondin1 CM (EWR) [20]. 1 µM Jagged-1 (AnaSpec Inc. 61298) was added to the medium for the first 48 h. 1 µM estradiol (E2) and 2.5 µM progesterone (Pg) were added to EWR medium as indicated. Organoid culture medium was changed every 48 h. Organoid numbers were counted after 10–13 days of cultivation. To passage organoids, medium was aspirated and Matrigel digested by incubating in 500 µl of Matrigel Recovery Solution (BD Bioscience) for 1 h at 4°C. Organoids released from Matrigel were collected and single cells were obtained by digestion with 0.25% trypsin-EDTA at 37°C for 5 min, followed by gentle pipetting. Single cells were then replated in Matrigel as above. The ability of Wnt3a CM and R-spondin1 CM to stimulate organoid formation was calibrated *versus* commercial recombinant Wnt3a (R&D 1324-WN/CF) and R-spondin1 (R&D 4645-RS/CF) proteins.

2.8. Histology, immunofluorescence and immunohistochemistry

Organoids were fixed in 4% PFA for 30 min at room temperature, washed with PBS, transferred to 70% ethanol, then processed for paraffin embedding. 5 μ m sections on Superfrost Plus Slides were baked at 60°C for 1 h prior to staining. Mammary glands were fixed in 4% PFA for 18–24 h prior to paraffin embedding. Tissue sections from #4 mammary glands were deparaffinized, rehydrated through graded ethanols, and stained with hematoxylin and eosin (H&E). For immunohistochemistry and immunofluorescence, sections were stained with the following antibodies using standard procedures. Primary antibodies were: rat polyclonal anti-Keratin 8 (ab15160, Abcam, 1:400 dilution), rabbit polyclonal anti-Keratin 14 (Covance; PRB-155P, 1:500 dilution), goat polyclonal anti-c-Kit (Troma 1, Developmental Studies Hybridoma Bank, Iowa; 1:100 dilution), rabbit polyclonal anti-Keratin 10 (Covance; PRB-159P, 1:1000 dilution). Secondary antibodies were peroxidase-conjugated Alexa-488-conjugated or Alexa-594-conjugated. Envision System-HRP (DAB) For Use With Mouse Primary Antibodies Kit was used for immunohistochemistry. Staining for β -catenin (BD Transduction Labs, cat#610153, 1:100 dilution) was performed as described [21] and hematoxylin counter staining was performed to visualize nuclei. Images were captured using Zeiss Axio wide-field microscopy or a Leica TCS SP5 confocal microscope.

2.9. In situ hybridization

The QuantiGene ViewRNA *in situ* Hybridization Tissue 2-plex Assay Kit (QVT0012) was utilized according to manufacturer's instructions using *Lgr5* (mouse VB1-10818) and *GAPDH* (mouse VB1-10150) probes from Affymetrix. *In situ* hybridization for *GAPDH* was used to confirm mRNA integrity of all samples. Nuclei were counterstained with DAPI.

2.10. Statistical analysis

Values are shown as mean $\pm$ SD or SEM as indicated. Student's *t* test, or one-way analysis of variance (ANOVA) was used to effect comparisons. A *P* value $\leq$ 0.05 was considered significant.

3. Results

3.1. Characterization of *Lgr5*⁺ cells from mammary glands of *Lgr5-lacZ* mice

In initial studies we examined the abundance of *Lgr5*-expressing cells in mouse mammary epithelium. While a previous study analyzed *Lgr5* cell content using *Lgr5-EGFP-IRES-creERT2* reporter mice [5], we employed the *Lgr5-lacZ* knock-in strain, considered a more accurate reporter of the prevalence of *Lgr5* expression in other tissues [22,23]. Individual #3 and #4 mammary glands were surgically removed from *Lgr5-lacZ* mice, and processed to generate single cell suspensions enriched in mammary epithelial cells. This epithelial cell-enriched population was incubated with the fluorogenic beta-galactosidase substrate, di- β -D-galactopyranoside (FDG), and fluorescent mouse mammary cells were identified by flow cytometry, as described [24]. Only a trace number of FDG-positive cells were detected in the mammary gland of wild type (WT) C57BL/6 mice (0.5% in Fig. 1A), representing non-specific background. In contrast, N10% of mammary epithelial cells of *Lgr5-lacZ* mice were

FDG-positive (Fig. 1B shows a representative study). Collated data from 3 replicate experiments indicate that $Lgr5-lacZ^+$ cells represent $12 \pm 1\%$ (mean $\pm$ SEM) of the total mammary epithelial cell-enriched population (Fig. 1H). This number is significantly higher than the 0.26% $Lgr5^+$ cell abundance reported in *Lgr5-EGFP* reporter mice [11]. We therefore performed a similar flow cytometry analysis of the *Lgr5-EGFP* strain. In our experiments, the proportion of $Lgr5-EGFP^+$ cells in mammary epithelium was $1.0 \pm 0.4\%$ (Fig. 1C), consistent with the published result. Published data indicate that GFP expression in the intestines of *Lgr5-EGFP-IRES-creERT2* mice is “variegated” [22,23]. We confirmed these data in the intestines of *Lgr5-EGFP-IRES-creERT2* mice in our study (Supplementary Fig. S1B), and further showed GFP expression in mammary glands is variegated (Supplementary Fig. S1D). In contrast, X-gal staining showed consistent high level $Lgr5$ expression throughout both organs (Supplementary Fig. S1A, S1C). Moreover, in situ hybridization for $Lgr5$ mRNA in *Lgr5-EGFP-IRES-creERT2* and *Lgr5-lacZ* mice confirm that 10–12% of mammary ductal cells in both strains are $Lgr5^+$ (Supplementary Fig. S2A, S2B). These data indicate that the *Lgr5-lacZ* knock-in strain represents the more accurate reporter of mammary $Lgr5$ expression, as published for small intestine [22,23].

Subsequent studies examined the relationship between $Lgr5$ expression and surface markers previously associated with MaSCs. As reported [4], Lin^- mammary epithelial cells, which represent 98.5% of the mammary epithelial cell-enriched population (Fig. 1D), are divisible into three sub-populations based on differential expression of CD24 and CD49f. These populations are recognized as enriched for basal cells ($CD24^{low/med} CD49f^{high}$), luminal cells ($CD24^+ CD49f^{low/med}$) and stromal cells ($CD24^- CD49f^-$) (Fig. 1E). Upon examining compartmentalization of the $Lgr5^+$ population, $98 \pm 2\%$ of murine mammary $Lgr5^+$ cells were found to be $CD24^{low/med} CD49f^{high}$ basal cells (Fig. 1F). Among $CD24^{low/med} CD49f^{high}$ basal cells, reported as enriched for MaSCs [4], ~40% were $Lgr5^+$ in the representative experiment shown in Fig. 1G. In 3 replicate experiments the mean ($\pm$ SEM) value was $35 \pm 6\%$ (Fig. 1H). Of note, Protein C receptor ($Procr$) $^+$ and $Lgr5^+$ basal sub-populations show no significant overlap (Supplementary Fig. S3A–S3F), consistent with data obtained using the *Lgr5-EGFP-IRES-creERT2* model [12]. Our finding that the vast majority of $Lgr5^+$ cells are contained within the $CD24^{low/med} CD49f^{high}$ basal sub-population is consistent with previous data obtained using the *Lgr5-EGFP-IRES-creERT2* model [11].

3.2. $Lgr5^+$ cells preferentially form colonies in vitro

To investigate the in vitro expansion capacity of $Lgr5^+$ cells, equal numbers of $Lgr5^+$ and $Lgr5^-$ mammary epithelial cells were cultured in Matrigel with medium containing EGF, Wnt3a R-spondin1 (EWR) [20]. These culture conditions were designed to establish Wnt-dependent organoids from $Lgr5^+$ cell populations obtained from other mammalian organs [20]. Both $Lgr5^+$ and $Lgr5^-$ mammary cells formed primary colonies *in vitro* in EWR medium (Fig. 2A, B), but $Lgr5^+$ cells did so with more than 2-fold greater efficiency. The colony-forming capacity of $Lgr5^+$ and $Lgr5^-$ populations were $13 \pm 1\%$ and $6 \pm 2\%$, respectively ($P < 0.01$; Fig. 2C). Moreover, the size and shape of colonies derived from $Lgr5^+$ and $Lgr5^-$ cells differed. The average size of colonies derived from $Lgr5^+$ cells was 1.9-fold that of $Lgr5^-$ cells at day 12 ($P < 0.05$; Fig. 2D). In addition, $Lgr5^+$ cells formed

more branched colonies than Lgr5⁺ cells ($P < 0.05$; Fig. 2E), suggesting that Lgr5⁺ cells may have enhanced ability to differentiate into cells other than basal cells [20]. Note that while R-spondin did not impact Lgr5⁺ cell colony-forming efficiency, it facilitated formation of branched colonies (Supplementary Fig. S4A–S4C).

To examine the contribution of Lgr5⁺ mammary cells to the colony-forming capabilities of the MaSC-enriched basal cell population, Lin[−]CD24^{low/med}CD49f^{high} cells were fractionated into Lgr5⁺ and Lgr5[−] cells. Lgr5⁺ cells were more efficient in forming primary colonies than Lgr5[−] cells (Fig. 2F). Critically, although Lgr5[−] cells could also expand into primary colonies, their colony-forming efficiency rapidly decreased by an order of magnitude with successive passaging (from 5% to 0.5% over 6 passages). In contrast, the colony-forming efficiency of Lgr5⁺ cells was maintained at ~10% over serial passaging (Fig. 2G). Importantly, the percentage of Lgr5⁺ cells within colonies derived from single Lgr5⁺ cells was $14 \pm 2\%$, similar to the $12 \pm 1\%$ found in normal mammary ducts. In contrast, Lgr5[−] cell-derived organoids contained few Lgr5⁺ cells (<4%) in early passages, and 1–3% at later passages, consistent with published data that indicate Lgr5[−] derived clones can re-express Lgr5 after culturing, albeit in small numbers [25]. When Lgr5[−] cells are sub-fractionated into Lgr5[−] luminal cells and Lgr5[−] basal cells, Lgr5[−] luminal cells do not generate sustainable colonies while Lgr5[−] basal cells do so at low frequency (Supplementary Fig. S5), consistent with the Lgr5[−] basal population containing other mammary stem cells, such as Procr⁺ cells. These findings indicate that Lgr5⁺ cells display robust self-renewal during serial colony culture, a property of legitimate stem cells.

3.3. Single Lgr5⁺ cell-derived colonies represent mammary organoids

Histochemical analysis of sectioned colonies derived from single Lgr5⁺ cells revealed two distinct 3-dimensional structures. The majority of Lgr5⁺ cell-derived colonies, ~80%, were solid (Fig. 3A), whereas ~20% displayed duct-like lumens (Fig. 3B), highly reminiscent of normal *in vivo* mammary duct structure (Fig. 3C). Most LacZ-stained Lgr5⁺ cells line the outer cell layers of both solid and luminal colonies (Fig. 3D, E), comparable to normal ducts *in vivo* (Fig. 3F).

In situ hybridization assays for *Lgr5* were also performed to corroborate Lgr5⁺ cell localization within colonies, since validated antibodies that detect mouse Lgr5 in histologic sections are not currently available. These studies confirmed that Lgr5 preferentially localizes to the outer cell layers of both solid and hollow colonies (Fig. 3G, H), and the outer basal layer of normal mammary ducts (Fig. 3I). In fact, Lgr5⁺ cells represent $38 \pm 3\%$ of the total cells in the outer cell layer (Fig. 3G, H), a value indistinguishable from the $35 \pm 6\%$ incidence of Lgr5⁺ cells in the basal compartment of murine mammary ducts detected by FDG/LacZ staining and flow cytometry (Fig. 1H).

Consistent with these observations, solid colonies derived from a single Lgr5⁺ cell display the basal cell marker keratin 14 (K14) in the cell population encircling the periphery, whereas the luminal cell marker keratin 8 (K8) is present in cells in the colony interior (Fig. 3J). In a small portion of the hollow colonies, a bi-layer structure was observed, again with a K14⁺ outer cell layer and a K8⁺ inner cell layer (Fig. 3K). This bi-layer structure is similar

to that of normal mammary ducts in which basal cells (K14⁺) reside in the outer layer, while luminal cells (K8⁺) form the inner layer (Fig. 3L).

We also characterized the expression pattern of c-Kit, a marker of mammary luminal progenitor cells [26]. In solid colonies, the majority of c-Kit⁺ cells localize to the interior layers (Fig. 3M). In colonies with lumens, c-Kit⁺ cells were again present in the inner cell layer (Fig. 3N), consistent with the expression pattern in normal mammary ducts (Fig. 3O). *In situ* hybridization for *Lgr5* and immunostaining using basal (K14) and luminal (K8) markers on passaged organoids showed that passaged organoids maintain their phenotype and structure (Supplementary Fig. S6). Additionally, consistent with *Lgr5* as a Wnt-target gene [5], 31 ± 6% of the cells in the basal layer display active nuclear β-catenin, while >90% of the luminal layer cells display transcriptionally inactive cytoplasmic/membrane-bound β-catenin (Fig. 3P–R). In summary, colonies derived from single *Lgr5* cells display multiple differentiated mammary epithelial populations that spontaneously self organize into structures with topology and abundance similar to intact mammary ducts. Furthermore, the phenotype of the hollow colonies corresponds closely to that of ducts in intact mammary glands, suggesting these colonies with a lumen represent “mini-duct” organoids. The studies further show that *Lgr5*⁺ cells display self-renewing and differentiation capabilities of mammary stem cells *in vitro*.

3.4. A ventral-dorsal *Lgr5* gradient during post-natal development and pregnancy

As MaSCs are known to be responsive to steroid hormone stimulation [27], we examined the impact of puberty and pregnancy on the population of *Lgr5*⁺ stem cells in *Lgr5-lacZ* mammary glands. For these studies, *Lgr5* expression was initially assessed in whole-mount X-gal-stained mammary glands from 8-week old *Lgr5-lacZ* mice (Fig. 4A, B). We observed *Lgr5* predominantly expressed in mammary ducts located in the ventral and central (nipple-proximal) zones of young adult mammary glands. In the ventral zone, 29 ± 6% (mean ± SEM) of epithelial cells were *Lgr5*⁺, while 15 ± 3% of cells were positive in the central zone. In contrast, only 2 ± 1% of cells were positive in the dorsal zone (Fig. 4C). *Lgr5*⁺ cells were largely absent from the distal ends of ducts, as reported [11]. Consistent with higher efficiency of *Lgr5*⁺ cells to form primary organoids than *Lgr5*[−] cells, epithelial cultures derived from the ventral zone could more efficiently generate organoids in Matrigel than those from the other zones (Fig. 4D).

In subsequent studies, we examined the pattern of *Lgr5* expression in post-natal development of mammary glands during puberty, adulthood and pregnancy. In control WT C57BL/6 mice, no LacZ-positive mammary epithelial cells were detected in mammary glands of 16 week old adult virgin mice, nor in those of other ages (Fig. 5A and data not shown), demonstrating low background staining. In glands from 5 week old *Lgr5-lacZ* pubertal mice, as expected, the ductal trees were still undergoing expansion and terminal end buds (TEBs) were located in the central zone [28]. At this stage, *Lgr5* expression was largely restricted to the ventral zone and no *Lgr5*⁺ cells were observed in the TEBs (Fig. 5B). In mammary glands from adult virgin mice of various ages, in which ducts extend towards the edge of the dorsal zone (Fig. 5C, D), *Lgr5* expression extends from the ventral zone to the central zone, but not into the dorsal zone. These results indicate a ventral-to-dorsal graded

pattern of *Lgr5* expression during post-natal mammary development with *Lgr5* expression expanding from the ventral zone through the central zone, but essentially absent in the adjacent dorsal zone.

During pregnancy, mammary epithelium undergoes additional lobuloalveolar differentiation in preparation for lactation. In mammary glands from pregnant WT mice, only slight non-specific staining was detected in the fat pad (Fig. 5E). In contrast, mammary glands of P8.5 *Lgr5-lacZ* mice, displayed a blue signal stronger than in virgin mice (Fig. 5F). Higher magnification and representative sections from each zone counterstained with fast red (lower panels of Fig. 5F) again revealed a ventral-to-dorsal gradient of *Lgr5*⁺ cell abundance. Moreover, examination of paraffin sections counterstained with fast red showed that *Lgr5* is not only expressed in $23 \pm 9\%$ and $20 \pm 7\%$ of the epithelial cells of ventral and central zones, respectively, but also weakly in $4 \pm 2\%$ of those in the dorsal zone, while no *Lgr5* was seen in alveoli. A similar pattern and abundance of *Lgr5*⁺ cells was maintained in P12 and P14 mammary glands (Fig. 5G, H).

3.5. *Lgr5*⁺-derived organoids are responsive to reproductive hormones

To test whether comparable changes to intact pubertal/pregnant murine mammary glands are reproducible in culture, we incubated organoids derived from single *Lgr5*⁺ cells in EWR medium containing estradiol (E2) and progesterone (Pg). Hormone treatment induced a two-fold increase in organoid size (Fig. 6A–D, quantified in 6E). Phenotypic changes were also observed.

Although we noted a limited number of organoids with lumens in the untreated group ($23 \pm 4\%$; Table 1), incidence of such luminal organoids increased significantly upon hormone treatment ($41 \pm 3\%$). Furthermore, hormone-treated luminal organoids displayed significantly larger luminal diameter (Fig. 6A–D, quantified in F). Responsiveness of organoids to steroid hormones is consistent with ductal expansion and alveologenesis mediated by these hormones during post-natal mammary development [29].

In histologic sections of hormone-treated organoids with lumens, epithelial cells demonstrated orderly differentiation into squamous epithelial cells centrally, which often contained keratohyaline granules within their cytoplasm. Additionally, there was prominent keratin pearl formation within the lumina of these organoids, consistent with squamous differentiation, a phenomenon noted in a variety of breast pathologies [30–34]. Keratin 10 (*K10*), normally highly enriched in the suprabasal epidermis, was found in the interior of hormone-treated solid organoids (Fig. 6H), as well as in the center of luminal organoids with squamous differentiation (Fig. 6I) and in islands of cells floating within luminal secretions, but was rare in untreated controls (Fig. 6G). Consistent with this phenotype representing a form of basal cell (trans)differentiation, the *K14*⁺ cell population, normally confined to the outer basal cell layer of organoids (Fig. 3G, H and Fig. 6J), extended into the inner cell layers after hormone treatment (Fig. 6J, K), co-localizing with *K10*⁺ cells (see floating cellular islands within secretions). That this population represents a differentiated non-stem cell basal population is evidenced by the fact that *K10/K14* expansion into the inner organoid layers was not accompanied by increased numbers of centrally located cells displaying nuclear β -catenin (data not shown).

4. Discussion

The current investigation adapts principles and techniques derived from the study of the intestinal Lgr5⁺ stem cell for the purpose of addressing stem cell properties of the mammary Lgr5⁺ cell, a topic of controversy. Mammary organoids were grown from isolated Lgr5⁺ mammary cells in Matrigel, the substratum of choice for growth of large and small intestinal organoids, and in a growth factor cocktail containing EGF, Wnt3a and R-spondin, designed to optimally activate the endogenous Wnt program in stem cells displaced from their *in vivo* niche. The result of this experimental design is the development of the first mammary organoid culture originating from single Lgr5⁺ mammary cells.

Multiple lines of evidence indicate that the mammary Lgr5⁺ cell is a stem cell capable of forming colonies that represent functioning organoids. Single Lgr5⁺ cells display the properties of self-renewal and multipotency. In this regard, organoids derived from single cells manifest at least four distinct cell populations: Lgr5⁺ and Lgr5⁻ basal cells and c-Kit⁺ and c-Kit⁻ luminal cells. Furthermore, a portion of the organoids spontaneously organize into a ductal structure in which basal cells are at the exterior and luminal cells line an apparent lumen, closely mimicking the structure of a normal mammary duct. Evidence that these organoids represent functional duct-like structures derives from their responsiveness to estrogen and progesterone treatment.

Only two prior studies have examined the role of mammary Lgr5⁺ cells in colony formation. After fractionating the CD24⁺ CD29^{high} MaSC-enriched population into Lgr5⁺ and Lgr5⁻ cells, Zeng and co-workers reported that Lgr5⁺ cells did not manifest colony-forming capability, whereas Lgr5⁻ Protein C receptor⁺ cells were able to do so [12]. In contradistinction, depletion of the Lgr5 population from primary cultures of mammary epithelial cells derived from Lgr5-DTR:GFP mice by treatment with diphtheria toxin [11] resulted in complete loss of mammosphere-forming potential. In contrast to the current investigation, neither of these studies reported data derived from organoids grown in the presence of R-spondin.

The existence of mouse MaSCs was first demonstrated by the capacity of fragments from mammary gland to regenerate whole mammary glands upon transplantation into cleared fat pads [1]. Subsequent studies showed that epithelium from all segmental branches of the adult mouse mammary tree can successfully regenerate whole glands [35], suggesting that MaSCs are widely distributed [36]. The active proliferation capability of cap cells in the TEBs suggested that MaSCs might reside in the tip of ducts during pubertal development [37]. However, we did not observe Lgr5⁺ cells in the TEBs. Furthermore, recent multiscale *in situ* analysis shows that large ducts in the mouse mammary ventral compartment contain a significantly larger reservoir of putative stem cells than the dorsal compartment [38]. These data are consistent with our observations that as nascent ducts invade the mammary fat pad during pubertal development the relative abundance of Lgr5⁺ cells is manifestly higher in ventral ducts than in dorsal, and that there is a corresponding gradient in the ability of cells from the ventral to dorsal compartments to generate sustainable organoids.

Development of the mammary gland is under control of hormones and cell-fate determining signaling pathways. The estrogen-EGF-FGF and GH-IGF-1 signaling cascades regulate pubertal ductal morphogenesis [39], while progesterone-Wnt and progesterone-RANKL signaling cascades promote ductal side branching and alveolar morphogenesis in mature mammary glands [16,40,41]. These mechanisms might explain how *Lgr5*⁺ cells grow into ductal organoids in the presence of EGF and Wnt3a *ex vivo*. Furthermore, evidence indicates that estrogen and progesterone enhance effects of both EGF and Wnt signaling pathways [42]. Consistent with this interaction, larger organoids with wider lumens occur upon steroid hormone treatment, suggesting that our organoids might represent a model of hormonally-regulated ductal development.

Squamous metaplasia of mammary epithelium is a well-documented phenomenon in a variety of benign breast pathologies [30–34], and in transgenic mouse models in which high levels of Wnt/ β -catenin signaling are generated in mammary epithelium [43,44]. It is notable that human breast cancers that display squamous differentiation exhibit a poor prognosis [45]. Until recently the potential relevance of this phenomenon to normal physiology was unexplored. Brugge and colleagues, however, while examining the impact of inactivation of the pro-apoptotic BCL-2 family member Bim on mammary morphogenesis noted that central clearing of developing ductal structures was significantly delayed [46]. This delay was dependent on the activation of a compensatory duct-clearing system involving squamous differentiation of basal cells, considered by her group as an alternate, caspase-independent form of cell death. Further investigation by this group using 3D structures originating from MCF-10A and HMECtert cell lines noted that detachment of mammary epithelium from the matrix predisposed to squamous cell differentiation. Our data in *Lgr5*⁺ cell-derived mammary organoids are consistent with this notion as squamous transdifferentiation appears to occur in the expanding core of our organoids upon steroid hormone-induced proliferation, a location that physically separates cells from the matrix.

Further, detection of K10⁺/K14⁺ islands of cells within the luminal keratin pearl secretions is consistent with the notion of squamous transdifferentiation representing an alternate form of cell death regulating lumen formation. It should be noted that we do not see evidence of apoptotic death of epithelial cells in our organoid cultures as measured by staining for activated caspase 3 (data not shown).

While it is not possible to determine from the current data whether the source of the reproductive hormone-dependent expansion of K14⁺ basal cells into the organoid interior, and the squamous metaplasia, is due to the *Lgr5*⁺ or *Lgr5*[−] basal cells on the organoid periphery, nonetheless the steroid hormone dependence of these events suggest organoids display a functioning stem cell niche. Mammary basal cells do not contain reproductive steroid hormone receptors. Rather, impact of reproductive hormones is considered, as nicely detailed by Zeng and co-workers [42], to be indirect relating to the estrogen/progesterone-dependent presentation of Wnt to basal stem cells by luminal cells. In this regard, supplementation of medium with Wnt and R-spondin would likely bypass requirement for this interaction, although it remains possible that such microenvironmental interaction remains intact. Alternatively, other hormone-dependent luminal-basal cell interactions may be driving the impact of reproductive hormones on organoid restructuring. Irrespective of

mechanism, Sorof and co-workers showed fresh human mammary tissue in organ culture is more likely to undergo squamous metaplasia if derived from patients during the last 10 days of their menstrual cycle when estrogen/progesterone levels are high. This group concluded that reproductive hormones predispose to mammary epithelial transdifferentiation, and provided evidence that this event was mediated by cyclic adenosine 3'5'-monophosphate [47]. Future studies will be directed at deciphering these interactions at the cellular levels in the context of signal transduction.

We propose that the estrogen/progesterone-responsive, self-organizing mammary duct-like organoid derived from single murine Lgr5⁺ mammary cells as described here represents a “mini-breast” in cell culture. Our results thus support mammary Lgr5⁺ cells as legitimate mammary stem cells. We predict that this system will find use in the community of investigators interested in mammary development and stem cell biology.

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Fig. 1.

Characterization of Lgr5⁺ cells from mouse mammary glands. (A,B) Isolation of mouse mammary Lgr5⁺ cells. Mouse mammary glands #3 and #4 from 8 to 12 week old WT (A) or *Lgr5-lacZ* (B) virgin female mice were surgically resected, finely minced, collagenase treated, trypsin digested, and enriched for epithelial cells by negative selection. After staining with FDG, epithelial cells were analyzed for Lgr5 content by flow cytometry. Shown is one representative of 5 independent studies. (C) Percentage of Lgr5⁺ cells in *Lgr5-EGFP-IRES-creERT2* mice. (D, E) To further characterize Lgr5⁺ cells, the Lin⁻ population (TER119⁻), which represents 98.5% of the enriched epithelial cells (D), was fractionated by expression of the cell surface markers CD24 and CD49f by flow cytometry into three sub-populations (E): CD24^{low/med}CD49f^{high} basal cells, CD24⁺CD49f^{low/med} luminal cells and CD24⁻CD49f⁻ stromal cells. The majority of Lgr5⁺ (98.5%) cells were Lin⁻CD24^{low/med}CD49f^{high} basal cells (F) and constituted approximately 40% of all basal cells (G). (H) Percentage of Lgr5⁺ cells in the enriched epithelial population (as in B) and in the basal population (as in G). Data (mean ± SEM) are collated from 3 independent studies.

Fig. 2.

Lgr5⁺ cells preferentially initiate organoid growth *in vitro*. Lgr5⁺ (A) and Lgr5⁻ (B) populations were isolated as in Fig. 1B and equal numbers of cells were cultured in Matrigel with medium containing EGF, Wnt3a, and R-spondin1 (EWR) for 12 days. Organoid-forming efficiency (C) of the two populations, mean size of organoids (D), and extent of branching (E) were compared. Data (mean ± SD) are from 3 independent studies. In (F), organoid-forming efficiency was measured using Lgr5⁺ cells isolated from the basal cell population as in Fig. 1F. Data (mean ± SEM) are from 2 independent studies performed in quadruplicate. (G) Serial passage of organoids derived from Lgr5⁺ and Lgr5⁻ cells. At the end of each 2-week passage, organoids were disrupted with trypsin, single cell suspensions were stained with FDG as in Fig. 1B, and Lgr5⁺ and Lgr5⁻ populations were replated. Scale bar: 200 μm. Data (mean ± SEM) are from 2 independent studies, each with 4–6 replicates.

Fig. 3.

Differentiation of organoids derived from single *Lgr5*⁺ cells. *Lgr5*⁺ cell-derived organoids cultured in Matrigel with EWR medium were heterogeneous: most of them, ~80%, were solid (A,D,G,J,M,P), while ~20% had a lumen (B,E,H,K,N,Q), as shown in H&E-stained sections (A, B). Sections of normal mammary ducts are shown for comparison (C,F,I,L,O). X-gal-stained *Lgr5-lacZ*⁺ cells line the outer cell layers of both solid and luminal colonies (D,E) and in normal ducts (F). *In situ* hybridization for *Lgr5* RNA reveals basally distributed *Lgr5*⁺ cells in organoids (G, H) and in normal mammary ducts (I). Immunostaining for K14

and K8 shows the location of basal and luminal cells, respectively, in a typical solid organoid (J), organoid with lumen (K) and mouse mammary duct (L). Immunostaining for c-Kit shows the distribution of luminal progenitor cells in solid organoids (M), organoids with lumen (N) and normal mammary ducts (O). Nuclear and cytoplasmic β -catenin distribution in Lgr5⁺ cell-derived organoids is shown in (P, Q). Purple and brown double positive cells (arrow) indicate cells with nuclear β -catenin. Percentage of cells showing nuclear β -catenin in the outer and inner organoid cell layers is quantified in (R). Data (mean $\pm$ SD) are from 2 independent studies, examining 4 organoids per experiment. Scale bars: 20 μ m.

Fig. 4.

Ventral-dorsal gradient of $Lgr5^{+}$ cell abundance in mammary glands correlates with organoid-forming ability of different zones. (A) $Lgr5^{+}$ cell distribution in young adult mice. Whole-mount X-gal staining of mammary glands from 8 week old *Lgr5-lacZ* mice was performed as in [13]. Representative sections subsequently stained with fast red are shown. Subdivision of ventral (v), central (c) and dorsal (d) zone $Lgr5^{+}$ epithelial cell fractions is shown schematically in (B), and quantification of $LacZ^{+}$ cell content is shown in (C). Data (mean $\pm$ SEM) are from 3 glands. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (D) Organoid-forming efficiency of epithelial cells from the different zones of the young adult mammary gland. The epithelial-enriched population isolated from each zone was plated in Matrigel containing EWR and quantified after 12–14 days (D). Data (mean $\pm$ SEM) are derived from 1 representative of 3 independent studies using 4–6 replicates per zone.

Fig. 5.

Preferential expression of *Lgr5* in ventral ducts of the mammary gland during post-natal development, adulthood, and pregnancy. Whole-mount X-gal staining was performed as in Fig. 4 on mammary glands from 16 week old virgin WT mice (A), and *Lgr5-lacZ* transgenic mice of 5 weeks (B), 8 weeks (C) and 32 weeks (D) of age. The lower panels of figures A–D show 100× magnification of the ventral, central, and dorsal zones. Representative sections from each zone counterstained with fast red are shown in the lowest panel of figures A–D. X-gal staining was also performed on mammary glands from pregnant WT mice at pregnancy day 12 (E), and *Lgr5-lacZ* transgenic mice on pregnancy day 8.5 (F), day 12 (G) and day 14 (H). Lower panels show 100× magnification and representative sections counterstained with fast red.

Fig. 6. Hormone treatment alters organoid structure and induces squamous differentiation. Bright field images of organoids cultured in EWR medium (A) or EWR plus E2 and Pg (B). H&E-stained sections of organoids cultured in EWR (C) or in EWR plus hormones (D). Arrows in (C, D) identify organoid lumens. Organoid size (E) and lumen size (F) of treatment groups are shown. Data (mean $\pm$ SEM) are from 2 independent studies. (G–L) Immunofluorescent

staining for K10 in organoids treated with EWR only (G, J) or EWR + E2 + Pg (H, I), and immunostaining for K14 and K8 (J, K, L). Scale bars: 20 μm . * $P < 0.05$, ** $P < 0.01$.

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Table 1

Percentage of luminal organoids increases after hormone treatment. Lgr5⁺ cells were cultured with EWR medium or EWR medium plus E2 and Pg. At 12–14 days of culture, organoids were fixed in 4% PFA, embedded in paraffin, and 5 µm slides were stained with H&E. Luminal and solid organoids were quantified.

	Untreated	Hormone treated
Total organoid number	81	103
Luminal organoid number	19	42
% luminal organoids	23.4%	40.8% *

* $P = 0.017$ vs. untreated (Fisher's exact test).