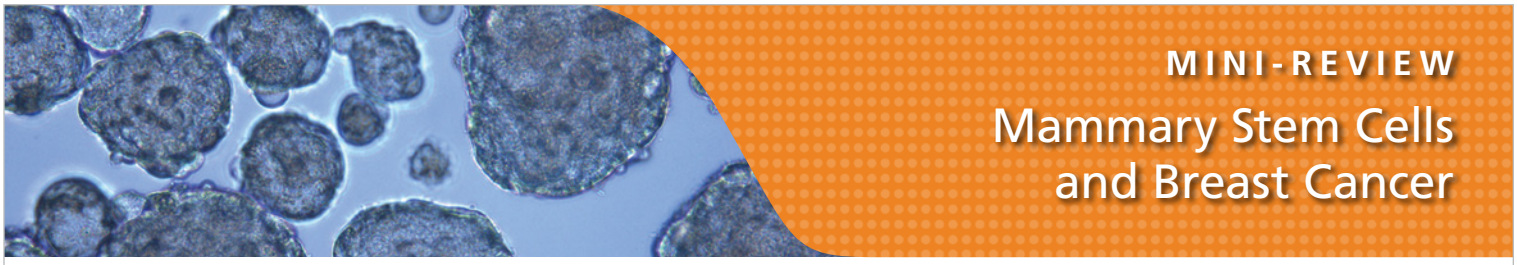

MINI-REVIEW

Mammary Stem Cells and Breast Cancer

John Stingl, PhD | Associate Director

Organization of the Mammary Epithelium

The mammary gland is composed of a series of branched epithelial ducts that drain alveolar structures during lactation, with the epithelium embedded within a fibrous and fatty stroma. The gland is a dynamic organ that undergoes extensive morphological changes through development, puberty, pregnancy, lactation and involution. The mammary epithelium in the resting (non-pregnant) gland is made up of two general lineages of epithelial cells: luminal cells that surround a central lumen, and basally-positioned myoepithelial cells that contract during lactation and forces milk to be ejected from the gland. The luminal cell compartment can be further subdivided into two sub-lineages: estrogen receptor (ER)-expressing cells and ER$^-$ alveolar precursor cells.[1–4] These latter cells proliferate during pregnancy under the influence of progesterone and prolactin to generate daughter cells that will synthesize and secrete milk during lactation.[3,5] Maintenance of the mammary epithelium during all of these stages is via mammary stem/progenitor cells, although how these cells are organized as a hierarchy is still not fully understood.

Identification of Mammary Stem and Progenitor Cells

Over 50 years ago, Kenneth DeOme and colleagues described a model system in which transplantation of normal mammary epithelial tissue segments from a donor mouse into the epithelium-free (cleared) mammary fat pad of a juvenile recipient mouse led to the regeneration of the entire organ.[6] Subsequent studies showed that transplantation of any segment of the mammary epithelium, including a single cell, into the cleared fat pad could generate the ductal and lobular components of the mammary epithelium.[7–9] Later studies then demonstrated that single flow-sorted cells could regenerate the mammary epithelium[10,11], and that these mammary repopulating units (MRUs) were largely restricted to the basal cell compartment.[1,10,11] Analogous studies in which purified luminal and basal human mammary epithelial cells were transplanted into immune-deficient mice reveals that the MRUs in the human breast also have a basal phenotype.[12,13] Thus, based on this early work, it was perceived that mammary stem cells have a basal phenotype and multilineage potential, and that luminal cells are a more differentiated cell compartment derived from the basal layer. However, a landmark paper published in 2011 from Cedric Blanpain's laboratory challenged this theory. The Blanpain lab used an inducible lineage-tracing strategy to show that in the intact homeostatic mammary gland, the luminal and basal cell compartments are maintained by their own lineage-restricted stem cells.[14] The authors argued that transplantation of purified cells as single cell suspensions is not a valid physiological assay since it involves disruption of stem cell niches, and that assessing cells in isolation in the absence of their niche may not permit cells to exhibit their stem cell behavior. The conclusion that the basal and luminal cell compartments are maintained by their own stem cells is supported by another manuscript which used inducible lineage-tracing to track the fate of smooth muscle actin (SMA)-expressing myoepithelial cells. In this latter manuscript the myoepithelial cells, which have historically been considered to be terminally differentiated, were observed to have all detectable MRU activity and function as basal-restricted stem cells.[15]

The presence of these basal-restricted stem cells in the postnatal gland has proven to be controversial since research from other groups, which also used a lineage-tracing approach, detected the presence of multilineage basal stem cells.[16–18] These multipotent basal stem cells have an epithelial to mesenchymal (EMT) phenotype and express the Wnt target gene protein C receptor (Procr).[17] A number of theories have been proposed to account for the discrepancy between the different reports; these include differences in labelling efficiency, how the samples are prepared for microscopic analysis, how the clones are scored (e.g., in two vs. three dimensions) and the lineage-specificity of the trace.[19,20] However, many of these discrepancies can be resolved if one keeps a sense of scale regarding the proliferation rates of these different types of stem cells. The mouse mammary epithelium undergoes an approximate doubling in cell number (and subsequent apoptosis) during the 4-5 day-long estrus cycle.[21,22] However, the cellular output of Procr$^+$ basal stem cells appears to be relatively low since clones derived 6 weeks after initiation of a trace are composed of 10 cells or less.[17] This indicates that Procr$^+$ cells have little contribution to day-to-day cell turnover during normal tissue homeostasis; instead, their contribution is only significant over much longer time scales.

A key question in mammary stem cell biology concerns the identity of the luminal stem cells. As previously mentioned, the luminal cell compartment can be broadly divided into two lineages: estrogen receptor$^+$ (ER$^+$) cell lineage and the ER$^-$ milk lineage. A number of recent manuscripts have used either mathematical modelling of cell proliferation in the mammary epithelium or inducible lineage tracing to demonstrate that the ER$^+$ lineage and the milk lineage are maintained by their own lineage-restricted stem cells[3,5,22–24],

although the identity of the stem cell that maintains each lineage, if it exists as a discreet subtype of cell, is not known.

Phenotypic Profiles of Different Subsets of Mammary Epithelial Cells

The ability to identify distinct subsets of mammary cells can permit their functional and phenotypic characterization. In the mouse, multipotent stem cells have a basal (EpCAM^{low}CD49f^{high}) phenotype and express the Wnt target gene Procr.[2,17] It has been recently reported that basal cells that co-express Lgr5 and tetraspanin 8 are also highly enriched in MRUs, have an embryonic stem cell features, and may lie at the apex of the mammary epithelial cell hierarchy, although it is not clear if these cells are the same as Procr^{+} cells.[25] The identity of basal-restricted stem cells is not known, other than they have a basal/myoepithelial phenotype.[15] Among the luminal cells, ER^{+} cells have an EpCAM^{high}CD14^{-}CD29^{low}CD49b^{-}CD49f^{low} CD61^{-}Sca1^{high}Prominin 1^{high} phenotype, whereas the ER^{-} milk lineage cells have an EpCAM^{high}CD14^{+}CD29^{low}CD49b^{+}CD49f^{low} CD61^{+}Sca1^{low}Prominin 1^{low} ALDEFLUOR^{bright} phenotype.[1,2,4] It should be noted that the markers CD14, CD49b, CD61, Prominin 1 and ALDEFLUOR™ are largely redundant with each other, although subtle differences are observed between different mouse strains.[2] The milk lineage cells are also characterized by expressing high levels of Elf5, a transcription factor that specifies alveolar cell fate[26], and slightly lower levels of proteins associated with luminal cell differentiation.[2] These Elf5^{+} alveolar precursors generate milk producing alveolar cells during lactation, with each alveolus composed of the progeny from multiple precursor cells.[16]

In the human, basal cells have an EpCAM^{low}CD49f^{high} phenotype (Figure 1), milk lineage cells have an EpCAM^{high}CD49f^{high}c-KIT^{+}ALDEFLUOR^{+} phenotype, and ER^{+} lineage cells have an EpCAM^{high}CD49f^{-} phenotype.[2,12,27,28] The milk lineage in the human mammary epithelium expresses much higher basal cell features (e.g., keratins 5/14) than corresponding cells in the mouse.[2]

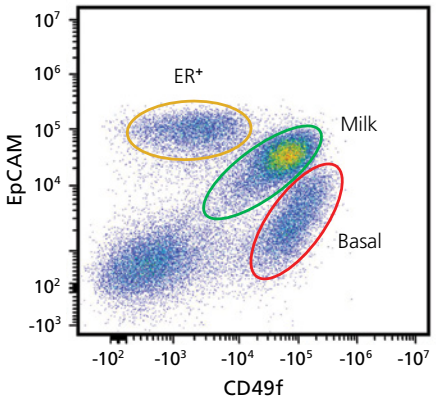

Figure 1. A flow cytometry dot plot showing the distribution of EpCAM and CD49f among freshly dissociated normal human breast cells. The different epithelial cell lineages are indicated.

Cell Culture Models for Mammary Epithelial Cells

When human mammary epithelial cells are seeded into culture, both the basal cells and the milk-lineage cells proliferate; these latter cells are commonly referred to as "luminal progenitors" because of their high clonogenicity in vitro.[2,27] Cell populations enriched for ER^{+} cells do not grow when cultured in commonly used mammary cell media, and not surprisingly, ER^{+} tumour cells do not proliferate under these conditions either. In fact, human breast tumours are notoriously difficult to grow in vitro, and the ones that do grow are typically ER^{-} and are only derived from the most aggressive tumours.[29] It has recently been described that ER^{+} mammary epithelial cells have distinct growth factor requirements from milk lineage and basal cells, although it is currently not known if these reformulated culture conditions promote the growth of primary ER^{+} breast tumours in adherent culture.[30]

Growing mammary cells in serum-free media in ultra-low attachment dishes to detect mammosphere-forming cells is commonly used as a surrogate assay to detect mammary stem cells, with the basis of this assay being that only stem/progenitor-like cells have the ability to survive anoikis in suspension culture (Figure 2).[31] Although this assay does not detect all stem cell activity since stem cells of the ER^{+} lineage do not proliferate in this culture system, it is a useful tool for detecting stem-like cells with basal features, including luminal progenitor (milk lineage) cells and/or basal cells.[32] However, the assay does have some scientific merit since it has been shown in a large study involving over 300 patient-derived breast cancer samples that mammosphere-forming efficiency is positively correlated to tumour progression, with the metastatic tumours, particularly those that are ER^{-}, having significantly higher mammosphere-forming efficiencies than early-stage tumours.[33]

Although cell growth in suspension culture may be somewhat physiologically relevant, one limitation of the mammosphere assay is that the resultant spheres often do not recapitulate the morphology of the parental tissue from which the cells were derived.[31,32,34] To circumvent this, there is a great interest in developing organoid culture systems for growing mammary epithelial cells in a more physiologically relevant manner. Organoid culture, which relies on culturing primary epithelial cells within a reconstitute basement membrane matrix such as Matrigel™, has proven to be immensely successful in promoting the growth, maintenance and proper differentiation of a variety of epithelial stem cells, including intestinal, prostate, pancreatic, hepatic, and lung stem cells.[35,36] Several recent manuscripts have now reported the growth of both human and mouse mammary cells in organoid culture.[37–39] The challenge going forward with mammary organoid cultures is standardizing these protocols to increase reproducibility between labs, being able to propagate the stem cells long-term while maintaining a normal karyotype, and maintaining tumours such that they recapitulate the in vivo counterparts from which they were derived.

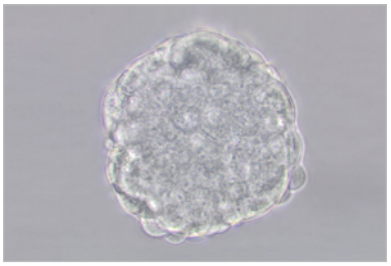

Figure 2. An MCF-7 mammosphere grown in MammoCult™

The Cellular Context of Breast Cancer

At least five distinct molecular subtypes of breast cancer have been identified through gene expression profiling studies; these are the luminal A, luminal B, HER2, basal-like and claudin^{low} subgroups of tumours.[40] The luminal B signature is essentially a luminal A signature, except with higher levels of cell proliferation.[41] When different subsets of normal mammary epithelial cells are purified and their gene expression patterns compared to those obtained from human breast tumours, the ER$^+$ cells have gene expression signatures that most closely resemble luminal A and B tumours, the milk lineage resemble the basal-like breast cancers, whereas the Lgr5$^+$Tetraspanin 8$^+$ primitive stem cells resemble the claudin^{low} tumours.[2,12,25] The HER2 subset has no clear association with the normal epithelial cell types, although it is known that over-expression of HER2 in mammary epithelial cells in vitro does expand the ALDH$^+$ milk lineage subpopulation.[42] A simplistic way to interpret the cellular origins of breast tumours is to assume that tumour phenotype is merely a reflection of the cell of origin of the tumour. The ER lineage is a likely candidate as a cell of origin for ER$^+$ breast cancers since these cells are the most proliferative cells in the normal mammary epithelium and they contain a stem cell population; however, formal demonstration that these cells can function as a cell of origin for this type of tumour has yet to be shown. In some cases, tumour phenotype may just reflect the phenotype of the cell of origin, however this is likely not to be true for all tumours since oncogene expression can alter lineage cell fate in mammary epithelial cells.[43,44]

The observation that basal-like breast cancers have gene signatures that most resemble those obtained from the milk lineage rather than the basal cells is counter-intuitive. Keratins 5/14 were initially characterized in the mouse mammary gland where these proteins are almost exclusively restricted to the basal layer.[45] Subsequent studies immunostained human breast tumours to detect the expression of these "basal-associated" keratins and demonstrated that approximately 20% of tumours stained positive. Thus, this subset of tumours was termed "basal-like" breast cancers.[46] However, the inference that these tumours resemble the basal cells of the normal human breast is incorrect since keratins 5/14 localize to both the basal cells and the milk lineage cells, the latter which are a subset of luminal cells of the human breast. Additionally, these tumours, like all breast tumours, also express luminal-associated keratins. It is important to note that basal-like breast cancers rarely express the true basal/myoepithelial cell markers p63 and α-smooth muscle actin, and thus don't resemble myoepithelial cells.[47]

Do Breast Cancer Stem Cells Exist?

In 2003 a manuscript was published that identified a subpopulation of human breast tumour cells that were more tumourigenic when transplanted into immune-deficient recipient mice than all the other cell subpopulations.[48] These putative cancer stem cells (CSCs) had a CD44$^+$CD24$^-$ phenotype, which is a phenotype that corresponds to basal cells in the normal human breast.[2] In 2007 it was reported that high expression of ADLH1 is a marker of both normal and malignant breast stem cells.[49] Unexpectedly, it was observed that the ALDH1$^+$ phenotype, as detected using the ALDEFLUOR™ Kit, had minimal overlap with the CD44$^+$CD24$^-$ CSC phenotype reported previously. In hindsight this makes sense because ALDH1 identifies the milk lineage, whereas the CD44$^+$CD24$^-$ phenotype identifies the basal cells.[2,28] Thus, it appears that in breast cancer CSC-like cells can have more than one phenotype. In recent years, the existence of CSCs has increasingly been brought into question.[50–52] There are multiple reasons for this, but a main recurring problem in the identification of CSCs typically relies on purifying different subsets of cells, and then transplanting them at limiting dilutions into recipient mice, or growing them as tumourspheres. However, we know now that in the normal mammary gland, such assays do not detect all stem cell subpopulations, and it would be unrealistic to expect these same assays to faithfully detect all tumour stem cell subpopulations. Considering that multiple stem cell populations exist in the normal mammary gland, it is not unreasonable to expect that multiple stem cell populations could exist within individual breast tumours. A more reliable approach for studying the hierarchical organization of breast tumours would be to use a lineage-tracing approach in mouse models of breast cancer or in patient-derived xenografts, a strategy which has recently been used for studying intestinal tumours.[53,54]

Implications of the Different Mammary Epithelial Cell States on Treatment

As described above, mammary epithelial cells in the normal gland exist in three semi-stable cell states: ER$^+$, milk (alveolar precursor) and basal (myoepithelial). The ER$^+$ cells express the highest levels of luminal cell differentiation, the milk lineage has features of both luminal and basal cells (albeit both at low levels), whereas the basal cells are entirely devoid of any luminal features, but have some mesenchymal properties. In the normal homeostatic mammary gland, these lineages are very stable and there is very little

interconversion of cells from one lineage to another. However, in the case of cancer where there is dysregulated gene expression and cell niches are disrupted, these cells become very phenotypically plastic where any subpopulation of cells can recapitulate the entire parental population.[43,44,55,56] An EMT transition in the context of the normal mammary gland is a transition from a more luminal cell state to a more basal cell state, whereas a MET transition is vice-versa. Such cellular plasticity poses a problem for therapy because there is increasing evidence of a strong selective pressure (e.g., drug therapy) that targets one specific lineage of cells, eventually just selects for other cell types in a Darwinian fashion. For example, in a recent study using patient-derived breast tumour xenografts, treatment with the anti-estrogen drugs tamoxifen or fulvestrant, which target the ER-expressing cells, eventually selects for the $ER^{-}ALDH^{+}$ milk lineage cells.[57] Considering that milk lineage cells are dependent on Notch signaling[58], it is no surprise that treatment of tumours with both tamoxifen or fulvestrant and a Notch inhibitor had synergistic effects in inhibiting tumour growth.[57] The selection of milk lineage cells during anti-estrogen treatment is also supported by in vitro studies[59] and by studies that identify ELF5, which specifies alveolar cell fate, as a key regulator of endocrine resistance.[60]

Tumour cellular heterogeneity makes treating cancer very difficult. However, by understanding the types of cells that are present within the normal mammary epithelium and the molecular mechanisms that regulate their behavior, we can now develop strategies to efficiently target these cells, One can envision that therapies in the near future will rely on a combination of drugs to target multiple cell populations independently, or will be used to drive cells into a single stable targetable cell state for eradication.

References

1. Sleeman KE et al. (2007) J Cell Biol 176(1): 19–26.
2. Shehata M et al. (2012) Breast Cancer Res 14(5): R134.
3. Chang TH et al. (2014) Breast Cancer Res 16(1): R1.
4. Asselin-Labat ML et al. (2007) Nat Cell Biol 9(2): 201–209.
5. Tao L et al. (2015) Stem Cell Reports 5(1): 60–74.
6. Deome KB et al. (1959) Cancer Res 19(5): 515–520.
7. Hoshino K & Gardner WU. (1967) Nature 213(5072): 193–194.
8. Daniel CW et al. (1968) Proc Natl Acad Sci U S A 61(1): 53–60.
9. Kordon EC & Smith GH. (1998) Development 125(10): 1921–1930.
10. Shackleton M et al. (2006) Nature 439(7072): 84–88.
11. Stingl J et al. (2006) Nature 439(7079): 993–997.
12. Lim E et al. (2009) Nat Med 15(8): 907–913.
13. Eirew P et al. (2008) Nat Med 14(12): 1384–1389.
14. Van Keymeulen A et al. (2011) Nature 479(7372): 189–193.
15. Prater MD et al. (2014) Nat Cell Biol 16(10): 1-7-950.
16. Rios AC et al. (2014) Nature 506(7488): 322–327.
17. Wang D et al. (2015) Nature 517(7532): 81–84.
18. van Amerongen R et al. (2012) Cell Stem Cell 11(3): 387–400.
19. Rios AC et al. (2016) Breast Cancer Res 18(1): 116.
20. Wuidart A et al. (2016) Genes Dev 30(11): 1261–1277.
21. Fata JE et al. (2001) Biol Reprod 65(3): 680–688.
22. Giraddi RR et al. (2015) Nat Commun 6: 8487.
23. Rodilla V et al. (2015) PLoS Biol 13(2): e1002069.
24. Wang C et al. (2017) Cell Rep 18(12): 2825–2835.
25. Fu NY et al. (2017) Nat Cell Biol 19(3): 164–176.
26. Oakes SR et al. (2008) Genes Dev 22(5): 581–586.
27. Stingl J et al. (2001) Breast Cancer Res Treat 67(2): 93–109.
28. Eirew P et al. (2012) Stem Cells 30(2): 344–348.
29. Ethier SP. (1996) J Mammary Gland Biol Neoplasia 1(1): 111–121.
30. Fridriksdottir AJ et al. (2015) Nat Commun 6: 8786.
31. Dontu G et al. (2003) Genes Dev 17(10): 1253–1270.
32. Smart CE et al. (2013) PLoS One 8(6): e64388.
33. Eyre R et al. (2016) J Mammary Gland Biol Neoplasia 21(3–4): 99–109.
34. Pastrana E et al. (2011) Cell Stem Cell 8(5): 486–498.
35. Drost J & Clevers H. (2017) Development 144(6): 968–975.
36. Barkauskas CE et al. (2017) Development 144(6): 986–997.
37. Sachs N et al. (2017) Cell.
38. Jamieson PR et al. (2017) Development 144(6): 1065–1071.
39. Zhang L et al. (2017) Cell Signal 29: 41–51.
40. Prat A & Perou CM. (2011) Mol Oncol 5(1): 5–23.
41. Cheang MC et al. (2009) J Natl Cancer Inst 101(10): 736–750.
42. Korkaya H et al. (2008) Oncogene 27(47): 6120–6130.
43. Koren S et al. (2015) Nature 525(7567): 114–118.
44. Van Keymeulen A et al. (2015) Nature 525(7567): 119–123.
45. Smith GH et al. (1990) Cell Growth Differ 1(4): 161–170.
46. Gusterson BA et al. (2005) Breast Cancer Res 7(4): 143–148.
47. Livasy CA et al. (2006) Mod Pathol 19(2): 264–271.
48. Al-Hajj M et al. (2003) Proc Natl Acad Sci U S A 100(7): 3983–3988.
49. Ginestier C et al. (2007) Cell Stem Cell 1(5): 555–567.
50. Quintana E et al. (2008) Nature 456(7222): 593–598.
51. Shackleton M et al. (2009) Cell 138(5): 822–829.
52. Kern SE & Shibata D. (2007) Cancer Res 67(19): 8985–8988.
53. Shimokawa M et al. (2017) Nature 545(7653): 187–192.
54. de Sousa e Melo F et al. (2017) Nature 543(7647): 676–680.
55. Chaffer CL et al. (2011) Proc Natl Acad Sci U S A 108(19): 7950–7955.
56. Gupta PB et al. (2011) Cell 146(4): 633–644.
57. Simoes BM et al. (2015) Cell Rep 12(12): 1968–1977.
58. Bouras T et al. (2008) Cell Stem Cell 3(4): 429–441.
59. Haughian JM et al. (2012) Proc Natl Acad Sci U S A 109(8): 2742–2747.
60. Kalyuga M et al. (2012) PLoS Biol 10(12): e1001461.