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Breast Cancer: Multiple Subtypes within a Tumor?

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Abstract

Breast cancer is a heterogeneous disease and stratification of tumors is paramount to achieve better clinical outcomes. While it is common to stratify and treat breast tumors as a single entity, insights from studies on intra-tumoral heterogeneity and cancer stem cells raise the possibility that multiple breast cancer subtypes may co-exist within a tumor. A role for plasticity in driving dynamic conversions between breast cancer subtypes is proposed and the clinical implications would be a need for combinatorial therapeutic strategies that account for the discrete disease entities and their plasticity. Accordingly, the advent of single-cell technologies will be crucial in enabling the diagnosis and stratification of distinct disease subtypes down to the cellular level.

Keywords

Intra-tumoral heterogeneity; Plasticity; Breast Cancer; Cancer Stem Cells

Breast cancer stratification and its role in guiding therapeutic decisions

Breast cancer is a complex disease that displays a large degree of inter- and intra- tumoral heterogeneity [1–4]. For that reason, a tailored approach is necessary to elicit the best responses in patients when administering treatment modalities. Histological stratification of breast cancers based primarily on the expression of estrogen receptor (ER), progesterone receptor (PR) and ERBB2 receptor (HER2) has been useful and laid the foundation for classification of breast cancers. In fact, breast cancer is a prototypic tumor in which the initial molecular sub-classification has led to improved outcomes, by guiding the administration of targeted therapeutics such as hormonal therapy (e.g. Tamoxifen) and HER2-targeted therapy (e.g. Trastuzumab). While histological stratification is still a common practice, technological advances have unraveled further complexities with the emergence of at least five distinct molecular subtypes (i.e. Luminal A, Luminal B, Her2-enriched, Basal-like and Normal-like) based on gene expression clustering [1]. Building on this, the combined genomic/transcriptomic analyses of breast cancers have resulted in identification of ten distinct breast cancer subtypes based on integrated clusters [2]. In line

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with these advancements, efforts to further segregate some of the established histological subtypes have also been carried out for ER-negative and triple-negative breast cancers [5, 6]. These studies have identified at least four distinct subtypes of ER-negative breast cancer and six triple-negative breast cancer subtypes respectively. The progress made in these areas highlights the intricate complexity of breast cancer subtypes and it is likely that more defined and precise characterizations based on new parameters (e.g. metabolites) will be uncovered in the future. Notably however, a gap exists between research methods and current clinical practice. Although intrinsic subtyping of breast cancer has been employed in the research setting for more than a decade [1], PAM50 genomic tests (e.g. Prosigna) have not been utilized specifically for breast cancer subtyping in the clinic. On the other hand, it would also be crucial to be able to identify and continuously develop targeted therapeutics to match and improve the outcomes of each emerging subtype. Progress in the ability to identify breast cancer subtypes in precise detail, along with the availability of therapeutics directed against each breast cancer subtype's Achilles heel could then enable more personalized approaches that are in line with precision oncology. Here we review current evidence that supports the idea of multiple breast cancer subtypes co-existing within a tumor, with an emphasis on cell state plasticity as a driving force behind the emergence of distinct disease entities. Consequently, we discuss the clinical implications of co-existing breast cancer subtypes and a prospective role for single-cell technologies in enabling diagnosis of breast cancer at the cellular level.

Cancer stem cells: A source of non-genetic heterogeneity

The concept of cancer stem cells (CSCs) has been around for many decades [7] and is still evolving [8]. A key tenet that has remained consistent is the functional heterogeneity in terms of tumor initiating capacity that can be observed between tumor cells [9–11]. In breast cancers, although tumor cells are organized in a hierarchical manner where CSCs exhibit features of stem/progenitor cells [11], there is plasticity which allows the acquisition of CSC traits by bulk tumor cells [12]. Accordingly, it is conceivable that dynamic conversions as a result of differentiation and plasticity can give rise to tumors with cells that correspond to distinct differentiation states of the normal mammary gland hierarchy [13, 14]. Due to the plasticity of tumor cells, therapeutic strategies will need to consider all the cell types within the hierarchical structure of the tumor and not just CSCs alone, since re-emerging CSC populations can arise from bulk tumor cells [15, 16].

It is also worth noting that the hierarchical structures in breast tumors need not necessarily be confined to a binary organization consisting of CSCs and bulk tumor cells. A continuum of differentiation states that are reflective of the normal mammary hierarchy (Figure 1) is plausible and would result in hierarchical heterogeneity within breast tumors [11, 14]. With regards to this, multiple independent studies have observed the presence of minimally overlapping distinct CSC populations within tumors based on the expression of established CSC markers such as $CD44^{hi}/CD24^{-}$, $ALDH^{+}$, $CD133^{+}$ or $CD29^{hi}/CD61^{+}$ [13, 17–19]. The heterogeneity of the observed CSC populations could be a representation of the diversity in normal mammary stem/progenitor cells that are spatially and temporally regulated [20–31]. This is in line with the view that there can be “mesenchymal-oriented breast CSCs” and “epithelial-like breast CSCs” which are more similar to adult mammary stem cells

(aMaSCs) and fetal MaSCs (fMaSCs), respectively [22, 31]. Although a common view in the CSC paradigm is that cells with the least differentiated state (at the apex of the hierarchy) would exhibit the most malignant traits (e.g. increased tumorigenic potential), this may be a generalization that need not necessarily hold true in every case. In fact, there are reports of more differentiated luminal breast cancer cells exhibiting equal tumor initiating capacity as basal/stem-like breast cancer cells [13, 19]. Further insights and elucidation of the normal mammary hierarchy, which are extensively covered by these reviews [20, 21, 32], will be vital to the understanding of various distinct CSC populations in breast tumors. Additionally, comprehension of the intrinsic and niche factors that govern each of the normal mammary stem/progenitor cell states may shed light on the vulnerabilities of distinct CSC populations. This is especially important since there are indications that distinct CSCs may have differential susceptibilities to targeted therapies against CSC-associated pathways such as Notch, Stat3 and TGF-beta [13, 33].

As a whole, the CSC concept has brought about urgency to account for intra-tumoral heterogeneity when it comes to administration of therapeutics [11]. Nevertheless, the emergence of distinct breast CSC populations that can co-exist within tumors adds to this complexity [13, 17, 18]. By extension, the same underlying principles of stratifying bulk tumors to account for inter-tumor heterogeneity can be applied to guide the administration of therapeutics for heterogeneous breast CSCs and hierarchical heterogeneity within a tumor. However, a key difference would be the requirement for diagnosis of breast tumors at the cellular level rather than the tumor as a bulk entity. Some of the potential single-cell technologies that may enable such diagnoses will be discussed in a later section.

Differentiation states of normal mammary hierarchy and intrinsic molecular subtypes of breast cancer

The idea that distinct breast cancer subtypes each correspond to a differentiation state of mammary cells represents a convergence point between normal mammary gland biology and breast cancer biology (Figure 1) [32, 34]. Although cancer cells of a particular differentiation state may only be 'caricatures' and not exactly resemble their normal counterparts [4], the ability to recognize a differentiation state that is most similar could still be helpful, just as with the stratification of patients based on intrinsic molecular subtypes [1]. Conceptually, if this is applied to the observation of hierarchical heterogeneity/heterogeneous CSC populations within breast tumors [13, 17, 18], it can be inferred that multiple breast cancer subtypes can co-exist within a tumor. Moreover, if we assume that the hierarchical heterogeneity/heterogeneous CSC populations exist in a dynamic equilibrium that is governed mainly by differentiation and plasticity, it is possible that breast tumors consist of a mixture of inter-converting breast cancer subtypes.

Plasticity of breast cancer subtypes

While this may be a provocative notion, there are several studies which support the argument that breast cancer cells can interconvert between distinct disease subtypes. Integral to this notion is a study where isogenic MCF-7 cells contextually displayed gene expression patterns of either luminal-A cell lines or basal-like cell lines, when injected intra-ductally

through the teat or into the mammary fat pad of female SCID/Beige mice respectively [35]. This highlights the possibility of breast cancer subtype plasticity [35] and a role for the tumor microenvironment in regulating it. Most recently, Ror2 (an alternative Wnt signaling receptor) has been shown to negatively regulate distinct claudin-low sub-populations within TP53-null mammary tumors that were classified as basal-like [36]. This study illustrates the co-existence of claudin-low and basal-like subtypes within tumors and the potential for plasticity between them through balancing canonical and alternative Wnt signaling [36]. Studies on circulating tumor cells (CTCs) from patients with primary breast tumors classified as ER⁺/Her2⁻ also indicate the potential for breast cancer subtype plasticity [33]. Isolated CTCs that were Her2⁺ and Her2⁻ exhibited distinct phenotypes and therapeutic vulnerabilities but were plastic and able to inter-convert, illustrating the plasticity in Her2 expression, one of three markers frequently employed in histological subtype stratification [33]. All these examples illustrate reversible conversions in breast cancer subtypes.

In the case of comparisons between primary tumors and corresponding metastases, there will be spatial and temporal factors that could potentially influence subtype switching. It is well documented that biopsies from geographically separated tumor areas display marked sub-clonal diversity [37]. Longitudinal studies that involve serial assessment of multiple tissue and liquid biopsies also reflect changes in sub-clonal populations that correspond to disease progression and differential responses to therapy [38]. Importantly, there is evidence that demonstrates intrinsic subtype switching in breast cancer brain metastases relative to primary tumors [39]. In this particular study, molecular changes in Her2 and intrinsic subtype switching to Her2-enriched subtypes can be associated with brain metastasis, indicating that the brain micro-environment may favor the progression of Her2 driven malignancies. In a separate study, PAM50 subtype classification of 123 paired primary and metastatic breast tumors revealed that subtype switching does indeed occur predominantly in cases where the primary tumors were classified as luminal A, luminal B or Her2-enriched [40], albeit these subtype switches could be driven by other mechanisms such as clonal evolution rather than plasticity. Notwithstanding that, evidence for a hierarchical model in breast cancer metastases has been reported [41]. Single cell analyses of metastatic cells implicates a stem-cell gene expression pattern in the initiation of metastasis, which is ensued by differentiation into more luminal-like cells that drive advanced metastatic disease [41]. One may view this as plasticity between a stem/basal-like disease subtype and a more differentiated/luminal disease subtype that occurs through the metastatic cascade.

Implications of multiple breast cancer subtypes within a tumor

If we view the hierarchical heterogeneity within breast tumors as a culmination of distinct disease subtypes, the direct clinical implication would be the application of similar stratification strategies as per the heterogeneity observed between patients (Figure 2, Key Figure). Consequently, combinations of the respective therapeutic agents that are most effective for each of the subtypes present can be utilized together to minimize the probability of resistant residual populations from recurring. With this viewpoint in mind, it has been shown that Trastuzumab, when administered in an adjuvant setting, can limit the progression of ER⁺/Her2⁻ luminal xenografted tumors [42]. The authors ascribed these positive responses in luminal Her2⁻ tumors to the effects on sub-populations of Her2⁺ CSCs [42].

These observations are in agreement with the existence of distinct Her2⁺ and Her2⁻ breast cancer subtypes co-existing within a tumor [33] and also illustrate the potential benefits of administering targeted therapeutics against sub-populations that do not fall under the classification of the bulk tumor subtype. In line with this, the therapeutic potential of targeting Her2⁺ CTCs with trastuzumab as an adjuvant treatment in Her2⁻ early breast cancer patients is being examined in an ongoing phase-2 clinical trial, TREAT-CTC (NCT01548677), exemplifying the use of liquid biopsies in a clinical setting [43]. The impetus for classification of such sub-populations as separate disease subtypes rather than painting them with a broad brush with the general term CSCs is mainly due to the existence of distinct CSC populations [13, 17–19]. As mentioned before, there could be CSCs that are ‘caricatures’ of normal fMaSCs, aMaSCs, unipotent stem/progenitors and parity induced-mammary epithelial cells (PI-MECs) [20–31].

An alternative approach to combinatorial targeting of distinct breast cancer subtypes is to limit the plasticity within tumors. The epithelial-to-mesenchymal transition (EMT) process has been shown to enable the acquisition of stem-like properties in normal and malignant mammary cells, indicating that the EMT process could be key in cell state plasticity [12, 22]. Protein Kinase C alpha (PKCα) has been shown to be a central signaling node that regulates EMT and could potentially be targeted to curb plasticity, alongside other EMT inducing pathways such as TGF-beta, WNT and STAT3 [44, 45]. Interestingly, the claudin-low subtype of breast cancer is enriched with an EMT gene signature and stem cell-like features [34]. These observations raise the possibility that the EMT program could also potentially drive the plasticity of breast cancer cells towards the claudin-low subtype, thus contributing to the emergence of multiple breast cancer subtypes within tumors. Another potential class of therapeutic targets that could be instrumental in regulating cell state plasticity are epigenetic regulators [46]. As an example, histone-deacetylase inhibitors (e.g. suberoylanilide hydroxamic acid, abexinostat) have been shown to promote differentiation of breast cancer cells and limit the number of CSCs within tumors [47, 48]. The tumor microenvironment is also an important factor that contributes to the plasticity of cancer cells by establishing niches. Specifically, extra-cellular matrix (ECM) components such as periostin, ECM-receptors such as discoidin domain receptor 1 (DDR1) and respective downstream signaling molecules such as focal adhesion kinase (FAK) have been shown to be important in inducing cancer-stem cell phenotypes such as metastasis initiation and drug resistance [49–52]. The milieu of other tumor-associated cell types which include fibroblasts, macrophages and endothelial cells could also contribute to the plasticity of tumor cells by producing paracrine or juxtacrine signals that induce a particular cell state and have been described in a recent review [53].

Cooperativity between distinct disease subtypes in breast tumors

It is worth noting that although we have placed emphasis on non-genetic mechanisms as a driving force towards breast cancer subtype heterogeneity within a tumor, it is very likely that there can be genetic underpinnings. As pointed out in other reviews, [4, 32] clonal evolution and hierarchical heterogeneity are not mutually exclusive models, and are likely to be concurrently exerting their respective influences within breast tumors (Figure 3). Implicit in this view of multiple breast cancer subtypes is the potential for interactions between the

disparate disease types, as applies generally to intra-tumoral heterogeneity (i.e. genetic clonal heterogeneity, epigenetic heterogeneity and noise-driven heterogeneity), which have been articulately reviewed [4, 32, 54]. These interactions may be cooperative, disruptive or neutral in nature, and instances of ‘altruistic’ clones which support the growth of breast tumors as a whole have been elegantly shown [55–57]. In addition, the cooperativity between distinct cell types can also drive invasion and metastasis [58]. It has been shown in MMTV-PyMT driven mammary tumors that luminal cell clusters invade together with leader cells that express K14 and exhibit basal features [58]. Interestingly in this case, the cooperating cell types can interconvert in response to the extracellular matrix, and enter the blood circulation as clusters prior to the formation of metastases [59]. Hence, knowledge of the entities present within a tumor could also be informative because the interactions between disease subtypes may bring about unanticipated characteristics (i.e. the whole is greater than the sum of its parts).

A perspective that heralds single-cell diagnostics?

Despite the potential benefits of subtyping breast cancer at the cellular level, there are certain practical challenges to the implementation of this concept. Recent advances in single-cell technologies may aid in addressing these perspectives and perhaps usher in its implementation. Critical to this may be the adaptation of these technologies to existing workflows in breast cancer diagnosis (e.g. the customary pathological analysis of formalin-fixed paraffin embedded (FFPE) tissues or CTC isolation from liquid biopsies). Namely, single-cell sequencing of DNA/RNA, single cell epigenomic methods (e.g. HI-C, ATAC-seq), mass cytometry, mass cytometry imaging, multiplexed ion beam imaging (MIBI) and multiplexed fluorescence barcoding hybridization methods are the rapidly developing modalities that could be instrumental and the technical aspects of each methodology are described in more detail in the respective reviews [60–64]. In the case of FFPE based diagnosis, mass spectrometry based techniques have been employed to spatially unravel molecularly distinct tumor subpopulations with clinically relevant attributes [65, 66]. There is also potential for integration of multiple ‘omics’ platforms, since some of these mass-spectrometric methods are also amenable to parameters such as metabolites [67]. Beyond the ability to tease out the heterogeneity between tumor populations, the distribution and abundance of various stromal populations could also be mapped out concurrently with these methods. This is important because stromal composition can provide prognostic value [68].

Concluding remarks

In summary, we have put forward a perspective that breast cancers may consist of multiple subtypes within a tumor and these separate disease entities could potentially be dynamic due to the plasticity of breast cancer cells. Application of single-cell technologies for diagnostic purposes will be essential in such a scenario and may provide better guidance when deciphering combinatorial treatment regimens. However, it is worth noting that a substantial gap exists between research methods and current clinical practice. As such, there are still practical and technical challenges that need to be addressed, if single-cell diagnostics were to be implemented in the clinic (see outstanding questions).

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References

1. Perou C, et al. Molecular portraits of human breast tumours. *Nature*. 2000; 406:747–752. [PubMed: 10963602]
2. Curtis C, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012; 486(7403):346–52. [PubMed: 22522925]
3. Network CGA. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012; 490(7418):61–70. [PubMed: 23000897]
4. Marusyk A, et al. Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer*. 2012; 12(5):323–34. [PubMed: 22513401]
5. Teschendorff AE, et al. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol*. 2007; 8(8):R157. [PubMed: 17683518]
6. Lehmann BD, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011; 121(7):2750–67. [PubMed: 21633166]
7. Furth J, Kahn M. The transmission of leukemia of mice with a single cell. *Am. J. Cancer*. 1937; 31:276–282.
8. Nguyen LV, et al. Cancer stem cells: an evolving concept. *Nat Rev Cancer*. 2012; 12(2):133–43. [PubMed: 22237392]
9. Al-Hajj M, et al. Prospective identification of tumorigenic breast cancer cells. *PNAS*. 2003; 100(7):3983–3988. [PubMed: 12629218]
10. Lapidot T, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*. 1994; 367:645–648. [PubMed: 7509044]
11. Brooks MD, et al. Therapeutic Implications of Cellular Heterogeneity and Plasticity in Breast Cancer. *Cell Stem Cell*. 2015; 17(3):260–71. [PubMed: 26340526]
12. Mani SA, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008; 133(4):704–715. [PubMed: 18485877]
13. Yeo SK, et al. Autophagy Differentially Regulates Distinct Breast Cancer Stem-like Cells in Murine Models via EGFR/Stat3 and Tgf β /Smad Signaling. *Cancer Res*. 2016; 76(11):3397–410. [PubMed: 27197172]
14. Yeo SK, Guan JL. Hierarchical heterogeneity in mammary tumors and its regulation by autophagy. *Autophagy*. 2016; 12(10):1960–1961. [PubMed: 27624535]
15. de Sousa e Melo F, et al. A distinct role for Lgr5(+) stem cells in primary and metastatic colon cancer. *Nature*. 2017; 543(7647):676–680. [PubMed: 28358093]
16. Shimokawa M, et al. Visualization and targeting of LGR5(+) human colon cancer stem cells. *Nature*. 2017; 545(7653):187–192. [PubMed: 28355176]
17. Hwang-Verslues WW, et al. Multiple lineages of human breast cancer stem/progenitor cells identified by profiling with stem cell markers. *PLoS One*. 2009; 4(12):e8377. [PubMed: 20027313]
18. Liu S, et al. Breast Cancer Stem Cells Transition between Epithelial and Mesenchymal States Reflective of their Normal Counterparts. *Stem Cell Reports*. 2014; 2(1):78–91. [PubMed: 24511467]
19. Kim J, et al. Tumor initiating but differentiated luminal-like breast cancer cells are highly invasive in the absence of basal-like activity. *Proc Natl Acad Sci U S A*. 2012; 109(16):6124–9. [PubMed: 22454501]

20. Visvader JE, Stingl J. Mammary stem cells and the differentiation hierarchy: current status and perspectives. *Genes Dev.* 2014; 28(11):1143–58. [PubMed: 24888586]
21. Lloyd-Lewis B, et al. Mammary Stem Cells: Premise, Properties, and Perspectives. *Trends Cell Biol.* 2017
22. Wahl GM, Spike BT. Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity. *NPJ Breast Cancer.* 2017; 3:14. [PubMed: 28649654]
23. Van Keymeulen A, et al. Distinct stem cells contribute to mammary gland development and maintenance. *Nature.* 2011; 479(7372):189–93. [PubMed: 21983963]
24. van Amerongen R, et al. Developmental stage and time dictate the fate of Wnt/ β -catenin-responsive stem cells in the mammary gland. *Cell Stem Cell.* 2012; 11(3):387–400. [PubMed: 22863533]
25. de Visser KE, et al. Developmental stage-specific contribution of LGR5(+) cells to basal and luminal epithelial lineages in the postnatal mammary gland. *J Pathol.* 2012; 228(3):300–9. [PubMed: 22926799]
26. Fu NY, et al. Identification of quiescent and spatially restricted mammary stem cells that are hormone responsive. *Nat Cell Biol.* 2017; 19(3):164–176. [PubMed: 28192422]
27. Rios AC, et al. In situ identification of bipotent stem cells in the mammary gland. *Nature.* 2014; 506(7488):322–7. [PubMed: 24463516]
28. Davis FM, et al. Single-cell lineage tracing in the mammary gland reveals stochastic clonal dispersion of stem/progenitor cell progeny. *Nat Commun.* 2016; 7:13053. [PubMed: 27779190]
29. Scheele CL, et al. Identity and dynamics of mammary stem cells during branching morphogenesis. *Nature.* 2017; 542(7641):313–317. [PubMed: 28135720]
30. Boras-Granic K, et al. Embryonic cells contribute directly to the quiescent stem cell population in the adult mouse mammary gland. *Breast Cancer Res.* 2014; 16(6):487. [PubMed: 25467960]
31. Spike BT, et al. A mammary stem cell population identified and characterized in late embryogenesis reveals similarities to human breast cancer. *Cell Stem Cell.* 2012; 10(2):183–97. [PubMed: 22305568]
32. Sreekumar A, et al. The mammary stem cell hierarchy: a looking glass into heterogeneous breast cancer landscapes. *Endocr Relat Cancer.* 2015; 22(6):T161–76. [PubMed: 26206777]
33. Jordan NV, et al. HER2 expression identifies dynamic functional states within circulating breast cancer cells. *Nature.* 2016; 537(7618):102–106. [PubMed: 27556950]
34. Prat A, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res.* 2010; 12(5):R68. [PubMed: 20813035]
35. Sflosmos G, et al. A Preclinical Model for ER α -Positive Breast Cancer Points to the Epithelial Microenvironment as Determinant of Luminal Phenotype and Hormone Response. *Cancer Cell.* 2016; 29(3):407–22. [PubMed: 26947176]
36. Roarty K, et al. Ror2-mediated alternative Wnt signaling regulates cell fate and adhesion during mammary tumor progression. *Oncogene.* 2017
37. Yates LR, et al. Subclonal diversification of primary breast cancer revealed by multiregion sequencing. *Nat Med.* 2015; 21(7):751–9. [PubMed: 26099045]
38. Murtaza M, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nat Commun.* 2015; 6:8760. [PubMed: 26530965]
39. Friedigkeit N, et al. Intrinsic Subtype Switching and Acquired ERBB2/HER2 Amplifications and Mutations in Breast Cancer Brain Metastases. *JAMA Oncol.* 2017; 3(5):666–671. [PubMed: 27926948]
40. Cejalvo JM, et al. Intrinsic Subtypes and Gene Expression Profiles in Primary and Metastatic Breast Cancer. *Cancer Res.* 2017
41. Lawson DA, et al. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. *Nature.* 2015; 526(7571):131–5. [PubMed: 26416748]
42. Ithimakin S, et al. HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: implications for efficacy of adjuvant trastuzumab. *Cancer Res.* 2013; 73(5):1635–46. [PubMed: 23442322]

43. Ignatiadis M, et al. Liquid biopsy-based clinical research in early breast cancer: The EORTC 90091-10093 Treat CTC trial. *Eur J Cancer*. 2016; 63:97–104. [PubMed: 27289552]
44. Tam WL, et al. Protein kinase C alpha is a central signaling node and therapeutic target for breast cancer stem cells. *Cancer Cell*. 2013; 24(3):347–64. [PubMed: 24029232]
45. Pattabiraman DR, Weinberg RA. Tackling the cancer stem cells - what challenges do they pose? *Nat Rev Drug Discov*. 2014; 13(7):497–512. [PubMed: 24981363]
46. Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med*. 2013; 19(11):1438–49. [PubMed: 24202396]
47. Munster PN, et al. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. *Cancer Res*. 2001; 61(23):8492–7. [PubMed: 11731433]
48. Salvador MA, et al. The histone deacetylase inhibitor abexinostat induces cancer stem cells differentiation in breast cancer with low Xist expression. *Clin Cancer Res*. 2013; 19(23):6520–31. [PubMed: 24141629]
49. Malanchi I, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*. 2011; 481(7379):85–9. [PubMed: 22158103]
50. Gao H, et al. Multi-organ Site Metastatic Reactivation Mediated by Non-canonical Discoidin Domain Receptor 1 Signaling. *Cell*. 2016; 166(1):47–62. [PubMed: 27368100]
51. Hirata E, et al. Intravital imaging reveals how BRAF inhibition generates drug-tolerant microenvironments with high integrin β 1/FAK signaling. *Cancer Cell*. 2015; 27(4):574–88. [PubMed: 25873177]
52. Luo M, et al. Mammary epithelial-specific ablation of the focal adhesion kinase suppresses mammary tumorigenesis by affecting mammary cancer stem/progenitor cells. *Cancer Res*. 2009; 69(2):466–74. [PubMed: 19147559]
53. Bussard KM, et al. Tumor-associated stromal cells as key contributors to the tumor microenvironment. *Breast Cancer Res*. 2016; 18(1):84. [PubMed: 27515302]
54. McGranahan N, Swanton C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell*. 2017; 168(4):613–628. [PubMed: 28187284]
55. Marusyk A, et al. Non-cell-autonomous driving of tumour growth supports sub-clonal heterogeneity. *Nature*. 2014; 514(7520):54–8. [PubMed: 25079331]
56. Cleary AS, et al. Tumour cell heterogeneity maintained by cooperating subclones in Wntdriven mammary cancers. *Nature*. 2014; 508(7494):113–7. [PubMed: 24695311]
57. Zhang M, et al. Intratumoral heterogeneity in a Trp53-null mouse model of human breast cancer. *Cancer Discov*. 2015; 5(5):520–33. [PubMed: 25735774]
58. Cheung KJ, et al. Collective invasion in breast cancer requires a conserved basal epithelial program. *Cell*. 2013; 155(7):1639–51. [PubMed: 24332913]
59. Cheung KJ, et al. Polyclonal breast cancer metastases arise from collective dissemination of keratin 14-expressing tumor cell clusters. *Proc Natl Acad Sci U S A*. 2016; 113(7):E854–63. [PubMed: 26831077]
60. Bendall SC, Nolan GP. From single cells to deep phenotypes in cancer. *Nat Biotechnol*. 2012; 30(7):639–47. [PubMed: 22781693]
61. Di Palma S, Bodenmiller B. Unraveling cell populations in tumors by single-cell mass cytometry. *Curr Opin Biotechnol*. 2015; 31:122–9. [PubMed: 25123841]
62. Schwartzman O, Tanay A. Single-cell epigenomics: techniques and emerging applications. *Nat Rev Genet*. 2015; 16(12):716–26. [PubMed: 26460349]
63. Bheda P, Schneider R. Epigenetics reloaded: the single-cell revolution. *Trends Cell Biol*. 2014; 24(11):712–23. [PubMed: 25283892]
64. Ullal AV, et al. Cancer cell profiling by barcoding allows multiplexed protein analysis in fine-needle aspirates. *Sci Transl Med*. 2014; 6(219):219a9.
65. Abdelmoula WM, et al. Data-driven identification of prognostic tumor subpopulations using spatially mapped t-SNE of mass spectrometry imaging data. *Proc Natl Acad Sci U S A*. 2016; 113(43):12244–12249. [PubMed: 27791011]

66. Giesen C, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. *Nat Methods*. 2014; 11(4):417–22. [PubMed: 24584193]
67. Ly A, et al. High-mass-resolution MALDI mass spectrometry imaging of metabolites from formalin-fixed paraffin-embedded tissue. *Nat Protoc*. 2016; 11(8):1428–43. [PubMed: 27414759]
68. Saleh SM, et al. Identification of interacting stromal axes in triple-negative breast cancer. *Cancer Res*. 2017

Glossary box

Mammary epithelial hierarchy

hierarchical association between distinct cell types of varying differentiation states, in the normal mammary gland.

Intra-tumoral heterogeneity

phenotypic variations between cells within a tumor that can be driven by genetic or non-genetic mechanisms.

Hierarchical heterogeneity

variation in cell states which correspond to the mammary epithelial hierarchy between cells within a tumor.

Plasticity

refers to the ability of cancer cells to reversibly interchange between distinct cell states.

Luminal

refers to the inner layer of the mammary glandular structure. Luminal breast cancers are subtypes which express genes that are more closely associated with cells of the inner/luminal layer of the mammary gland.

Basal-like

Basal-like breast cancers are subtypes which have gene expression patterns that are reminiscent of the outer/basal cells of the mammary gland.

Mammary Stem Cells (MaSCs)

multipotent mammary cells that can give rise to all the other specialized mammary cell types within the gland.

Cancer Stem Cells (CSCs)

cells within tumors which exhibit increased tumorigenic potential and certain characteristics of normal stem/progenitor cells.

Circulating Tumor Cells (CTCs)

tumor cells which have disseminated from the primary tumor and are found in the vascular/lymphatic system.

PAM50 subtypes

a 50-gene intrinsic subtype classifier for breast cancers with prognostic significance

Trends Box

- Observations of distinct co-existing cancer stem cell (CSC) populations within breast tumors constitute the need to shift from a simple binary view of the tumor hierarchy (CSC and bulk tumor cells) towards a spectrum of varying differentiation states.
- Emerging evidence points to the potential existence of distinct breast cancer subtypes within a tumor and the possibility of these discrete disease subtypes to inter-convert through cell state plasticity.
- The advent of single-cell technologies could potentially enable cancer diagnosis at the cellular level, creating a new dimension where precision medicine is tailored down to the cell and not just the individual patient.

Outstanding questions Box

- What are the factors that drive the co-existence/plasticity of distinct disease entities? Both cell intrinsic and/or tumor micro-environmental factors are likely to contribute. Identification of such factors could provide targetable solutions against the plasticity/heterogeneity.
- What would be the best strategy to develop more effective therapies based on the proposed hypothesis? Targeting a common denominator for all the subtypes, targeting the Achilles heel of each subtype in combination or inducing a more homogeneous population (differentiation therapy) to sensitize tumors?
- How much benefit can be gained from knowing the composition of distinct disease subtypes versus diagnosing the bulk tumor as a whole? The benefits will need to substantially outweigh the costs, if more precise diagnostic measures are to be implemented.
- If multiple breast cancer subtypes co-exist within a tumor, what is the best way of sampling a patient's tumor? How much sample would be needed to obtain a fair representation of the distinct subtypes or would CTCs be sufficient?
- Insights from normal mammary gland biology and its hierarchy could be instrumental in better defining distinct breast cancer subtypes and potential susceptibilities for each.

Figure 1. Association between differentiation states of the mammary gland hierarchy and intrinsic breast cancer subtypes

Depiction of differentiation states within the normal mammary gland hierarchy during embryonic development as well as in the postnatal mammary gland. Corresponding intrinsic breast cancer subtypes which are most closely associated with each state are illustrated (on the right).

Figure 2, Key Figure. Multiple breast cancer subtypes within a tumor and its therapeutic implications

Illustrated are individual tumors (Tumors A–D) with cells occupying varying levels of the differentiation hierarchy within a tumor. Distinct differentiation states are illustrated in separate colors. Based on the presumption that each differentiation state is associated most closely with a particular breast cancer intrinsic subtype, this would imply that multiple breast cancer subtypes can co-exist in a tumor. As such, combinatorial therapeutic approaches which account for each disease subtype present may be required for improved efficacy of treatment.

Figure 3. Integrated model of clonal evolution along with hierarchical heterogeneity

An illustration of intra-tumoral genetic heterogeneity as a result of clonal evolution (Each circle with varying color represents a different genetic subclone). In addition to the clonal diversity, each subclone may exhibit plasticity across a spectrum of differentiation states (Note: hierarchy and differentiation states are illustrated below each subclone, and subclones may exhibit plasticity across a different range of differentiation states). This integrated model illustrates the potential interplay between clonal evolution and hierarchical heterogeneity, where genetic sub-clones may have cells that span varying spectra of the differentiation hierarchy.