Meeting report

It's all in the details: methods in breast development and cancer

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Abstract

The inaugural European Network for Breast Development and Cancer (ENBDC) meeting on 'Methods in Mammary Gland Development and Cancer' was held in Weggis, Switzerland last April. The goal was to discuss the details of techniques used to study mammary gland biology and tumourigenesis. Highlights of this meeting included the use of four-colour fluorescence for protein co-localisation in tissue microarrays, genome analysis at single cell resolution, technical issues in the isolation of normal and tumour stem cells, and the use of mouse models and mammary gland transplantations to elucidate gene function in mammary development and to study drug resistance in breast cancer.

Introduction

There are several meetings devoted to breast cancer research, some of which include talks on normal breast development. In contrast, there is no meeting specifically dedicated to discussing methods used in breast development and cancer. A new group, the European Network for Breast Development and Cancer (ENBDC), has initiated an annual meeting specifically devoted to presenting and discussing methods in the mammary gland field. The first meeting was organised in Weggis, Switzerland last April. First-year graduate students as well as novices in the field of breast development and cancer were encouraged to attend. This inaugural meeting encompassed discussions on breast cancer histopathology, tumour-initiating cells, animal models and normal breast stem cells.

Methods in human and mouse breast pathology (Chair: Torsten Stein)

The first session included David Robertson from the Breakthrough Breast Cancer Research Centre in London and Dr Kim Jensen from Cambridge University. Robertson presented the latest developments in multi-colour fluorescent imaging using formalin-fixed paraffin-embedded (FFPE) sections. FFPE archives around the world add up to a large database of tumour samples, but standard staining using chromogenic substrates has several limitations, especially the inability to target multiple proteins simultaneously and the limited intracellular resolution. Immunofluorescence potentially provides increased resolution and allows a multi-colour approach. However, FFPE material often displays a high level of auto-fluorescence. Using an optimised protocol and confocal laser microscopy, Robertson was able to dramatically reduce this background fluorescence, allowing the use of four-colour fluorescence for cellular and intracellular co-localisation studies on FFPE tissue microarrays [1]. His latest protocols will be published on the ENBDC website [2].

Kim Jensen from Fiona Watt's laboratory also presented work on studying multiple genes in small samples. He has developed a technique for full genome microarray analysis on RNA amounts equivalent to that from a single cell using a PCR-based amplification step. This allowed him to study mRNA expression profiles of single flow sorted epidermal stem cells. In this way, Lrig1 was identified as a marker for epidermal stem cells that keeps these cells in a quiescent state [3]. Jensen further showed that Lrig1-positive cells define a distinct subpopulation in the hair follicle that can give rise to all epidermal cell lineages, as well as to cells of the sebaceous gland and the interfollicular epidermis [4]. This powerful technique thus allows the study of the expression profiles of very small numbers of cells, including from isolated cap cells of the mammary terminal end bud or from very small flow sorted mammary cell populations.

ENBDC = European Network for Breast Development and Cancer; ER = estrogen receptor; FFPE = formalin-fixed paraffin-embedded; K5 = keratin 5.

Cancer stem/progenitor cells of the breast (Chair: Rob Clarke)

In the second session, Dr John Stingl from the Cancer Research UK Cambridge Research Institute spoke about the detection and analysis of mammary gland stem and progenitor cells. These cells are detected by their ability to generate ductal-lobular outgrowths when transplanted into immune-compatible mice and by their ability to generate colonies *in vitro*. Stingl reviewed some limitations of these assays, with particular emphasis on how variable they can be with minor changes in protocol. He presented results from his laboratory on how to minimize variability and increase the efficiency of these assays. For example, the enzymatic dissociation of mammary glands in growth factor-depleted versus growth factor-rich media results in the differential yield of mammary stem and progenitor cells, with stem cells preferring growth factor-depleted conditions and the progenitor cells favouring growth factor-rich conditions. As well, the detection frequency of stem cells can be increased approximately sixfold by the inclusion of Matrigel™ within the transplant inoculum.

The second speaker, Dr Gabriela Dontu from King's College, University of London, talked about estrogen receptor (ER) expression in stem and progenitor cells from normal and malignant breast epithelium. Dontu proposed that no one stem cell marker is useful for all breast cancers and that there may be specific markers useful for different breast cancer subtypes, of which there are at least five. For example, aldehyde dehydrogenase expression determined by the fluorescent substrate Aldefluor is useful for enriching stem cells in normal and some malignant breast samples but is low in cell lines and absent in many ER-positive cancers [5]. Dontu argued that researchers should beware of several common misconceptions concerning cancer stem cells. In reality, there are unlikely to be universal markers, the cancer stem cell population may not be infrequent (up to 25% in melanoma) [6], there will be genetic and phenotypic heterogeneity within a tumour, not all tumours will be hierarchical, and the cancer stem cell model is compatible with, and not distinct from, the clonal evolution model.

Animal models for studying breast cancer (Chair: Jos Jonkers)

In the third session, Dr Sven Rottenberg from the Netherlands Cancer Institute talked about drug responses and therapy resistance in conditional mouse models of breast cancer. Resistance to chemotherapy is a central unsolved problem in breast cancer treatment, since most patients die of disseminated tumours that are resistant to all forms of therapy. To tackle clinically relevant mechanisms of drug resistance, Rottenburg used the K14*cre;Brca1*F/F;*p53*F/F mouse model for *BRCA1*-associated breast cancer [7], and found acquired resistance to cytotoxic drugs (doxorubicin, docetaxel) [8] and to the poly(ADP-ribose) polymerase (PARP) inhibitor AZD2281, which induces synthetic lethality in *BRCA1*-deficient tumours [9]. The only resistance mechanism identified thus far is upregulation of the P-glycoprotein multidrug efflux transporter. Although no resistance was observed for cisplatin, tumours could not be eradicated and consistently re-grew from small tumour remnants. These remnants were not enriched in tumour-initiating cells, but contained beta-galactosidase-positive and Ki-67-negative cells, suggesting (therapy-induced) senescence or dormancy as escape mechanisms.

The second speaker, Dr Vida Vafaizadeh from the Georg-Speyer-Haus in Frankfurt am Main, Germany, investigated the role of Stat5 in mammary gland development and tumourigenesis via *ex vivo* transduction of primary mammary epithelial cells with lentiviral vectors encoding a Stat5-specific short hairpin RNA (shStat5ab) or a constitutively active Stat5 variant (cS5F). The transduced mammary epithelial cells were transplantated into cleared fat pads to reconstitute functional glandular epithelium. Downregulation of Stat5 did not affect the outgrowth of primary ducts, but resulted in thinner ducts, reduced side-branching and impaired alveologenesis. Conversely, constitutive activation of Stat5 caused hyperproliferation of epithelial cells, thickening of the ducts and precocious alveolar development in nulliparous mice, indicating that Stat5 activity regulates the emergence of mature alveolar cells from luminal progenitors. The persistent activation of Stat5 during the involution stage prevents apoptosis of the epithelial cells and causes formation of tumours that also express activated Stat3.

Studying normal mammary stem cells (Chairs: Matthew Smalley and Mohamed Bentires-Alj)

In the final session, Dr Matthew Smalley from the Breakthrough Breast Cancer Research Centre discussed the preparation and flow cytometric analysis of primary mammary epithelial cells, including stem cells. His group uses the CD24, Sca1 and CD49f markers to isolate four lineages of the mammary gland: basal stem cells, basal myoepithelial cells, ER-positive luminal cells and ER-negative luminal cells [10]. The critical steps for optimal isolation of the different lineages and confirming the identity of the isolated subpopulations were the topics of Smalley's presentation. In particular, he focussed on the validation of antibodies used for flow cytometric cell separation. He argued that gating based on controls in which one antibody is left out of the staining cocktail ('fluorescence minus one' controls) are critical. He also noted that different fluorochromes conjugated to the same antibody clone may give different flow cytometric patterns, as may different clones against the same antigen. Hence, antibody titration to saturation must be carried out for each new fluorochrome combination. Finally, to assess the quality of the whole process, each population isolated must be characterised by testing expression of cell-type specific markers and *in vitro* and *in vivo* functional assays [10,11].

The second speaker, Dr Marina Glukhova from the Institut Curie in Paris, discussed the usefulness of the keratin 5 (K5) promoter active in the basal cells from various stratified epithelia for targeting transgenes to the basal cell layer of the mammary epithelium. Glukhova's lab crossed transgenic mice expressing Cre recombinase under the control of the K5 promoter with mice presenting conditional alleles for β1 integrin gene (*itgb1*F/F). In virgin mutant mice, β1 integrin was efficiently deleted from the basal cell layer, whereas luminal cells remained β1 integrin-positive. The deletion of the *itgb1* gene completely abolished the regenerative potential of the mammary epithelium, suggesting that β1 integrins are essential for the maintenance of the functional stem cell population [12]. Surprisingly, in pregnant mutant mice, luminal cells in the alveoli stained negative for β1 integrin, suggesting that they originated from K5-positive basal cells. Further analysis showed that whereas wild-type basal cells divide parallel to the basement membrane and their progeny therefore remained in the basal layer, the orientation of basal division was random in glands from K5*cre*;*Itgb1*F/F mice. This led to cell fate changes explaining the presence of progeny from the basal cells in the luminal layer. Further, using the K5 promoter, this lab has obtained transgenic mice presenting a constitutive activation of Wnt/β-catenin signalling in basal mammary epithelial cells. These mice develop hyperplastic mammary lesions presenting characteristics similar to those of human breast basal-type carcinomas.

Conclusion

The meeting proved a very valuable forum for the dissemination of ideas and experiences relating to commonly used, but sometimes poorly understood, techniques in mammary cell biology. Talks by speakers from outside the mammary cell biology field also helped highlight, especially to younger researchers, potential new avenues for exploration. Planning is already beginning for the 2010 meeting, which, it is hoped, will prove as stimulating as in 2009.

Competing interests

The authors declare that they have no competing interests.

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References

1. Robertson D, Savage K, Reis-Filho JS, Isacke CM: **Multiple immunofluorescence labelling of formalin-fixed paraffin-embedded (FFPE) tissue.** *BMC Cell Biol* 2008, **9:**13.
2. **European Network of Breast Development and Cancer Labs** [www.enbdc.org]
3. Jensen KB, Watt FM: **Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence.** *Proc Natl Acad Sci USA* 2006, **103:**11958-11963.
4. Jensen KB, Collins CA, Nascimento E, Tan DW, Frye M, Itami S, Watt FM: **Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis.** *Cell Stem Cell* 2009, **4:**427-439.
5. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS: **Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature.** *Cancer Res* 2009, **69:**1302-1313.
6. Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, Morrison SJ: **Efficient tumour formation by single human melanoma cells.** *Nature* 2008, **456:**593-598.
7. Liu X, Holstege H, van der Gulden H, Treur-Mulder M, Zevenhoven J, Velds A, Kerkhoven RM, van Vliet MH, Wessels LF, Peterse JL, Berns A, Jonkers J: **Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer.** *Proc Natl Acad Sci USA* 2007, **104:**12111-12116.
8. Rottenberg S, Nygren AO, Pajic M, van Leeuwen FW, van der Heijden I, van de Wetering K, Liu X, de Visser KE, Gilhuijs KG, van Tellingen O, Schouten JP, Jonkers J, Borst P: **Selective induction of chemotherapy resistance of mammary tumors in a conditional mouse model for hereditary breast cancer.** *Proc Natl Acad Sci USA* 2007, **104:**12117-12122.
9. Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, Derksen PW, de Bruin M, Zevenhoven J, Lau A, Boulter R, Cranston A, O'Connor MJ, Martin NM, Borst P, Jonkers J: **High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs.** *Proc Natl Acad Sci USA* 2008, **105:**17079-17084.
10. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ: **Dissociation of estrogen receptor expression and *in vivo* stem cell activity in the mammary gland.** *J Cell Biol* 2007, **176:**19-26.
11. Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ: **CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells.** *Breast Cancer Res* 2006, **8:**R7.
12. Taddei I, Deugnier MA, Faraldo MM, Petit V, Bouvard D, Medina D, Fassler R, Thiery JP, Glukhova MA: **Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells.** *Nat Cell Biol* 2008, **10:**716-722.