Breast Cancer RESEARCH

MEETING REPORT

The devil is in the methods: lineage tracing, functional screens and sequencing, hormones, tumour-stroma interactions, and expansion of human breast tumours as xenografts

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Abstract

The meeting of the European Network for Breast Development and Cancer (ENBDC) on 'Methods in Mammary Gland Development and Cancer' has become an annual international rendezvous for scientists with interests in the normal and neoplastic breast. The third meeting in this series, held in April-May 2011 in Weggis, Switzerland, focussed on functional screens and sequencing, hormones, lineage tracing, tumor-stroma interactions and the expansion of human breast tumours as xenografts.

Introduction

The third international meeting of the European Network for Breast Development and Cancer (ENBDC) again promoted the sharing of protocols and ideas between groups working on breast development and cancer. Graduate students, postdocs and research associates were encouraged to attend. The following topics were covered in depth: functional screens and sequencing, hormones, lineage tracing, the propagation of human breast tumours as xenografts and tumour-stroma interactions.

Functional screens and sequencing (Chair: Momo Bentires-Alj)

Chris Lord from the Breakthrough Breast Cancer Research Centre at the Institute of Cancer Research in London presented examples of the power of genome-wide functional screens. First, his group combined tamoxifen treatment of breast cancer cells *in vitro* and a small interfering RNA (siRNA) screen for kinases in an approach to identify events leading to tamoxifen sensitivity and tamoxifen resistance. They identified low cyclin-dependent kinase (CDK)10 expression as an important mediator of resistance to endocrine therapy in breast cancer. Knockdown of CDK10 blocked its inhibitory effect on ETS2, which in turn induced transcription of c-Raf and led to activation of the ERK/mitogen-activated protein kinase (MAPK) pathway and resistance to tamoxifen [1]. They also used a pooled genome-wide small hairpin RNA (shRNA) screen in the presence or absence of tamoxifen, coupled with massively parallel sequencing, and identified groups of genes the silencing of which increased sensitivity (for example C10orf72, C15orf55/*NUT*, *EDF1*, *ING5*, *KRAS*) or resistance (for example, *BAP1*, *CLPP*, *GPRC5D*, *NAE1*, *NF1*) to tamoxifen [2]. Second, they used a synthetic lethal unbiased shRNA screen for identifying sensitizers to a poly (ADP-ribose) polymerase (PARP) inhibitor in *BRCA1* wild-type breast cancer cells. Knockdown of *RAD51D*, a novel ovarian cancer susceptibility gene, dramatically increased cell death upon PARP inhibition [3]. Third, they used a kinome-wide siRNA screen to identify genetic dependencies of breast cancer cell lines. Characterization of the genetic dependencies of breast tumour cell lines with different tumorigenic mutations demonstrated a synthetic lethality between loss of *PTEN* and the inhibition of the mitotic checkpoint kinase TTK/MPS1, which suggests a novel therapeutic approach [4]. These studies warrant further in-depth validation and mechanistic studies, which could reveal clinically relevant combined therapies or novel targets for cancer.

David Adams from the Wellcome Trust Sanger Institute described the efforts made by his team to find driver mutations in mouse models of cancer. First, they carried out insertion mutagenesis in $Apc^{fl x/+}$/*Ah-Cre* mice using *Sleeping Beauty* insertional mutagenesis technology, followed by deep sequencing of insertion sites to identify genes collaborating with loss of the tumour

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suppressor *Apc* in bowel cancer and driving tumour formation. They found that inactivation of the H3K4 methyltransferase *Mll3* induces the Wnt pathway and increases tumorigenesis, either alone or in concert with loss of *Apc* [5]. Second, he summarized their efforts to sequence the genomes of mouse models of breast cancer (including models of lobular and basal-like breast cancer), which led to the identification of patterns of rearrangement in the different models and to candidate driver cancer genes. Preliminary data on these studies have recently been published [6].

Hormones
(Chairs: María dM Vivanco and Momo Bentires-Alj)

Cathrin Brisken (Swiss Institute for Experimental Cancer Research/EPFL Lausanne) spoke on the 'Genetic dissection of hormonal control in mammary gland development'. Tissue recombination techniques have shown that while oestrogen targets the mammary epithelium to invoke ductal elongation, progesterone is required for ductal side branching. Results from the Brisken lab showed that progesterone drives proliferation in two waves: first, cyclin D1 is required for the proliferation of some progesterone receptor $(PR)^+$ cells; second, PR^- cells in particular proliferate through a Receptor activator of nuclear factor kappa-B ligand (RANKL)-dependent mechanism [7]. These results highlight the relevance of RANKL in the mediation of progesterone function, since it rescues the $PR^{-/-}$ phenotype. Importantly, they demonstrate a key role of progesterone in inducing proliferation by a paracrine mechanism. In addition, by modulating the expression levels of DeltaNp63 in primary human breast epithelial cells, they showed that this transcription factor is required to confer a basal cell phenotype. Notably, Notch, which reduces DeltaNp63 expression, can shift the balance towards a luminal phenotype [8]. Furthermore, *in vivo* ablation of Notch signalling demonstrated that it is not possible to establish and/or maintain luminal cells in the absence of Notch. These findings reveal antagonistic effects of Notch and p63 on cell fate determination in the mammary epithelium.

Maria Vivanco from CIC bioGUNE in Bilbao spoke on 'Hormonal regulation in human breast stem cells' focusing on the effects of oestrogen on breast stem cells. She showed that expression of the stem cell markers Nanog, Oct4 and Sox2 is higher in stem cells isolated from normal or breast tumour tissue and can be reduced by serum-induced differentiation or treatment with oestrogen, whilst tamoxifen had the opposite effect. Vivanco further demonstrated that overexpression of each individual stem cell gene is sufficient to increase both the number of stem cells, as measured by mammosphere formation, aldehyde dehydrogenase activity and the cell-surface phenotypes EMA^+CALLA^+ and $CD44^+CD24^{-/low}$, and their invasion capacity; these are characteristics of tumourigenesis and poor prognosis [9]. Interestingly, this led to reduced oestrogen receptor (ER) expression, which is in agreement with the reported absence or low expression of ER in breast stem cells. The results indicate that oestrogen reduces the pool of breast stem cells, providing a plausible explanation for several clinical observations, namely that ER^+ tumours coincide with a better prognosis than ER^- tumours, and that women on hormone-replacement therapy using oestrogen alone have a lower risk of breast cancer than those taking oestrogen plus progestin, where the risk is elevated. Finally, this report emphasised that future therapies should combine treatments directed at diminishing tumour bulk with new strategies to eliminate cancer stem cells whilst sparing normal stem cells [10].

Lineage tracing
(Chair: John Stingl)

Nick Barker from the Institute of Medical Biology in Singapore summarized his work and that of his former colleagues in the Hans Clever's lab on characterizing mouse intestinal stem cells. They demonstrated the importance of Wnt as a key regulator of intestinal epithelial cell homeostasis. Wnt factors, which are produced by epithelial cells in the intestinal crypt, are essential for Paneth cell differentiation, which in turn function as the stem cell niche and also have antimicrobial properties. Wnt is also a mitogen for transit-amplifying (TA) cells and for maintaining the intestinal stem cell population. Lgr5 (also known as Gpr49), which is an orphan G protein-coupled receptor, was identified as a Wnt target gene and found to have a unique distribution within the epithelium, with expression restricted to basal columnar cells of the crypt. Elegant genetic lineage tracing experiments in mice targeting $Lgr5^+$ cells revealed the capacity of these cells to maintain all of the different lineages of the intestinal epithelium in the long-term, thus fulfilling the functional requirements of intestinal epithelial stem cells. Fate mapping of individual stem cells using multicolour reporter mice revealed that intestinal stem cells predominantly divide symmetrically and that daughter cells stochastically adopt stem or TA cell fates. Selective deletion of the adenomatous polyposis coli (*APC*) gene in $Lgr5^+$ cells demonstrated that aberrant activation of Wnt signalling in these cells causes aggressive adenoma growth throughout the intestine. In contrast, loss of APC in TA cells resulted only in the generation of microadenomas, whose growth rapidly stalled. These results demonstrate the likely stem cell origin of colorectal cancer in this mouse model. Interestingly, mouse intestinal tumours also contain a minor population of $Lgr5^+$ cells that reside in a Paneth cell niche, although it is not clear at this stage

whether these cells function as colon cancer stem cells. Barker also presented data demonstrating that clonal outgrowths can be generated and passaged in Matrigel cultures in the absence of a cellular stromal niche, and that cells from these structures have the potential to contribute to the colonic epithelium when introduced into suitable recipient mice.

Propagation of human breast tumours as xenografts (Chair: John Stingl)

The participants of the meeting were invited to take part in a group discussion on the xenotransplantation of human breast tumours into immunodeficient mice. The ability to propagate primary human tumours in mice is a powerful tool for studying breast cancer stem cells, as well as for breast tumour modelling and other aspects of breast cancer cell biology. Unfortunately, human breast tumours, particularly those that are ER^+, are notoriously difficult to engraft into mice. During this group discussion, participants shared their experiences of the dissociation and transplantation of human breast tumour cells into mice and several factors critical for successful engraftment were identified. These include (1) minimal tissue dissociation in order to minimize the toxicity of collagenase over-digestion, (2) the preferred use of NOD scid gamma (NSG; NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ JAX) mice, (3) co-implantation of cells with Matrigel, and (4) patience, since tumour formation may take up to 6 months.

Tumour-stroma interactions (Chair: Rob Clarke)

Akira Orimo (Paterson Institute for Cancer Research, University of Manchester, UK) discussed a method that allows the experimental generation of cancer-associated fibroblasts (exp-CAFs) from human mammary fibroblasts using a co-implantation tumour xenograft model. Human mammary fibroblasts, co-implanted with breast carcinoma cells into immunodeficient nude mice, were extracted from breast tumour xenografts. These fibroblasts were shown to increasingly acquire the ability to promote carcinoma growth during tumour progression. Exp-CAFs also included large numbers of myofibroblasts, which are a hallmark of activated fibroblasts present in wound healing and chronic fibrosis. Taken together, these findings suggested that exp-CAFs in their tumour-promoting myofibroblastic phenotypes resemble primary CAFs prepared from breast carcinomas dissected from breast cancer patients [11]. Moreover, Orimo demonstrated that the establishment of autocrine signalling loops mediated by transforming growth factor-β and stromal cell-derived factor-1 cytokines is responsible for the induction and maintenance of CAFs' tumour-promoting myofibroblastic ability, thereby indicating the evolution of resident mammary fibroblasts to tumour-promoting CAFs during the course of tumour progression. The roles of exp-CAFs in promoting tumour metastasis were also investigated.

Clare Isacke (Breakthrough Breast Cancer Centre, London, UK) described approaches to high-throughput *in vivo* RNA interference screens for identifying novel determinants of the breast cancer metastatic process. The pros and cons of different biological systems in which to conduct a screen, of RNA interference libraries, of the methodologies for read-out and analysis, and of different approaches to validate potential hits were described.

The third annual ENBDC methods meeting served as a valuable international forum for exchanging protocols and ideas relevant to mammary gland development and cancer. The 2012 ENBDC methods meeting will be held in Weggis on April 13-15 [12].

Abbreviations

ENBDC, European Network for Breast Development and Cancer; ER, oestrogen receptor; exp-CAF, experimentally generated cancer-associated fibroblast; PARP, poly (ADP-ribose) polymerase; PR, progesterone receptor; RANKL, Receptor activator of nuclear factor kappa-B ligand; shRNA, small hairpin RNA; siRNA, small interfering RNA; TA, transit-amplifying.

Competing interests

The authors declare that they have no competing interests.

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