Mammary gland biology and breast cancer

Conference on Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer Progression

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Introduction

As a model system for biological investigations, the mouse mammary gland is as versatile as the *Drosophila* eye. This comparison does not reflect any ignorance of comparative anatomy, but rather it highlights the multitude of possibilities that the mammary gland offers to experimental biologists. The mammary gland has many unique developmental features: it goes through embryonal, puberty, pregnancy, lactation and post-lactation phases, and many of the genetic determinants that regulate cell specification, proliferation, differentiation, survival and death have been studied in great detail. Growth, differentiation and regression of the mammary epithelial cells occur repeatedly in the adult organism. Each cycle of pregnancy, lactation and involution is accompanied by massive cellular proliferation, functional differentiation and cell death. These developmental stages are regulated by epithelial stromal cell interactions, and by systemically and locally acting steroid and peptide hormones (Hennighausen & Robinson, 2005). Genetic analyses can be carried out using tissue-specific knockout mice, or genetically modified primary cells can be transplanted into fat pads cleared of endogenous epithelium. These experimental strategies have been recently complemented by the isolation and genetic manipulation of adult mammary stem cells. The basic scientific aspects of mammary gland biology are not only fascinating but also central to our understanding of breast cancer, which is at present the most common cancer in women in the Western world. Extensive research is underway to being carried out the basic biology of the mammary gland and to use this information in the fight against the disease.

Mammary epithelial cell organization

The mammary gland consists of ductal cells, milk-producing alveolar epithelial cells that are embedded in stromal connective tissue and the mammary fat pad. Mammary epithelial cells organize into three-dimensional structures, which are strongly dependent on a polarized morphology, specialized cell-cell contacts and specific attachments to an underlying basement membrane. Mechanical forces and signalling from neighbouring cells also influence cellular organization (Fig 1). These features are necessary for the proper control of cell proliferation, survival, differentiation, migration and milk-protein

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The EMBO Molecular Medicine Conference on Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer Progression was held jointly with a meeting of the COST B20 (European cooperation in the field of scientific and technical research)—a group of about 35 scientists from 23 European countries who have been meeting regularly during the past five years to discuss questions about mammary gland development, function and cancer.

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secretion (Bissell *et al*, 2002; O'Brien *et al*, 2002; Rodriguez-Boulant & Salas, 1989). Transformed mammary epithelial cells lose their ability to form organized ducts or alveoli. At the meeting, extracellular matrix interactions, cell–cell interactions and the signalling pathways that govern the organization of mammary epithelial cells were discussed.

Adhesive interactions between epithelial cells and extracellular matrix components, particularly laminin, regulate many functional properties of normal mammary epithelial cells. C. Streuli (Manchester, UK) reported that laminin, and its $\beta 1$ -integrin receptor, are crucial for triggering expression of the milk-protein gene β -casein (Naylor *et al*, 2005). Streuli used knockout mice and three-dimensional cell-culture model systems to show that $\beta 1$ -integrin, the small GTPase Rac1, and a downstream mediator of integrin signalling, the integrin-linked kinase (ILK), are essential for signal integration between the integrin and prolactin receptors. Dominant-negative Rac1 inhibited prolactin signalling, whereas constitutively activated Rac1 rescued differentiation in both $\beta 1$ -integrin-null and ILK-null cells.

Stromal epithelial interactions are required for the development and maintenance of tissue homeostasis. Malignant transformation of the breast is associated with definable changes in the extracellular matrix composition and organization, which results in progressive increases in tissue stiffness. *In vitro* manipulation of the extracellular matrix and an increase in stiffness destabilizes cell–cell adherens junctions, compromises cell polarity, modifies cytoskeletal organization, increases the cell proliferation rate and cell survival, and alters gene expression profiles. Data presented by V. Weaver (Philadelphia, PA, USA) suggest that these changes arise from integrin aggregation, which facilitates focal adhesion maturation through enhancement of Rho-dependent cell contractility and extracellular-signal-regulated kinase (ERK)-induced increases in myosin tension. Growth-factor-transformed mammary epithelium that exerts abnormally high integrin-generated tension reverted its phenotype when the myosin-dependent force was normalized. Matrix stiffness also altered the responsiveness of the epithelium to exogenous death cues. Thus, tissue homeostasis and apoptotic responsiveness seem to be functionally linked to tension homeostasis through integrin-growth-factor receptor–GTPase crosstalk.

Many growth factors are synthesized as transmembrane precursor proteins, which can become liberated and mobilized through the action of proteases. P. Kenny (Berkeley, CA, USA) analysed malignant breast cancer cells driven by an autocrine loop. Mobilization of two growth factors—amphiregulin and transforming growth factor α (TGF α)—was found at the earliest stages of tumour progression and was dependent on TGF α -converting enzyme (TACE) protease activity. Inhibition of this protease resulted in downregulation of epidermal growth factor receptor (EGFR) tyrosine kinase activity and phenocopied the inhibition of EGFR activity by Iressa® (gefitinib, AstraZeneca, London, UK). This enzyme inhibitor reverts the malignant phenotype in three-dimensional culture assays. TACE inhibition had similar consequences for the transcriptional profile as EGFR or mitogen-activated-protein (MAP)/ERK kinase (MEK) inhibition. Thus, preventing ligand mobilization by inhibiting TACE, and consequently EGFR signalling, might be a potential therapeutic strategy for breast cancer.

Madin–Darby canine kidney (MDCK) epithelial cells and primary mouse mammary epithelial cells can be used as *in vitro* models to study cellular pathways necessary for polarized assemblies.

Fig 1 | Factors governing mammary epithelial cell architecture. Normal mammary epithelial cells organize into spheroids called 'acini', which are characterized by the presence of a central, hollow lumen surrounded by polarized epithelial cells. This three-dimensional organization regulates cell proliferation, migration and differentiation, and is strongly dependent on specific cell–cell and cell–matrix interactions. Disruption of these interactions or changes in mechanical stress owing to alterations in the extracellular matrix can interfere with the signalling pathways that govern cellular architecture.

S. Muthuswamy (Cold Spring Harbor, NY, USA) found that activation of ErbB2, an oncogenic receptor tyrosine kinase, initiates the disruption of epithelial architecture at the apical–lateral border. This leads to a loss of apical polarity through the formation of a novel ErbB2–Par6 complex after disruption of the Par6–aPKC (atypical protein kinase C) polarity complex, which controls the establishment of the apical–lateral border. F. Martin (Dublin, Ireland) observed that inhibition of MAP kinase c-Jun N-terminal kinase (JNK) impaired cell polarization and lumen clearance during acinus formation. JNK inhibition allowed sustained phosphorylation of ERK, cell proliferation, cell survival and expression of epithelial–mesenchymal transition markers. Martin showed that protection from JNK inhibition could be conferred by inhibiting EGFR function and could be reversed by inhibiting ERK phosphorylation.

Cell populations must coordinate migration, proliferation and apoptosis—both spatially and temporally—to create organized multicellular tissues. For epithelia, three-dimensional culture models provide a method to explore the potential mechanisms that underlie this orchestration and have shown that extracellular factors

Fig 2 | Steps in metastasis. During the metastatic process, cancer cells proceed through a series of distinct steps to form an overt, secondary tumour. Cells detach from the primary tumour, invade adjacent tissue and then enter the lymphatic or circulatory system. Size constraints cause the cells to be arrested in distant organs, where they extravasate and enter the surrounding microenvironment. At this point, the cell might proliferate to form a clinically detectable metastasis or it might remain dormant as a single cell or micrometastasis. The specific factors governing the fate of disseminated cells are as yet unknown; however, it is thought to be largely dependent on favourable interactions between the tumour cell and its microenvironment. (Adapted from Chambers *et al*, 2002; Chambers & Matrisian, 1997.)

interact with an intrinsic differentiation programme to specify the architecture of epithelial tissues.

Invasion and metastasis

Breast cancer has the potential to spread to almost any region of the body, with the bone, lung and liver representing the most common secondary locations. However, as treatment options for advanced metastatic breast cancer are limited, this stage of the disease is responsible for the highest number of breast cancer deaths. Metastasis is a complex process composed of numerous distinct steps involving progressive alterations in the tumour cell, as well as tumour-induced alterations of the secondary microenvironment (Welch *et al*, 2000). Intravasation initiates metastasis and involves the escape of cancer cells from the primary tumour into the circulatory or lymphatic system, through which they are transported to distant organs. The movement of tumour cells in the circulation is typically arrested by the first capillary bed encountered, at which point the tumour cells must exit the circulatory system, enter the surrounding tissue and proliferate to form an overt, clinically significant

metastasis (Yoshida *et al*, 2000). However, the ability of the disseminated cells to survive and proliferate at the new site is dependent on favourable interactions between the tumour cell and the ectopic microenvironment (Chambers *et al*, 2002). Understanding the basic biology of each step in the metastatic process is essential for the development of improved therapeutic strategies (Fig 2).

Matrix-degrading proteases have long been suspected to have a role in breast cancer development and progression. Applying time-lapse microscopy, H. Yamaguchi (New York, NY, USA) discovered the ability of metastatic breast cancer cells to form specialized structures called invadopodia, which degrade the extracellular matrix. These ventral membrane protrusions are enriched in various proteases, adhesion molecules and membrane-remodelling proteins, and are thought to have a role in intravasation (Yamaguchi *et al*, 2005). B. Fingleton (Nashville, TN, USA) also emphasized the importance of proteases in breast cancer with data from a study of matrix metalloproteinase 9 (MMP-9)-null mice, which indicate that although the protease has no impact on primary tumour growth, it is crucial for the survival and establishment of distant metastases. Further analysis revealed that MMP-9 expression is localized to inflammatory cells and is coupled to an increase in vascular endothelial growth factor availability (Acuff *et al*, 2006). This study illustrates the importance of normal cells in tumour development and progression. These cells are typically conscripted by cancer cells and induced to secrete numerous factors—including chemokines, proteases and integrins—which allow the tumour cells to adapt to their environment (Woodhouse *et al*, 1997). MMP-9 released by tumour-activated inflammatory cells might help to prime the secondary site by creating a permissive, pre-metastatic niche for tumour cell growth (Kaplan *et al*, 2005).

Genes that regulate metastasis often function through inappropriate expression, as opposed to oncogenes and tumour suppressor genes the function of which is typically affected by mutations. G. Berx (Ghent, Belgium) highlighted the ability of transcription factors to activate programmes that drive invasion and migration. Berx showed that the transcription factors Smad-interacting protein 1 (SIP1), Snail and Slug bind the promoter region of E-cadherin and other junctional genes and repress their transcription in a coordinated fashion. Downregulation of E-cadherin through aberrant transcriptional regulation is a hallmark of tumour progression, resulting in epithelial-mesenchymal transition by disrupting cell-cell adhesion (Vandewalle *et al*, 2005). Thus, E-cadherin has been identified as a crucial metastasis suppressor through its role in the maintenance of epithelial cell interactions.

Other genes and pathways are also involved in metastasis. A. Burger (Baltimore, MD, USA) described a new, potential tumour suppressor gene—insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1)—which was found to show loss of heterozygosity in 50% of invasive breast cancers. This gene is also associated with inactivation of the retinoblastoma protein, cyclin E overexpression and increased proliferation of oestrogen-receptor-negative tumours (Burger *et al*, 2005).

Many studies have shown that a large number of cancer cells are shed from primary tumours; however, only a minute proportion of these cells survive the circulatory system, reach a secondary site and grow to form an overt metastasis. In fact, many of the disseminated cells persist in secondary organs as dormant, solitary cells or micrometastases (Chambers *et al*, 2002). A. Chambers (London, Ontario, Canada) presented fascinating intravital videomicroscopy

images that attested to the inefficiency of the metastatic process and the fate of disseminated tumour cells. However, although improvements in imaging technology have allowed the visualization of these dormant cells, their role in cancer progression is still uncertain. It might be that they represent quiescent populations awaiting activation or non-dividing, metabolically active cells responsible for priming the environment for incoming tumour cells.

Biomarkers in breast cancer

The mechanisms of tumour progression have been studied by enhanced imaging techniques on a macroscopic level, and by gene expression profiling of cancer cells on a molecular level. A major focus in the field of breast cancer research has been the search for new cancer biomarkers, which accurately diagnose and monitor disease, indicate recurrence, signify metastatic potential and predict therapeutic response. Oestrogen and progesterone receptors have been used as biomarkers for many years and are being complemented by human EGF receptor 2 (HER2)/NEU expression—a clinical biomarker as well as a therapeutic target (Tetu & Brisson, 1994).

New biomarkers are required to predict a patient's response to therapies, but only a few have gained US Food and Drug Administration approval in the recent past. The majority of node-negative breast cancer patients do not benefit from adjuvant chemotherapy. To spare such patients from this treatment, markers that differentiate between indolent and metastatic disease are required. M.J. Duffy (Dublin, Ireland) presented urokinase plasminogen activator (uPA) as a marker for metastatic potential, particularly when analysed in conjunction with its inhibitor PAI-1 (Duffy, 2002). By using human breast tumour samples linked to clinicopathological data and disease endpoints, D. Brennan (Dublin, Ireland) proposed that high-throughput proteomic techniques, such as tissue microarrays, offer an ideal platform for rapid validation of putative biomarkers.

However, single biomarkers might not be informative in a complex disease such as breast cancer. Global screening approaches, such as DNA microarrays and proteomic profiling techniques, have allowed the comparison of the entire gene expression patterns or proteomes of normal and malignant tissues. This has led to the identification of molecular 'signatures' with both prognostic and predictive value (Aparicio *et al*, 2000; Brennan *et al*, 2005). This method of disease classification is thought to be more specific and sensitive than the use of single markers as it takes into account the heterogeneity of breast cancer. On the basis of DNA microarrays, T. Sorlie (Oslo, Norway) has classified five molecular subtypes of breast cancer, which are defined by distinct patterns of gene expression (Sorlie *et al*, 2003). These subtypes show differences in metastatic potential, response to therapy and survival rates, and can thus be defined as different disease entities. They are detectable at an early stage of breast cancer; a re-analysis of previous datasets has confirmed their significance (Sorlie *et al*, 2006). R. Millikan (Chapel Hill, NC, USA) presented a population-based study examining the prevalence of these breast cancer subtypes (Carey *et al*, 2006) and their association with race and menopausal status. His results also indicate that each subtype can be associated with specific environmental or personal risk factors.

DNA microarray analysis has identified patterns of gene expression that can predict responses to different therapies, the likelihood of recurrence and metastatic potential of breast cancers (Brennan

et al, 2005). The potential to exploit these advances for patient-specific analysis and treatment is being pursued. R. Bernards (Amsterdam, The Netherlands) introduced the potential of RNA interference (RNAi)-based screens for analysing the mechanism of tumour progression and the mode of action of anti-cancer agents. RNAi screens are being used to identify genes that, when inactivated, cause resistance to conventional drugs or have a lethal effect on the tumour cell. This concept of 'synthetic lethality' might help to identify improved therapeutic targets (Brummelkamp *et al*, 2006).

Breast cancer susceptibility genes, such as breast cancer 1 (BRCA1), provide possible therapeutic opportunities in the context of synthetic lethality. P. Mullan (Belfast, UK) presented data showing how wild-type BRCA1 expression in breast tumours can cause resistance to DNA-damaging chemotherapy through the inhibition of apoptosis after BRCA1 and nuclear factor κ B (NF κ B) interaction. Alternatively, resistance might arise through transcriptional regulation as a result of BRCA1 and c-Myc interaction, which enforces DNA-damage cell-cycle checkpoints. This study also showed how low-level expression or mutation of BRCA1 can sensitize tumours to chemotherapy. Thus, BRCA1 could be used as a predictive biomarker of response to this treatment (Kennedy *et al*, 2004). Noting the similarities between BRCA1 mutant tumours and the basal subtype of breast tumours, Mullan's group also identified a transcriptional mechanism by which downregulation of BRCA1 could result in basal gene expression (Kennedy *et al*, 2005). Given that somatic mutations are rare in sporadic basal breast cancer, M. Esteller (Madrid, Spain) suggested promoter hypermethylation as a possible mechanism by which BRCA1 could be downregulated. Hypermethylation of promoter sequences, which inhibits the transcription of the affected gene, is a common feature of many human cancers (Alvarez *et al*, 2005; Esteller *et al*, 2000).

Therapeutic implications and future directions

Targeted therapies and rational molecular interventions should ideally result from detailed insights into the processes of tumour initiation and progression. Breast cancer treatment acts as a model for how these aims can be implemented. The effects of oestrogen on mammary gland development and the progression of breast cancer have been known for many years, and the oestrogen receptor was exploited as a drug target long before its molecular mechanisms as a transcriptional regulator were understood. Endocrine therapies, on the basis of oestrogen antagonists such as tamoxifen, that compete with oestrogen for binding to the receptor or, more recently, aromatase inhibitors that block the biosynthesis of oestrogen have been of tremendous clinical value (L. Martin, London, UK).

Despite these impressive benefits, there is room for improvement. For example, the mechanisms that underlie the resistance of tumour cells to tamoxifen treatment must still be elucidated. This might be revealed by the detailed molecular analysis of the high-molecular-weight protein complexes that assemble and disassemble at regular intervals on oestrogen-receptor-regulated promoter sequences (F. Gannon, Heidelberg, Germany). These insights offer many new points of intervention in oestrogen-induced gene transcription beyond receptor–ligand interactions. However, unconventional drugs might be required to make use of these insights. For example, it might be necessary to disrupt protein–protein or protein–DNA interactions, functions that low-molecular-weight compounds cannot achieve. The use of target-specific peptides, delivered into cells in constrained conformations, provides a strategy to achieve this goal (B. Groner, Frankfurt, Germany).

Finally, mammary stem cells could provide new perspectives for understanding the origin of tumours and possibly for preventive and therapeutic measures (M. Smalley, London, UK). The identification of adult breast epithelial stem cells, their relation to breast cancer stem cells, the contribution of tumour stem cells to drug responsiveness, tumour growth and recurrence after treatment are issues that will dominate discussions in the future. They will keep the scientific thrill at least on a par with investigations on the *Drosophila* eye.

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