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SHORT COMMUNICATION

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Method for the generation and cultivation of functional three-dimensional mammary constructs without exogenous extracellular matrix

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Abstract During puberty, pregnancy, lactation and post-lactation, breast tissue undergoes extensive remodelling and the disruption of these events can lead to cancer. In vitro studies of mammary tissue and its malignant transformation regularly employ mammary epithelial cells cultivated on matrigel or floating collagen rafts. In these cultures, mammary epithelial cells assemble into three-dimensional structures resembling in vivo acini. We present a novel technique for generating functional mammary constructs without the use of matrix substitutes.

Keywords Mammary epithelial cell · Breast tissue · Mammary acini · Breast cancer · Three-dimensional culture · Mouse (C57bl/6×CBA/F1)

Introduction

In 2000, an estimated 1.05 million new cases of breast cancer were diagnosed worldwide (Parkin 2001), placing it second only to lung cancer in cancer incidence. This high frequency is believed to be partially a result of the highly active nature of normal breast tissue. During puberty, pregnancy, lactation and post-lactation, breast tissue undergoes extensive remodelling and functional change. The underlying processes of proliferation, migration, differentiation and apoptosis require precise control during these periods of change and their disruption can lead to the development of cancer (Ip and Darcy 1996; Park et al. 2000). As such, in vitro systems in which disruptions can be induced and examined are useful.

Commonly employed methods for the cultivation of in vitro functional three-dimensional mammary constructs involve the cultivation of isolated mammary epithelial cells (MEC) on matrigel or floating collagen rafts (Petersen et al. 1992; Ip and Darcy 1996; Debnath et al. 2003). In these cultures, MEC assemble into three-dimensional structures resembling in vivo acini. However, matrigel and collagen gels may exert subtle but significant effects on cellular behaviour through the presence of modulators of growth and differentiation and through the non-native structure, composition and abundance of extracellular matrix (ECM) elements (Ip and Darcy 1996). Using the hanging-drop method of spheroid cultivation (Kelm et al. 2003), we are able to generate in vitro mammary acini constructs in the absence of matrix substitutes.

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Materials and methods

Isolation and cultivation of murine MEC

The MEC were isolated as previously described (Pullan and Streuli 1996). Briefly, pregnant C57bl/6×CBA/F1 mice were sacrificed 17 days post-coitus. Their mammary glands were removed and digested in serum-free media containing 0.2% collagenase solution (type III, Worthington) for 90 min. The resulting cell slurry was filtered through a 100-μm sieve. The filtrate was washed by centrifugation and transferred to tissue culture dishes for 1 h to allow the differential adherence of fibroblasts. Unattached cells were then transferred into tissue culture flasks and cultivated in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (JRH), 10 μg/ml insulin (Sigma, St. Louis, Mo., USA), 10 ng/ml recombinant epidermal growth factor (rEGF, Chemicon) and 10 μg/ml hydrocortisone (Sigma), at 37°C, 5% CO_2 and 98% humidity.

Hanging-drop cultivation

The MEC were dissociated from monolayers by incubation in 0.05% EDTA for 5–7 min followed by trypsination and enumerated by trypan blue counting. The cell suspension was used to initiate hanging-drop cultures as previously described (Kelm et al. 2003). Aliquots of 20 μl cell suspension were dispensed into each well of a 60-well mini tray (Nunc) and the tray was inverted. Initial seed densities between 50 cells/well and 1,000 cells/well were evaluated. Hanging-drop cultures were incubated as described above with the addition of 6 μl fresh media at day 6. For cultures in which milk production was desired, 2 μg/ml (final) ovine prolactin (oPRL, National Hormone and Peptide Program, USA) was also added at day 6.

Immunofluorescence microscopy

At day 12, hanging-drop MEC cultures were harvested and fixed in 4% paraformaldehyde for 15 min. Samples were blocked and permeablised in PBS with 6% bovine serum albumin (BSA), fraction V, and 0.1% Triton X-100 for 4 h. Primary labelling was achieved by overnight incubation with rabbit anti-mouse laminin (Chemicon) or rabbit anti-mouse milk antibodies (Accurate Chemical and Scientific Corporation, Westbury, N.Y., USA), at dilutions of 1:100 in PBS + 1% BSA and 0.1% Triton-X100. Following washes in PBS, AlexaFluor647 goat anti-rabbit antibody (Molecular Probes) was incubated with the cultures for at least 4 h in the same buffer solution as for the primary antibodies. Samples were washed, RNase-treated for 20 min, counterstained with propidium iodide (Molecular Probes) and mounted in 80% glycerol solution. Alternatively, nuclei were labelled in a single step with TOPRO-3 (Molecular Probes). Visualisation was accomplished on a BioRad Radiance 2000 confocal laser scanning microscopy system.

Results and discussion

Functional mammary acini structures are commonly cultivated from isolated MEC on a matrix substitute. Cells attach to the matrix, develop into a multicellular structure by proliferation and undergo central apoptosis resulting in a central luminal space (Blatchford et al. 1999; Debnath et al. 2003).

Here, we describe a novel method for generating functional mammospheres in the absence of exogenous matrix. When cultivated as hanging-drops, cells initially settled at the air–medium interface, collected into small clusters and developed into aggregates (Fig. 1a–d). From approximately day 6 onwards, constructs displayed an organised morphology when viewed by phase-contrast microscopy, similar to the morphology observed when cultivated on matrix substitutes (Fig. 2a). TOPRO-3 labelling revealed that the constructs were hollow and surrounded by a single cell layer (Fig. 2b).

We have successfully formed spheroidal constructs from freshly isolated MEC and from MEC maintained up to five passages. A single passage tends to remove cell clumps and debris, thus improving construct yield. In our hands, passaging MEC on standard tissue culture plastic beyond passage 2 leads to reduced viability and an increase in debris; this is associated with a reduced yield. We have also formed spheroidal constructs from MEC that had been frozen in complete growth medium with 10% dimethyl-sulfoxide. Again, however, reduced viability and an increase in debris reduced the yield of well-formed constructs. In the absence of dead cells and debris, i.e. with MEC from the first or second passage, the yield is greater than 50 spheroidal constructs in a 60-well plate.

Spheroidal constructs have been formed by using an initial seed density of as few as 50 cells/well to as many as 1,000 cells/well. The initial seed density affects the final construct diameter. Constructs produced from an initial seed of 50 cells/well have an average diameter of 83 μm (±23%), whereas constructs produced from an initial seed of 1,000 cells/well have an average diameter of 137 μm (±19%).

Spheroidal constructs of approximately 100 μm in size are formed from an initial seed density of 100 cells/well. As is the case with the similarly sized matrix-based constructs, handling these small constructs during multiple labelling is cumbersome and many are lost in processing. This problem can be overcome by using an initial cell density of 1,000 cells/well. Whereas the resulting constructs deform somewhat during processing, the larger constructs are more readily labelled with minimal loss.

Labelling these larger constructs with antibodies against the basement membrane glycoprotein laminin and a nuclear

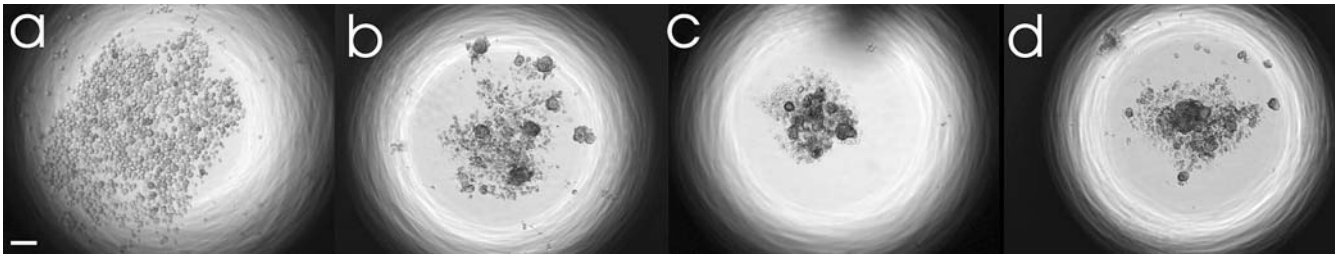

Fig. 1 MEC in hanging-drop culture initially settle at the medium/air interface (**a** 30 min) and subsequently collect into small aggregates (**b** 24 h). These aggregates agglomerate into larger structures (**c**, **d** 72 h and 120 h, respectively). *Bar* 100 μm

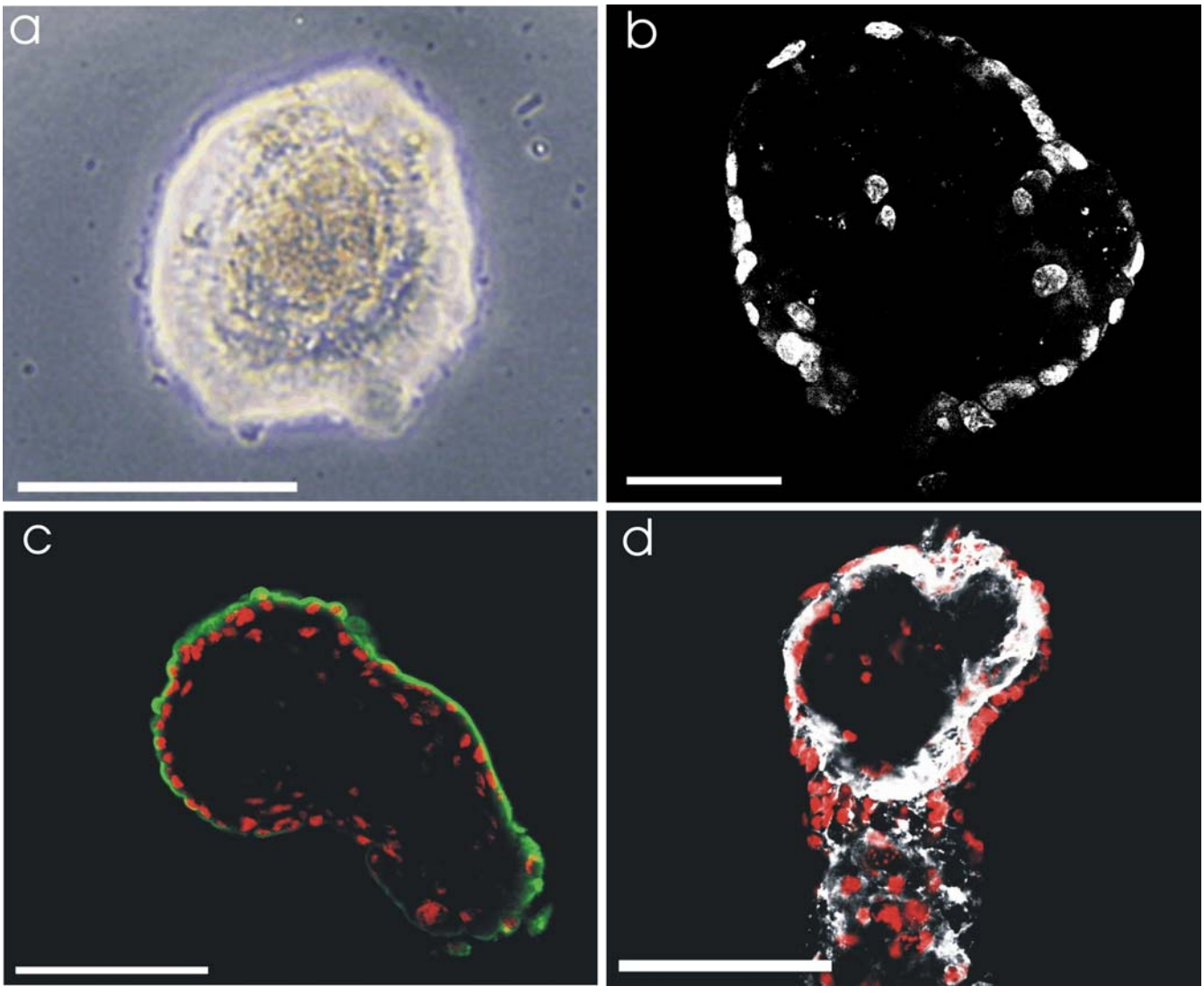

Fig. 2 **a** Hanging-drop mammary constructs generated from 100 cells/well at day 11. **b** TOPRO-3 stained nuclei (*white*) show that structures are comprised of a single layer of cells. **c** Three-dimensional mammary constructs are composed of a layer of cells (*red* nuclei) surrounding a central luminal space. Laminin is secreted towards the basal surface of the structures (*green*). **d** When cultivated with oPRL, milk (*white*) is detected within cells and the luminal space. Multicellular spheroids made from 3T3 cells were used as a negative control for milk production, and no signal was detected. *Bars* 100 μm

stain revealed that the resulting structures were hollow, consisting of a layer of MEC covered with laminin (Fig. 2c).

Under appropriate hormonal stimulation, mammary acini cultivated on matrix substitutes produce and secrete milk proteins into the central space (Ip and Darcy 1996). Constructs generated by the hanging-drop method also display this functional characteristic, with milk proteins being detectable within cells and the luminal space (Fig. 2d).

Interaction with the ECM is critical to the development and function of mammary acini (Petersen et al. 1992; Boudreau et al. 1995; Blatchford et al. 1999; Jolivet et al. 2001; El-Sabban et al. 2003; Taddei et al. 2003) and a crucial aspect in the development of malignancies (Park et al. 2000; Wiseman and Werb 2002). Although existing methods attempt to re-establish this interaction through the use of exogenous ECM substitutes, we have demonstrated that MEC have the capacity to produce their own ECM. This native ECM is sufficient for the development/maintenance of a functional phenotype and might more accurately portray conditions in vivo.

In addition to avoiding the use of non-native matrix substitutes, the hanging-drop format permits individual controlled manipulation of the culture environment. This is of benefit in co-culture applications in which the contribution of different populations can be readily defined. For example, for studying MEC/stroma interactions, a defined number of fibroblasts can be introduced to each well resulting in reproducible multi-component constructs (data not shown).

Although we have employed media supplemented with 10% fetal bovine serum in the cultivation of our constructs, previous experience with other cell types has shown that, in many cases, the amount of serum can be significantly reduced and, in several instances, serum-free cultivation is possible. We present the method in its cur-

rent form as a platform for the further development of "matrix"-free systems for the cultivation of mammary acini and other primary tissue constructs.

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