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Unanticipated Reversal of Polarity in Primary Human Mammospheres Cultured in Ultra-Low Attachment Plates

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In experiments to determine susceptibility of primary human mammary cells to infection with HIV type 1 as part of HIV breast milk pathogenesis studies,¹ our intentions were to block HIV receptor binding, halt viral replication, or prevent endocytosis using pharmaceutical preparations. For the purposes of these studies, we obtained human mammary epithelial cells (MECs) from consenting reduction mammoplasty patients, digested the tissue, and enriched for BerEp4⁺ MECs per published protocols.^{2–6} Our initial series of experiments were conducted by culturing enriched MECs on tissue culture plates or on transwell membranes (BD/Falcon, Franklin Lakes, NJ).¹ Recently, we extended these studies to three-dimensional (3D) mammary epithelial models by culturing BerEp4⁺ MECs either in Matrigel™ (BD Biosciences, Franklin Lakes, New Jersey)⁷ or on ultra-low attachment plates (Corning®, Corning, New York)⁴ to allow for formation of spherical structures possessing similar properties to those *in vivo*. 3D culture of MECs is utilized frequently for *in vitro* studies of epithelial stem cell dynamics, epithelial differentiation, and response to experimental drugs (as reviewed by Elliot and Yuan⁸).

In vivo, mammary spheres are sites of milk production. The outer surface of milk-producing spheres, or alveoli, contains basement membrane (BM) proteins, and is the surface in closest proximity to the bloodstream. The apical surface, comprising milk-secreting villi, deposits milk constituents into the milk duct or lumen.⁹ We chose the ultra-low attachment protocol as one of our culture methods because of the fact that we were interested in selecting for survival and clonal expansion of mammary stem and progenitor cells,⁴ and anticipated that our 3D cultures under ultra-low attachment conditions would demonstrate similar polarity.

Here, we report a novel observation that culturing BerEp4⁺ MECs from three distinct patients under ultra-low attachment conditions for 20 days in complete mammary epithelial growth medium (Lonza, Rockland, Maine) resulted in mammosphere formation in which the polar orientation was opposite to that observed *in vivo* and in MECs cultured in Matrigel™ (BD Biosciences). These observations were visualized by transmission electron microscopy (TEM) (Fig. 1). Apical villi and tight junctions were readily observed on the outer surface of the spheres. Conversely, the luminal surface was smooth and appeared to contain a protein layer, suggesting the presence of a BM with accompanying proteins.

Within the spheres, we observed both secretory MEC, with a general cuboidal/rectangular shape, and myoepithelial MEC, which possessed elongated morphology and actin filaments.

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However, cells appeared disorganized within the sphere and distinct layers of luminal epithelia and myoepithelia were not observed. We also noted that presence of villi on the outer spherical surface was independent of the cell subtype present on the outer most surface.

In order to further ensure that we were visualizing “true” mammospheres, that is, cells of multipotent lineage with self-renewing capacity, and not acinar structures directly derived from patient tissue, we conducted additional *in vitro* experiments. We repeated culturing primary spheres in ultra-low attachment plates from patient cells previously stored in liquid nitrogen. This was followed by dissociating spheres into single-cell suspension using Accumax™ solution (Innovative Cell Technologies, San Diego, California), and reseeded single cells on fresh ultralow attachment plates for secondary sphere culture. In all cases, cells from primary spheres were able to seed secondary spheres, indicating that a subset of the cells retained their self-renewing capacity. Primary cells from patients, as well as primary spheres and secondary spheres were then analyzed by quantitative PCR for relative abundance of the luminal epithelial marker, CK19, as well as the basal/ myoepithelial markers CK17 and CK14. A recognized marker of basal epithelia, Δ Np63, is also required for the preservation of replicative capacity in epithelial stem cells.^{10,11} Therefore, Δ Np63 mRNA levels were evaluated as a surrogate for mammary-stem-cell-enriched populations. We found that although results from patient samples differed slightly, mRNAs for luminal, basal, and stem cell markers were present in secondary mammospheres (Fig. 2), confirming the primary spheres’ multipotent and self-renewing capacity.

The mechanisms underlying the changes in polarity are unclear; however, previous studies of 3D cultures of primary cells and established cell lines from mouse and human suggest that culturing stem/progenitor cells on substrata such as Matrigel™ (BD Biosciences) induces acinar structures with superior cell differentiation as defined by phenotype^{4,12} (and as reviewed by Elliot and Yuan⁸).

Immunostaining mammospheres for apical and BM surface proteins and examining them using confocal microscopy likely would have saved us a great deal of time and expense. In prior experiments, we had used primary antibody fluorophore staining for BerEp4 to confirm that the spheres were of epithelial origin. However, we were particularly interested in viewing pathogen attachment or endocytosis, and chose TEM as the ideal means for observation, without first staining for apical and BM proteins.

We are not aware of published literature in which mammospheres cultured in ultra-low attachment plates have been characterized by TEM. Our observations may be of interest to the greater research community, depending on the experimental application. For example, for our purposes, it is of critical importance to ensure that mammosphere morphology is similar to that *in vivo* when applying pathogens or pharmaceuticals to these culture conditions, as either pathogen attachment and/or drug absorption may be affected. However, if spheres are completely dissociated to single-cell suspension prior to exposure to experimental conditions, orientation of apical and basolateral surfaces may be irrelevant. Because we did not expect the spheres cultured in this manner to be reversed in orientation, we submit this *Lesson Learned* to disseminate our findings.

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Figure 1.

Human primary mammary epithelial cells cultured in ultra-low attachment plates result in alveolar structures with reversed polarity. (a) Cross-section of a portion of mammosphere structure containing several mammary cells, as observed by TEM. Particulate visualized around the outer surface is debris, possibly from ultra-low attachment plate surface coating. MECs, mammary epithelial cells; L, luminal surface. (b) Enlargement from outer surface. V, villi; TJs, tight junctions. (c) Enlargement from inner surface. L, luminal; BM, basement membrane.

Figure 2.

Cellular heterogeneity within mammospheres reflects the developmental potency of sphere-initiating cells. (a) Gene expression analysis from cells from primary MEC preparation #52 that were BerEp4 enriched (blue), grown as primary mammospheres (red), or grown as secondary mammospheres (green) indicate expression of luminal epithelial and basal/myoepithelial markers. (b) Gene expression analysis from cells from primary MEC preparation #42 that were BerEp4 enriched (blue), grown as primary mammospheres (red), or grown as secondary mammospheres (green) indicate expression of luminal epithelial and basal/myoepithelial markers. (c) Melting curve data from the quantitative PCR analysis indicate that all messages were amplified with high fidelity.