Received: 12 March 2023 | Revised: 30 March 2023 | Accepted: 12 April 2023

DOI: 10.1002/mba2.52

HIGHLIGHT

MedComm–BIOMATERIALS AND APPLICATIONS WILEY

Biomimetic hydrogel supporting baths as an alternative to initiate and maintain breast tumor-derived organoids culture

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Funding information
H2020 European Research Council, Grant/Award Number: ERC Starting Grant 848325

Prince et al.[1] have been exploring cancer pathophysiology approaches by successfully culturing in vitro patient-derived tumor organoids (PDOs). Indeed, cancer remains one of the world's most devastating diseases, with a substantial and growing global burden. In 2019, there were an estimated 23.6 million new cancer cases and 10.0 million cancer deaths globally. Since 2010, these have represented a 26.3% increase in new cases and a 20.9% increase in deaths.[2] From those, breast cancer is still the most common cancer worldwide and has a mortality-to-incidence ratio of 15%. It holds highly intrinsic tumor genetic/phenotypic variations and intratumor heterogeneity, making each patient with breast cancer unique.

The current breast cancer in vitro models to study drug development and native pathophysiological mechanisms have shown an absence of patient specificity, limited structural integrity, stability over the cell culture period, and limited recreation of the complex three-dimensional tumor microenvironment, which almost exclusively depends on the culture of PDOs in basement membrane extract (BME) systems.[3] Hydrogels have emerged as a viable method for promoting the development and maintenance of organoids in vitro in recent years. In fact, biomimetic hydrogels, by emulating the extracellular matrix (ECM), can create a more physiologically realistic microenvironment for cells while providing physical support, hence enhancing their growth, development, and function. Recently, Prince et al.[1] proposed a biomimetic hydrogel that supports the initiation and growth of PDOs, EKGel, as an alternative culture system for BME, such as Cultrex, which are commercially available matrices derived from mouse tumor that contains high levels of ECM proteins (e.g., laminin, collagen IV).

EKGel is a nanofibrillar hydrogel that consists of Schiff base crosslinks between aldehyde groups on the surface of cellulose nanocrystals (a-CNCs) and amine groups of lysine residues in gelatin (Figure 1A). On one hand, the arginine–glycine–aspartate integrin receptor-binding motif, present in gelatin, is also present in native ECM proteins, which facilitate cell–matrix interactions. On the other hand, the network of fibers formed by the rod-like a-CNCs have similar dimensions (43 ± 17 nm) to in vivo tumor collagen fibrils. EKGel showed a Darcy permeability, which measures the convection-driven transport, more than two orders of magnitude larger than the reported values for BME (1.9×10^{-11} cm^2), due to its significantly larger pores. By changing a-CNC concentration (0.5–3.75 wt%), EKGel obtained Young's

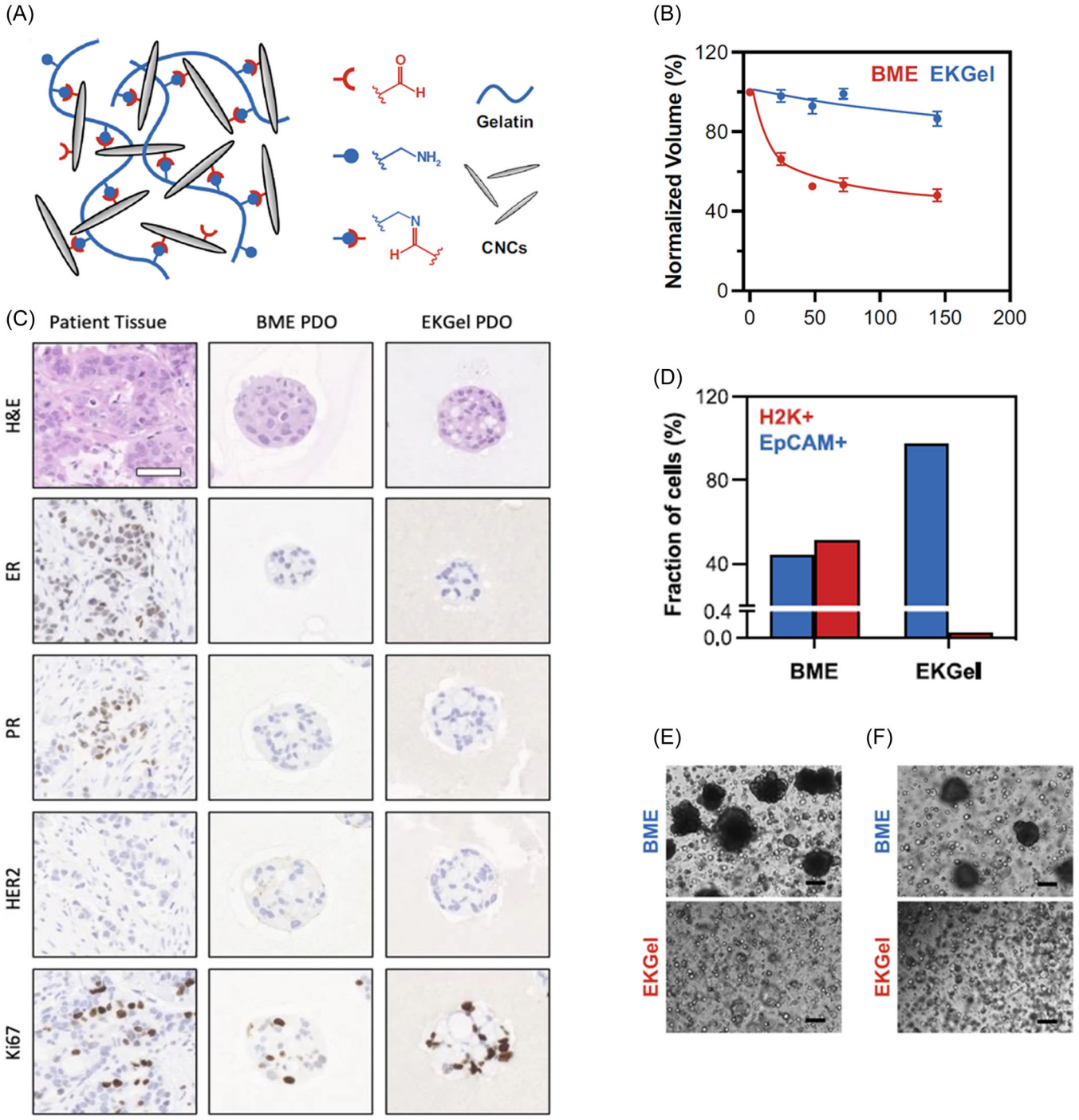

FIGURE 1 (A) Schematic representation of the EKGel networks. (B) Temporal volume loss of EKGel and BME under continuous perfusion of cell culture medium. (C) Hematoxylin and eosin staining, and immunohistochemistry of PDOs grown in BME and EKGel and of their respective primary tissue. (D) Comparison of the fraction of cells that are H2K+ or EpCAM+ for PDXO initiated in BME and EKGel. (E) Brightfield microscopy images of undepleted PDX cells 14 days after plating in BME or EKGel and (F) mouse cell-depleted PDX sample from the same tumor plated in BME or EKGel for 14 days in culture. Scale bars: (C) 100 μm, (E, F) 100 μm. Copyright Permission from Prince et al.[1] BME, basement membrane extract; PDO, patient-derived tumor organoid.

modulus (24–3738 Pa) within the stiffness range of ECM in breast tumor biopsies (1.2–3.7 kPa). Furthermore, EKGel was more stable under shear-induced stress (14% relative volume reduction) than BME (60% relative volume reduction), Figure 1B. The imine covalent crosslinks present on EKGel, which hydrolyze over time, contribute to a slow degradation, whereas BME is rapidly degraded in culture. This indicates the potential application of EKGel in microfluidic organoid-on-a-chip models, which is a significant benefit relative to BME.

To compare PDOs initiation and expansion in either EKGel or BME, isolated breast cancer cells with different receptor statuses and tissue sources were encapsulated on both systems. After 2 h of gelation, the cell-laden hydrogel was overlaid with breast cancer organoid media and the cells were cultured for 2–3 weeks. The observed PDO initiation and maintenance were similarly representative of in vivo breast tumor in many aspects on both systems. PDOs consisted of spherical clusters of human epithelial cells, with no statistically significant differences in shape or size diameter. Moreover, actively dividing cells were present after >2 weeks of culture, as indicated by Ki67 positive staining (Figure 1C). Results demonstrated that EKGel is suitable for long-term passage and maintenance of breast PDOs since the organoid diameters for each organoid line were consistent over four passages. Regarding histologic features, such as the relative abundance of eosinophilic to amphophilic cytoplasm, cytoplasmic vacuolization, and chromatin pattern, both types of PDOs showed similar results to the parental tumor (Figure 1C). To assess the gene expression differences between PDOs grown in different matrices, RNA sequencing of cells isolated either from organoid cultures or the corresponding primary tumor was performed. Results showed high similarity between PDOs formed on different growth matrices (average Spearman's correlation = 0.89) and between PDOs and their originating tumors (average Spearman's correlation = 0.83). The six genes that were found to be differently expressed did not show to be important to breast tumor progression.

Regarding the chemosensitivity of PDOs, no significant differences compared to commonly used drugs, such as doxorubicin and paclitaxel, were demonstrated. Moreover, xenografts generated from PDOs were treated with paclitaxel, demonstrating similar results for organoids growth rates cultured in both systems.

Furthermore, EKGel showed to be very important to prevent the contamination and overgrowth of murine cells upon the initiation of patient-derived xenograft-derived organoids (PDXOs), which is commonly observed in BME. Often, for long-term PDXO cultures, a mouse cell depletion step is required to minimize the chances of murine host cells overtaking the organoid. While PDX grown in BME had 47% of the samples contaminated, of the same 17 PDXO grown in EKGel, none presented larger dark clusters of mouse cells, as observed in Figure 1F. The absence of such contamination was confirmed by flow cytometry and immunofluorescence staining. The sample initiated in BME was highly positive for H2K (mouse cells), while this marker was only residual on the sample plated in EKGel (Figure 1D). This was only confirmed for noncancerous mouse cells, as the proliferation of mouse mammary tumor cells in BME and EKGel was similar. This can be justified by the presence of murine-derived bioactive molecules in BME,[3] which can promote a higher proliferation of murine noncancerous cells that depend on growth factors as opposed to tumor cells that can escape this dependence. Moreover, when plating the cells directly in EKGel without the mouse cell depletion step, no mouse cell contamination was observed on the patient-derived xenograft (PDX) tumors analyzed, bringing up an interesting advantage of EKGel over BME (Figure 1E,F).

In summary, this comparative study enabled the validation of EKGel as a support bath for PDOs culture. Importantly, the initiation rate, growth, tumorigenicity, drug response, histopathological properties, and gene expression patterns of the breast PDOs were consistent between EKGel and BME. No major alterations were observed when comparing these properties with the corresponding source tissue. Besides the similarity obtained between these systems, EKGel allowed a decrease in batch-to-batch variability, showed high stability, and provided controlled mechanical properties that can be fine-tuned to improve PDO initiation rates in the future (e.g., test the impact of different EKGel stiffnesses on PDO culture). Moreover, since EKGel can suppress the overgrowth of normal mouse cells, this system can be explored to also suppress overgrowth and contamination by normal human cells, which is a huge challenge for PDO in vitro cultures. Taking into consideration the CNC surface chemistry, the hydrogel can play a relevant role in the active modulation of the biological microenvironment by adding biologically relevant structural proteins (e.g., fibronectin), growth factors, and cytokines of human origin, which are present in the ECM.[4] For example, this can be achieved by the incorporation of platelet lysate to the gel, as previously performed, to improve the biological activity of natural polymers.[5] Thus, EKGel has the potential to serve as a platform for different organoid models, enabling the creation of in vitro systems for translational research that capture intra- and interpatient heterogeneity, another step towards personalized cancer medicine.

AUTHOR CONTRIBUTIONS

Jhenifer Oliveira: Conceptualization (supporting); writing—original draft (lead); writing—review and editing (lead). **Mariana Pereira**: Conceptualization (supporting); writing—original draft (equal); writing—review and editing (equal). **Bárbara B. Mendes**: Conceptualization (equal); writing—original draft (equal); writing—review and editing (equal). **João Conde**: Conceptualization (equal); writing—original draft (equal); writing—review

and editing (equal). All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the European Research Council (ERC) Starting Grant (ERC-StG-2019-848325 to João Conde) and (DAI/2021/14 to Jhenifer Oliveira) and the Fundação para a Ciência e Tecnologia FCT Grant (PTDC/BTM-MAT/4738/2020 to João Conde) and (2022.07775.PTDC to Bárbara B. Mendes).

CONFLICT OF INTEREST STATEMENT

João Conde is a cofounder and shareholder of TargTex S.A. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Not applicable.

ETHICS STATEMENT

Not applicable.

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How to cite this article: Oliveira J, Pereira M, Mendes BB, Conde J. Biomimetic hydrogel supporting baths as an alternative to initiate and maintain breast tumor-derived organoids culture. *MedComm – Biomater Appl*. 2023;2:e52. doi:10.1002/mba2.52