

# Biomimicking of the Breast Tumor Microenvironment

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**Abstract** The tumor microenvironment is well known to play a role in sustaining malignant transformation of tissue, tumor progression, and in drug responsiveness; however, much remains unclear about the interplay between tumor cells, the extracellular matrix, and stroma cells. The extracellular matrix has been shown to elicit both biochemical and biophysical signaling, and matrix rigidity is an important microenvironmental parameter in the regulation of cellular behavior. Thus, tissue engineering and the development of novel biomaterials that mimic mechanical and topological properties of tumor stroma and can cope with the effect of mechanical forces are promising approaches to study this interplay. New in vitro

tools to investigate the effect of mechanical signals on breast cancer cell aggressiveness and drug sensitivity include genipin-crosslinked gelatin hydrogel scaffolds with adjustable degrees of stiffness.

**Keywords** Breast cancer · Extracellular matrix · 3D tumor tissue model · Mechanical characterisation · MALDI mass spectrometry · Glycans

## Introduction

### Microenvironment and Cancer Progression

In recent years, the role of the microenvironment in maintaining tissue specificity and organ structure and in promoting or inhibiting progression to malignancy has been widely recognized [1•]. The mammary gland is a dynamic tissue composed of epithelial cells and surrounding stroma which not only modulates the normal development of the gland but also actively participates in its malignant transformation, contributing to tumor phenotype and disease progression. This suggests that the tumor tissue goes beyond the properties of the tumor epithelium itself and requires interconnections with the surrounding microenvironment [2, 3]. The growing interest in deciphering the role of the tumor microenvironment in cancer progression is reflected by several recent studies based on gene expression profiling of tumor stroma [4–6]. For example, extensive gene expression changes have been observed in the stroma associated with ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC), suggesting the co-evolution of the tumor adjacent stroma with epithelium even before tumor invasion and supporting the important role of stromal changes in the transition from pre-invasive to invasive tumor growth [4]. Moreover, stromal characteristics have been

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shown to provide novel biological and clinically relevant insights into breast cancer progression [5, 6]. These studies demonstrate that the tumor microenvironment is an important player in tumorigenesis. Analyses of expression patterns of genes encoding extracellular matrix (ECM) molecules have shown that stromal expression patterns can vary among breast carcinomas and may be clinically quite independent of the intrinsic characteristics of neoplastic cells [7, 8]. Recently, we identified an ECM gene expression signature (ECM3) in ~40 % of breast carcinomas that defines an independent group of tumors and has prognostic significance related to tumor differentiation status, stratifying a subgroup with poor prognosis only within the most undifferentiated grade III tumors [9, 10]. The extracellular matrix is the major player of this niche composed of a large collection of biochemically distinct components including proteins, glycoproteins, proteoglycans, and polysaccharides with different physical and biochemical properties [11]. Communication between cells and the microenvironment occurs through a complex cascade of molecular signals generated by cell-matrix interactions as well as by the interplay between epithelial, stromal, and other organ-specific cell types (i.e., fibroblasts, adipocytes, myoepithelial cells, immune cells) [1•]. The cooperation between the mechanical microenvironment and the intrinsic cell state plays a key role in tumor progression through mechanical responsive sensors such as integrins, focal adhesion kinase, and cytoskeletal molecules able to elicit a specific cellular response [12, 13, 14•].

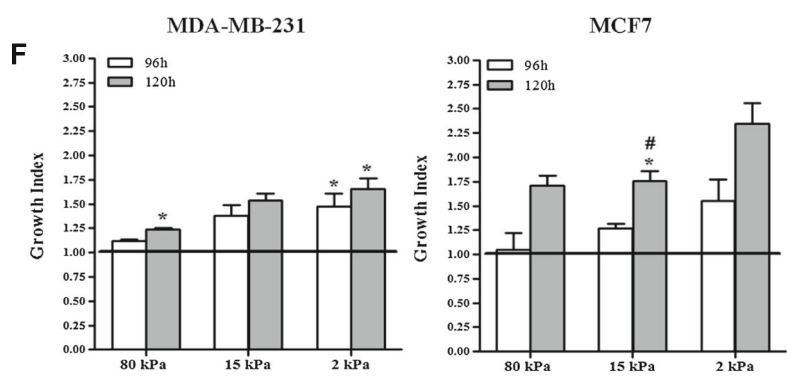
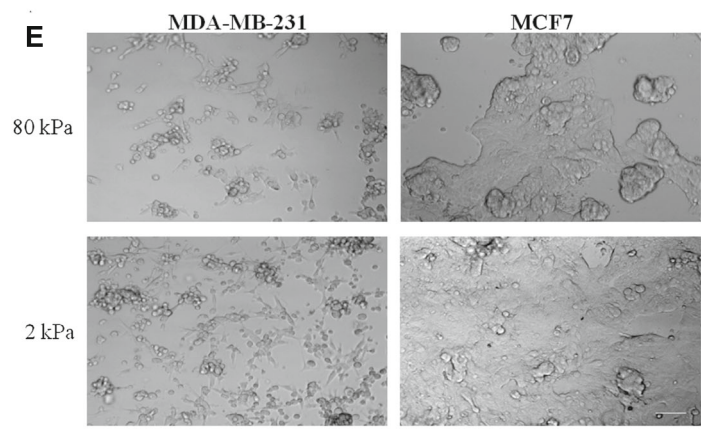
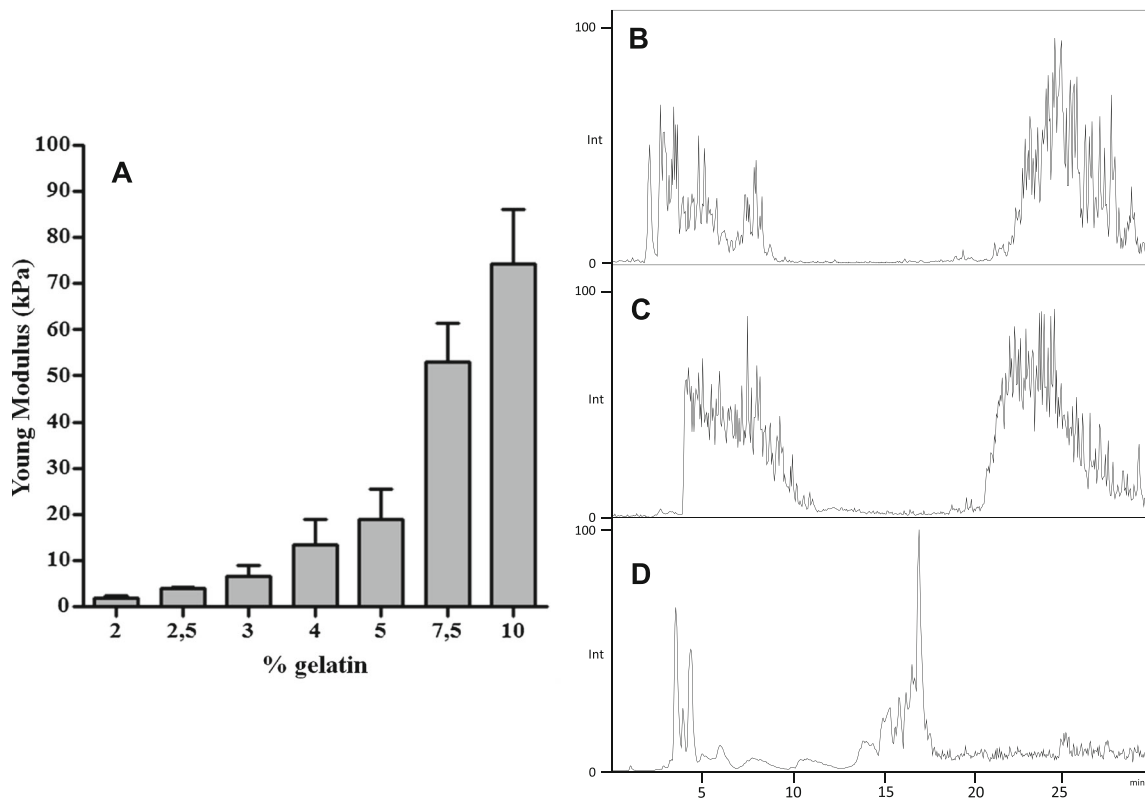
Alteration of ECM biochemical properties during microenvironment remodeling induced by changes in protein abundance and post-translational modifications potentiates the oncogenic effect of various signaling pathways (e.g., ERK, PI3K, TGF- $\beta$ , and RhoA/Rac signaling), representing relevant cancer hallmarks [15]. In addition, the architecture and other physical characteristics of tumor-associated ECM may affect the properties of the stroma to change tissue rigidity. Epithelial cancers are characterized by an altered tissue tensional homeostasis that reflects the increment of cell-generated forces in transformed cells, an increased compression due to the solid-state pressure exerted by the expanding tumor mass, and matrix stiffening associated with a desmoplastic response [13, 16]. The malignant transformation of normal breast tissue is associated with significant matrix remodeling that triggers progressive stiffening [2]. Indeed, rigidity of breast cancer stroma is typically 10-fold higher than that in normal tissue [17]. Models able to reproduce tumor complexity can provide tools for tumor-stroma interaction studies to pinpoint the molecular mechanisms involved in aggressiveness and resistance to therapy.

### Biological Tools Mimicking the Tumor Microenvironment

Traditional in vitro culture platforms to study the role of the ECM in cancer have their limitations, and cancer biologists

look with growing interest to the field of tissue engineering as a promising approach to obtaining “functional” in vitro tumors [18, 19] for a better understanding of chemical and mechanical interactions between tumors and the microenvironment [20]. One avenue is through the development of novel biomaterials that mimic biophysical, mechanical, and topologic properties of tumor stroma [21•]. Current approaches to tissue engineering have focused on hydrogel materials displaying ECM-like biophysical properties that provide dynamic microenvironments for cell fate regulation [18, 22]. Natural hydrogels are derived from or are themselves components of the ECM, such as Matrigel, collagen and fibrin scaffolds, whereas synthetic hydrogels are typically composed of polymers whose representative biomaterial is polyethylene glycol (PEG). Natural hydrogel have been widely used for 3D microenvironment mimicking to support tumor growth and angiogenesis, since they can be proteolytically degraded and remodeled by most cultured cell types [18]. Matrigel is a basement membrane-derived hydrogel containing laminin as the primary component. Extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, Matrigel is characterized by high cytocompatibility and cell adhesion sites and can change its physical properties over time [22, 23]. Collagen hydrogels, based on the most abundant ECM protein, are rich in cell-interactive ligands and thus able to reproduce a fibrous architecture similar to collagen structures of native ECM and to provide a bioactive microenvironment for cell culture; several different crosslinking methods are available to obtain different structures [19, 24, 25]. Fibrin hydrogels, obtained via polymerization of fibrinogen with thrombin and calcium ions,

**Fig. 1** **a** Young modulus of gelatin-genepein scaffold as a function of gelatin percentage. **b** Base peak chromatogram of glycans released after PNGase F treatment of **b** 2 kPa, **c** 15 kPa, and **d** 80 kPa gelatin scaffold. PNGase F (Promega, Milano, Italy) digestion was carried out on the gelatin layer for 16 h at 37 °C using 3 U/ $\mu$ L of enzyme in ammonium bicarbonate buffer (pH 8.4). Released glycans were analyzed by LC-MS/MS. Mass spectra were recorded using an Amazon Speed ETD mass spectrometer (Bruker Daltonics, Bremen, Germany) interfaced to an Easy-nLC chromatograph (Proxeon, Waltham, MA, USA). Glycans were separated by reverse-phase C18 chromatography and eluted on a 30-min ACN/0.1 % formic acid (buffer B) gradient (Thermo, Waltham, MA, USA). **e** Representative areas of MDA-MB-231 and MCF7 cells cultured on 80 and 2 kPa scaffolds in 24-well plates for 120 h. Images were acquired with an optical microscope (Nikon Te-S) at  $\times 10$  magnification (scale bar: 100  $\mu$ m). **f** Histograms showing growth index of cells on scaffolds at different time points from cell seeding using the alamarBlue assay. At each time point, fluorescence intensity was read at 30 min and at 2.5 h, when alamarBlue was added to the medium, using Tecan ULTRA Plate Reader XFLUOR4 version (MTX Lab Systems, Inc., VA, USA) at excitation 535 nm and emission 590 nm. The 72-h time point was considered as reference to calculate the growth index. #0.01 <  $p$  < 0.05 compared with the 72 h condition; \*0.01 <  $p$  < 0.05 compared with the 80 kPa scaffold at the 120-h time point. Data are representative of one of three independent experiments in duplicate determinations. (Error bars=SD)



have also been widely used due to their nano/macro-fibrous architecture mimicking native ECM [26]. Increasing attention has focused on the development of synthetic hydrogels that present a time of proteolytic degradability, cell adhesion sites, and matrix stiffness similar to those during neo-tissue development; PEG-based hydrogel materials, characterized by a non-bioreactive structure coated with bioactive molecules (e.g., cell adhesion peptide, RGD sequence) or crosslinked with biopolymers (e.g., collagen, gelatin), fulfill those requirements [22]. Other hydrolytically degradable polymers in the polyester group, such as poly(lactic acid) or its poly(lactic-co-glycolic acid) (PLG) copolymer, have also been widely used due to their non-cytotoxic degradation products and their tunable matrix mechanical properties [27].

The ECM elicits not only biochemical but also biophysical signaling, and matrix stiffness is an important microenvironmental parameter that cooperates in regulating cell behavior both in promoting a malignant phenotype and in affecting response to therapy [16, 17, 28, 29]. Thus, the development of new *in vitro* tools able to cope with the effect of mechanical forces is essential both to study tumor progression and to foster the discovery of anti-tumor drugs. To date, the most widely used substrates to generate scaffolds for assessment of the mechanical effects on tumor cells have been the flexible polyacrylamide substrates coated with collagen I [30–32], collagen I or collagen I and fibronectin [33, 34], collagen I hydrogels [19], and growth factor-reduced Matrigel [35]; however, those studies showed that the stiffness increment perturbs fibril topology and matrix pore size, introducing confounding biophysical cues and affecting the ECM natural topology. These side effects are relevant and affect the sensing on matrix organization, rendering them unsuitable for studies on breast carcinoma cells. Recently, a new self-assembling peptide hydrogel (SAP) has been described that recreates a native-like ECM fibril topology with minimal variability in pore size [36]. This hydrogel was able to influence the organization of mammary epithelial cell colonies with cell dissemination into the surrounding matrix; however, despite the high performance in recreating mechanical ECM properties, this system shows mechanical fragility, limiting its use for high-throughput analyses, e.g., for the screening of novel therapeutics designed to target tumor cells or modify tumor ECM.

### Genipin-Crosslinked Gelatin Hydrogel Scaffolds for Breast Cancer Cell Line Culture

To reproduce the cell environment, a scaffold must mimic the biochemical, mechanical, and topological features of the ECM matrix. In recent years, efforts have focused on the use of natural polymers, principally based on ECM proteins and their derivatives, to produce scaffolds that mimic these ECM features. Tunable mechanical properties are especially important

when developing tissue-specific scaffolds, where alterations in such features can be introduced by physical, enzymatic, or chemical crosslinking [37–39]. For example, gelatin is normally soluble in cell culture media but must be crosslinked to obtain a 3D self-consistent scaffold. To achieve this, different crosslinkers have been used, either synthetic such as glutaraldehyde or natural such as genipin, derived from gardenia fruit and non-toxic [40].

We have developed genipin-crosslinked gelatin hydrogel scaffolds to study their potential usefulness in analyzing the role of stiffness in the behavior of breast carcinoma cells according to their intrinsic molecular characteristics and in high-throughput assays to screen for new therapeutics. Genipin was chosen among other crosslinking agents to be sure of crosslinking reaction in the inner parts of the final samples, as indicated by blue coloring upon its spontaneous reaction with amino acids and proteins and because it is less toxic of other crosslinkers. The genipin-fixed scaffold resists enzymatic degradation at a level comparable to that of glutaraldehyde-fixed tissue, but genipin is less cytotoxic than glutaraldehyde [41, 42]. These scaffolds were generated with stiffness values between 2 and 80 kPa based on the typical value of 2–4 kPa reported for the Young acellular tumor extracellular matrix [43] and on the 16 kPa to 130 kPa increase in the elastic modulus with progression of invasive breast carcinoma [44, 45].

As shown in Fig. 1a, we are able to modulate the elastic modulus of scaffolds by changing the gelatin concentration, with increased stiffness upon increasing concentrations of gelatin, to mimic the tumor tissue microenvironment. In the context of structure characterization, the profile of exposed N-linked oligosaccharides is a peculiar feature of genipin scaffolds with different gelatin concentrations (2, 4, and 10 % corresponding to 2, 15, and 80 kPa, respectively). Gelatin matrix crosslinked with genipin treated with PNGase F and analyzed by LC-MS/MS revealed a different profile of glycans, depending on the gelatin concentration (Fig. 1b–d). While the base peak chromatograms of cleaved oligosaccharides from 2- and 15-kPa samples exhibited similar profiles, the 80-kPa sample appeared different, suggesting that a more rigid support can induce changes in exposed glycans or that the glycan moiety plays an active role in matrix polymerization contributing to the polymer tridimensional structure and stiffness [46]. A better mechanistic understanding of the relationship between the bio-mimicked microenvironment and cell behavior awaits an in-depth characterization of the glycan surface exposed to protein interactions in scaffolds with different gelatin concentrations.

Evaluation of our hydrogel materials as new tools for cells in culture using two human cell lines representative of two breast cancer molecular intrinsic subtypes, i.e., MDA-MB-231 for triple-negative and MCF7 for luminal A tumors, showed that both cell lines survived and grew on scaffolds



with different stiffness (an elastic modulus of 2, 15, and 80 kPa), with significant and trend toward slowing proliferation as stiffness increased in MDA-MB-231 and MCF7, respectively (Fig. 1e, f). Furthermore, our genipin-crosslinked gelatin hydrogel scaffolds affected breast cancer cell behavior, inducing a more aggressive phenotype in both cell lines regardless of their intrinsic characteristics, according to their elastic modulus values. In MDA-MB-231 cells, a 2- to 3-fold increase in their migration potential was observed as a function of increased stiffness, while MCF7 cells, which are unable to migrate after culture in plastic plates, were induced to migrate when cultured on the stiffest scaffolds.

## Conclusions

The established role of the tumor microenvironment in cancer progression, which mainly relies on biochemical properties, architecture, and other physical properties of tumor-associated ECM, has led to new approaches in tissue engineering to study the cancer-ECM relationship. The genipin-crosslinked gelatin hydrogel scaffold, which mimics not only biochemical but also mechanical forces of the tumor cell microenvironment, represents an available model in which it is possible to modulate stiffness. This scaffold could represent effective tools to investigate the effect of mechanical signals on tumor cell aggressiveness and drug sensitivity.

## Compliance with Ethics Guidelines

**Conflict of Interest** Marta Giussani, Carmelo De Maria, Vasso Michele, Francesca Montemurro, Tiziana Triulzi, Elda Tagliabue, Cecilia Gelfi, and Giovanni Vozzi declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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