Mammary Tissue-Derived Extracellular Matrix Hydrogels Reveal the Role of the Irradiated Microenvironment in Breast Cancer Recurrence

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Abstract

Radiation therapy (RT) is essential for triple negative breast cancer (TNBC) treatment. However, patients with TNBC continue to experience recurrence after RT. Extracellular matrix (ECM) alternations in healthy breast tissue induced by radiation and its role in tumor recurrence are still unknown. In this study, we evaluated the structure, molecular composition, and mechanical properties of irradiated murine mammary fat pads (MFPs) and developed ECM hydrogels from decellularized tissues to assess the effects of RT-induced ECM changes on breast cancer cell behavior. Irradiated MFPs were characterized by increased ECM deposition and fiber density compared to unirradiated controls, which may provide a platform for cell invasion and proliferation. ECM component changes in collagens I, IV, and VI, and fibronectin were observed following irradiation. Encapsulated TNBC cell proliferation was enhanced in irradiated ECM hydrogels, and morphology analysis indicated an increase in invasive capacity. In addition, TNBC cells co-cultured with macrophages in irradiated ECM hydrogels exhibited further increases in cell proliferation. Our study establishes that the ECM in the irradiated microenvironment promotes TNBC invasion and proliferation that is enhanced in the presence of macrophages. This work represents an important step toward elucidating how changes in the ECM after RT contribute to breast cancer recurrence.

Keywords: Radiation therapy; Breast cancer recurrence; ECM hydrogels; Proliferation; Invasion; Macrophages

1. Introduction

Breast cancer is the second leading cause of cancer related mortality in women [1]. One widely used treatment for patients with breast cancer is radiation therapy (RT), which typically enhances antitumor effects and eliminates residual tumor cells after surgery and chemotherapy. However, approximately 20% of breast cancer patients suffer locoregional recurrence after initial treatment, especially in triple-negative breast cancer (TNBC) where patients have higher mortality compared to other subtypes [2,3]. Over 13% of treated TNBC patients suffer local recurrence at the primary site [4]. Previous work has shown that radiation can induce excess macrophage infiltration and ultimately tumor cell recruitment under immunocompromised conditions, suggesting that recurrence following RT may be dependent on immune status [5,6]. Therefore, understanding mechanisms behind recurrence post-RT in TNBC is critical for improved patient survival.

The extracellular matrix (ECM) in the tumor microenvironment plays a major role in tumor progression and metastasis. ECM structure, composition, and mechanical properties have profound effects on cell and tissue phenotype. Recent studies have revealed that ECM deposition is altered by RT-induced fibrosis and that tumor cells grow differently on ECM depending on the tissue origin [7,8]. We are therefore motivated to determine the effect of the irradiated ECM on tumor and immune cell behavior, which has not yet been explored. A variety of synthetic hydrogels have been designed to study the impact of ECM on cell behavior. However, these synthetic systems have many limitations in recapitulating the native ECM. Decellularized ECM (dECM) hydrogels prepared from mammalian organs retain the composition of the original tissue and are considered promising biological scaffolds for cell growth and tissue regeneration. For example, dECM hydrogels have been engineered from multiple organs, including adipose tissue, dermis, or bladder, and then applied to cell culture *in vitro* or as natural injectable materials to repair and reconstruct tissues *in vivo* [9–11]. In this study, we developed dECM hydrogels to replicate the *in vivo* adipose tissue microenvironment and study the influence of the irradiated ECM on tumor recurrence *in vitro*.

We hypothesized that RT-induced ECM alterations may contribute to local recurrence by facilitating tumor cell growth and invasion. To test this hypothesis, we first analyzed the structure, composition, and mechanical properties of murine mammary fat pads (MFPs) following irradiation. We then fabricated dECM hydrogels from MFPs and evaluated the role of the irradiated ECM on tumor and immune cell behavior. This work represents a crucial step toward elucidating how modulation of the ECM after RT contributes to breast cancer recurrence.

2. Methods and Materials

2.1 Animals

Animal studies were performed in accordance with institutional guidelines and protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee. 8-10-week-old female Nu/Nu mice were obtained from Charles River Laboratories. The mice were allowed free access to standard diet and water and maintained under a 12 hr light/12 hr dark cycle.

2.2 Preparation of MFP-derived dECM hydrogels

MFP-derived dECM was prepared as previously described [12]. In brief, MFPs were harvested from sacrificed Nu/Nu mice using CO_2 asphyxiation followed by cervical dislocation and were then irradiated at a dose of 20 Gy *ex vivo* using a cesium source. After two days incubation in a 37 °C/ 5% CO_2 incubator, MFPs were stored at -80 °C. Thawed MFPs were treated with 0.02% w/v trypsin/0.05% w/v EDTA (Gibco) in deionized water for 1 hr, 3% v/v Triton-X-100 (Sigma-Aldrich) for 1hr, 4% w/v deoxycholic acid (Frontier Scientific) for 1 hr, 1% v/v penicillin-streptomycin (Gibco) at 4 °C overnight, and 4% v/v ethanol/0.1% v/v peracetic acid (Sigma-Aldrich) for 2 hr. MFPs were then treated to 4x 15 min washes of phosphate-buffered saline (PBS) (Quality Biological, Inc.), 100% n-propanol (Fisher Scientific) incubation for 1hr, and 4x 15 min washes of deionized water. Decellularized and delipidated MFPs were then frozen and lyophilized for use in hydrogel preparation. The lyophilized MFP ECM samples were ground into a powder and mixed into 0.1 M hydrochloric acid–pepsin solution (1 mg/mL) (Sigma-Aldrich) at a concentration of 20 mg/mL, and enzymatically digested for 48 hr under a constant stir rate at room temperature. The digestion was ended by titration to pH 7.4 with 1 M NaOH and a 10x PBS solution bringing the pre-gel solution to 1x PBS. The dECM hydrogel is liquid at room temperature and solidifies after 30 min incubation at 37 °C.

2.3 Cell lines

GFP- and luciferase-labeled 4T1 mouse mammary carcinoma cells were obtained from Dr. Laura Bronsart (Stanford University) in December 2017. Unlabeled 4T1 cells were obtained from ATCC. MDA-MB-231 human breast cancer parental cells were obtained from Dr. Amato Giaccia (Stanford University) in August 2011. MDA-MB-231 cells were transduced with retrovirus particles encoding for the expression of firefly luciferase gene. All cells were cultured at 37°C and 5% CO_2. Bone marrow derived macrophages (BMDMs) were isolated from the femurs of 8-10-week-old female Nu/Nu mice [13,14]. All cell lines tested negative for *Mycoplasma* contamination with the MycoAlert Mycoplasma detection kit (Lonza). 4T1 cells were cultured in in RPMI-1640 (Gibco),

MDA-MB-231 cells were cultured in DMEM (Gibco), and both were supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) (Sigma-Aldrich). Isolated BMDMs were cultured in IMDM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 10 ng/mL macrophage colony stimulating factor (MCSF)(Gibco), and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) for 7 days for maturation into macrophages.

2.4 Cell culture in dECM hydrogels

GFP- and luciferase-labeled 4T1 and unlabeled 4T1 cells were seeded in dECM hydrogels at a concentration of $1x10^5$ or $5x10^5$ cells/mL of pre-gel solution, respectively. GFP- and luciferase-labeled MDA-MB-231 cells were encapsulated in dECM hydrogels at a concentration of $5x10^5$ or $10x10^5$ cells/mL of pre-gel solution. 100 µL of the gel-cell solution was added into each well of a 16-well chamber slide (Thermo Fisher Scientific). Cells were thoroughly mixed in the pre-gel solution and then incubated at 37°C for 30 min. 100 µL of complete RPMI media for 4T1 cells or complete DMEM media for MDA-MB-231 cells was added to each well. After culturing at 37 °C for 48 hr, cell proliferation was measured by adding luciferin (0.167 mg/mL) to the wells for 10 min and performing bioluminescence imaging (Caliper LifeSciences IVIS Lumina Series III). The GFP-labeled cells were also visualized using fluorescence microscopy (Leica DMi8) at 0, 24, and 48 hr after gelation.

BMDMs were seeded in dECM hydrogels at concentrations of $5x10^5$ or $10x10^5$ cells/mL of pre-gel solution. 100 µL of gel-cell solution was added into each well of a 16-well chamber slide. Cells were thoroughly mixed in the pre-gel solution and then incubated at 37 °C for 30 min. 100 µL of complete IMDM media were added to each well, and cells were cultured at 37°C for 48 hr. For co-culture experiments, BMDMs ($10x10^5$ cells/mL) were stained with CellTrace™ Far Red Cell Proliferation Kit (Thermo Fisher Scientific) immediately before culturing in 100 µL pre-gel solution with GFP- and luciferase-labeled 4T1 cells ($5x10^5$ cells/mL). 50 µL of complete IMDM media, 50 µL of complete RPMI (Gibco) media, and 10 ng/mL MCSF were added to each well after gel formation and subsequently incubated at 37 °C for 48 hr. Both cell lines were visualized using fluorescence microscopy at 0, 24, and 48 hr post-gelation. Proliferation of 4T1 cells was quantified by bioluminescence measurements at 48 hr. Cell number per field for 4T1 cells and BMDMs were quantified using ImageJ (National Institutes of Health).

2.5 Live/Dead assay

Cell viability was examined by the LIVE/DEAD™ Cell Viability Assay Kit (Molecular Probes, Inc.) 48 hr after gelation. In brief, encapsulated unlabeled 4T1 cells or BMDMs were washed twice in Dulbecco's PBS (DPBS) (Gibco). 100 µL of DPBS containing 1 µM calcein AM (excitation/emission = 494/517 nm) and 2 µM ethidium homodimer (excitation/emission = 528/645 nm) was then added to the wells and measured using a Varioskan plate reader (Thermo Fisher Scientific) after 30 min. Cells were then visualized by fluorescence microscopy (Leica DMi8).

2.6 Scanning electron microscopy (SEM) tissue preparation and quantification

MFP structure was examined using SEM (Quanta 250 E-SEM), and fiber network characteristics were quantified using a previously developed image analysis algorithm run on MATLAB software [15]. MFPs were fixed in cold 2% v/v glutaraldehyde with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) for 4 hr followed by one wash in the same buffer. Fixed tissues were placed in 1% aqueous osmium tetroxide solution (OsO_4) (Electron Microscopy Sciences) for 1 hr and then washed twice with milli-Q water. MFPs were dehydrated in a graded series of alcohol (50, 70, 90, 100% ethanol in deionized water) for 10 min per wash and then left in 100% ethanol (Thermo Fisher Scientific) overnight at 4°C. After three additional 45 min changes in 100% ethanol, MFPs were slowly critical point dried (Tousimis). After drying, tissues were sputter coated (Ted Pella, Inc.) with a 4.5 nm thick gold/palladium alloy coating and imaged with SEM. A complete set of fiber network descriptors was collected from SEM images of each MFP: fiber alignment, node density (number of fiber intersections per mm^2), and fiber diameter. Fiber alignment was described through the normalized orientation index where 0% represents a randomly organized (isotropic) network while 100% represents a completely aligned (anisotropic) network. Porosity was described through the mean of the pore size histogram (mm^2). Automated extraction of these fiber architectural features was achieved with an algorithm, which has been previously described in detail [15]. Briefly, the SEM image is digitally processed by a cascade of steps, including equalization with a 3x3 median filter, local thresholding through the Otsu method, thinning, smoothing, application of morphological operators, skeletonization, binary filtering for Delaunay network refinement, and ultimately the detection of fiber network architecture and its descriptors.

2.7 Raman spectroscopy

MFPs were fixed in 10% neutral buffered formalin (NBF) for 24 hr at 4 °C and submerged in 30% sucrose (Sigma-Aldrich) for 48 hr at 4 °C. The fixed samples were then embedded in OCT and cut into 5 µm sections onto Raman grade CaF_2 disks (Sigma-Aldrich). The Raman measurements

were taken with 5 s exposure time using a Renishaw inVia Raman microscope system with a 785 nm laser that delivered ~30 mW of power. A ×100 objective lens was used to focus a laser spot on the surface of samples. Raman spectra were analyzed by a custom MATLAB (R2019b) code to perform smoothing and biological fluorescent background subtraction. The spectra were first smoothed by using the Savitzky and Golay filter with fifth order and coefficient value of 15. Modified polynomial fit method was then performed to remove the background fluorescence by using a 7th order polynomial with a threshold of 0.0001. Afterwards, Raman spectra were normalized using min-max normalization method. Principal components analysis (PCA) was performed by using the MATLAB built-in "PCA" function. Two principal components were plotted to discriminate two groups.

2.8 Atomic force microscopy (AFM)

MFPs or dECM hydrogels were cut into 1mm x 1mm x 1mm pieces, and each specimen was immobilized on 22 mm x 22 mm coverslip with a thin layer of two-component fast drying epoxy glue (Devcon). All AFM indentations were performed using a Bruker inverted optical AFM and silicon nitride triangle cantilever with a 10 µm diameter borosilicate spherical tip (205 mm-long DNP-S10 triangular silicon nitride cantilevers, resonance frequency (air) f =18 kHz, nominal cantilever spring constant k = 0.08 Nm, tip radius = 5 µm, Bruker). The exact spring constant (k) of the cantilever was determined before each experiment using the thermal tune method and the deflection sensitivity was determined in fluid using glass substrates as an infinitely stiff reference material. Force-distance (FD) measurements of excised tissues were performed in a fluid cell. Samples were indented with a calibrated force of 5 nN and the Hertz model of impact was used to determine the elastic properties of the tissue.

2.9 Luminex multiplex immunoassay

Conditioned media (CM) from GFP- and luciferase-labeled 4T1 cells cultured in dECM hydrogels was collected after 2 days incubation. Supernatant collected following centrifugation at 1,100 rpm to remove cells was stored at -80 °C until processed. Three replicates were collected independently. All samples were evaluated using a mouse 32-plex Affymetrix kit (Eve Technologies Corporation). **Supplementary Table S1** lists all cytokines analyzed.

2.10 Histological analysis and immunofluorescence (IF)

MFPs were removed from 8-10-week-old female Nu/Nu mice and irradiated to a dose of 20 Gy *ex vivo* using a cesium source. After 48 hr incubation at 37 °C in complete RPMI media, control

and irradiated MFPs were fixed in 10% NBF for 48 hr, paraffin-embedded, and sectioned (5 μm). For hematoxylin and eosin (H&E) staining (Sigma-Aldrich), standard procedures were followed, including deparaffinization, hydration, staining with hematoxylin, and counterstaining with eosin. For Masson's trichrome staining (Thermo Fisher Scientific), sample preparation included deparaffinization, rehydration, staining with Weigert's iron hematoxylin and Biebrich scarlet-acid fuchsin, differentiation in phosphomolybdic-phosphotungstic acid solution, and staining in aniline blue solution. For IF staining, tissue sections (5 μm) were deparaffinized followed by antigen retrieval using 1X citrate buffer. After blocking in 10% goat serum, sections were incubated with primary antibodies, including anti-collagen I (1:200, Thermo Fisher Scientific), anti-collagen IV (1:200, Thermo Fisher Scientific), anti-collagen VI (1:200, Abcam) and anti-fibronectin (1:400, Thermo Fisher Scientific) overnight at 4°C. Fluorescently labeled secondary antibodies (Alexa Fluor 488) were used to stain tissues for 1 hr at room temperature followed by applying Sudan Black B (Acros Organics) to reduce autofluorescence. Sections were mounted using ProLong glass antifade mountant with NucBlue stain (Thermo Fisher Scientific) and imaged using a fluorescence microscope. For F-actin staining, cells after 48 hr incubation in dECM hydrogels were fixed in 10% NBF for 10 min and washed with PBS. Cell membranes were permeabilized by 0.1% Triton X-100 in PBS for 5 min and washed by PBS. Cells were then incubated with phalloidin (Phalloidin-iFluor 594, Abcam) for 1 hr at room temperature and washed by PBS. For cortactin staining, cells after fixation and permeabilization by similar methods were blocked with 5% NGS in PBS for 1 hr at room temperature. Cells were incubated with anti-cortactin (1:50, Novus Biologicals) primary antibody in 1% BSA/PBS overnight at 4 °C followed by three washes in PBS. Cells were then incubated with secondary antibodies (goat anti-rabbit IgG Alexa-Fluor 488; Thermo Fisher Scientific) for 1 hr at RT and washed with PBS. For Ki67 staining, BMDMs were fixed, permeabilized, and blocked followed by incubation with anti-Ki67 (1:200, Abcam) primary antibody in 1% BSA/PBS overnight at 4 °C followed by three washes in PBS. Cells were then incubated with secondary antibodies (goat anti-rabbit IgG Alexa-Fluor 488; Thermo Fisher Scientific) for 1 hr at room temperature and washed with PBS. Chambers were removed and mounted on a glass slides with ProLong glass antifade mountant with NucBlue stain.

2.11 Patient data analysis

We used The Cancer Genome Atlas (TCGA) to obtain patient mRNA expression. The TCGA TARGET GTEx study was accessed through the University of California Santa Cruz's Xena platform (https://xenabrowser.net/) on May 3rd, 2022 to evaluate how gene expression of *Col4A3BP* and *Col4A2-AS1* in the normal adjacent tissue of breast cancer patients correlated

with patient outcome. The Xena visualization tool was used to generate Kaplan-Meier curves (https://xenabrowser.net/, n = 113 patients). To investigate breast cancer patient gene expression, we accessed mRNA data from the TCGA cBioPortal (PanCancer dataset, n = 1,082 patients) on May 10th, 2022. From these data, correlations between collagen, invadopodia, and secreted cytokine mRNA expression were determined. We also interrogated publicly available datasets though KMplotter (kmplot.com), which is a meta-analysis tool. Breast cancer patients were filtered based on ER, PR, and HER2 positivity. TNBC patients (n= 126 patients) were used for analysis and stratified based on high or low expression of C-C motif chemokine ligand (*CCL2*) with data reported as probability of survival.

2.12 Statistical analysis

In each experiment, obtained data are presented as the mean ± 95% confidence limit. Differences among more than two groups were tested using one-way analysis of variance (ANOVA), and Student's t-tests were performed to compare differences between two groups. $p<0.05$ was considered significant. All analyses were performed using GraphPad Prism 9. A custom MATLAB code was used to perform smoothing and biological fluorescent background subtraction of Raman spectra. Smoothing of the data was done by following the Savitzsky and Golay method with fifth order and coefficient value of 33. A modified polynomial fit method was performed to subtract the background fluorescence. A polynomial with seventh order was used to fit the Raman spectra with threshold of 0.0001.

3. Results

3.1 Structure and composition of control and irradiated MFPs

SEM images of control and irradiated MFPs revealed more ECM fibers covering irradiated adipose cells than unirradiated adipose cells as well as thinner and denser fibers after radiation (**Fig. 1A-D**). We subsequently quantified ECM fiber network characteristics using fiber network analysis software to confirm our qualitative observations [15] (**Fig.1E-H**). Collagen deposition was evaluated by the fiber covered area ratio parameter, which increased significantly after radiation ($p<0.01$) (**Fig. 1E**). Visual inspection of the algorithm output showed accurate automatic detection of the fiber network for MFPs (**Supplementary Fig. S1A**). Both fiber diameter and pore size decreased significantly in irradiated MFPs ($p<0.05$) (**Fig. 1F-G**). The node density of irradiated MFPs also increased significantly (**Fig. 1H**). However, the porosity and orientation index showed no significant changes between control and irradiated samples (**Supplementary Fig. S1B-C**).

We then used Raman spectroscopy to evaluate the biochemical composition changes following irradiation of MFPs (**Supplementary Fig. S2A**). The peaks at 1300 cm^{-1} are from lipids, which are responsible for vibrations of $-(CH_2)_n-$ in-plane twist and fatty acids [16,17]. The peaks at 1265 cm^{-1} (amide III) and 1650 cm^{-1} (amide I) are from the collagen matrix [18,19]. **Supplementary Figs. S2B-C** show that collagen/lipid ratios (both amide I/lipid and amide III/lipid) increased significantly in MFPs ($p<0.01$) after RT, confirming the increase in ECM deposition observed using SEM images. In an effort to elucidate the biochemical changes between irradiated and control samples, we used PCA to simplify the complexity of high-dimensional data while retaining relevant information necessary for classification [20–22]. In a two-dimensional PC scatter plot, the first principal component (PC1) and the second principal component (PC2) showed a clear separation between the control and irradiated groups with a variance level of 29.36% for PC1 and 9.66% for PC2, again confirming compositional changes induced by RT (**Supplementary Fig. S2D**). Normalized Raman spectra peak analysis validated this difference, especially for the collagen peaks (**Supplementary Table S2**).

3.2 Mechanical properties and ECM component analysis

SEM images and Raman spectroscopy results indicated that ECM fibers increased in irradiated MFPs. To characterize the impact of increased ECM fibers on tissue biomechanical properties, AFM was used to determine the stiffness of MFPs. FD measurements (**Supplementary Fig. S3**) were taken to determine Young's moduli [23]. The range of elastic moduli for control and irradiated breast tissue is shown in **Fig. 2A** and **Fig. 2B**. The histogram of stiffness values from control MFPs reveals a bimodal stiffness distribution with two prominent peaks at 0.2 kPa ('peak 1') and 0.6 kPa ('peak 2') (**Fig. 2A**). In comparison, irradiated MFPs exhibit a bimodal stiffness distribution with two prominent peaks at 0.4 kPa ('peak 1') and 1 kPa ('peak 2'). At values higher than 2 kPa, a broadening in the distribution indicates a marked mechanical heterogeneity across the sample. The average Young's modulus for irradiated MFPs was 2.4-fold greater than control MFPs (**Fig. 2B**). H&E staining confirmed an increase in overall ECM in irradiated MFPs (**Supplementary Fig. S4**). To determine how ECM components in normal breast tissue impact patient outcomes, we obtained mRNA expression data from the normal adjacent tissue of patients with breast cancer from TCGA datasets. We found that high mRNA expression of collagen type IV alpha-3-binding protein *(Col4A3BP)* and collagen type IV alpha-2 antisense RNA 1*(Col4A2-AS1)* were correlated with poor overall survival (**Fig. 2C**). *Col4A3BP* phosphorylates the non-collagenous domain of the α3 chain of collagen IV and is associated with EMT and mesh-like collagen IV formation [24] while *Col4A2-AS1* is a long non-coding RNA for collagen IV α2 that is associated with the growth and

metastasis of breast cancer cells [25]. These associations led us to investigate specific changes in ECM components in irradiated breast tissue. Following IF staining, we found that collagen I, collagen IV, collagen VI, and fibronectin (FN) were increased in irradiated murine breast tissue (**Fig. 2D**). These data show that RT modulates the expression of ECM subcomponents and leads to changes in ECM deposition and stiffness, which may influence tumor behavior and negatively impact patient survival in breast cancer.

3.3 dECM hydrogels

Motivated by the RT-induced increase in ECM deposition that may provide a platform for cell adhesion and invasion, we next utilized a decellularization technique to extract ECM, develop dECM hydrogels, and encapsulate cells to observe their behavior. We used an immunodeficient model to replicate *in vivo* conditions relevant to recurrence [6]. Murine MFPs were decellularized by a series of mechanical, chemical, and enzymatic methods [12]. The final volume of decellularized MFPs was 10–20% of the original adipose tissue. H&E staining confirmed the removal of cell nuclei while preserving tissue structure following decellularization (**Fig. 3A**). We also verified the retention of ECM using Masson's trichrome staining as well as lipid removal using perilipin staining. IF staining showed that decellularization of MFPs retained post-RT trends in collagen I, collagen IV, collagen VI, and FN expression (**Supplementary Fig. S5**). The above results indicate that most of the extracellular components were preserved while the cells and lipids were removed from decellularized MFPs. To evaluate the structural and stiffness characteristics of control and irradiated dECM hydrogels, SEM and AFM were performed. SEM was utilized to determine the architecture of dECM hydrogels (**Fig. 3B**). Automatic fiber quantification of SEM images showed the parameters of fiber diameter, node density, and pore size were altered in irradiated dECM hydrogels (**Fig. 3C**), which parallels the changes observed in irradiated MFPs. Furthermore, AFM measurements showed that irradiated dECM hydrogel stiffness was significantly higher than those for control hydrogels. Together, these data demonstrate the integrity of ECM after decellularization and that the changes in dECM hydrogels between control and irradiated samples are consistent in control and irradiated MFPs.

3.4 Proliferation and invasion of breast cancer cells in dECM hydrogels

We encapsulated 4T1 murine TNBC cells in dECM hydrogels and evaluated their response to RT-induced ECM changes after 48 hr (**Fig. 4A**). A Live/Dead viability assay showed that dECM hydrogels are not cytotoxic to 4T1 cells (**Supplementary Fig. S6A-B**). The ratio of live to dead cells was higher in the irradiated group, indicating 4T1 cell proliferation in the irradiated dECM

hydrogels (**Supplementary Fig. S6C**). Additionally, IF images and bioluminescence were used to quantify the proliferation of GFP- and luciferase-labeled 4T1 cells in dECM hydrogels (**Fig. 4B**). 4T1 cell proliferation increased significantly in irradiated dECM hydrogels compared to unirradiated controls ($p<0.05$). MDA-MB-231 human TNBC cells also showed enhanced proliferation in the irradiated microenvironment (**Supplementary Fig. S7A-C**), suggesting that irradiated dECM hydrogels promote tumor cell proliferation.

We further visualized the cytoskeletal properties of 4T1 cells embedded in the dECM hydrogels. The driving force for cancer invasion into their surrounding microenvironment as well as numerous cellular physical processes is through localized actin filaments [26–29]. Therefore, we stained for F-actin using phalloidin, and cellular morphology was quantified in the irradiated dECM hydrogels (**Fig. 4C-D**). Elongation index and form factor can be used as measures of cell morphology, where high elongation index and low form factor indicate higher invasiveness [30,31]. Cells in the irradiated group increased their elongation index ($p<0.05$) and reduced their form factor significantly ($p<0.05$), suggesting cell invasiveness increases in the irradiated microenvironment. We also examined invadopodia, which are protrusive structures that can remodel the ECM and facilitate invasive migration [32–34]. The colocalization of F-actin and cortactin are considered reliable indicators for invadopodia [35]. F-actin and cortactin colocalization revealed significantly more invadopodia in 4T1 cells within irradiated hydrogels ($p<0.05$), indicating a higher invasive capacity of 4T1 cells influenced by an irradiated microenvironment (**Fig. 4C-D**). Tyrosine kinase substrate 4 (TKS4) and WASP interacting protein (WIP) are key molecular components that modulate invadopodia extension [36]. Clinical data from TCGA showed an association between the gene expression of collagen subtypes IV and VI and TKS4 (*SH3PXD2B*) or WIP (*WIPF*) in patients with breast cancer (**Supplementary Fig. S8A-B**), indicating high invadopodia formation may be the result of increased microenvironmental collagen expression.

3.5 4T1 cell and BMDM interactions in dECM hydrogels

We collected CM from 4T1 cells encapsulated in the dECM hydrogels and analyzed cytokine secretion using a Luminex multiplex immunoassay. Factors with a 1.5-fold or greater increase in mean fluorescence intensity (MFI) are shown (**Fig. 5A**), with granulocyte macrophage colony-stimulating factor, CCL2, and interleukin 6 (IL-6) having the highest expression. To understand the relationship between ECM components, invadopodia, and cytokine secretion in a broader clinical context, TCGA analysis revealed a correlation between *Col4A4* and *CCL2* mRNA expression (**Fig. 5B**). We also found a correlation between *Col4A4* and *IL6* expression (**Fig. 5C**), indicating the increased collagen IV levels in breast tissue after RT may elevate the secretion of

cytokines in tumor cells. Further analysis of the TCGA PanCancer dataset showed *WIPF1* was associated with *CCL2* mRNA expression (**Fig. 5D**). This clinical data suggests a link between increased collagen IV deposition in the tumor microenvironment, invadopodia regulation, and CCL2 expression, which mirrors our findings that tumor cell cytokine secretion is enhanced in response to the increase in collagen IV after RT, which may facilitate invasion via invadopodia expression. To investigate the link between CCL2 secretion and patient outcomes in TNBC, we utilized publicly available datasets through KMplotter. Among TNBC patients, high CCL2 expression was associated with poor patient survival (**Fig. 5E**). These data indicate that RT-induced collagen IV deposition may lead to elevated levels of CCL2 in recruited tumor cells, ultimately decreasing patient survival.

Both GM-CSF and CCL2 expression are associated with macrophage infiltration [37], where GM-CSF has specifically been implicated in post-RT macrophage and circulating tumor cell infiltration [5,6]. We therefore evaluated how macrophages behave in irradiated dECM hydrogels and how they interact with tumor cells. We isolated BMDMs from Nu/Nu mouse femurs and seeded them in dECM hydrogels. IF imaging and quantification of Ki67 staining showed increased BMDM proliferation in the irradiated microenvironment (**Fig. 5F-H**). We then co-cultured GFP-labeled 4T1 cells and BMDMs stained with CellTrace Far Red to evaluate differences in their interactions within irradiated and unirradiated microenvironments. Fluorescence images and bioluminescence measurements demonstrated an increase in 4T1 cell proliferation when co-cultured with macrophages in the irradiated hydrogels. It is noteworthy that this increased proliferation is 2-fold higher when compared to the proliferation of 4T1 cells cultured alone (**Fig. 4B**), suggesting that macrophages further promote tumor cell proliferation (**Fig. 5I-L**). BMDMs also exhibited increased proliferation in the irradiated microenvironment when co-cultured with 4T1 cells (**Fig. 5M**).

4. Discussion

The high incidence of local recurrence following RT is associated with poor overall survival in TNBC patients, and previous work suggests a link between radiation damage and TNBC recurrence in immunocompromised patients and mouse models [6,38]. Understanding how irradiation modulates the microenvironment is necessary for evaluating drivers of recurrence. We first analyzed structural and compositional alterations of irradiated breast tissue and showed more ECM deposition, reduced fiber diameter and pore size, and increased stiffness following irradiation (**Figs. 1-2**). We determined that collagen IV-associated changes in normal tissues predicted worse outcomes in breast cancer patients, and we confirmed an increase in multiple

ECM components, including collagen IV, in MFPs post-RT. As physicochemical cues dictated by changes in ECM deposition modulate numerous biological functions, we developed dECM hydrogels as models of the irradiated microenvironment to determine the impact of RT-induced ECM changes on tumor cell behavior (**Fig. 3**). We showed that normal tissue radiation damage may lead to a favorable environment for TNBC recurrence by influencing both tumor and immune cells (**Figs. 4-5**). Our study is the first to recapitulate the irradiated ECM microenvironment, and this model can be used as a platform for studying radiation response and recurrence mechanisms.

Immune cell infiltration to injured sites leads to the recruitment and activation of fibroblasts, resulting in ECM remodeling and, when prolonged, fibrosis [7,39–41]. The ECM in the injured microenvironment is usually denser and mechanically stiffer than normal ECM, and the physical characteristics of the newly deposited fibers are critical in affecting the stiffness of the microenvironment that the cells sense [42–44]. These findings are consistent with our SEM results showing excess ECM deposition and denser and thinner ECM fibers in irradiated samples, and our AFM studies confirmed that the stiffness of irradiated tissue increased due to the altered ECM. The stiffness range of human breast tissue is approximately 0.5-1 kPa [45], which is in the range of our findings.

Changes in ECM density and stiffness are not just attributed to fiber size, but are also impacted by the molecular makeup of the matrix [43]. Approximately 300 proteins present in the ECM have been reported to mediate cellular behaviors and tissue development, and collagen is the major fibrous protein which constitutes up to 90% of the ECM [46]. 28 types of collagen have been described [47], and collagens I, IV, and VI are the most relevant subtypes of collagens in the microenvironment of mammary tissue. A major function of collagens I, IV, and VI is to provide structural support for cells and enrich the cell surface with growth factors and cytokines that are important in tumor progression. Increased collagen IV after chemotherapy has been shown to drive TNBC cell invasion [48]. Collagen VI is secreted by adipocytes and is known to regulate breast tumor development and facilitate TNBC cell migration [49,50]. In addition to collagens, other prominent fibrous proteins including elastins, fibronectins, and laminins are related to cellular dynamics [51]. Fibronectins are crucial for attaching cells to matrix and guiding cell migration and have been reported to be upregulated in the RT-induced fibrosis process [52]. This agrees with our results showing that FN quantity increased in irradiated MFPs. We also showed that changes in collagen deposition may provide more tensile strength and support tumor cell retention and invasion through direct structural changes and cell-protein contact.

Variations in ECM density, composition, and stiffness have been correlated with tumor progression and recurrence [51]. In this study, we aimed to isolate the impact of post-RT changes in ECM on tumor and immune cell behavior while still recapitulating *in vivo* conditions through the development of irradiated mammary tissue-derived dECM hydrogels. Collagen- and Matrigel-based hydrogels have been widely used to study cell behavior, but they have limitations due to the absence of native tissue structure or site-specific bioactive molecules such as exogenous growth factors and peptides [53,54]. The advantages of component integrity and biologically active constituent matrix molecules make dECM hydrogels a promising method to mimic the irradiated microenvironment [55]. Our results confirm the preservation of ECM components following the decellularization process to form our hydrogels. dECM hydrogels have been previously fabricated from various tissue sources, including adipose tissue [10,56], liver [57], lung [58], dermis, and urinary bladder [9], with hydrogel mechanical properties ranging from 0.1 to 40 kPa [9,59], consistent with our results. The pore size, fiber density, and fiber orientation in ECM hydrogels have previously been demonstrated to lead to variations in mechanical properties [60,61]. Dermal dECM hydrogels have a higher fiber node density than urinary bladder dECM hydrogels and a higher storage modulus, indicating a link between fiber node density and mechanical strength [62]. Our SEM images and fiber analysis showed that fibers in irradiated dECM hydrogels were denser compared to unirradiated dECM hydrogels, which correlated to increased stiffness. Changes in ECM stiffness have been shown to impact cell motility [63], and we showed that the increased stiffness in dECM hydrogels after radiation had a profound effect on tumor cell invadopodia formation and the switch to cellular morphology indicative of increased invasiveness. Tissue-specific ECM has also been shown to influence tumor cell growth [64] directly through engagement with integrins [65], and our results showed that tumor cell proliferation was increased when encapsulated within irradiated dECM hydrogels. We determined that breast cancer cell interactions with the irradiated ECM promote proliferation and invasion, which builds on previous findings linking the recruitment of tumor cells to irradiated sites to recurrence under immunocompromised conditions [6].

Immune cells are recruited to RT-damaged sites by inflammatory mediators and secrete profibrotic cytokines such as tumor necrosis factor alpha, interleukin 13, and transforming growth factor-β during fibrosis [7]. Macrophages are known to infiltrate irradiated normal tissue [66], and the presence of inflammatory macrophages has been correlated to recurrence and poor overall survival in breast cancer patients [67]. We did not model macrophage recruitment in this study but instead showed that the irradiated microenvironment supports macrophage growth

through tumor secreted factors. Although increases in tumor cell proliferation occur without macrophages in irradiated dECM hydrogels, the extent of cancer cell proliferation is greater during co-culture with macrophages. This suggests that the irradiated ECM influences tumor-immune cell interactions and may promote a pro-recurrent niche. This agrees with previous *in vivo* findings [5,6,68] showing that excess macrophage infiltration attracts circulating tumor cells and indicates that cross-talk with immune cells plays a pivotal role in tumor cell survival in irradiated tissues.

Overall, we developed a mammary-derived dECM hydrogel to model normal tissue radiation response, which will lead to the discovery of novel targets for reducing TNBC recurrence and increasing breast cancer patient survival following therapy. We replicated *in vivo* conditions using dECM hydrogels to study the influence of the irradiated breast tissue on TNBC recurrence. Although our MFPs were irradiated *ex vivo*, which may have different outcomes compared to *in vivo* radiation where cell infiltration may further alter ECM remodeling, this model is valuable for analyzing the direct effect of radiation on changes in the ECM within the mammary gland. Future studies will evaluate the impact of *in vivo* RT on ECM-linked TNBC recurrence.

5. Conclusions

We demonstrated that dECM hydrogels are an effective tool to study the normal tissue radiation response. ECM structural and compositional changes induced by radiation facilitated tumor cell proliferation and invasion. In addition, we showed that macrophages enhance TNBC cell proliferation in the irradiated microenvironment, which may drive TNBC recurrence following therapy.

Declaration of Competing Interests

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data will be made available on request.

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Figures

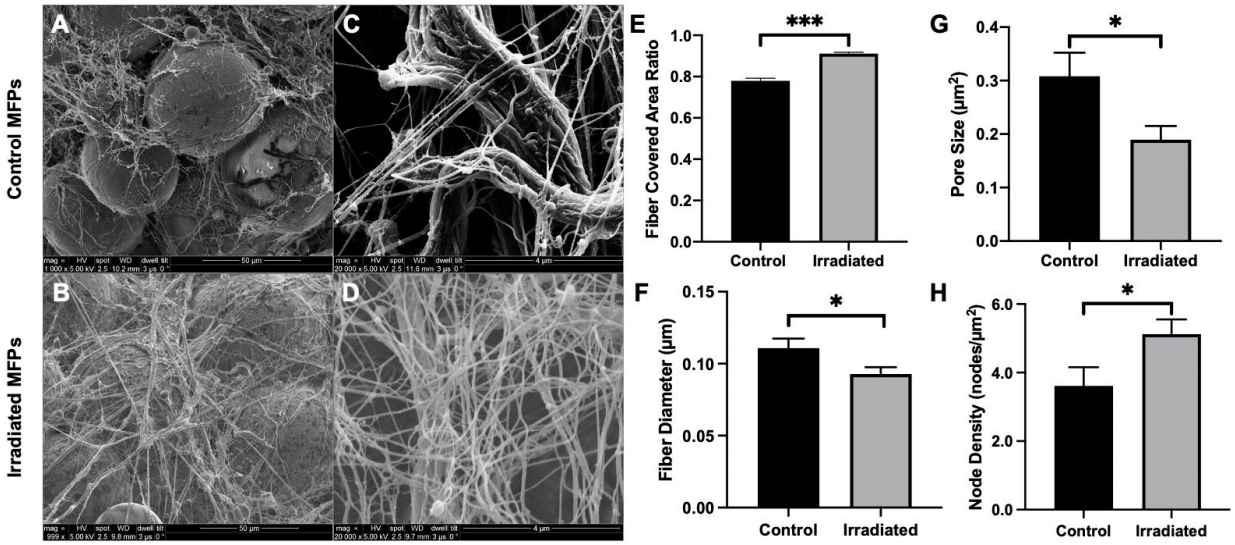

Figure 1. Structure of control and irradiated MFPs by SEM and fiber network analysis. SEM images of unirradiated control (**A**) and *ex vivo* irradiated (20 Gy) (**B**) MFPs from Nu/Nu mice (n = 10) at 1,000x, and control (**C**) and irradiated (**D**) MFPs (n = 10) at 20,000x magnification. SEM images were analyzed using an automated fiber tracking algorithm to determine the fiber covered area ratio (**E**), average fiber diameter (**F**), pore size (**G**), and node density (**H**). Statistical significance was determined by Student's t-tests with *$p<0.05$ and ***$p<0.001$. Error bars show the 95% confidence limit.

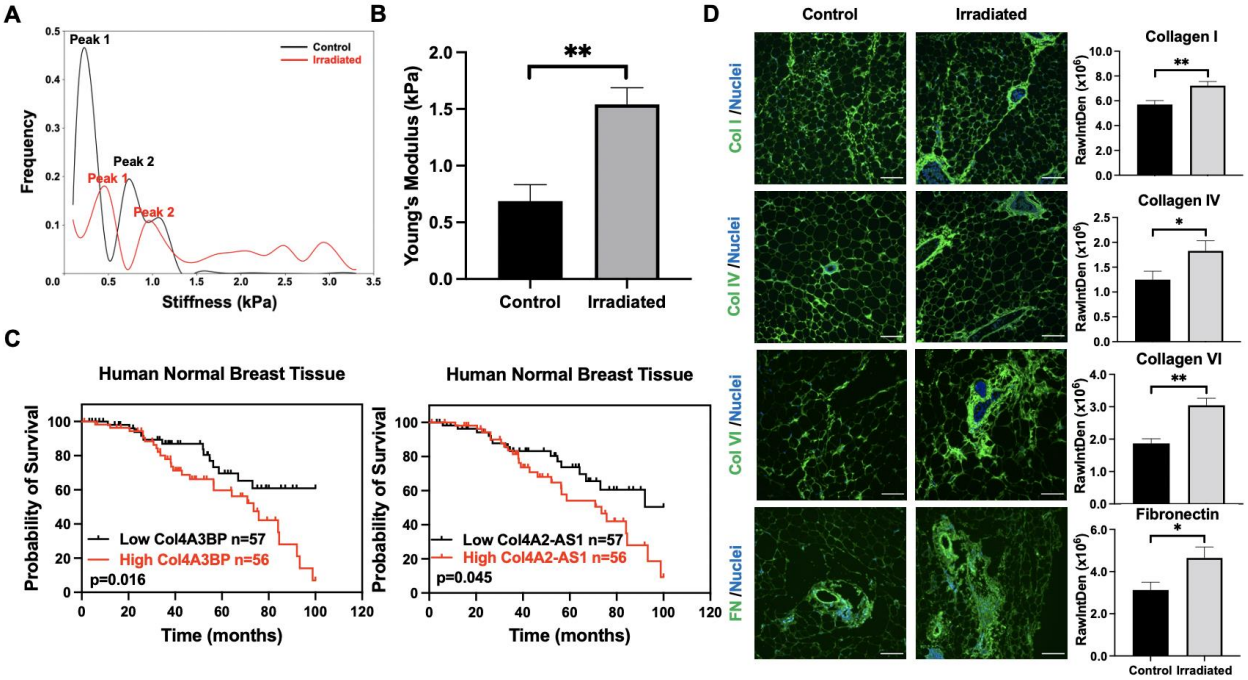

Figure 2. MFP irradiation enhances tissue stiffness and ECM deposition. (**A**) Stiffness distribution of control and *ex vivo* irradiated (20 Gy) MFPs from Nu/Nu mice (n = 4). (**B**) AFM analysis of the Young's modulus of control and *ex vivo* irradiated MFPs using the Hertz model. (**C**) Kaplan-Meier curves for patients with low versus high mRNA expression of *Col4A3BP* and *Col4A2-AS1* (n = 113 patients) in the tumor-adjacent normal tissue from the TCGA database. Immunofluorescence (IF) was performed to detect collagen I, collagen IV, collagen VI, and fibronectin in MFPs, and the corresponding quantification of fluorescence intensity is shown in (**D**). Scale bars are 100 µm. Statistical significance was determined by Student's t-tests with *p<0.05 and **p<0.01. Error bars show the 95% confidence limit.

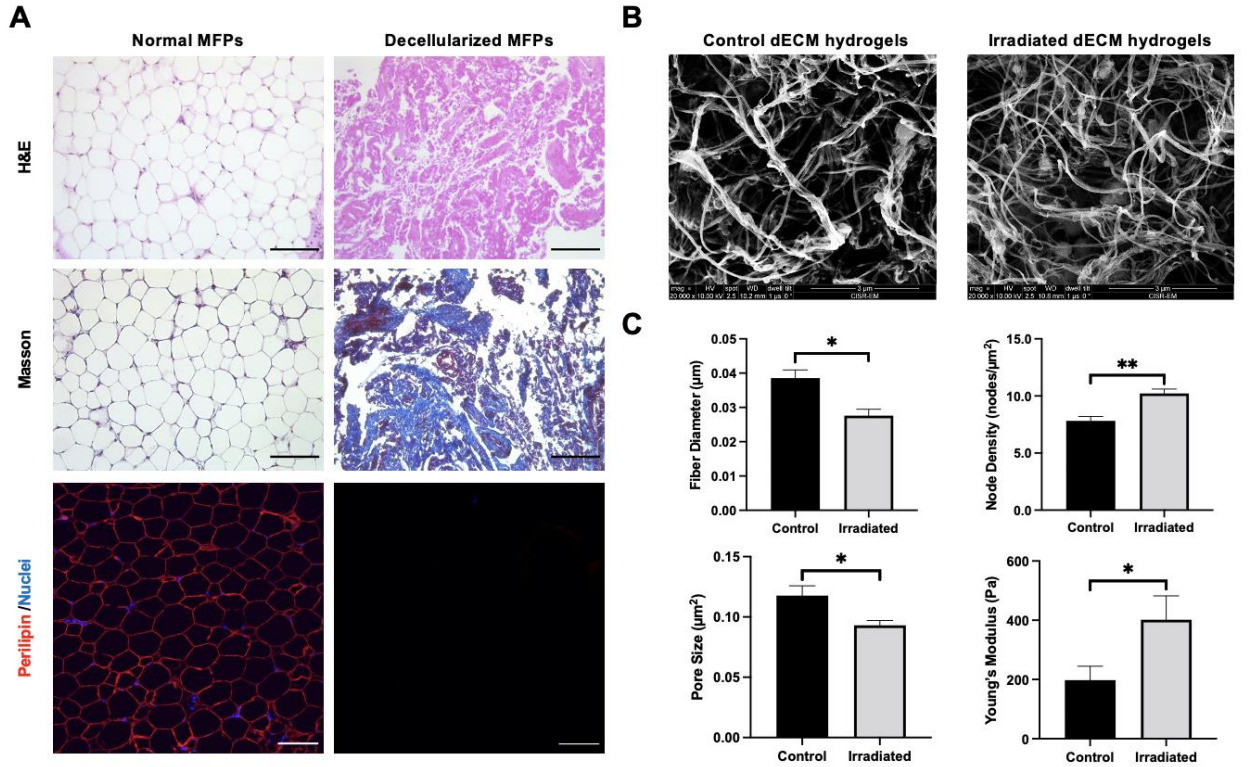

Figure 3. Characterization of dECM hydrogels from Nu/Nu mice. (**A**) H&E, Masson's Trichrome, and perilipin (red) and nuclear (blue) staining of pre- and post-decellularized control MFPs confirmed decellularization and delipidation. Scale bars are 100 μm. SEM images of control and irradiated dECM hydrogels at 20,000x magnification (**B**), and the average fiber diameter, node density, pore size, and mechanical properties determined by AFM were evaluated (**C**). Statistical significance was determined by Student's t-tests with *$p<0.05$ and **$p<0.01$. Error bars show the 95% confidence limit.

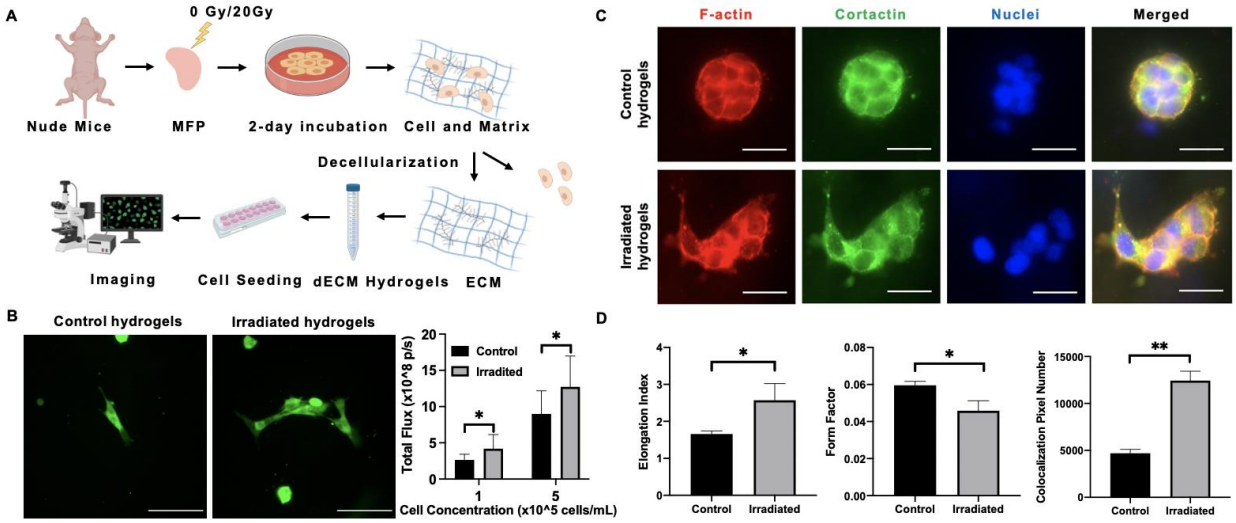

Figure 4. 4T1 cell proliferation and invasion are enhanced in irradiated dECM hydrogels. (**A**) Schematic of experimental workflow. (**B**) Fluorescence images of GFP- and luciferase-labeled 4T1 cells in dECM hydrogels from Nu/Nu mice (n = 3 for each condition) and bioluminescence quantification. (**C**) IF staining of F-actin (red), cortactin (green), nuclei (blue) and merged images for 4T1 cells embedded in control and *ex vivo* irradiated dECM hydrogels for 48 hr. Scale bars are 100 μm. (**D**) Elongation index and form factor based on F-actin staining and colocalization pixel number as determined by cortactin-F-actin overlap were analyzed. Statistical significance was determined by Student's t-tests with* $p<0.05$ and **$p<0.01$. Error bars show the 95% confidence limit.

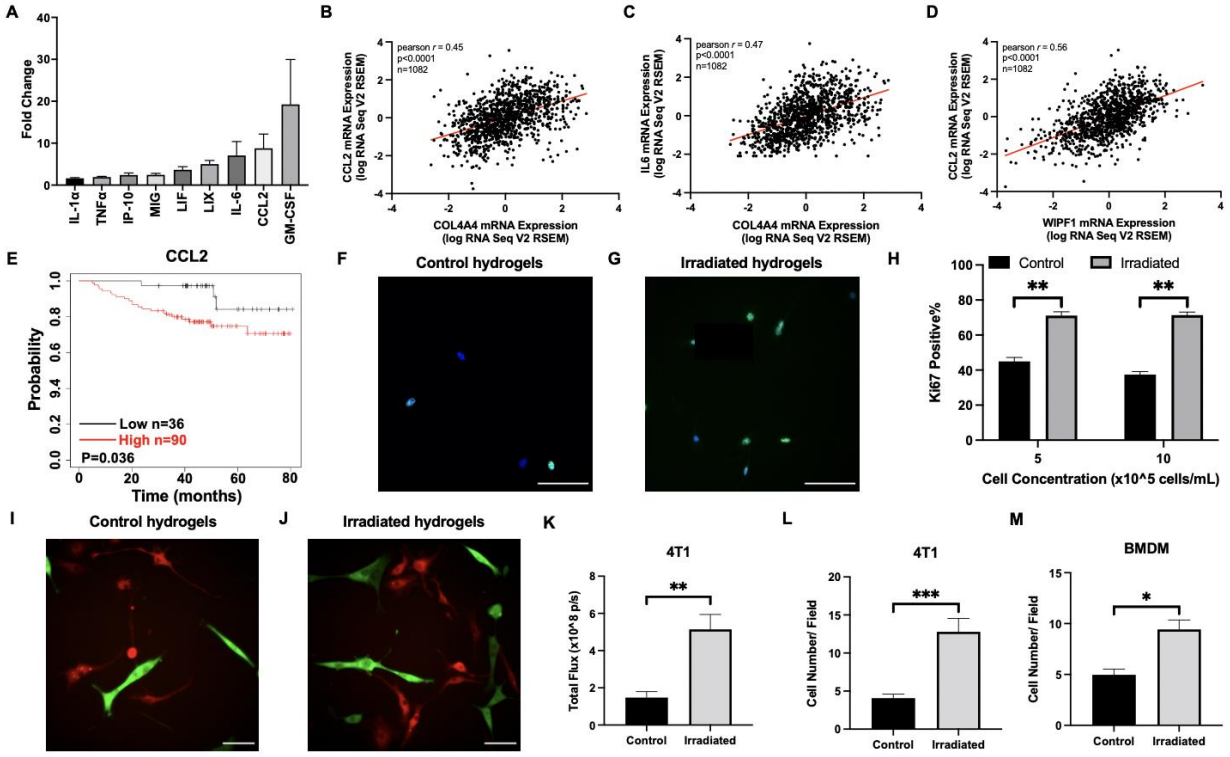

Figure 5. Irradiated microenvironments promote worse outcomes in patients and enhance tumor cell proliferation and tumor-macrophage interactions. (**A**) A Luminex multiplex immunoassay was performed to determine the secreted cytokine profile of CM from 4T1 cells cultured in irradiated dECM hydrogels (n = 3). Data presented is mean fluorescence intensity (MFI) fold change compared to cytokine secretion from 4T1 cells cultured in control dECM hydrogels. Only factors with a 1.5-fold or greater increase in MFI are shown. **Supplementary Table S1** defines abbreviations. Correlation of *Col4A4* and *CCL2* mRNA expression (**B**), *Col4A4* and *IL6* mRNA expression (**C**), and *WIPF1* and *IL6* mRNA expression (**D**) from TCGA analysis (PanCancer dataset) in 1,082 patients with breast tumors. Pearson correlation shown. (**E**) Kaplan-Meier curves of TNBC patients (n = 126 patients) with low versus high mRNA expression of *CCL2* from KMplotter. Fluorescence images of Ki67 positive (green) BMDMs in control (**F**) and irradiated (**G**) dECM hydrogels with the corresponding quantification in (**H**). Nuclei are stained blue. Fluorescence images of co-cultures of GFP-labeled 4T1 cells and BMDMs stained with CellTrace Far Red in control (**I**) and irradiated (**J**) dECM hydrogels with the corresponding bioluminescence quantification in (**K**). Cell number per field for 4T1 cells (**L**) and BMDMs (**M**) was quantified. Scale bars are 50 µm. Statistical significance was determined by Student's t-tests with *p<0.05, **p<0.01, and ***p<0.01. Error bars show the 95% confidence limit.