The Journal of Pathology: Clinical Research
J Pathol Clin Res; January 2019; **5:** 40–52
Published online 1 November 2018 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/cjp2.115

ORIGINAL ARTICLE

ColXα1 is a stromal component that colocalizes with elastin in the breast tumor extracellular matrix

Yihong Wang[1]*, Shaolei Lu[1], Jinjun Xiong[2], Kamaljeet Singh[2], Yiang Hui[1], Chaohui Zhao[1], Alexander S Brodsky[1], Dongfang Yang[1], Grant Jolly[1], Madhu Ouseph[1], Christoph Schorl[3], Ronald A DeLellis[1] and Murray B Resnick[1]

[1]*Department of Pathology and Laboratory Medicine, Rhode Island Hospital and Lifespan Medical Center, Warren Alpert Medical School of Brown University, Providence, RI, USA*
[2]*Department of Pathology, Women and Infants Hospital, Warren Alpert Medical School of Brown University, Providence, RI, USA*
[3]*Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI, USA*

**Correspondence to: Yihong Wang, Department of Pathology, Rhode Island Hospital and Lifespan Medical Center, 593 Eddy Street, Providence, RI 02903, USA. E-mail: ywang6@lifespan.org*

Abstract

The tumor microenvironment regulates tissue development and homeostasis, and its dysregulation contributes to neoplastic progression. Increased expression of type X collagen α-1 (ColXα1) in tumor-associated stroma correlates with poor pathologic response to neoadjuvant chemotherapy in estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2)-positive breast cancers. Evaluation of ColXα1 expression patterns suggests a potential connection with elastin fibers. To investigate the possible interaction between ColXα1 and elastin, we evaluated the expression of ColXα1 in relation to elastin fibers in normal breast tissue, ductal carcinoma *in situ*, and invasive breast carcinomas at cellular and subcellular levels. Our findings demonstrate that ColXα1 colocalizes with elastin in invasive breast cancer-associated stroma by immunohistochemistry, immunofluorescence, and electron microscopy. In 212 invasive breast carcinomas, this complex was aberrantly and selectively expressed in tumor extracellular matrix in 79% of ER+/HER2−, 80% of ER+/HER2+, 76% of ER−/HER2+, and 58% of triple negative breast cancers. In contrast, ColXα1 was generally absent, while elastin was present perivascularly in normal breast tissue. ColXα1 and elastin were coexpressed in 58% of ductal carcinoma *in situ* (DCIS) in periductal areas. In mass-forming DCIS with desmoplastic stroma, the complex was intensely expressed in periductal areas as well as within the tumor-associated stroma in all cases. Our data suggest that the breast carcinoma neoplastic process may involve aberrant expression of ColXα1 and elastin in the tumor microenvironment emerging early at the DCIS stage. Enrichment of these complexes in tumor-associated stroma may represent a stromal signature indicative of intrinsic differences between breast cancers. These findings shed light on investigation into the role of aberrant collagen complex expression in tumorigenesis and tumor progression which may be leveraged in therapeutic and theranostic applications.

Keywords: tumor microenvironment; type X collagen α-1; ColXα1; elastin; extracellular matrix; breast cancer; neoadjuvant

Received 12 March 2018; Revised 5 September 2018; Accepted 7 September 2018

Conflict of interest statement: YW, ASB and MBR declare that a patent application has been approved 7/2017 titled as Collagens as Markers For Breast Cancer Treatment to ASB, YW, and MBR of Rhode Island Hospital, A Lifespan-Partner (Application No. 15/187279). SL, JX, KS, YH, CZ, DY, GJ, MO, CS, and RAD have nothing to disclose.

Introduction

Neoplastic progression is attributed to the accumulation of somatic mutations in epithelial cells. Neoplastic behavior is also influenced by the tumor microenvironment that includes the extracellular matrix (ECM), blood and lymphatic vasculature, inflammatory cells, and fibroblasts [1]. The ECM contributes to diverse functions. ECM dynamics are tightly regulated to ensure normal development, physiology, and robustness of organ systems. When such control mechanisms are disrupted, the resultant deregulation contributes to the initiation and progression of cancer [2].

The ECM is composed of a collection of biochemically and structurally diverse components, each with diverse subcategories contributing to various physical and biochemical properties. Some ECM proteins, including fibrillar collagens and elastin, form fibrils from protein monomers contributing to tensile strength and viscoelasticity of the tissue [3,4]. In tumors including head and neck, cervical, esophageal and prostate cancer, stromal properties have been shown to affect disease progression and patient prognosis [5–9]. Although the specific pathophysiologic processes driving collagen reorganization in breast cancer remain unclear, the cross-linked and orientated collagen in cancer tissue is a reliable marker associated with poor survival, regardless of tumor grade, size, subtype, estrogen receptor (ER) expression, progesterone receptor (PR) expression, and nodal status [10]. High stromal content predicts poor survival in triple negative breast cancer [11]. Increased collagen VI deposition stimulates cancer cell proliferation [10,12]. ColVα2 and ColXIα1 are highly expressed in invasive ductal carcinoma compared to ductal carcinoma *in situ* (DCIS) and are involved in triggering cancer cells to disseminate [13,14]. Together, these studies lend credence to the influence of collagen deposition in cancer growth and metastasis.

Type X collagen α-1 (ColXα1) is a short-chain collagen, typically found underlying endothelial cells and in the hypertrophic zone of cartilage during endochondral ossification where it participates in calcifying cartilage formation [15]. ColXα1 is encoded by the *COL10A1* gene, which is expressed by hypertrophic chondrocytes. Mutations in *COL10A1* are associated with Schmid-type metaphyseal chondrodysplasia and Japanese-type spondylometaphyseal dysplasia [16]. We previously found that increased expression of ColXα1 was predictive of poor pathologic response in neoadjuvant-treated ER+/HER2+ breast tumors [17]. Although increased stromal collagen content has been clinically documented in breast cancers, its specific pattern of distribution and relationship to the malignant epithelial component and other ECM components is poorly understood.

Elastin is normally expressed in significant quantities in skin, lung, cartilage, and large arteries. Elastin fibers provide recoil to tissues subjected to repeated stretching motions. Importantly, elastin stretching is limited by tight association with collagen fibrils [18]. Together, collagen, elastin, and other ECM proteins such as fibronectin and tenascin influence cellular behavior including the promotion of fibroblast migration during wound healing, tumor growth, and metastasis [19,20].

The ECM associated with breast carcinoma is comprised of large aggregates of elastin fibers, known as elastosis [21–23]. Elastin can be cleaved into small peptide fragments, which can affect cellular processes including apoptosis, chemotaxis, and metastasis [24,25]. ColXα1 exhibits a patchy pattern of expression in breast tumors which is reminiscent of the elastosis patterns. We hypothesized that elastin and ColXα1 colocalize. To test this hypothesis, publically available data were collected and analyzed using Oncomine (Thermo Fisher Scientific, Waltham, MA, USA) for *COL10A1* and elastin. Normal breast tissue, DCIS, and breast tumors were then examined through immunohistochemical, immunofluorescent, and electron microscopic techniques to assess ColXα1 and elastin expression and localization.

Materials and methods

Case selection

With institutional review board approval at Rhode Island Hospital (467617-9) and Women Infants Hospital (797108-3), human tissues from 2009 to 2017 were obtained for study. We evaluated 52 normal breast specimens from 26 reduction mammoplasties, 51 DCIS, and 212 breast cancer specimens (Table 1). The DCIS group included low, intermediate, and high nuclear grade lesions. Forty-three cases were DCIS with associated calcifications with some showing necrosis and normal appearing stroma. Eight were mass-forming exhibiting stromal changes resembling desmoplasia akin to those found in invasive cancer. The invasive tumors included breast cancers of all four molecular subtypes.

Pathological evaluation

For patients treated with neoadjuvant chemotherapy (NAC), pathologic response was assessed by the AJCC cancer staging and residual cancer burden (RCB) score [26,27]. The RCB system stratifies patients into classes I, II, and III [RCB class 0 is synonymous with having achieved a pathological complete response (pCR); on-line calculator is available at http://www.mdanderson.org/breastcancer_RCB]. Patients who achieved a pCR or minimal residual disease (RCB class I) were considered good responders, while RCB class II and III were considered poor responders. For patients treated with primary surgery followed by adjuvant treatment, the outcome measure in this analysis was progression-free survival, defined as time from

Table 1. Patient demographic data

Characteristic	Ductal carcinoma *in situ* (%)	Invasive carcinoma (%)
No. of patients	51	212
Age (years)		
<50	14 (27.5)	62 (29.2)
≥50	37 (72.5)	150 (70.8)
Nuclear grade		
Low	13 (25.5)	–
Intermediate	23 (45.1)	–
High	15 (29.4)	–
Nottingham grade		
I	–	15 (7.1)
II	–	76 (35.8)
III	–	121 (57.1)
ER		
Positive	45 (88.2)	106 (50.0)
Negative	6 (11.8)	106 (50.0)
HER2		
Positive	–	104 (49.1)
Negative	–	108 (50.9)

surgery to documented progression or death. Patients who had no recurrence or death were considered good responders and patients who had recurrence or death were defined as poor responders. Overall survival (OS) was not analyzed due to the low overall death rate.

Bioinformatics

COL10A1 and elastin gene expression in breast cancer or cancer stroma was interrogated through Oncomine (www.oncomine.com, December 2017, Thermo Fisher Scientific) using filters including Gene name, 'Cancer versus Normal Analysis', and 'Breast Cancer'. Curated breast cancer studies in Oncomine were selected. Analyses were focused on studies with normal tissue, with or without DCIS, and invasive cancer. Both whole tumor tissue extract and stroma-only studies were included.

Chi-square analyses were used to evaluate the correlation of ColXα1 and elastin expression with patient outcome. Statistical analyses were performed using JMP 13.0 (SAS, Cary, NC, USA).

Immunohistochemistry and expression scoring

Four-micron sections were cut from formalin-fixed paraffin-embedded (FFPE) tissue blocks, heated at 60 °C for 30 min, deparaffinized and rehydrated. These were then subjected to antigen retrieval by heating in epitope retrieval buffer in a 95 °C water bath for 45 min. The slides were incubated with either mouse monoclonal antibodies or rabbit polyclonal antibodies for 30 min at room temperature. Anti-ColXα1 (1:50, Clone X53, eBioscience/Affymetrix, San Diego, CA, USA) and anti-alpha elastin (1:200, polyclonal, Abcam, Cambridge, MA, USA) were used for immunohistochemistry (IHC). Immunoreactivity was detected using the DAKO EnVision method according to the manufacturer's recommended protocol. Peri- and intra-tumoral stromal staining for ColXα1 and elastin were scored as 0, 1+, 2+, and 3+ as previous described [17]; briefly, 0 for absent staining, 1+ for <5% stroma tissue, 2+ for 5–10% of stroma tissue, and 3+ for >10% of stroma tissue. All scoring was performed blinded to the diagnosis. Developing cartilage and cartilaginous tumors were used as positive controls while normal bone and breast tissue were used as negative controls. Cases with a ColXα1 score 0 and elastin score 1–3+ or *vice versa* were not considered as coexpression or discordant expression patterns.

Immunofluorescence

Immunofluorescence (IF) was performed on 4-µm FFPE sections mounted on Superfrost Plus slides. After rehydrating tissue sections, citrate buffer was used for heat-induced epitope retrieval. The sections were incubated with 0.1% Triton X-100 for 15 min, then with 2% bovine serum albumin (BSA) for 30 min at room temperature. Sections were probed with anti-ColXα1 and anti-elastin overnight at 4 °C. After three washes with phosphate buffered saline (PBS), the tissue was incubated for 1 h in the dark with FITC-conjugated secondary goat anti-rabbit and goat anti-mouse antibodies (Thermo Fisher Scientific). The tissue sections were then washed three times with PBS and covered with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Immunostaining was observed under a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at ×400 magnification. Developing cartilage/cartilaginous tumors were used as positive controls and normal bone and breast tissue were used as negative controls.

Electron microscopy

Post-embedding double labeling immunogold (colloidal-gold) electron microscopy was performed as follows. Specimens were fixed in 0.1% glutaraldehyde in sodium cacodylate (0.1 M: pH 7.4) at 37 °C for 3 h, washed in the same buffer, then dehydrated in 70–100% ethyl alcohol, placed in LR White resin (hard grade) for 2–3 h with at least two changes of resin, and then placed in a 50 °C oven overnight.

Semi-thin sections (1 μm) were cut and examined under a light microscope for areas of interest. Pale gold ultra-thin sections were then cut on a Reichert-Jung Ultracut E ultramicrotome (Reichert, Depew, NY, USA) and collected on nickel grids. Sections were blocked with 0.4% BSA in PBS with 2% normal goat serum for 45 min, incubated overnight at 37 °C in polyclonal anti-elastin and anti-ColXα1 antibodies at 1:400 and 1:50 dilutions respectively. They were subsequently washed and placed in their respective secondary antibodies: IgG conjugated to 10 nm and 25 nm gold labels diluted 1:30 with 0.2% BSA in PBS for 90 min. Staining with uranyl acetate was performed and the tissue was examined under a Philips CM10 electron microscope. Digital images were acquired using a Gatan Erlangshen ES 1000 W camera (Gatan, Pleasanton, CA, USA).

Results

COL10A1 gene expression is increased in DCIS and invasive breast cancer

COL10A1 gene expression data were extracted from five studies examining whole tumor and three studies that assayed tumor cell and stroma separately [23,28–34]. Concurrently, elastin expression levels were studied. The data are summarized in Table 2. All eight studies showed significantly increased *COL10A1* gene expression in DCIS and higher expression in invasive cancer compared to normal control breast tissue. In contrast, elastin gene expression levels were similar between normal breast tissue, DCIS, and invasive cancer. When whole extracts of tumor tissue were examined, *COL10A1* expression levels increased 4.37–6.79-fold and 5.87–-43.5-fold in DCIS and invasive cancers respectively. In studies evaluating only stroma, *COL10A1* expression level increased 82.6-fold and 10.8–132-fold in DCIS and invasive tumors respectively.

ColXα1 and elastin expression in normal breast tissue

Since ColXα1 is expressed in developing cartilage and chondrogenic tumors [16], ColXα1, elastin IHC, and elastic special stains (Verhoeff's elastic stain) were first evaluated in normal mature bone and cartilage as well as in cartilaginous tumors including one cystic teratoma with cartilage, one enchondroma, and three well-differentiated chondrosarcomas. ColXα1 showed strong positive staining in portions of developing cartilage, enchondroma, and well-differentiated components of chondrosarcomas. Mature cartilage and the dedifferentiated component of chondrosarcoma lacked expression. In contrast, elastin was absent in cartilaginous tumors and present in the mature cartilage matrix as well as the cystic teratoma with developing cartilage.

Fifty-two normal tissue specimens were evaluated to define the ColXα1 and elastin expression patterns in normal breast tissue. ColXα1 was largely negative in normal breast with only rare faint staining in a perivascular or periductal distribution in a subset of cases. In contrast, elastin staining was prominent and extensively stained structures in normal breast tissue including the vasculature and occasional surrounding ducts and lobules (Figure 1A,B). These observations indicate a lack of association between elastin and ColXα1 expression in normal breast tissue.

ColXα1 and elastin expression in DCIS by IHC

The 51 DCIS cases studied included 43 non-mass-forming DCIS cases with normal-appearing stroma and 8 mass-forming DCIS cases with desmoplastic stromal reaction. Compared to normal breast tissue, there was a quantitatively significant increase in staining for ColXa1 and elastin in the DCIS cases ($p < 0.001$). In the 43 non-mass-forming DCIS cases with normal appearing stroma, there was no expression of ColXa1 in the stroma. Elastin in these cases was identified in perivascular and scant periductal patterns resembling those of normal breast stroma. However, in the expanding periductal areas of DCIS, ColXα1 was expressed in a periductal distribution in all cases. We noted that periductal ColXα1staining was heterogeneous around DCIS. In some cases, staining was sparse, involving few periductal foci while in others staining was extensive involving all periductal areas. Among the 51 cases, 20 scored 1 (39.2%), 10 scored 2 (19.6%), and 3 scored 3 (17.6%). In contrast, stromal ColXα1 expression was present in all eight cases with mass-forming DCIS. In those cases, ColXα1 was not only present in periductal areas as seen in non-mass forming DCIS, but also in DCIS-associated reactive desmoplastic stroma in seven of eight cases. Elastin was coexpressed in 30 of 43 non-mass-forming DCIS cases and in all mass-forming DCIS cases. Elastin was coexpressed with ColXα1 in reactive desmoplastic stroma and periductal areas surrounding the expanding DCIS (Table 3 and Figure 1C–E).

ColXα1 and elastin are coexpressed in all molecular subtypes of invasive breast carcinoma

We evaluated 212 invasive breast cancer cases with both ColXα1 and elastin staining, including 56

Table 2. Expression of *COL10A1* and elastin genes in breast cancer and its stroma

			COL10A1			Elastin		
Study	Index	Normal	DCIS	Invasive lobular	Invasive ductal	DCIS	Invasive lobular	Invasive ductal
TCGA breast [28]	Fold change	1		15.9	43.5		1.59	−1.01
	P value			7.22E−43	1.28E−52		6.52E−5	0.549
	Case number	61		36	389		36	389
Curtis *et al*, 2012 [29]	Fold change	1	6.79	6.68	8.74	1.09	1.262	1.00
	P value		2.63E−5	1.72E−57	3.32E−223	0.046	1.75E−4	0.375
	Case number	144	10	148	1556	10	148	1556
Richardson *et al*, 2006 [30]	Fold change	1			13.6			1.012
	P value				1.05E−14			0.473
	Case number	7			40			40
Turashvili *et al*, 2007 [31]	Fold change	1		19.4	12.9		2.84	−1.10
	P value			0.006	0.005		0.098	0.583
	Case number	10		10	10		10	10
Radvnyi *et al*, 2005 [32]	Fold change	1	4.37	5.87	7.16	3.71	2.88	1.22
	P value		0.042	0.021	0.013	0.122	0.107	0.393
	Case number	7	3	7	31	3	7	31
Ma *et al*, 2009 [33]*	Fold change	1	82.6		132	1.03		1.02
	P value		1.43E−8		2.89E−6	0.298		0.379
	Case number	14	11		9	11		9
Finak *et al*, 2008 [23]*	Fold change	1			16.8			4.35
	P value				1.25E−26			9.55E−9
	Case number	6			53			53
Karnoub *et al*, 2007 [34]*	Fold change	1			10.8			1.30
	P value				5.80E−4			0.225
	Case number	15			7			7

The table summarizes eight studies reanalyzed through Oncomine [23,28–34]. The first five studies are based on the entire tumor including both epithelial and stromal components. In the remaining three studies, stromal tissue was dissected out and the analyses were based solely on stroma. All the studies used normal tissue as control. The gene expression levels of *COL10A1* and elastin in DCIS and/or invasive cancers were compared to the levels in normal tissue. Expression levels in tumor are presented as fold changes of normal tissue (whose fold change was set as one). *P* values are listed below the fold change to illustrate statistical significance.

*Only the expression in stroma was studied in these reports.

ER+/HER2−, 50 ER+/HER2+, 54 ER−/ HER2+, and 52 triple negative tumors.

In invasive carcinoma, a significant increase of ColXα1 and elastin in the tumor-associated stroma was readily apparent in all histological grades and molecular subtypes.

ColXα1 and elastin were coexpressed in the stroma of invasive carcinomas in 155 of the 212 (73.1%) cases. The distribution of expression among the different molecular subtypes is summarized in Table 3. Twenty-three tumors (23/212, 10.9%) lacked expression of either ColXα1 or elastin. Thirty-four of 212 (16.1%) cases were discordant in ColXα1 and elastin expression. Among these, 5 cases expressed only ColXα1 while 29 cases expressed only elastin. In 22 of the 34 discordant cases, the discrepancy between elastin and ColXα1 staining was minor with a difference in scores of 0 to 1. ColXα1 was expressed in a patchy pattern within the stroma (Figure 1F,G). In cases where DCIS was admixed with invasive tumor encased in dense stroma, ColXα1 was expressed in a periductal distribution similar to DCIS only cases. When elastin was coexpressed with ColXα1, it was present in a similar pattern as ColXα1 in tumor-associated stroma in the vast majority of the cases. In addition, elastin was also present in the intratumoral vasculature that demonstrated minimal to absent ColXα1 expression.

Outcome data were available in a subset of the invasive tumors (184 of 212). This included 92 neoadjuvant-treated cases and 92 primary surgery cases followed by adjuvant treatment and average follow up of 33 months (range 12–88 months). Tumors with different ColXα1 and elastin status correlated significantly with outcome in the neoadjuvant-treated patients ($p = 0.027$, see supplementary material, Table S1). Both ColXα1 expression and ColXα1/elastin complex were significantly correlated with outcome, that is, high ColXα1 alone or ColXα1/elastin expression correlated significantly with poor response in neoadjuvant-treated cases by Chi-square analysis ($p = 0.0127$ and 0.0274, respectively). Similar correlations were not identified in the adjuvant setting. When separating the tumors into different molecular subtypes, high expression of ColXα1 and/or elastin correlated with poor outcome (p ranges from 0.0487 to 0.0735) in ER+/HER2− and

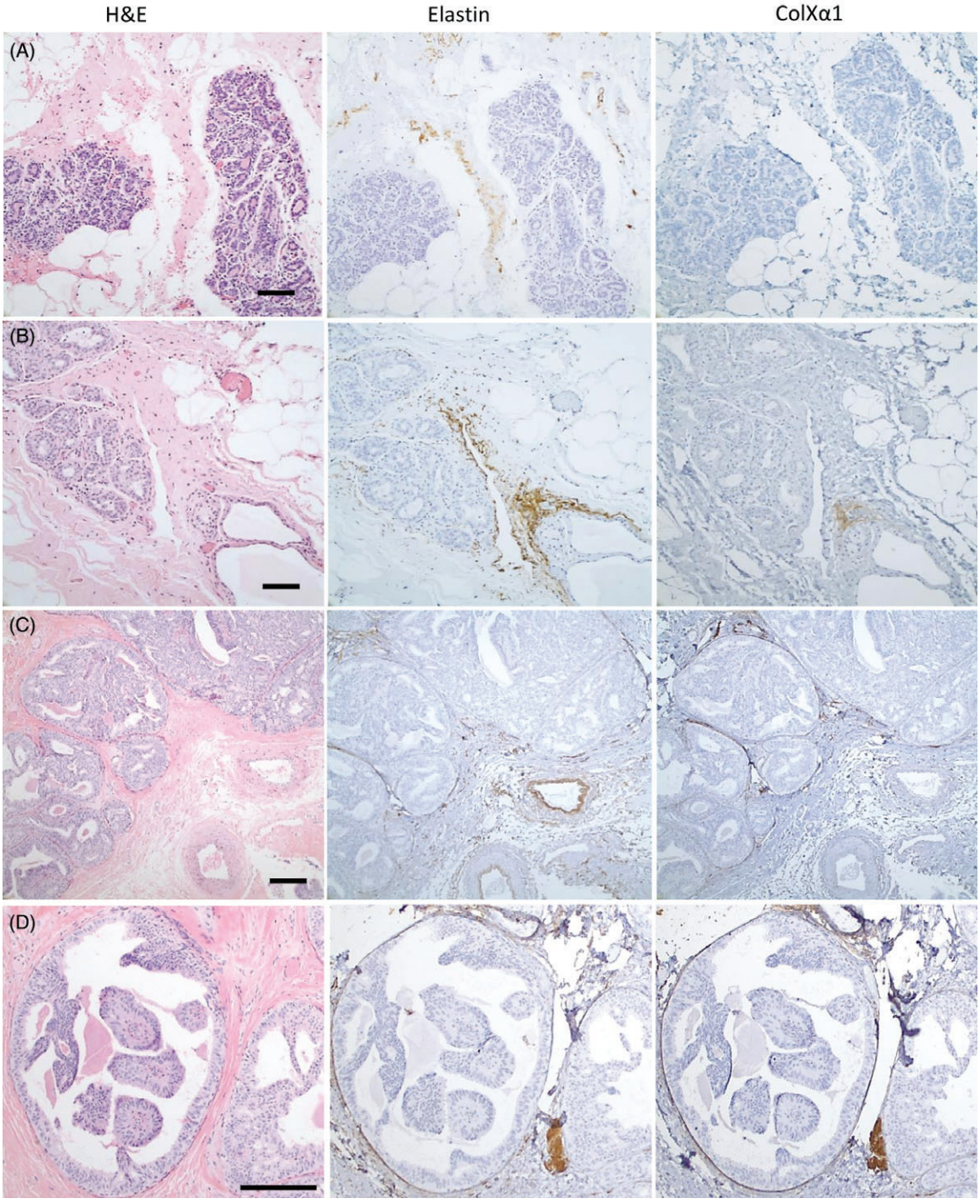

Figure 1. Elastin and ColXα1 immunohistochemistry staining of normal breast and breast tumors. (A,B) In normal breast stroma, ColXα1 was largely negative by IHC apart from occasional perivascular and periductal faint staining. Elastin was present around normal structures including periductal, lobular, and perivascular areas. Scattered elastin staining was also present in normal breast stroma. (C,D) Non-mass-forming DCIS, D at high magnification: ColXα1 and elastin expression in a periductal pattern. Elastin highlighted the vessels while ColXα1 was negative. (E) Mass-forming DCIS: ColXα1 and elastin coexpression in a periductal and stromal pattern of distribution. (F,G) ColXα1 and Elastin were strongly expressed in a similar patchy distribution in an ER+/HER2+ breast cancer (F) and a triple negative breast cancer (G). (H) Invasive ductal carcinoma with DCIS. ColXα1 and elastin expression were present in a periductal pattern and within the stroma. Scale bars = 10 μM.

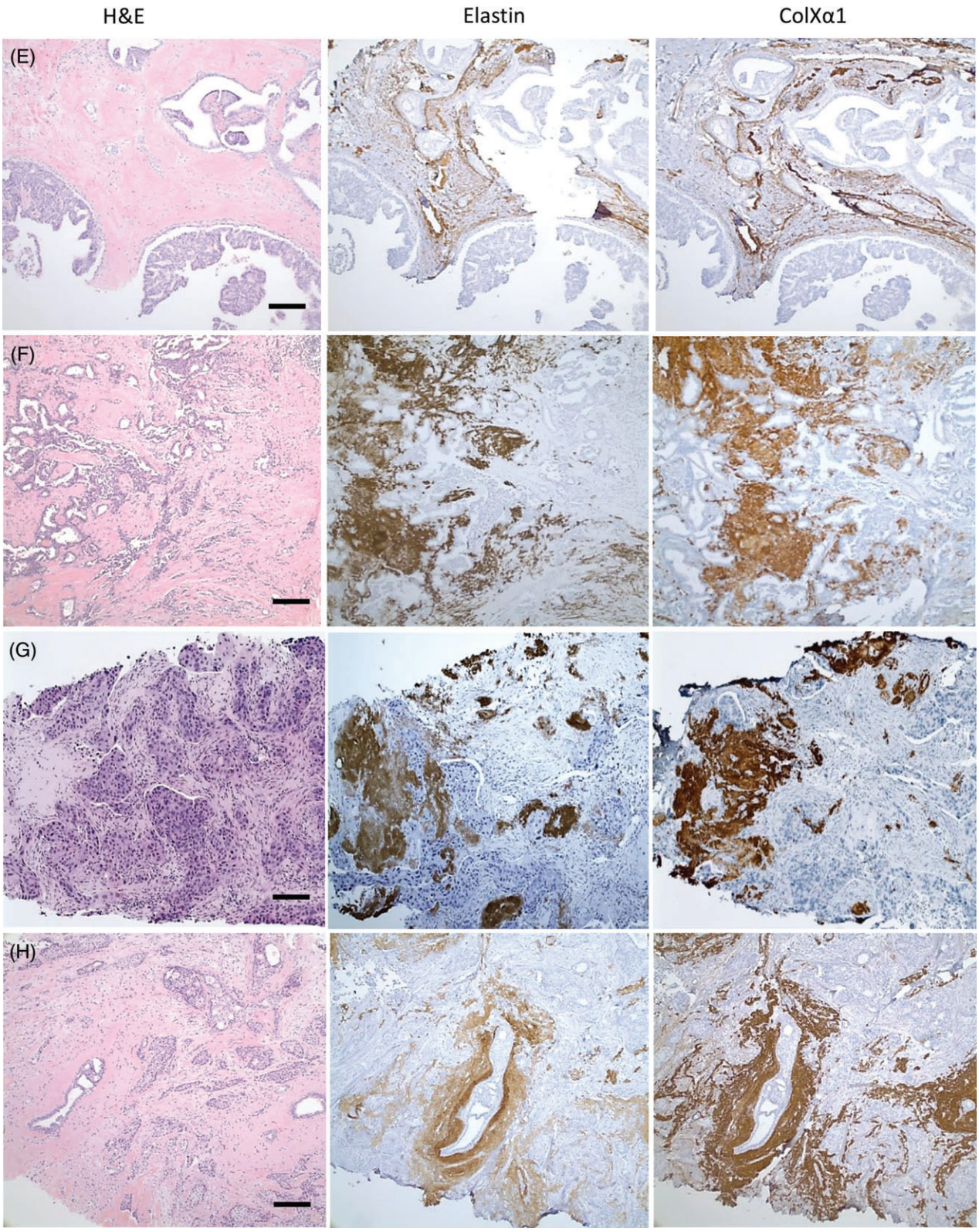

Figure 1. (Continued)

Table 3. Elastin and ColXα1 expression in DCIS and invasive carcinomas

Subtypes (*n*)	Elastin −/ ColXα1− (%)	Elastin +/ ColXα1+ (%)	Elastin −/ ColXα1+ (%)	Elastin +/ ColXα1− (%)
Normal (52 specimens in 26 patients)	11 (21.2)	0	0	41 (78.8)
DCIS (51)	1 (2.0)	30 (58.8)	9 (17.7)	11 (21.6)
Invasive (212)				
ER+/HER2− (56)	5 (8.9)	44 (78.6)	2 (3.6)	5 (8.9)
ER+/HER2+(50)	3 (6.0)	40 (80.0)	3 (6.0)	4 (8.0)
ER−/HER2+ (54)	5 (9.3)	41 (76.0)	0 (0)	8 (14.2)
ER−/HER2− (52)	10 (19.2)	30 (57.7)	0 (0)	12 (23.1)
All invasive subtypes (212)	23 (10.9)	155 (73.1)	5 (2.4)	29 (13.7)

ER+/HER2+ groups, although the patient numbers in each group were small (49 and 46, respectively). Grouping the ER+ tumors irrespective of their HER2 status, ColXα1/elastin complex was associated with poor outcome ($p = 0.0466$ and $p = 0.0186$ respectively) (see supplementary material, Table S1).

ColXα1 and elastin colocalize in breast tumor-associated stroma by IF and immunoelectron microscopy

The distribution and colocalization of ColXα1 and elastin in the breast tumor ECM was determined using IF (Figure 2). ColXα1 and elastin was patchily distributed in the ECM similar to patterns seen by IHC. ColXα1 staining colocalized with elastin throughout the ECM. Elastin appeared to be expressed more extensively in ECM without ColXα1. Tumor stroma and perivascular areas were also replete with elastin. Stromal areas expressing ColXα1 and elastin were used for immunoelectron microscopy (IEM) to elucidate subcellular morphology and localization. With IEM, we identified irregular amorphous aggregates of material that stained with both anti-ColXα1 and anti-elastin antibodies (Figure 3). This revealed that elastin is not deposited in the ECM as fibrils. Rather, it forms amorphous aggregates of polymerized tropoelastin. ColXα1, a nonfibrillar collagen, was also

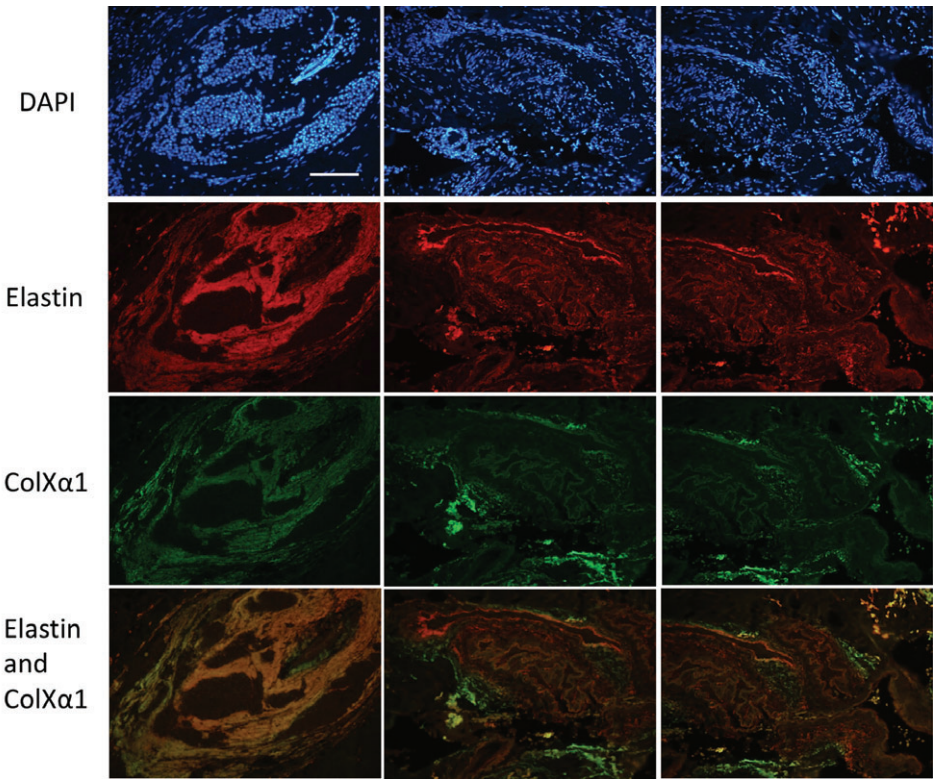

Figure 2. Immunofluorescence staining of elastin and ColXα1 in tumor-associated stroma. DAPI highlights the tumor; elastin and ColXα are distributed and colocalized in tumor-associated stroma. Intratumoral vessels stain with elastin but not ColXα1. Scale bar = 10 μM.

identified colocalizing with elastin in the amorphous material located near the fibroblast. Bundles of collagen fibrils consisting of type I collagen were also seen in the stroma.

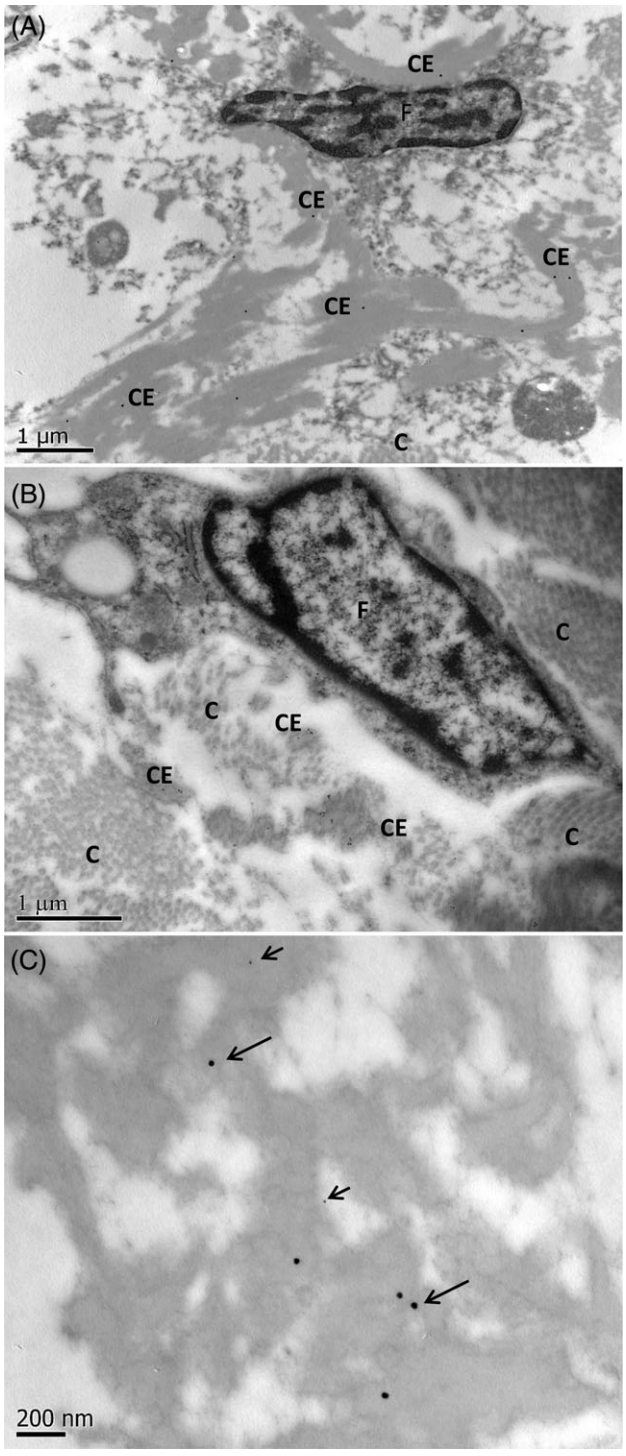

Figure 3. Legend in next column.

Discussion

The tumor microenvironment plays an integral role in the neoplastic process. Tumorigenesis and progression are influenced by biochemical and biomechanical properties of the tumor microenvironment [35]. The ECM in the tumor microenvironment serves as the scaffold upon which tissue is organized. Along with vasculature and immune cells, it provides the framework and critical cues that direct cell growth, survival, differentiation and migration. Thus, a dynamically evolving tumor microenvironment modulates tumor cell behavior [36,37].

During our previous study of ColXα1 expression, we observed a possible overlap with putative elastin fibers. Oncomine data analysis demonstrated that *COL10A1* RNA was significantly increased in both the tumor and tumor-associated stroma. We then systematically combined traditional pathologic review with immunohistochemical, immunofluorescent, and IEM techniques to investigate stromal ColXα1 and elastin organization in normal and neoplastic breast tissue. These observations endorse our hypothesis that most ColXα1 expressed in breast tumors is associated with elastin. However, not all of the elastin is associated with ColXα1. Overlapping ColXα1 and elastin expression was only observed in breast cancers, not in normal breast or normal tissues that exclusively express elastin, suggesting that this colocalization is specific for neoplastic transformation.

ColXα1 is a collagen type specific to the chondrocyte lineage. Functional studies predicate its importance in maintaining the stem cell pool and driving differentiation [38]. This study found that ColXα1 is virtually absent in normal breast tissue but is expressed in a variable periductal distribution pattern surrounding areas of DCIS. In 58% of the cases, ColXα1 expression was accompanied by elastin. In non-mass-forming DCIS cases, ColXα1 is recruited to

Figure 3. Immunoelectron microscopy of ColXα1 and elastin localization in the tumor microenvironment using double labeling. The micrograph shows a ColXα1 and elastin complex (CE) as patchy amorphous material in the extracellular space near a fibroblast nucleus which is double stained with gold conjugated anti-ColXα1 antibody (10-nm gold particles) and gold conjugated anti-elastin antibody (25-nm gold particles). The field also contains collagen fibrils (C) and fine cytoplasmic extension of the fibroblasts (F) responsible for elaboration of the extracellular constituents. Original magnification (×25 000). Low (A), intermediate (B), and high (C) magnifications are shown. Larger particles – elastin (long arrows) and smaller particles – ColXα1 (short arrows) are identified in (C).

the periductal region of DCIS despite the absence of histologically apparent stromal reaction. In mass-forming DCIS with reactive stroma [39], ColXα1/elastin complex is expressed in a pattern similar to that of the stroma associated with invasive cancer. This aberrant expression of ColXα1 raises the possibility that it may herald the initiation of neoplastic stromal transformation. Predicting tumor progression is an unresolved issue and a clinical concern. While histologic factors such as nuclear grade and hormone receptor expression have been extensively scrutinized, less is understood regarding the impact of ECM alterations. Our observations endorse the hypothesis that ColXa1 in the tumor microenvironment of preinvasive lesions is an indicator of neoplastic progression. In pancreatic adenocarcinoma, Shi *et al* demonstrated that low-grade pancreatic intraepithelial neoplasms show a marked increase in periductal collagen deposition [40]. It is possible that increased activation of fibroblasts in tumor-associated stroma may result in the collagen remodeling within the tumor microenvironment that we observed. Prior investigations have adduced that cancer-associated fibroblasts are the principal source of fibrillar collagens, other ECM proteins, and soluble factors promoting growth, invasion, metastasis, and survival of cancer cells [41–43].

As a component of vasculature and ECM in normal stroma, elastin exhibited changes associated with the neoplastic process. Uchiyama *et al* identified 'breast cancer elastosis' as condensed accumulations of irregularly arranged small amorphous elastin components associated with only a few microfibrils [44]. These amorphous components were ill-defined and occasionally associated with spiraling collagen fibrils and cellular debris. We demonstrated that ColXα1 and elastin are both patchily distributed and colocalized in the invasive breast cancer-associated stroma. Ultrastructurally, the colocalized ColXα1/elastin complexes appeared as amorphous irregular clumps that are comparable to the elastosis described by Uchiyama *et al* Using radioactive labeling and HPLC methodology, Kao *et al* found that the synthesis of collagen and elastin increased by 50 and 70% in desmoplastic breast cancer stroma on a per-cell basis [45]. However, unlike *COL10A1*, elastin RNA expression levels were not increased in DCIS or invasive carcinoma compared to normal tissue when analyzed through Oncomine. This may be attributable to the presence of elastin in normal breast tissue as a component of vascular and periductal structures. Accruing evidence suggests that focally aligned collagen at the stroma–cancer interface guides the migration of cancer cells away from the tumor and toward the vasculature during the metastatic cascade [46]. An increase in collagen deposition or ECM stiffness, alone or in combination, up-regulates integrin signaling and thus promotes cell survival and proliferation [47,48]. The quantitative changes in elastin within breast carcinoma stroma may be negligible. However, given its colocalization with ColXα1, elastin may undergo redistribution thus contributing to the neoplastic process.

There is emerging interest in stratifying breast carcinoma patients on the basis of stromal characteristics. Studies of invasive breast cancer-associated stroma have furnished conflicting prognostic results between the molecular subtypes of breast cancer. Downey *et al* reported that high stromal contents are associated with improved outcomes in ER-positive breast cancers [49]. However, these portend an adverse prognosis in triple negative tumors [11]. Dennison *et al* recently discovered that a particular stromal protein signature in breast carcinoma is associated with a highly differentiated phenotype [50]. The outcome analysis in our study identified significant correlation between ColXα1/elastin expression in the neoadjuvant-treated tumor but not in the adjuvant setting. Compared to neoadjuvant-treated cases, patients with early stage small tumors are treated with primary surgery and often have a good prognosis. Neoadjuvant-treated tumors are often of higher stage and histologic grade. Therefore, studying the predictive value of ColXα1 and/or elastin expression may be more useful in patients undergoing neoadjuvant treatment. We tried repeating the analysis in each molecular subtype. Our case numbers with available outcome data were relatively small for each subtype, but ER+ tumors (ER+/HER2+ and ER+/HER2−) showed a steady trend of correlation between higher ColXα1 and/or elastin expression and poor outcome. Further studies with larger patient numbers in each subtype and a longer follow-up period are needed.

Increased deposition of ECM components has also been described to enhance tumor progression. Overall, breast tumors exhibiting certain stromal biomarkers have a greater propensity for metastasis [11,51]. At the molecular level, Wnt7a was found to potentiate TGFβ, which correlates with stromal desmoplasia and poor prognosis [42]. Expression of matrix remodeling genes such as matrix-metalloproteinases and collagen cross-linkers is also predictive of a poor prognosis [52]. Therefore, expression patterns of factors within tumor-associated stroma may have therapeutic and theranostic applications. However, barriers to identifying effective anticancer therapies include the inability of *in vitro* cell culture models to recapitulate the complex three-dimensional (3D) tumor microenvironment for screening platforms to identify therapeutic candidates [53,54]. Novel complex 3D multicellular tumor models have shown promise in this application [55–58].

In summary, we demonstrate elastin and ColXα1 colocalization and plausible complexing as suggested by immunofluorescent and IEM ultrastructural assays. Our data support a model where ColXα1and elastin colocalization is specific to the neoplastic process. Progression of DCIS to invasive carcinoma may involve aberrant expression of ColXα1 and elastin in the tumor microenvironment. Enrichment of these complexes in tumor-associated stroma may represent a stromal signature indicative of fundamental biological and intrinsic differences between breast cancers. Accordingly, the success of cancer prevention and therapy may require an intimate understanding of reciprocal feedback processes between tumor cells and their associated stroma. These findings establish a foundation for investigation into the role of aberrant collagen complex expression in tumorigenesis and tumor progression that may be leveraged in future therapeutic and theranostic applications.

Acknowledgements

We thank Dr. Douglas Anthony (Pathologist-in-Chief of Lifespan Medical Center) for providing departmental research funding for this study. This study was also supported by the Molecular Pathology Core of the COBRE Center for Cancer Research Development funded by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P20GM103421.

Author contributions statement

YW and MBR conceived the study and prepared the manuscript. SL performed the Oncomine data analysis and statistical analyses. YW, JX, KS, CZ, and MO collected and reviewed all the cases in the study including morphological and immunohistochemical evaluation. DY and CS performed the molecular experiments. GJ performed electron microscopy experiments. RD, ASB, and HY participated in data reviewing and revising the manuscript. All authors read and approved the final manuscript.

References

1. Dolberg DS, Hollingsworth R, Hertle M, *et al*. Wounding and its role in RSV-mediated tumor formation. *Science* 1985; **230**: 676–678.
2. Lu P, Takai K, Weaver VM, *et al*. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb Perspect Biol* 2011; **3**: a005058.
3. Vakonakis I, Campbell ID. Extracellular matrix: from atomic resolution to ultrastructure. *Curr Opin Cell Biol* 2007; **19**: 578–583.
4. Daley WP, Peters SB, Larsen M. Extracellular matrix dynamics in development and regenerative medicine. *J Cell Sci* 2008; **121**: 255–264.
5. Marsh D, Suchak K, Moutasim KA, *et al*. Stromal features are predictive of disease mortality in oral cancer patients. *J Pathol* 2011; **223**: 470–481.
6. Liu J, Liu J, Li J, *et al*. Tumor-stroma ratio is an independent predictor for survival in early cervical carcinoma. *Gynecol Oncol* 2013; **132**: 81–86.
7. Wang K, Ma W, Wang J, *et al*. Tumor-stroma ratio is an independent predictor for survival in esophageal squamous cell carcinoma. *J Thorac Oncol* 2012; **7**: 1457–1461.
8. Underwood TJ, Hayden AL, Derouet M, *et al*. Cancer-associated fibroblasts predict for poor outcome and promote periostin-dependent invasion in oesophageal adenocarcinoma. *J Pathol* 2014; **235**: 466–477.
9. Ayala G, Tuxhorn JA, Wheeler TM, *et al*. Reactive stroma as a predictor of biochemical-free recurrence in prostate cancer. *Clin Cancer Res* 2003; **9**: 4792–4801.
10. Conklin MW, Eickhoff JC, Riching KM, *et al*. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am J Pathol* 2011; **178**: 1221–1232.
11. Moorman AM, Vink R, Heijmans HJ, *et al*. The prognostic value of tumour-stroma ratio in triple-negative breast cancer. *Eur J Surg Oncol* 2012; **38**: 307–313.
12. Iyengar P, Espina V, Williams TW, *et al*. Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. *J Clin Invest* 2005; **115**: 1163–1176.
13. Barsky SH, Rao CN, Grotendorst GR, *et al*. Increased content of Type V Collagen in desmoplasia of human breast carcinoma. *Am J Pathol* 1982; **108**: 276–283.
14. Vargas AC, McCart Reed AE, Waddell N, *et al*. Gene expression profiling of tumour epithelial and stromal compartments during breast cancer progression. *Breast Cancer Res Treat* 2012; **135**: 153–165.
15. Hulmes DJS. Collagen diversity, synthesis and assembly. In: *Collagen: Structure and Mechanics*, Fratzl P (Ed). Springer, New York, 2008; 12–47, Chapter 2.
16. Savarirayan R, Cormier-Daire V, Lachman RS, *et al*. Schmid type metaphyseal chondrodysplasia: a spondylometaphyseal dysplasia identical to the "Japanese" type. *Pediatr Radiol* 2000; **30**: 460–463.
17. Brodsky AS, Xiong J, Yang D, *et al*. Identification of stromal marker ColXa1 and tumor inflating lymphocytess as putative predictive markers of stromal marker of response to neoadjuvant therapy in hormone positive/HER2 positive breast cancer. *BMC Cancer* 2016; **16**: 274–287.
18. Wise SG, Weiss AS. Tropoelastin. *Int J Biochem Cell Biol* 2009; **41**: 494–497.
19. Trebaul A, Chan EK, Midwood KS. Regulation of fibroblast migration by tenascin-C. *Biochem Soc Trans* 2007; **35**: 695–697.

20. Tucker RP, Chiquet-Ehrismann R. The regulation of tenascin expression by tissue microenvironments. *Biochim Biophys Acta* 2009; **1793**: 888–892.
21. Kadar A, Tõkés A-M, Kulka J, *et al*. Extracellular matrix components in breast carcinomas. *Semin Cancer Biol* 2002; **12**: 243–257.
22. Bergamaschi A, Tagliabue E, Sørlie T, *et al*. Extracellular matrix signature identifies breast cancer subgroups with different clinical outcome. *J Pathol* 2008; **214**: 357–367.
23. Finak G, Bertos N, Pepin F, *et al*. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med* 2008; **14**: 518–527.
24. Duca L, Floquet N, Alix AJP, *et al*. Elastin as a matrikine. *Crit Rev Oncol Hematol* 2004; **49**: 235–244.
25. Rodgers UR, Weiss AS. Cellular interactions with elastin. *Pathol Biol* 2005; **53**: 390–398.
26. Sahoo S, Lester SC. Pathology of breast carcinomas after neoadjuvant chemotherapy: an overview with recommendations on specimen processing and reporting. *Arch Pathol Lab Med* 2009; **133**: 633–642.
27. Symmans WF, Peintinger F, Hatzis C, *et al*. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol* 2007; **25**: 4414–4422.
28. Tomczak K, Czerwinska P, Wiznerowicz M. The cancer genome atlas (TCGA): an immeasurable source of knowledge. *Contemp Oncol* 2015; **19**: A68–A77.
29. Curtis C, Shah SP, Chin SF, *et al*. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012; **486**: 346–352.
30. Richardson AL, Wang ZC, De Nicolo A, *et al*. X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell* 2006; **9**: 121–132.
31. Turashvili G, Bouchal J, Baumforth K, *et al*. Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. *BMC Cancer* 2007; **7**: 55.
32. Radvanyi L, Singh-Sandhu D, Gallichan S, *et al*. The gene associated with trichorhinophalangeal syndrome in humans is overexpressed in breast cancer. *Proc Natl Acad Sci U S A* 2005; **102**: 11005–11010.
33. Ma XJ, Dahiya S, Richardson E, *et al*. Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res* 2009; **11**: R7.
34. Karnoub AE, Dash AB, Vo AP, *et al*. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007; **449**: 557–563.
35. Meacham CE, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* 2013; **501**: 328–337.
36. Rombouts K, Carloni V. The fibrotic microenvironment as a heterogeneity facet of hepatocellular carcinoma. *Fibrogenesis Tissue Repair* 2013; **6**: 17.
37. Pickup MW, Laklai H, Acerbi I, *et al*. Stromally derived lysyl oxidase promotes metastasis of transforming growth factor-beta-deficient mouse mammary carcinomas. *Cancer Res* 2013; **73**: 5336–5346.
38. Gu J, Lu Y, Li F, *et al*. Identification and characterization of the novel Col10a1 regulatory mechanism during chondrocyte hypertrophic differentiation. *Cell Death Dis* 2014; **5**: e1469.
39. Monabati A, Sokouti AR, Noori SN, *et al*. Palpable ductal carcinoma in situ is Her-2 positive with high nuclear grade. *Int J Clin Exp Pathol* 2015; **8**: 3963–3970.
40. Shi C, Washington MK, Chaturvedi R, *et al*. Fibrogenesis in pancreatic cancer is a dynamic process regulated by macrophage-stellate cell interaction. *Lab Invest* 2014; **94**: 409–421.
41. Karagiannis GS, Theofilos P, Erdman SE, *et al*. Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. *Mol Cancer Res* 2012; **10**: 1403–1418.
42. Avgustinova A, Iravani M, Robertson D, *et al*. Tumour cell-derived Wnt7a recruits and activates fibroblasts to promote tumour aggressiveness. *Nat Commun* 2016; **7**: 10305.
43. Qiao A, Gu F, Guo X, *et al*. Breast cancer-associated fibroblasts: their roles in tumor initiation, progression and clinical applications. *Front Med* 2016; **10**: 33–40.
44. Uchiyama S, Fukuda Y. Abnormal elastic fibers in elastosis of breast carcinoma. UltrastructuraI and immunohistochemicaI studies. *Acta Pathol Jpn* 1989; **39**: 245–253.
45. Kao RT, Hall J, Stern R. Collagen and elastin synthesis in human stroma and breast carcinoma cell lines: modulation by the extracellular matrix. *Connect Tissue Res* 1986; **14**: 245–255.
46. Drifka CR, Tod J, Loeffler AG, *et al*. Periductal stromal collagen topology of pancreatic ductal adenocarcinoma differs from that of normal and chronic pancreatitis. *Mod Pathol* 2015; **28**: 1470–1480.
47. Wozniak MA, Desai R, Solski PA, *et al*. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J Cell Biol* 2003; **163**: 583–595.
48. Paszek MJ, Zahir N, Johnson KR, *et al*. Tensional homeostasis and the malignant phenotype. *Cancer Cell* 2005; **8**: 241–254.
49. Downey CL, Simpkins SA, White J, *et al*. The prognostic significance of tumour-stroma ratio in oestrogen receptor-positive breast cancer. *Br J Cancer* 2014; **110**: 1744–1747.
50. Dennison JB, Shahmoradgoli M, Liu W, *et al*. High intratumoral stromal content defines reactive breast cancer as a low-risk breast cancer subtype. *Clin Cancer Res* 2016; **22**: 5068–5078.
51. Provenzano PP, Eliceiri KW, Campbell JM, *et al*. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med* 2006; **4**: 38.
52. Neglia JP, FitzSimmons SC, Maisonneuve P, *et al*. The risk of cancer among patients with cystic fibrosis. Cystic fibrosis and cancer study group. *N Engl J Med* 1995; **332**: 494–499.
53. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* 2007; **8**: 839–845.
54. Hess MW, Pfaller K, Ebner HL, *et al*. 3D versus 2D cell culture implications for electron microscopy. *Methods Cell Biol* 2010; **96**: 649–670.
55. Vidi PA, Bissell MJ, Lelièvre SA. Three-dimensional culture of human breast epithelial cells: the how and the why. *Methods Mol Biol* 2013; **945**: 193–219.
56. Ye X, Luke BT, Wei BR, *et al*. Direct molecular dissection of tumor parenchyma from tumor stroma in tumor xenograft using mass spectrometry-based glycoproteomics. *Oncotarget* 2018; **9**: 26431–26452.

57. Belgodere JA, King CT, Bursavich JB, *et al*. Engineering breast cancer microenvironments and 3D bioprinting. *Front Bioeng Biotechnol* 2018; **6**: 66.

58. Wenzel C, Riefke B, Gründemann S, *et al*. 3D high content screening for the identification of compounds that target cells in dormant tumor spheroid regions. *Exp Cell Res* 2014; **323**: 131–143.

SUPPLEMENTARY MATERIAL ONLINE

Table S1. Differential tumor expression of ColXα1 and elastin