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Mechanical phenotype is important for stromal aromatase expression

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ABSTRACT

Evidence that aromatase expression in tumor-associated breast stroma is elevated, provides a rationale for use of aromatase inhibitors (AIs) in breast cancer treatment. However, regulation of local aromatase expression in cancer-free breast stroma is poorly understood. Recent clinical work indicates that stromal cells in dense breast tissue tend to express higher levels of aromatase than their counterpart from non-dense tissue. Consistent with the clinical observation, our cell culture-based study indicated that cell density, cell shape, and extracellular matrix (ECM) significantly induced stromal aromatase expression via a distinct signal transduction pathway. In addition, we identified a number of cell surface markers that are commonly associated with aromatase-expressing stromal cells. As mammographic density is one of the strongest and most prevalent risk factors for breast cancer, these findings provide a potential mechanistic link between alterations in tissue composition of dense breast tissue and increased stromal aromatase expression. Further exploration of the *in vitro* model system may advance understanding of an important problem in breast cancer biology.

1. Introduction

Estrogen biosynthesis in breast stroma is a significant source of estrogens in the tumor microenvironment [1–3]. For example, concentrations of local estradiol in breast tumors were similarly high in pre- and post-menopausal women [4,5]. Furthermore, the efficacy of aromatase inhibitors (AIs) for ER+ post-menopausal breast cancer clearly demonstrates the importance of aberrant extra-ovarian estrogen biosynthesis in breast cancer development and progression [6–8].

It has been well documented that tumor cell-derived factors such as cytokines and prostaglandin E_2 (PGE_2) stimulate stromal expression of aromatase (*Cyp19*), the rate-limiting enzyme in estrogen biosynthesis, in adipose stromal cells (ASCs) [9–12]. Subsequent promotion of the growth of estrogen-dependent tumor cells by elevated estrogen levels completes a "vicious cycle" for ER+ breast cancer progression [5,13]. Despite extensive studies of intratumoral stromal aromatase expression, relatively little is known about dysregulation of aromatase expression and local estrogen production in cancer-free microenvironment. However, there is a substantial individual-based variation in stromal aromatase expression [5,14,15]. Furthermore, forced expression of aromatase in mouse mammary gland results in hyperplasia [16] and increased incidence of chemical-induced tumor growth [17], supporting the notion that prolonged exposure to aberrant local estrogen biosynthesis can promote breast cancer.

Mammographic density refers to the extent of radiologically dense regions on a mammogram. Dense regions are enriched with epithelium and stroma [18]. The latter consists of various stromal cells and extracellular matrix (ECM) proteins [19,20]. In contrast, non-dense tissue is enriched with adipocytes. High mammographic density is a strong and prevalent risk factor for breast cancer [21–23]. Compared with those women with little or no mammographic density, women with mammographic density in more than 75% of the breast have 3–5 times higher risk of breast cancer [21,24–29].

The molecular cause for mammographic density-associated breast cancer risk remains poorly understood. Studies indicate that increased collagen density can promote cancer cell proliferation and invasiveness [30,31]. It has also been proposed that increased breast density could lead to aberrant production of hormones and growth factors, which in turn may stimulate proliferation of mammary epithelial cells [32–34]. Consistent with this notion, Vachon and coworkers recently showed that dense breast tissue expressed higher aromatase protein than non-dense tissue [35]. In the current study, we used cell culture models to investigate the potential impact of mechanical phenotype including cell number, cell shape, and ECM proteins on the aromatase-expressing capability of ASCs isolated from cancer-free individuals. Findings of our *in vitro* work

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Table 1
Characterization of cell surface marker expression in lyposuction- (top six) and reduction mammoplasty-derived stromal cells (bottom four).

	CD34	CD45	CD49d	CD90	CD140a	CD31	CD61	CD29
07L	71.39	1.48	0.58	98	0.04	0.26	1.51	96.69
08L	13.69	0.48	8.73	96.78	2	0.18	0.17	78.68
h6-17	12.9	0.73	3.98	96.22	1.68		0.64	
40504	13.31	0.87	23.68	98	0.38	0.71		80.64
h6-11	27	0.44	2.47	95.34	1.51		0.41	
41229	33.37	0.11	1.85	98.99	0.95			
BSC-5-10	14.97	0.56	0.12					
BSC5	84.56	5	12.45	96.98		0.27	7.44	79.4
BSC28	71.3	0.3	43.9	99.5	13.5	1.2		85.3
BSC29	2.4	0.5	1.8	98.2	3.7	3.1		50.4

offer some potential molecular and cellular explanations for the elevated aromatase expression in dense breast tissue.

2. Experimental

2.1. Cell culture

Primary human adipose stromal cells (ASCs) and breast derived stromal cells (BSCs) were isolated from patients undergoing abdominal lyposuction and reduction mammoplasty at the University of Virginia and University of Texas Health Science Center at San Antonio, respectively. The methods for tissue procurement and cell isolation were previously published and approved by the institutional Human Investigation Committees [36]. ASCs were cultured in DMEM/F-12 medium with 10% FBS and 1% antibiotic–antimycotic solution [36]. Sub-confluent and confluent ASC cultures were plated at 0.75×10^5 and 2×10^5/well in 6 well plates, respectively. To assess the effect of shape change, cells were cultured in ultra-low attachment plate (Corning) at 2×10^5/well in 6 well plates. For culturing ASCs in ECM, 2×10^5 cells were suspended in 125 μl of growth medium, mixed with the same volume of Matrigel (with low growth factor, Invitrogen) and cultured in 24 well plates for 2 days.

2.2. RNA isolation, cDNA preparation, and quantitative RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using the ImPrompII kit (Promega). Real-time PCR was carried out using the fluorescent dye SYBR-Green and an ABI 7900 Real-Time PCR System (Applied Biosystems). Primers used for GAPDH and aromatase were previously reported [37].

2.3. Gene knockdown with siRNA

Gene-specific knockdown by siRNA oligonucleotides were conducted using Lipofectamine reagent RNAiMAX (Invitrogen). The knockdown experiment was performed as previously described [38]. Briefly, cells at an approximate density of 60% were transfected with siRNA oligos at a final concentration of 10 nM and incubated for 72 h before they were harvested for RNA isolation.

2.4. Flow cytometry

For flow cytometry study, 5×10^5 cells were trypsinized and centrifuged for 3 min at 4000 rpm at 44 °C. They were subsequently resuspended in 100 μl of suspension buffer (1× PBS with 2% FBS), and incubated on ice with respective fluorescence-conjugated antibodies for 20 min. All antibodies were purchased from eBioscience. Samples were centrifuged, washed once with 1 ml suspension buffer, and suspended in 250 μl of suspension buffer. The cell surface marker analysis was performed by using a FACScalibur flow cytometer (Becton Dickinson).

3. Results

Primary ASCs used in published studies are usually isolated from reduction mammoplasty or lyposuction, and therefore are subject to individual-based sample variation. The lack of defined cell surface marker-based verification in standard protocols for ASC isolation could further contribute to inconsistency in laboratory findings. To address this technical caveat, we conducted an extensive marker-based characterization of primary ASCs from ten donors. As shown in Table 1, we found that aromatase-expressing ASCs isolated per our established protocol [36] reached at least 80% enrichment for the following cell surface markers: $CD31^-$, $CD45^-$, $CD61^-$, $CD140a^-$, $CD29^+$, and $CD90^+$. These markers provide useful tools for verifying the purity of primary aromatase-expressing ASCs.

Given that dense breast tissues are enriched with stromal cells and ECM proteins, we hypothesized that increased density of stromal cells and matrix proteins might alter the stromal expression of aromatase. Using primary ASCs from cancer-free individuals, we made the initial observation that high cell density increased aromatase mRNA level up to 500 fold (Fig. 1A) [15]. This was at least partly due to confluency-induced changes in cell shape, as physical alterations of cell shape by plating cells in low-attachment plates can also stimulate aromatase gene expression (Fig. 1B) [15]. The striking elevation in aromatase transcript upon cell density increase correlated with increases in aromatase protein and enzymatic activity [15]. Because the ASCs used in our study had not been exposed to any tumor milieu or changes in culture medium, our findings indicate that mechanical phenotype such as cell density and change in cell shape alone can markedly induce aromatase expression in ASCs. This can apparently occur in the absence of any tumor-associated factors or other exogenous stimuli. Using ASC-conditioned medium, we further showed that cell density-induced estrogen production in ASCs significantly stimulated estrogen-dependent transcription in ER-positive breast cancer cells [15].

Our published work on cell density and cell shape-induced aromatase expression was conducted in a 2-D system where ASCs were cultured on a plastic surface [15]. To explore the impact of ECM on stromal aromatase expression, we embedded ASCs in laminin-rich ECM (lrECM). When seeded with the same number, ECM-embedded ASCs express several orders of magnitude more aromatase mRNA than their counterparts cultured in 2-D (Fig. 1C). Interestingly, increasing cell density with a constant ECM concentration resulted in further elevation of aromatase transcription (Fig. 1D), indicating that the stimulatory effects of cell density and matrix are additive. Our published work identified IkB kinase β (IKKβ) as a key player in the cell density/shape-triggered aromatase induction [15]. IKKβ is a kinase that plays a critical role in chronic inflammation and cancer development [39,40]. Importantly, ECM-stimulated aromatase expression in rlECM-embedded ASCs is also dependent on IKKβ, as IKKβ knockdown substantially reduced the aromatase mRNA level in the 3D system (Fig. 2). Taken together, our findings support the notion that densities of stromal cells and