



Published in final edited form as:

J Mammary Gland Biol Neoplasia. 2020 March ; 25(1): 51–68. doi:10.1007/s10911-020-09446-3.

Inter-individual variation in response to estrogen in human breast explants

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Abstract

Background: Exposure to estrogen is strongly associated with increased breast cancer risk. While all women are exposed to estrogen, only 12% are expected to develop breast cancer during their lifetime. These women may be more sensitive to estrogen, as rodent models have demonstrated variability in estrogen sensitivity. Our objective was to determine individual variation in expression of estrogen receptor (ER) and estrogen-induced responses in the normal human breast.

Methods: Human breast tissue from female donors undergoing reduction mammoplasty surgery were collected for microarray analysis of ER expression. To examine estrogen-induced responses, breast tissue from 23 female donors were cultured *ex vivo* in basal or 10nM 17 β -estradiol (E2) media for 4 days.

Results: Expression of ER genes (*ESR1* and *ESR2*) increased significantly with age. E2 induced consistent increases in global gene transcription, but expression of target genes *AREG*, *PGR*, and *TGF β 2* increased significantly only in explants from nulliparous women. E2-treatment did not induce consistent changes in proliferation or radiation induced apoptosis.

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Conclusion: Responses to estrogen are highly variable among women and not associated with levels of ER expression, suggesting differences in intracellular signaling among individuals. The differences in sensitivity to E2-stimulated responses may contribute to variation in risk of breast cancer.

Keywords

Human breast explants; Estrogen Receptor; estrogen signaling

Introduction:

Epidemiological studies have identified lifestyle, environmental, and reproductive risk factors for breast cancer. Reproductive risk factors are linked to lifetime exposure to endogenous estrogens, including early menarche (RR = 1.3), late menopause (RR = 1.2–2.0), or having the highest quartile of serum estrogen levels (RR = 1.8 – 5.0) [1–4]. Exogenous estrogens and use of hormone replacement therapy containing both estrogen and progesterone also increases breast cancer risk (RR = 1.2) [5]. These risks factors would suggest that longer lifetime exposure to estrogen or periods of increased estrogen exposure increase breast cancer risk. Paradoxically, estrogen may also protect against breast cancer. While a full term pregnancy after age 35 increases breast cancer risk, a full term pregnancy before age 20 decreases breast cancer risk by up to 50% [6–9]. This protective effect of early pregnancy has been mimicked in rodent models with estrogen and progesterone treatment [10–13]. For postmenopausal women with breast cancer, high dose estrogen treatment can be an effective antitumor therapy [14, 15]. These contradictory outcomes to estrogen exposure suggest that high levels of estrogen can have antagonistic effects on breast cancer risk.

Estrogen acts in target tissues by binding to estrogen receptor dimers to initiate gene transcription [16–18]. There are two estrogen receptors: Estrogen Receptor alpha (ER α) and Estrogen Receptor beta (ER β). ER α protein is encoded by *ESR1*, located on chromosome 6q24–27 and ER β is encoded by *ESR2*, located on chromosome 14 q22–24. Gene transcription is modulated by the estrogen receptor dimer combination (ER α homodimers, ER β homodimers or heterodimers of ER α with ER β) and the recruitment of transcriptional co-activators or repressors [19]. In the breast, ER α expression is restricted to the luminal mammary epithelia, whereas ER β is expressed in both the luminal and basal epithelial cells and in the stroma [20]. The expression of ER α in the normal breast epithelium is positively correlated with risk of breast cancer [21, 22] and with age [23, 24]. The variation in ER α positivity among individuals, suggest that women vary, not only in their exposure to estrogen, but also their sensitivity.

All women are exposed to estrogens, but only a subset (~12%) are expected to develop breast cancer if their natural life expectancy is 80 years of age. Why are a subset of women more sensitive to their estrogens? Intracellular signals elicited by E2 may differ among women and determine the impact on breast cancer risk. Hormone studies using rodent models demonstrate variability to estrogen sensitivity in the mammary gland. Different strains of mice exhibit differences in proliferation, apoptosis and tumor development in

response to estrogen [25–27]. Likewise, in rat models, both the Copenhagen and Brown Norway rat are resistant to estrogen-mediated mammary tumors, while the ACI rat is susceptible [28].

Human models for estrogen response have relied primarily on population studies that have obtained human breast tissue at various life stages or different stages of the menstrual cycle. Several studies revealed wide variation in ER levels in breast tissues among women, but did not measure levels of estrogen or responses [29, 30]. 2D primary breast epithelial cell culture systems also have limitations because primary human breast epithelia lose estrogen receptor expression and consequently, their estrogen response [31, 32]. Estrogen receptor positive breast cancer cell lines, such as MCF7 and T47D cells, express high levels of estrogen receptor, and although exquisitely sensitive to estrogens, do not reflect responses of the normal breast. These estrogen receptor positive breast cancer cell lines have homogenous expression of ER α and they proliferate in response to estrogen-treatment [33, 34]. However, expression of ER α in normal breast epithelium is limited to a subset of cells and receptor expression does not overlap with cells that are actively proliferating [35, 36]. 3D human breast organoids are comprised of both basal and luminal epithelial cells and retain expression of estrogen receptors in a subset of cells [37], but are devoid of the endogenous stromal cells and connective tissues that may impact tissue responses. In contrast, human breast explants can be maintained in culture for a limited time (2–4 weeks), retain complex cell interactions and are responsive to steroid hormones *ex-vivo* [38–41]. The *ex-vivo* culture system eliminates confounding effects of other systemic hormones and factors.

Our goal was to determine variation of estrogen responses in women. Our first objective was to determine the range of endogenous estrogen receptor expression in a sample of women spanning 14 to 70 years of age. Our second objective was to utilize the *ex-vivo* human breast tissue explant model to examine the effects of a 4-day treatment with 10nM 17 β -estradiol (E2) in breast explants from individual women. Using this *ex-vivo* breast culture system, we can compare responses among women, as well as comparing specific responses within individual donor tissue cultured in basal or E2 media.

Transcriptional activation of genes containing estrogen response elements and formation of R-loops provide measures of estrogen-stimulated intracellular signaling. R-loops are DNA:RNA hybrids that form as a consequence of gene transcription [42, 43]. ER α has been shown to stimulate increases in R-loops providing a global indicator of E2-stimulated transcription [44, 45]. As a more specific indication of E2-mediated transcription, we also compared the expression of known estrogen receptor target genes (*AREG*, *PGR*, and *TGF β 2*) [46–48]. Changes in the proportions of cells with detectable levels of progesterone receptor (PR) and proliferating cell nuclear antigen (PCNA) provided additional measures of functional responses to E2-treatment. Previous work with human breast xenografts in mice [49, 50] and human breast explants cultured *ex-vivo* [39] demonstrated increases in both PR and proliferation. As a consequence of E2-mediated proliferation, apoptotic rates in the mammary epithelium may be altered. Eigeleine *et. al.* [39] determined that E2-treatment decreased spontaneous apoptosis the human mammary epithelium. However, in mice, radiation-induced apoptosis is increased in response treatment with estrogen and progesterone [51]. A prior pregnancy or treatment with exogenous estrogen and

progesterone at levels to mimic pregnancy also resulted in persistent increases in radiation induced apoptosis (spontaneous and radiation-induced) in the mammary epithelium of mice [13]. The human explant model system provides an opportunity to compare both spontaneous and radiation-induced apoptosis in human breast epithelium in response to E2 from both nulliparous and parous donors.

In the present study, significant age-related changes in estrogen receptors were observed in breast tissues. Although levels of *ESR1* and *ESR2* varied by greater than 80-fold in breast explants from donors, levels of estrogen receptor expression did not correlate with functional responses in the breast explant cultures. Tissue from nulliparous donors exhibited a greater sensitivity to E2 induced expression of target genes (*AREG*, *PGR*, *TGF β 2*), but expression was inconsistent among parous women. Levels of estrogen receptors do not appear to be rate-limiting for either induction of transcription-associated R-loops or expression of target genes. These data suggest that components of intracellular signal transduction differ among individuals resulting in variable patterns of gene expression and proliferation among individuals.

Materials and Methods

Sample Collection

Human breast tissue samples used for microarray analysis were collected from female donors, age 14–70 years, undergoing reduction mammoplasty surgery at Baystate Medical Center in accordance with the Institutional Review Boards at Baystate Medical Center and the University of Massachusetts, Amherst [52–54]. Donated tissue was snap frozen in liquid nitrogen and stored at -80°C at the Pioneer Valley Life Sciences Institute (PVLSI). Donor demographics and reproductive history were recorded after tissue collection. Human breast tissue for explants was gathered through the Rays of Hope Center for Breast Cancer Research at the PVLSI according to IRB approval #286173 and #132204. Tissue was collected from female donors ($n=23$) ages 18–62 years undergoing reduction mammoplasty surgery. Donor information including age and parity status were recorded.

Microarray Analysis

Microarrays were performed at the University of North Carolina at Chapel Hill as described previously [52–54]. Briefly, 100mg of frozen tissues were homogenized for RNA isolation using RNeasy kits. RNA quality and concentration were determined using an Agilent 2100 Bioanalyzer and a ND-1000 Nanodrop spectrophotometer. Two-color 4 \times 44K Agilent whole genome arrays were performed. Data are publicly available through the Gene Expression Omnibus (GSE:16113 and GSE:33526). When multiple probe sets for a gene were present, we used the probe with expression values for the most donor samples. Trends were analyzed using linear regression analysis in Graphpad Prism (GraphPad Prism version 7, La Jolla, CA).

Explant Culture

Fresh breast tissue was cut into approximately 1 \times 1 \times 4mm sections using a Stadie-Riggs microtome. For each donor, tissue sections were frozen or fixed immediately for time zero

(T=0) samples. The remaining sections were placed on top of surgical foam (Ethicon) in 60mm plastic dishes and cultured for a total of 7 days. The explants were kept in a humidified incubator with 5% CO₂ at 37°C. Explants were cultured in basal media for 3 days to clear endogenous hormones and then cultured in basal or E2 media for an additional 4 days. Basal culture media consisted of phenol red free DMEM:F12 (Sigma-Aldrich), 10% charcoal stripped FBS (FB-04, Omega Scientific), 10ng/mL human EGF (21–8356-U100, Tonobo Biosciences), and antibiotic-antimycotic (15240062, Gibco). E2 treatment media included basal media with the addition of 10nM 17 β -estradiol (E2758–250MG, Sigma-Aldrich). 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,4-triyl)trisphenol-treatment media (PPT; ER α specific agonist) or 7-Ethenyl-2-(3-fluoro-4-hydroxyphenyl)-5-benzoxazolol (ERB041; ER β specific agonist) included basal media with the addition of 200nM PPT or 200nM ERB041; respectively (Tocris, Minneapolis, MN). Half of the tissue from each donor was irradiated with 5Gy 6 hours prior to tissue collection on day 7 of culture. Half of the explant sections intended for histological endpoints were fixed in 10% NBF overnight and transferred to 70% ethanol before processing and paraffin embedding. The other half of the explant samples were snap frozen and stored at –80°C for later RNA isolation. Donor information for the explants used in this study are included in Table 1 and an overview of the breast explant model is detailed in Figure 2a.

RT-qPCR

RNA isolation was performed using TRIzol according to manufacturer's recommendation (15596018, ThermoFisher Scientific). cDNA was synthesized using 2 μ g of RNA with the Transcriptor First Strand cDNA synthesis kit (04–379-012–001, Roche). cDNA was diluted 1:10 before use in RT-qPCR. RT-qPCR was performed in a thermocycler (Mastercycler Eppgradient S model 5345, Eppendorf). See Supplemental Table 1 for primers used. Statistical analysis was performed using 2-way ANOVA in Graphpad Prism (GraphPad Prism version 7, La Jolla, CA). Expression is relative to mean control or to an inter run calibrator (IRC) consisting of pooled cDNA from a subset of T=0 (not cultured) samples.

Hematoxylin and Eosin Staining

Hematoxylin and eosin (H&E) staining were performed on paraffin-embedded 4-micron sections. Briefly, sections were deparaffinized in xylenes and rehydrated in graded ethanols. Sections were stained with Mayer's modified hematoxylin (S216–32OZ, Poly Scientific) and eosin phloxine alcohol working solution (S176–32OZ, Poly Scientific). After dehydration in graded ethanols and clearing with xylenes, slides were cover-slipped. Images were obtained under 200x magnification (BZ-X700 microscope, Keyence, Itasca, IL).

Promoter Methylation for ESR1 and ESR2

Donor tissues obtained from reduction mammoplasty conducted at Baystate Medical Center in Springfield, MA (IRB 132304) were minced and digested in digestion media with collagen immediately after collection as described [55]. Isolated mammary epithelia were enriched in primary culture in mammo-cult media. DNA extraction, bisulfite modification, PCR amplification and pyrosequencing were conducted as previously described [56]. Briefly, 2 μ l treated with DNA bisulfide modification reagents (ZymoResearch, Irving, CA). PCR products for each gene were generated using 1 μ l of bisulfite-modified DNA and

sequenced by Pyrosequencing PyroMark Q24 System (Qiagen Valencia, CA). Genomic target sequences for ER α and ER β are listed in supplementary table 2 [57, 58].

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin-embedded 4-micron sections using a DakoCytomation autostainer (Dako, Carpinteria, CA) and the antibodies listed in Supplemental Table 3. Sections were deparaffinized in xylenes, rehydrated in graded ethanols, and rinsed in phosphate buffered saline (PBS) before antigen retrieval. For ER β detection, antigen retrieval was performed in heated 0.01M citrate buffer for 10 minutes. Using the Acuity polymer detection system, primary antibody (1:200, Dako M7292 PPG5/10) incubation was performed at room temperature for 30 minutes, followed by incubation with horse-radish peroxidase (HRP) polymer and chromogen detection. For ER α , PR and PCNA detection the Envision HRP Detection system was used (Dako). Antigen retrieval was performed by heating in 0.01M citrate buffer (pH 6) for 10 minutes for PR and 20 minutes for ER α and PCNA. Primary antibody incubation was performed for 30 minutes using anti-ER α (1:20 MA5–13191, ThermoFisher) anti-PR (1:500 D8Q2J Cell Signaling), or anti-PCNA (1:10000 ab29 Abcam). Incubation with secondary antibody (K4001 or K4003, Dako) was performed according to manufacturer's instructions (Dako, Carpinteria, CA). Immunoreactivity was visualized with chromogen diaminobenzidine (DAB) incubation for 10 minutes. Sections were counterstained with hematoxylin. Images were obtained under 200x magnification (model BZ-X700 microscope). Positive cells were quantified (ImageJ software, <https://imagej.nih.gov/ij/>) as the percentage of positive luminal cell nuclei in a total of 600–1200 cells) [59].

Immunofluorescence

Freshly cut 4 μ M paraffin-embedded sections were deparaffinized/rehydrated (3X xylenes 5 min, 2X 100% ethanol for 5 min, 95% ethanol for 3 min, 70% ethanol for 3 min). Samples were rinsed with PBS. Antigen retrieval was performed by boiling slides in 0.1mM EDTA for 30 minutes. Samples were cooled to RT and treated with SSC 0.2X with gentle shaking at RT for 20 minutes. Samples were blocked in 5% BSA/PBS with 0.5% Tween-20 for 1 hr. Primary antibody incubation was done with monoclonal S9.6 antibody (1:100 ENH0001, Kerafast) or anti- γ H2AX (1:100, Cell Signaling #9718S) overnight at 4°C. After primary incubation, samples were washed 3 times with PBS containing 0.5% Tween-20 and then incubated with secondary antibody for 1 hr. Samples were washed 2–3 times with PBS containing 0.5% Tween-20 and then mounted with Vectashield mounting medium containing DAPI (H-1200, Vector Laboratories). Slides were imaged at 60X with Nikon A1 Spectral Confocal microscope in the Light Microscopy Facility and Nikon Center of Excellence at Umass Amherst. Analysis of S9.6 or γ H2AX foci per nucleus were calculated using Nikon analysis software.

TUNEL

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on 4-micron sections. Sections were deparaffinized in xylenes and rehydrated in graded ethanols. The Apoptag Plus Peroxidase in Situ Apoptosis Detection kit was used according to manufacturer's instructions to label apoptotic cells (S7101, MilliporeSigma). Sections

were rehydrated in ethanols and cleared in xylenes before adding cover slips. Images were taken at 200x (model BX40 microscope, Olympus, Tokyo, Japan) using a MicroPublisher 3.3RTV camera (QImaging, Surrey, British Columbia, Canada). Positive cells were quantified using ImageJ as the percentage of positive luminal epithelial cells.

RESULTS

Variation in estrogen receptor expression

Previous studies have demonstrated that ER α protein levels in normal human breast tissue increase with age. Because quantification of ER α expression by IHC is subjective due to heterogeneous distribution and intensity in the mammary gland, we used microarray expression data from normal breast tissue to examine the relationship between age of donor and mRNA levels of *ESR1* and *ESR2*. To account for variation in epithelial content between samples, cytokeratin 18 (*KRT18*) was used as a normalizer. Linear regression analysis of *ESR1* expression normalized to *KRT18* demonstrate that *ESR1* levels, although highly variable among women, significantly increase with age ($p < 0.0003$; Figure 1a), corresponding with previously reported ER α protein expression data. Variation in *ESR1* mRNA level was high, as indicated by a low R^2 -value for the line of best fit ($R^2 = 0.115$). *ESR2* expression normalized to *KRT18* (Figure 1b) in these same donors also revealed a significant increase in expression with age ($p < 0.0081$). There is a weak, but positive correlation between *ESR1* and *ESR2* (Pearson Correlation Coefficient 0.378, $p < 0.001$) in this data set. The data was also stratified by parity status to assess whether the effect of age was limited to one subgroup. There is no difference in *ESR1* expression between parous and nulliparous donors (Figure 1c) and age-related changes in expression were similar for both nulliparous and parous donors (Figure 1e $p = 0.064$ and 0.0016 , respectively). For *ESR2*, the overall expression was significantly higher among parous women (Figure 1d), but the effect of age did not reach statistical significance in either nulliparous or parous donors (Figure 1f; $p = 0.43$ and 0.12 , respectively). However, age is confounded with parity because there are significant differences in age between the nulliparous and parous donors (Supplementary Figure 1a and 1b; mean nulliparous = 26.46 ± 1.92 and mean parous = 44.45 ± 1.46 ; $p < 0.001$). Therefore, age is a major factor affecting levels of *ESR1* mRNA, but the apparent increase in *ESR2* expression (Figure 1b) is likely due to parity, rather than age.

While ER α expression is restricted to the luminal epithelial cells of the breast, ER β expression has been observed in luminal epithelial cells, myoepithelial cells, stromal cells, endothelial cells and lymphocytes. To examine ER β expression in luminal epithelial cells, we performed IHC using the PGG5/10 mouse anti-human ER β 1 monoclonal antibody in a subset of 45 samples (Figure 1g&h). Linear regression analysis of ER β positive luminal epithelial cells revealed a significant decrease in ER β protein expression with age ($p < 0.0288$). Both *ESR2* mRNA and ER β protein levels had a high level of variance (R^2 -values of 0.068 and 0.106, respectively). These data demonstrate age-related changes in expression of estrogen receptors, but that the effect is highly variable among individuals.

Promoter methylation at CpG sites silences gene expression and differences in promoter methylation for *ESR1* and *ESR2* may account for the differences in receptor expression between individuals. For instance, percent methylation will be lower in cells with high *ESR1*

expression and methylation will be higher in cells devoid of ER. This is demonstrated by comparing the high promoter methylation at 7 CpG sites at the *ESR1* promoter in MDA-MB-231 cells, which lack ER α , relative to the low *ESR1* promoter methylation in ER α -positive MCF7 (Figure 1i; last two samples) and ZR751 cells (supplementary Figure 2a). Likewise, comparison of 9 CpG sites in the promoter of *ESR2* demonstrates almost complete promoter methylation for ZR751 and a similar degree of promoter methylation for MCF7 and MDA-MB-231 breast cancer cells (Supplementary Figure 2b). Primary cultures of breast epithelial cells were used to interrogate methylation of these promoters. Comparing the 7 CpG promoter methylation sites for *ESR1* and the 9 CpG promoter methylation sites for *ESR2* in cells from 28 individuals spanning 16 to 61 years of age shows individual variation in the promoter methylation for both genes (Figure 1i & Supplementary Figure 2 c & d). Linear regression analysis shows no correlation with promoter methylation for either gene with age (Figure 1i) suggesting DNA methylation is not likely to be responsible for age-related changes in *ESR1* or *ESR2* mRNA. *ESR2* methylation was generally increased among women greater than 50 years of age, however two younger individuals also had elevated methylation of *ESR2*.

Human breast explant model for studying E2 responses in vitro

The analysis of *ESR1* and *ESR2* expression for figure 1 were performed from fresh tissue obtained from reduction mammoplasties. Some of these women were postmenopausal, but the majority were premenopausal and not controlled for stages of the menstrual cycle. Estrogen receptor expression has been reported to fluctuate with the menstrual cycle such that ER expression is higher at the follicular phase of the estrus cycle. There is also a general consensus that increasing E2 decreases the expression of ER α . The human breast explant system allows us to compare estrogenic responses in the breast tissues from each donor with their own basal control and without confounding systemic factors (Figure 2a). An additional benefit is that stromal, luminal, epithelial, myoepithelial, adipose and even immune cells are present in the culture, so cell interactions can still occur between these cell types. In attempting to restrict effects in explants due to growth factors, we have determined a requirement for EGF in the media; culturing in suboptimal explant conditions resulted in small rounded apoptotic nuclei in the ducts of epithelium that are indicative of tissue degradation (data not shown). In order to confirm that our culture conditions were not leading to degradation of the breast tissue, we compared tissue architecture from tissue that was fixed within two hours of surgery (T=0) to explant tissue after 7 days in culture (Figure 2 b–i). In the minimal media used for the explant samples in this study, we did not observe tissue degradation; nor did we detect gross apoptotic nuclei in the epithelial ducts. The explants retained normal breast architecture. Luminal epithelial cells maintained cuboidal shape and the surrounding stromal cells and adipocytes were intact. As reported by Russo J et al [60], the lobules of nulliparous individuals most often consisted of smaller lobular structures (Figure 2 b–e) whereas tissue from parous individuals had more complex Terminal Duct Lobular Unit (TDLU) structure (Figure 2 g–i) TDLU complexity (or lack of complexity) is retained by the tissue after 7 days in explant culture.

Optimization for gene expression in explant tissues

The expression of ER α protein in the human breast is heterogeneous, in both expression and intensity, and therefore, quantification is complicated. Patterns of distribution within individuals can be scattered or contiguous in the mammary epithelium of one lobule (Figure 3a), while neighboring lobules have absolutely no expression at all (Figure 3b). In addition, the explant fragments are small and tissue is limiting, specifically collagenous portions with good epithelial content. The combined heterogeneity and tissue limitations makes quantification of ER α immunohistochemistry unreliable in explant cultures. Therefore, we quantified *ESR1* and *ESR2* mRNA levels in response to estrogen treatment in the explant cultures. The breast tissue composition collected from the donors also varies greatly between individuals, which affects the amount of epithelium collected in each tissue fragment and the overall RNA yield. The breast tissue from some donors is enriched with collagenous stroma and epithelial cells, while the stroma of other donors has a greater adipose to collagenous tissue ratio and fewer epithelial cells. To account for differing amounts of epithelium between donors and between tissue fragments, we used *KRT18* as a normalizer for epithelial content. We prepared multiple fragments of flash-frozen un-treated breast tissue from three donors and used each piece in separate RNA isolations. We then used RT-qPCR to determine *ESR1* and *ESR2* normalized to *KRT18* (Figure 3 c & d). Donor 178 demonstrates how variable epithelial content of the tissue fragments can be. *KRT18* expression (light bars) in fragment 178b is ~6-fold greater and fragment 178d is ~2.7-fold greater than in fragment 178a. *ESR1* expression (striped bars) in the four fragments from donor 178 is also variable. However, when *ESR1* is normalized to *KRT18* (black bars), the *ESR1* expression is similar between the four fragments from the same donor. The tissue composition is less variable in donors 185 and 177, which have consistent *KRT18* and *ESR1* expression between fragments. However, comparison of *ESR1* normalized to *KRT18* shows that donor 177 had 3.8-fold higher expression of *ESR1* than donor 178 ($p < 0.01$) and 1.8-fold higher expression than donor 185 ($p < 0.05$; Figure 3d). Donor 185 had 2-fold higher expression than donor 178 ($p < 0.05$).

Both ER α and ER β contribute to the regulation of estrogen-stimulated responses in breast tissue. The promoters of amphiregulin (*AREG*) and progesterone receptor (*PGR*) have estrogen responsive elements that result in increased transcription, while transforming growth factor beta 2 (*TGF β 2*) is a direct ER target that may be transcriptionally repressed by E2 [61]. Expression of these genes may be modulated by ER β . Therefore, we wanted to compare the expression of each gene, each normalized to *KRT18*, in response to basal media, E2-treated media, or media treated with selective agonists for ER α (PPT) or ER β (ERB041) in tissue obtained from two different donors (Figure 3e). Basal expression of all 3 genes was significantly greater in donor 1 compared to donor 2, but the relative levels varied for each gene. For donor 1, only *PGR* expression was significantly increased by E2 whereas *AREG* and *PGR* expression were significantly increased by PPT ($p < 0.05$). Treatment with the ER β agonist ERB041 significantly decreased expression of all three genes in donor 1 ($p < 0.01$). Therefore, both ER α and ER β appear to participate in the regulation of these target genes. Although the basal expression of all three genes was very low in donor 2, E2 stimulated significant increases in *AREG* and *TGF β 2* ($p < 0.05$). PPT-treatment significantly increased all three genes: *AREG* by almost 40-fold, *PGR* by 24-fold and *TGF β 2* by 60-fold

($p < 0.001$). Whereas ERB041-treatment decreased gene expression in donor 1, expression of all 3 genes were increased by the ER β agonist in donor 2 ($p < 0.01$). These two donors demonstrate that basal gene expression differs between individuals as well as transcriptional responses mediated by ER α or ER β .

To confirm that *ESR1* expression is retained in the explant cultures, we compared normalized *ESR1* expression from two separate donors that had been cultured for 5 days in basal media (T5 Basal) or 10nM E2 (T5 E2) to the normalized *ESR1* expression in the fresh donor tissue (T=0; Figure 3f). *ESR1* levels were similar between the T=0 fresh tissue and tissue that had been cultured for five days, although there was a consistent 2-fold difference in normalized *ESR1* expression between tissue from the two different donors.

Transcriptional responses to estrogen

We used immunofluorescence for the S9.6 antibody against DNA:RNA hybrids as a measure of global gene transcription (Figure 4). There was no difference between the number of R-loop foci/nucleus between T=0 and the explants maintained in basal media (Figure 4c), indicating that basal transcription between the two conditions were similar. However, the E2-treated explants had significantly more R-loop foci/nucleus than either the T=0 tissues or the tissues cultured in basal media ($p < 0.01$). For each donor sampled, E2-treatment increased R-loop formation similarly relative to each basal control (compare representative Figure 4a with 4b), albeit donor 237 had significantly lower R-loop foci/nuclei due to E2-treatment than each of the other four E2-treated donor samples ($p < 0.01$; Figure 4d). These results indicate that 10nM E2-treatment activated significant increases in R-loop formation relative to basal levels, indicating greater transcription compared to the fresh tissue or the explants maintained in basal media.

E2-induced transcriptional activation of *AREG*, *PGR*, and *TGF β 2* were examined using RT-qPCR as more specific endpoints of E2-ER mediated transcriptional responsiveness (Figure 5a–c). Using 2-way ANOVA to account for donor variation, we compared expression levels between the basal and E2-treated samples. In our combined sample set of nulliparous and parous women, there were no significant changes in *AREG*, *PGR*, or *TGF β 2* expression induced by E2-treatment. However, after comparing responses from nulliparous and parous groups separately, we noted significant increases in E2-mediated expression in tissue from nulliparous donors. In nulliparous samples, mean *AREG* expression increased from 0.39 ± 0.23 to 1.14 ± 0.34 ($p < 0.05$; Figure 5a). *PGR* expression increased from 0.30 ± 0.15 to 1.53 ± 0.37 ($p < 0.06$; Figure 5b). *TGF β 2* expression increased from 0.46 ± 0.25 to 1.68 ± 0.34 ($p < 0.05$; Figure 5c). In parous samples, *AREG*, *PGR* and *TGF β 2* expression were not significantly increased and, in some individuals, expression decreased.

To determine if 4-day E2-treatment exerts a repressive effect on the estrogen receptors, we compared normalized *ESR1* and *ESR2* expression between explants that were either maintained in basal media or media with 10nM E2 from nulliparous and parous donors. RT-qPCR for normalized *ESR1* shows a trend of decreased expression in explants after 4-day E2-treatment relative to basal explants (Figure 6), but was not significant ($p = 0.1805$). Expression of *ESR1* was decreased or not changed in response to E2-treatment for 66% (8 of 12) donors, but increased for four donors. There was also no significant change in *ESR2*

expression between the basal or E2-treated explants, although two donors with high *ESR2* expression did demonstrate a reduction of *ESR2* expression after culturing in E2-media. Parity also did not affect *ESR1* and *ESR2* expression. Although E2-treatment did not repress expression of these receptors, we observed over 80-fold variation in *ESR1* expression and over 100-fold variation in *ESR2* expression between the explants maintained in basal media from this donor population. Pearson correlation analysis found no correlation between either *ESR1* or *ESR2* mRNA levels and fold-increase in expression of *AREG*, *PGR*, or *TGFβ2* with E2-treatment. However, *ESR1* is positively correlated with *ESR2* expression (0.702; $p=0.02$; Supplementary Figure 3). Therefore, levels of estrogen receptors do not appear to be rate-limiting in determining estrogen-induced transcriptional responses in breast tissues.

Functional responses to estrogen

We compared PR protein expression and proliferation in the human explants to assess functional responses of the E2-treatment *ex-vivo*. PR was detected in T=0 and cultured explants samples (Figure 7a). Quantification of PR positive luminal epithelial cells revealed 13-fold variation in PR expression level between donors in T=0, with a mean expression of 11.52% \pm 2.55 (Figure 7b). PR expression was maintained in the cultured explants. Basal control treated explants expressed mean PR levels of 7.9% \pm 1.38, which is not significantly decreased from T=0 samples. PR expression in explants treated with 10nM E2 were not different from basal-treated, but showed a significant decrease (3.44% \pm 0.94) compared to T=0 samples ($p<0.05$). Likewise, we did not detect significant differences in proliferation as measured by PCNA expression between T=0 (24.64% \pm 5.78), basal (17.21% \pm 3.6) or E2-treated (26.74 \pm 3.47) explants, although the E2-treated almost reached significance relative to basal (Figure 7c & d; $p = 0.067$). Linear regression analysis shows no significant change in proliferation with either age or parity for either the basal or E2-treated explants (Figure 7e). However, it is interesting to note, that some donors were insensitive to E2-mediated proliferation (black arrows, same color circle and square are close together) regardless of whether they had a high or low basal proliferation index. In particular, the oldest parous donor (58 yrs.) had the highest percent proliferation (basal and E2-treated), while the nulliparous donor of similar age (61 yrs.) had low basal and E2-treated proliferation. Both of these donor tissues were insensitive to the E2-mediated proliferation in culture. In contrast, explants from other donors were sensitive to E2-mediated proliferation (white arrows) with high percent PCNA positive cells (squares) relative to the basal-treatment (same color circles).

We irradiated half of the tissue from each explant sample with 5Gy ionizing irradiation (IR) 6 hours prior to collection in order to compare both spontaneous and radiation-induced apoptotic responses in human breast explants between basal and E2-treated. Irradiation significantly increased the percent apoptotic cells in basal-treatment from 1.36% \pm 0.34 to 4.53% \pm 1.2 and increased the number of apoptotic cells in the E2-treatment group from 1.14% \pm 0.33 to 2.94% \pm 0.55 (Figure 7f-h). Although radiation-induced apoptosis was greater than spontaneous apoptosis, E2-treatment did not enhance or reduce the apoptotic response in these explant samples. There was no significant difference in irradiation response between nulliparous and parous explants. In order to determine if there was also an E2-mediated accumulation of DNA damage due to IR, we quantified γ H2AX foci/nucleus

in each of the four explant treatments (Figure 7i–k). For this analysis, we quantified foci in epithelial cells (white arrow, E2 IR inset; Figure 7k) because they represent cells surviving with DNA damage, but not apoptotic nuclei (white arrowhead, basal IR inset; Figure 7j) because these cells are being eliminated. The apoptotic cells were often rounded with intense nuclear signal and usually located within the ductal lumen. Approximately 0.56 γ H2AX foci/nucleus were observed in the basal treated explants and γ H2AX was significantly increased to 1.06 foci/nucleus with IR ($p=0.02$). There was no significant increase in γ H2AX foci/nucleus for the E2-treatment (0.93 foci/nucleus) relative to the basal treatment. Although the E2+IR-treatment (2.3 foci/nucleus) was significantly greater than the basal no IR ($p=0.05$), it was not significantly different from the E2 no IR. Pearson correlation analysis of the functional responses to E2-treatment in the explants found that spontaneous apoptosis was correlated with proliferation (Correlation index 0.817; $p = 0.013$; data not shown), although none of the E2-mediated functional assessments were correlated with *ESR1* or *ESR2* expression.

Discussion:

Exposure to estrogen is a risk factor for breast cancer [1]. Extended lifetime exposures to estrogens, including early menarche, late menopause, or hormone replacement therapy post menopause are factors associated with breast cancer risk. Similarly, women with the highest quartile of serum E2 levels have increased risk relative to those with the lowest quartile of serum E2. Women vary in their estrogen exposure, but also in their sensitivity to estrogens. Increased breast density is also associated with increased risk of breast cancer and because density is positively correlated with higher serum estrogens [62], breast density may signify estrogen sensitivity. Likewise, specific genetic polymorphisms in the gene *ESR1*, including 908A/G (K303R) and 1608T/A (Y537N), code for an ER α protein that is hypersensitive to E2 [63] or constitutively active [64], respectively. These SNPs (and others) are associated with increased risk of breast cancer, resistance to anti-estrogens and poor prognosis [65–68].

Because E2 exposure and E2 sensitivity are associated with breast cancer risk, our goal was to utilize the breast explant model to normalize a panel of breast tissue specimens, from both pre-and post-menopausal women, to a specific dose of E2: either none (basal) or pregnancy levels (10nM). Our objective with these treatments was to establish ranges of E2 sensitivity in a population and determine if it was correlated to ER expression. To ascertain estrogen sensitivity, we compared E2-mediated transcription and proliferation. The effects of estrogen on radiation-induced apoptosis was also examined as an indicator of the potential to accumulate DNA damage and the bioactivity of the p53 tumor suppressor pathway.

Expression of ER α protein has been extensively studied in human breast tissue and the protein expression is highly variable, such that detection of any receptor positivity may be limited to between 35–60% of individual samples [29, 69, 70]. ER α detection and intensity are also highly variable within each individual specimen and one lobule may exhibit high ER expression while several neighboring lobules express none. Likewise, ER expression patterns throughout the tissue may be singular in a lobule, scattered, or a duct may express a contiguous pattern in several adjacent cells [71, 29]. Because explant tissue is limiting and immunohistochemistry for ER α is semi-quantitative, we sought to quantify *ESR1* and *ESR2*

expression in the treated and un-treated breast tissue explants to compare individual responses. Our data demonstrates considerable diversity in the estrogen receptor expression in the explant donor population. *ESR1* varies by greater than 80-fold and *ESR2* by greater than 100-fold among individual donors.

Previous studies quantifying immunohistochemical detection of ER α in the epithelial cells of normal human breast tissue demonstrate that ER α expression increases with age [22, 72, 73, 23], particularly among postmenopausal women. Through microarray analysis of normal human breast tissue from reduction mammoplasties, we observed that *ESR1* mRNA also increases with age. We observe a similar age associated increase for *ESR2* expression (Figure 1b), but the proportion of breast epithelial cells expressing ER β protein was decreased with age (Figure 1g). ER β is known to be expressed in stromal and basal epithelial cells in addition to luminal epithelial cells [20]. It has been reported that the epithelium: stroma ratio decreases with age [71]. Therefore, the increased proportion of stroma with age could account for the overall increase in *ESR2* mRNA when expressed relative to the luminal marker *KRT18* mRNA. There are some inconsistencies with regards to ER expression in parous versus nulliparous individuals. Some report that parity is associated with increased ER protein [74], but others report decreases in ER α and *ESR1* in samples from parous women [75]. We, however, did not find any differences in *ESR1* expression in the breast between nulliparous and parous individuals.

To modulate cellular sensitivity to hormone, negative-feedback of ligand-activated receptors represses the expression of their own receptors. The human explant system offers a unique opportunity to examine negative-feedback and repression of estrogen receptors because each E2-treated donor sample can be compared to its own basal control. E2-treatment did not significantly reduce *ESR1* or *ESR2* expression as a whole, but there was a trend towards decreased ER expression in 8 of the 12 donor tissues (Figure 6). Similar differences were observed among human breast cancer cell lines [76–80]. Other investigators demonstrate that MCF7 cells that have been maintained in media with serum will have E2-mediated repression of ER transcript. However, when MCF7 cells are maintained in media with serum depleted of estrogens for an extended time, they no longer repress ER transcript during estrogen-treatment. Additionally, the T47D breast cancer cell line does not repress ER transcript in the presence of estrogen. The literature demonstrates that the regulation of estrogen receptor transcript by E2 depends on the cell line and prior estrogen exposures and not all epithelial cells will repress ER transcripts in the presence of estrogen.

The estrogen receptors initiate ligand-dependent transcription in target cells [16–18]. As a consequence of estrogen-mediated transcription, DNA-RNA hybrids, referred to as R-loops, are formed [44, 42, 43]. In MCF7 cells, E2 stimulates R-loop formation at E2-responsive genes [44]. We have also demonstrated that E2-stimulates R-loop formation in T47D cells and in the mammary epithelium in mice [45]. MCF10A cells [44] and 76NTert cells [45] lack estrogen receptors and fail to increase R-loop formation in response to E2. But R-loops are induced by E2 in 76NTert cells that have been transduced with a doxycycline inducible ER α . Because E2-mediated increases in R-loop formation are dependent on ER-mediated transcription, we can use the S9.6 antibody to demonstrate global E2-ER-mediated gene transcription in the breast tissue. We detected a significant increase in estrogen-mediated R-

loop formation, signifying increased transcription in the E2-treated explants that was consistent among donors (Figure 4).

Although E2-treatment consistently increased global ER-mediated gene transcription in donors, the expression of specific estrogen response genes (*AREG*, *PGR*, and *TGFβ2*) was highly variable (Figure 3e and 5). *AREG* and *PGR* are induced in response to E2 in mice [46] and human breast cancer cell lines [47]. *TGFβ2* is also a direct target of ER and but E2 represses its expression in MCF7 cells [48]. Multiple components of the TGFβ pathway are also repressed by E2 and may be modulated by ERβ [61]. Therefore, we compared the expression of these estrogen response genes using ER specific agonists: PPT for ERα and ERB041 for ERβ. The ERα specific agonist induced expression of *AREG* and *PGR* in both sets of donor tissue and strongly induced *TGFβ2* expression for donor 2. The responses to the ERβ specific agonist were more surprising. For the donor with modest basal expression of the three genes, the ERβ specific agonist repressed expression of *AREG*, *PGR* and *TGFβ2*, agreeing with multiple reports that ERβ opposes the activity of ERα [81–83]. However, gene expression was increased for all three of the genes by the ERβ specific agonist in donor 2. In both of these donors, gene activation by E2, the agonist for both receptors, was more modest relative to either PPT or ERB041 alone and may be due to ratios between the two receptors in the breast tissue [19, 81].

Significant increases in *AREG* and *TGFβ2* mRNA were observed in the nulliparous subset, but not among the parous donor tissues (Figure 5). That we did not find significant increases in *PGR* was surprising because E2 has been shown to up-regulate *PGR* in estrogen responsive human organoids, whereas *AREG* was not [84]. Likewise, expression of *PGR* transcript is significantly greater in ER+ breast tumors taken from premenopausal women at phases of the menstrual cycle where serum E2 levels are highest [85]. Meng *et al.* proposed that in organoids, only a subset (7–10%) of cells are ER+, whereas ER expression is more homogenous in ER+ breast cancers. Therefore, there is likely a dilution effect that obscures detection of increases in estrogen responsive genes in the heterogeneous organoids, and likely in our more cellularly heterogeneous explants. More to the point, these genes were identified as ER targets in ER-positive breast cancer cell lines; cells with homogeneous expression of ERα. Human tissue has heterogeneous ERα expression. Therefore, the expression of these particular genes may be modest and more variable in relation to breast cancer cell lines. Although it is hypothesized that individuals with greater ER expression are more sensitive to estrogen, we found no correlation between *ESR1* and *ESR2* expression and E2-stimulated expression of *AREG*, *PGR*, and *TGFβ2*. Rather, genes that are consistently regulated by ER in breast cancer cell lines are more diversely regulated in the normal human breast, indicating the presence of “personal patterns” of ER-mediated transcription that differ among individuals.

The *ESR1* and *ESR2* expression also was not correlated to functional estrogen responses: PR protein expression, proliferation or apoptosis. Unlike ER which is detected in less than 50% of specimens, PR is readily detected in greater than 80% of human breast specimens by IHC [29, 30]. Human explants in nude mice had initial mean increases in PR expression in response to E2-treatment [49]. *In vitro* human explant experiments by Eigeliene *et al.* demonstrated E2-mediated increases in proliferation [39], although PR expression was not

changed relative to control. The PR expression in our E2-treated explants was also not different from basal-treated (Figure 7b). This agrees with human PR comparisons that demonstrate equivalent expression during both follicular and luteal phases of the menstrual cycle [29, 30].

Ki67 is used widely to quantify proliferation in human breast cancer. However, it is subject to inter-laboratory variability [86–88] and the range of Ki67 positive cells is very low (0.3–2.6%) in the normal breast [21]. Likewise, phospho-histone H3, an S/M-phase specific marker for proliferative index, is also very low (<0.05%) and in normal epithelium does not differentiate proliferation between E2-treated and control [41]. In the explant cultures, we found Ki67 expression was low (<2% of cells). Given the limited numbers of epithelial cells in the small fragments of explant tissues, it was not possible to accurately quantify differences between proliferation in basal and E2-treated. PCNA is a proliferation marker expressed more broadly across the S and G2 phases of the cell cycle [89, 90]. We used PCNA to mark proliferation in mammary epithelium from ovariectomized mice treated with estrogen and progesterone for 4 days. The percent PCNA positive in the E+P-treated mice (60%) is significantly increased relative to basal (<5%) [51]. In the human explants, PCNA positivity had a greater range amongst individuals (range 3.0–58.4%). In the explant cultures, there was a trend for an increase in PCNA-positivity in the E2-treated relative to basal control. Although several of the donors were insensitive to E2-mediated PCNA expression, most of the donor explants increased PCNA in response to E2 to varying degrees. Similar variability in proliferative responses to hormone-treatment of human breast tissue determined that ~35% responded to progesterone only, ~20% responded to estrogen only, ~34% responded to either hormone and ~10% were non-responsive [41]. It has also been reported that proliferation in the human mammary epithelium is reduced in parous individuals, declines with age and is correlated with increased serum progesterone during the luteal phase of the menstrual cycle [91–93]. However, E2, not progesterone, induced proliferation in human explants implanted in nude mice [49]. In the explant model, we did not find differences in percent PCNA-positive epithelial cells due to age (Figure 7e) or parity.

Parous BALB/c mice are more sensitive to radiation-induced apoptosis than nulliparous [13] and 4-day E2+P4-treatment enhances radiation-induced apoptosis [51]. In the human breast tissues, spontaneous apoptosis did not vary during the menstrual cycle [93, 91], although E2-treatment repressed spontaneous apoptosis in explants [39]. However, there was no difference in either spontaneous (no IR) or radiation-induced (+ IR) apoptosis in in the explants in response to E2 (Figure 7f). Although radiation enhanced apoptosis regardless of hormone treatment, there were no differences in apoptosis between nulliparous or parous donors. DNA damage induced by irradiation may accumulate in cells if E2 were to repress apoptosis. Additionally, E2 also induces DNA damage by producing free-radicals, forming DNA adducts, and causing DNA strand breaks [94–96]. ER-mediated formation of R-loops are also susceptible to DNA damage [44, 45]. Our results do show an increase in both apoptosis and DNA damage (γ H2AX foci/nucleus) due to IR in the breast explants. But neither apoptosis, nor DNA damage are increased by E2 alone. Likewise, there is no additional accumulation of IR-induced DNA damage in the human breast explants treated with E2. Therefore, from these two experiments we could conclude that E2-treatment for

four days neither induces significant DNA damage, nor does it repress apoptosis to allow the accumulation of epithelial cells with DNA damage.

These results demonstrate the diversity in expression of estrogen receptors in breast tissue among women. *ESR1* and *ESR2* expression increases significantly with age, following the previously reported trend for increased protein expression. E2-induces significant increases in global transcription-associated R-loops in the explants, but the pattern of gene transcription appears unique for each individual. ER-target genes were consistently induced in nulliparous explants, but expression was heterogeneous among tissues from parous women. Estrogen receptor mRNA expression is highly variable in the human breast, as is the estrogen response, and *ESR1* and *ESR2* expression were not correlated with either transcriptional responses or proliferation in the human breast. The data suggest that the complement of transcriptional co-activators and co-repressors may differ among individuals and modify the sensitivity and patterns of responses to estrogen in human breast epithelium. Differences in estrogen-induced transcriptional responses among women may be important for defining for whom exposure to endogenous estrogens or environmental xenoestrogens may pose a substantial risk for breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

Research reported in this publication was supported, in part, by the National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number U01ES026140 (DJJ, SSS) and R01ES015739 (DJJ). Funding was also provided by the Department of Defense under contract #W81XWH-15-1-0217 (DJJ) and the Rays of Hope Center for Breast Cancer Research (KAD, GMJ, DJJ).

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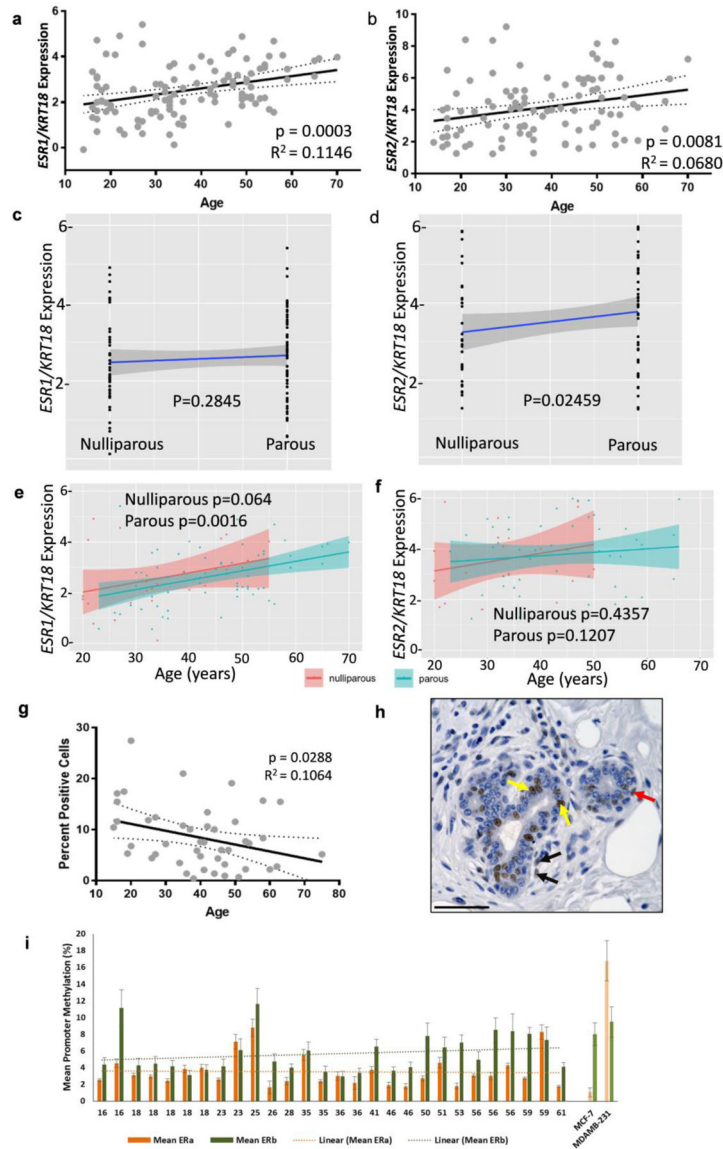


Fig. 1: Estrogen receptor expression diversity among women.

(a-b) Agilent microarray data showing relative (a) *ESR1* mRNA levels from 111 donor samples and (b) *ESR2* mRNA levels from 102 samples, normalized to *KRT18* expression. Linear regression analysis showed a significant increase in both *ESR1* ($P < 0.001$) and *ESR2* mRNA levels ($P < 0.01$) with age. Dotted lines indicate 95% confidence intervals and solid line indicates the best fit line. R^2 values for best fit lines are indicated. (c-d) Relative *ESR1* and *ESR2* expression in nulliparous and parous women. (c) There is no difference in relative normalized *ESR1* expression between nulliparous and parous donors. (d) *ESR2* expression is increased by parity. (e) Normalized *ESR1* expression increases with age in parous donors and is approaching significance in nulliparous donors. (f) Normalized *ESR2* expression does not increase with age for either nulliparous or parous donors. (g-h) IHC using ERβ antibody (PPG5/10) was performed on paraffin-embedded sections from 45 patient samples. (g) Quantification of ERβ positive ductal cells. Linear regression analysis of ERβ protein levels

shows a significant decrease in ER β with age ($P < 0.05$). 95% confidence intervals indicated by dotted lines and solid line indicates the best fit line. (h) Representative image of IHC. Arrows indicate representative positive cells: yellow arrows indicate positive luminal cells, red indicate positive basal cells, and black indicate positive stromal cells. Scale bar 50 μ M. (i) Methylation in the promoters for *ESR1* and *ESR2* show there is no association with promoter methylation and age for either *ESR1* or *ESR2*.

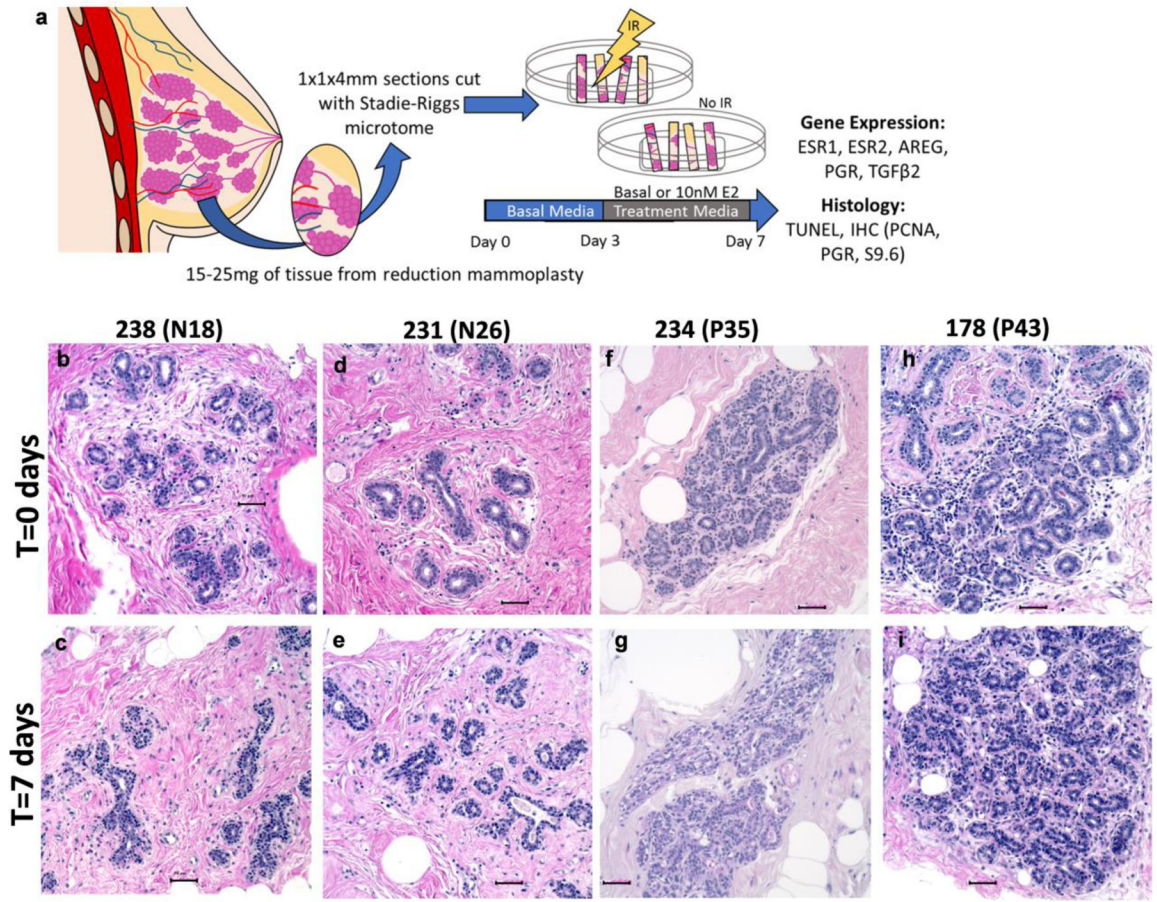


Fig. 2: Tissue architecture of human breast explants are intact after 7 days in culture.

(a) Overview of breast explant model. Tissue obtained from women undergoing reduction mammoplasties was sectioned using a Stadie-Riggs microtome. After clearing in Basal media for 3 days, explants were treated with Basal or E2 containing media for an additional 4 days. On day 7, half of the samples were subjected to 5Gy of irradiation 6 hours prior to tissue collection, and FFPE or freezing in liquid nitrogen for RNA analysis. (b-i) Representative hematoxylin and eosin staining of nulliparous (b-e) and parous (f-i) explant tissue. Comparison of fresh tissue T=0 (b,d,f,h) today 7 tissue (c,e,g,i) demonstrate that tissue architecture is maintained in culture conditions. Scale bars 50µm.

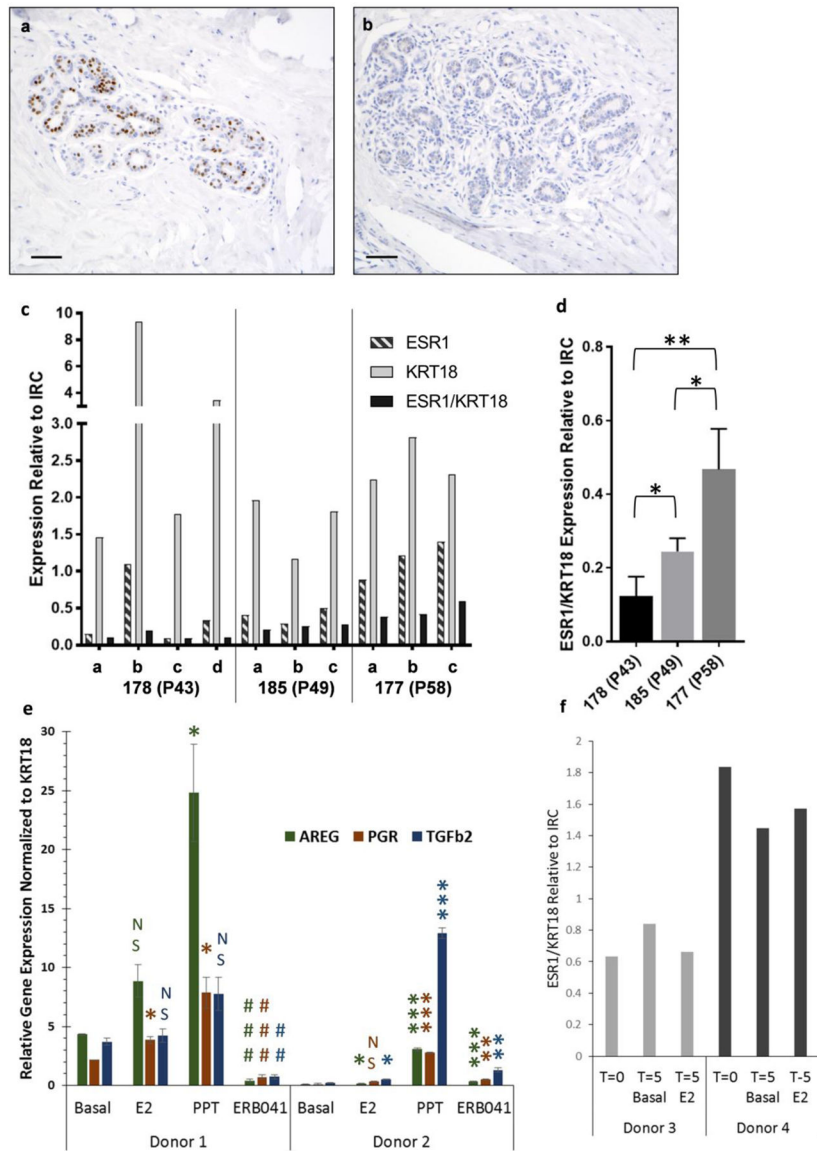


Fig. 3: Optimization for gene expression in explant tissues.

Representative ERα IHC for donor 234 T=0 of two different lobules from same section demonstrating (a) high ERα expression versus (b) no ERα expression. Scale bar is 50μM. (c) 3–4 separate 100ug pieces of flash-frozen tissue from three donors were treated as separate samples and used for RT-qPCR analysis of *ESR1*, *KRT18* and *ESR1* expression normalized to *KRT18*. (d) Normalized *ESR1/KRT18* expression demonstrates variation between donors. (e) Comparison of estrogen response genes (*AREG*, *PGR*, *TGFβ2*) in tissue from two donors (3 replicates per donor) collected after treatment with basal media, E2, PPT (ERα specific agonist) or ERB041 (ERβ specific agonist). Genes are normalized to *KRT18* and relative to inter run calibrator (IRC). (f) Tissue from two donors was collected at day 0 (T=0) and day 5 (T=5) for RT-qPCR analysis. *ESR1* levels normalized to *KRT18* and relative to a pool of fresh breast tissue which was used as an inter-run calibrator (IRC) are shown.

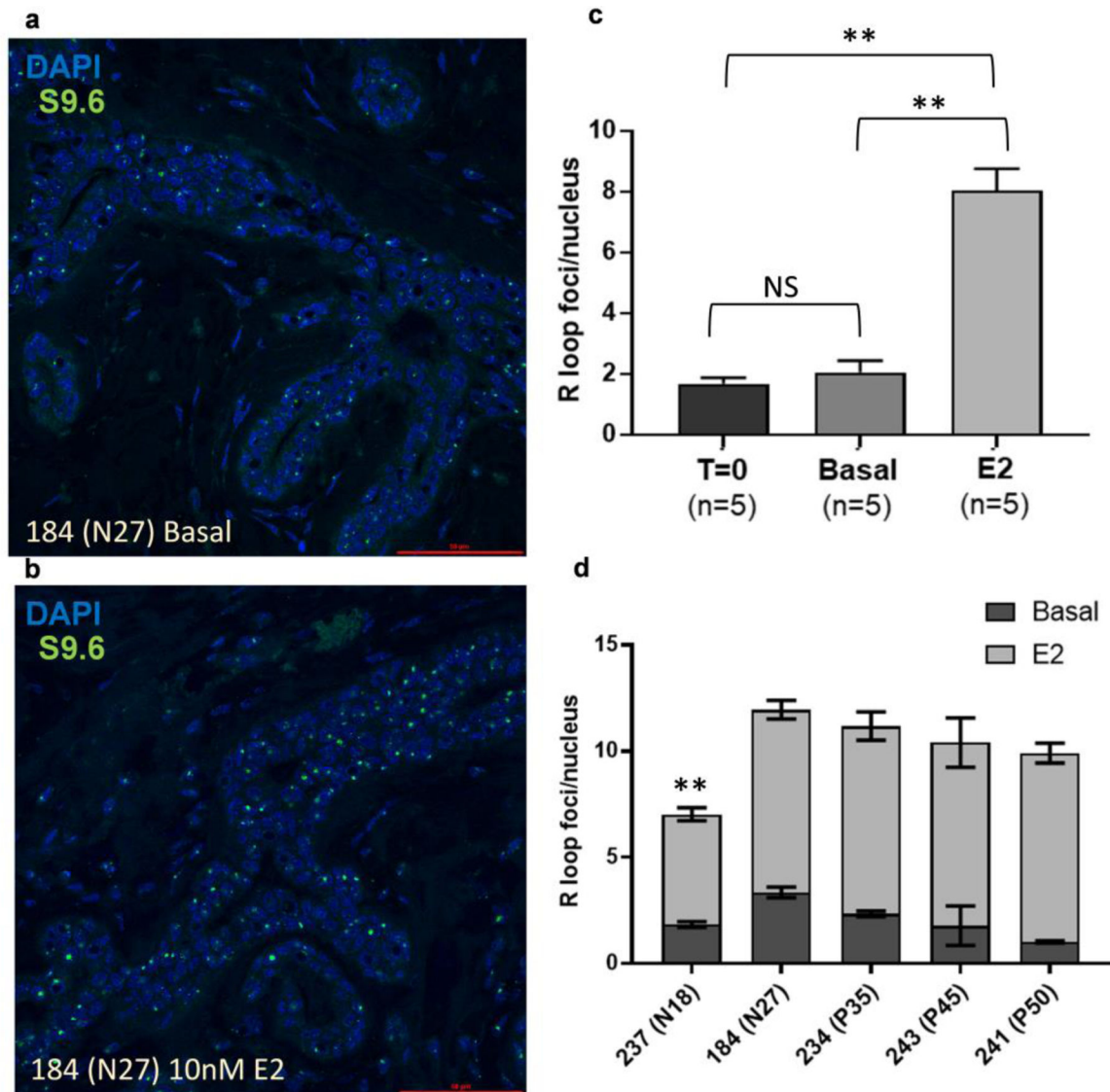


Fig. 4: E2-induces R-loop formation in human explants.

Representative images of S9.6 immunofluorescence in (a) basal and (b) E2-treated explants. (c) Quantification of R-loop foci per nucleus in basal and luminal epithelial cells demonstrates increased R-loop formation with E2-treatment relative to T=0 or basal explants (n =5 for T=0 and each treatment group). (d) R-loop foci per nucleus in Basal and 10nM E2 explants from 5 donors. Error bars indicate SEM. Significance was determined using ANOVA (** p<0.01).

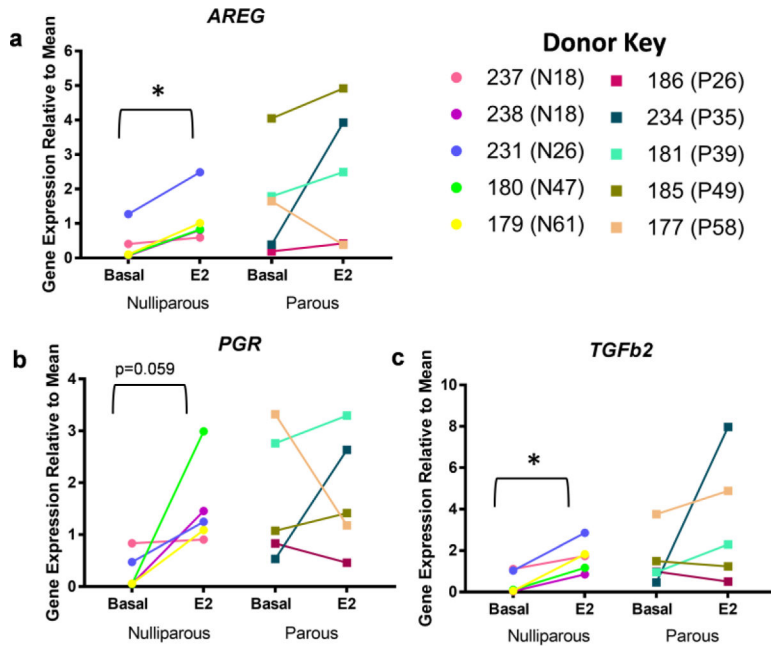


Fig. 5: *AREG*, *PGR*, and *TGFβ2* expression in nulliparous and parous explants. RT-qPCR analysis of basal and 10nM E2 treated explant samples for *AREG*, *PGR*, and *TGFβ2* using *KRT18* to normalize. Gene expression shown relative to basal mean. Circles represent nulliparous explants and squares parous explants. Parity status and age shown in brackets next to donor ID. 4 day E2 treatment significantly increased (a) *AREG* ($p < 0.05$), (b) increased *PGR* ($p = 0.0596$), and significantly increased (c) *TGFβ2* ($p < 0.05$) in nulliparous explants, but not in parous explants using 2-way ANOVA analysis.

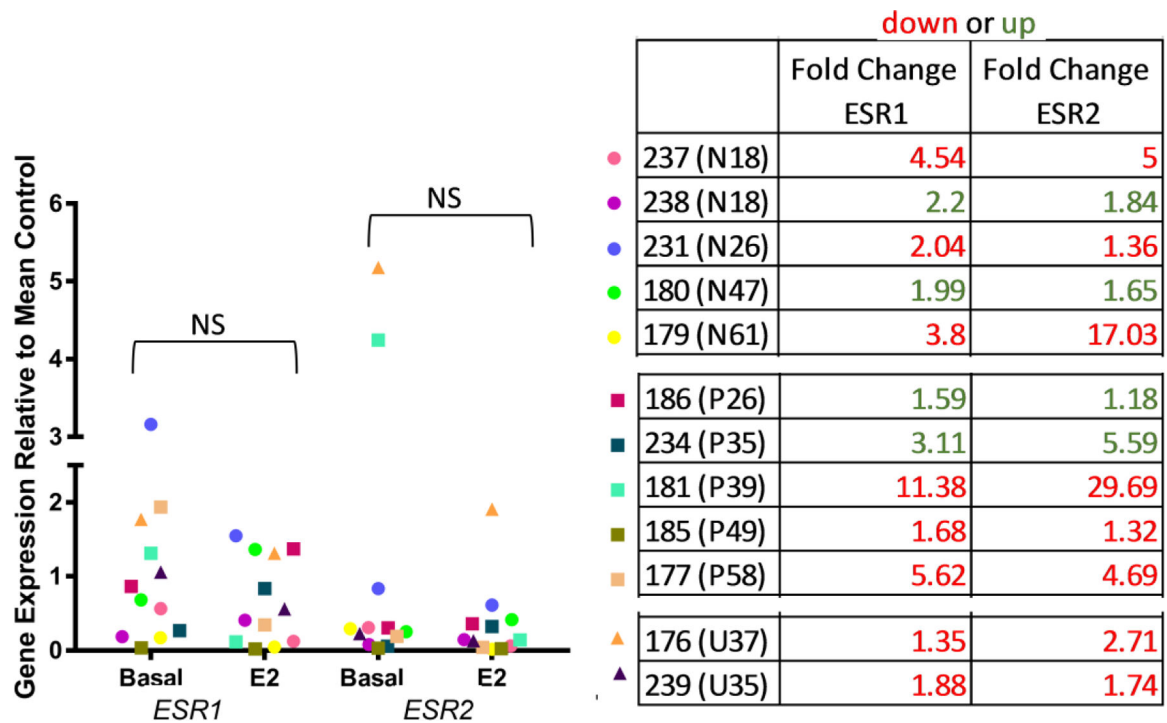


Fig. 6: *ESR1* and *ESR2* expression normalized to *KRT18* in nulliparous and parous explants. RT-qPCR analysis of basal and 10nM E2 treated explant samples for *ESR1* and *ESR2* using *KRT18* to normalize for differing amounts of epithelium. Gene expression shown relative to control mean. Circles represent nulliparous explants, squares parous explants, and triangles explants with unknown parity status. Parity status and age shown in brackets next to patient ID and individual fold change in gene expression is indicated (red is decreased expression in E2-treated; green is increased expression in E2-treated). No significant difference in *ESR1* or *ESR2* expression between basal and E2 treated explants ($P > 0.05$).

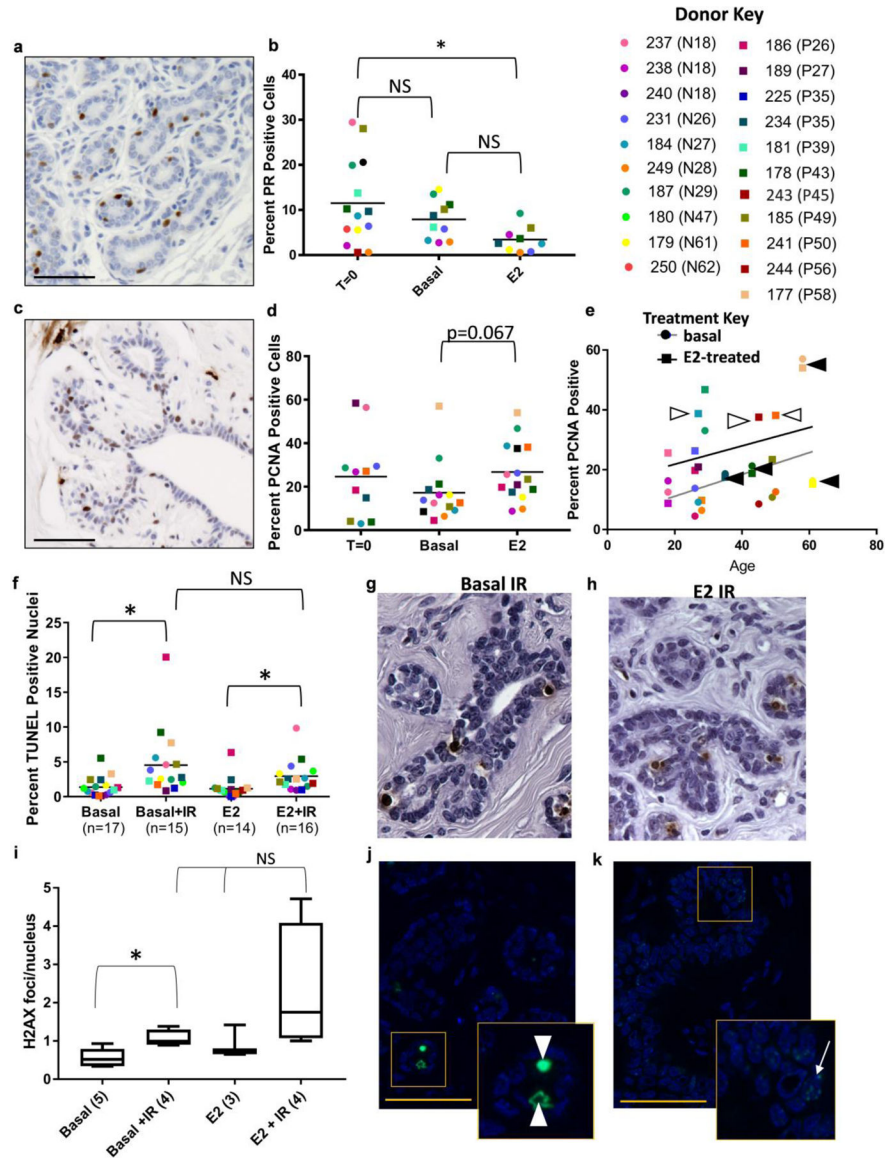


Fig. 7: Immunohistochemical analysis of E2-ER function in human breast explants. (a) Representative progesterone receptor and (b) quantification. (c) Representative PCNA with (d) quantification. (e) Linear Regression Analysis shows no significant changes in E2-mediated proliferation (PCNA) due to age. Each individual donor is indicated by a color (see key): square indicates E2-treated, circle indicates basal-treated. Black arrowhead identifies individuals that are insensitive to E2-mediated proliferation. White arrow identifies individuals that are highly sensitive to E2-mediated proliferation (compare to same color circle below square). (f) Quantification of radiation induced apoptosis (TUNEL) and representative images for basal IR (g) and E2 IR (h). (i) Quantification of γ H2AX foci/nucleus and representative images for basal IR (j) and E2 IR (k) with inset indicated. Scale bars are 50 μ M. Mean indicated in each column with a line. Donor key: Circles represent nulliparous explants and squares parous explants. Parity status and age shown in brackets next to patient ID.

Table 1:

Donor information. Donor number, age, parity status, and age at first birth for cultured breast explants. Mean age for nulliparous donor tissue is 33.4 years (sem +/-5.4); mean age for parous is 42 years (sem +/-3.5). There is no statistical difference in age between the nulliparous and parous donors (p=0.19).

Nulliparous		Parous		
Donor Number	Age	Donor Number	Age	Age at first birth
237	18	186	26	20
238	18	189	27	25
240	18	225	35	27
231	26	234	35	23
184	27	181	39	19
249	28	178	43	39
187	29	243	45	21
180	47	185	49	17
179	61	241	50	18
250	62	244	56	26
		177	58	21
		239	35	unknown
		176	37	unknown

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