17β-Estradiol Modulates the Expression of CD44 and CD326 in Estrogen-Sensitive Breast Cancer Cells

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Abstract

With the emergence of Molecular Targeted Therapy, the interest in studying immunogenetic components that act in carcinogenesis has grown. The role of the estrogen receptor (ER) in initiation and progression of breast cancer is well documented and the estrogen treatment may affect expression of proteins described as tumor stem cell biomarkers in estrogen-sensitive breast cancer. The aim of this study is to analyze the expression of CD44 and CD326 on MCF-7 (ER+) and MDA-MB-231 (ER-) cell lines treated with 17β-estradiol for different periods. Our results indicate that 17β-estradiol can modulate CD44 and CD326 expression in breast cancer cells that have functional estrogen receptors in a time dependent manner. To our knowledge, this is the first study to investigate the influence of 17β-estradiol on CD44 and CD326 expression in MCF-7 and MDA-MB-231 cell lines. Further investigations with primary patient samples and their cultures will enhance our knowledge on the effect of hormones on breast cancer.

Introduction

Breast cancer (BC) is the most prevalent tumor among women, accounting for 1 in 4 female cancers, and representing 6.6% of deaths from malignant tumors [1]. BC is a heterogeneous disease and its treatment depends on a molecular classification based on the presence of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) [2]. ER-positive BC further divides into Luminal A and Luminal B subtypes [3]. Generally, well-differentiated tumors with a relatively better prognosis are known to be ER-positive, which account for approximately 60–65% of primary breast cancers [4]. In contrast, triple-negative tumors that lack of ER, PR and HER2 expression account for 15% of all breast cancers are reported to be associated with higher mortality [5]. In a particular way, ER plays a key role in mediating endogenous hormones and therapeutic agents: upon ligand binding, ER modulates various genes' expression either directly by interacting with DNA or indirectly via other transcription factors [6]. There is also evidence that estrogen is capable of inducing BC cell proliferation and cell cycle progression by inhibiting proteins that control cell cycle [7].

Cell lines are valuable research tools for evaluating both the genetics and potential treatment options for BC [8]. Belonging to the luminal A molecular subtype, ER-positive and PR-positive MCF-7 cell line is a non-invasive cell line with low metastatic potential [9]. Being ER-positive cells, the MCF7 cell line depend on estrogen to proliferate. While ER expression is reported to be relatively weak in the parental line compared to tamoxifen-resistant sub-lines, parental MCF7 cells still express 17β-estradiol receptor [9]. On the other hand, poorly differentiated and invasive MDA-MB-231 cells have high metastatic potential and represent the triple negative BC phenotype [8].

With the discovery of cancer stem cells, studying cellular subpopulations expressing certain membrane proteins which are shown to be linked with breast cancer growth has gained importance [10, 11]. This hypothesis, which was first suggested for initiation of acute myeloid leukemia, is also applied to breast cancer [12]. In this context, $CD44^{+}/CD24^{low/-}$ cells in BC have shown to exert stem-cell like properties such as high proliferation potential and ability of differentiating while their growth rate decrease once

they are differentiated into other well differentiated cell types [13]. BC tumors are heterogeneous with both highly tumorigenic and non-tumorigenic populations, and it is crucial to target tumorigenic cells as these would lead to tumor relapse if they are not eradicated with an effective treatment [13]. Altered expression of anti-apoptotic proteins and certain membrane transporters or multiple drug resistance observed in normal stem cells may also be applied to this highly tumorigenic cell population observed in BC, suggesting targeting this population would be more efficient in cancer therapy [13].

CD44, a non-kinase transmembrane proteoglycan, is a multifunctional cell surface adhesion receptor that is widely expressed on various tissues as well as cancer cells and is a well-known marker for cancer stem cells [14]. CD44 binds to the extracellular matrix component hyaluronic acid, which is expressed by both stromal and cancer cells to initiate various signal pathways that leads to proliferation, migration and invasion [15]. Three CD44 variants, CD44v3, CD44v5, and CD44v6 have shown to be linked with BC metastases [16]. In addition, CD44 positivity, either alone or in combination with other stem cell markers is reported to be associated with tumorigenic potential [16].

Epithelial cell adhesion molecule (EpCAM, also CD326) is a transmembrane glycoprotein which is also involved in cellular signaling, proliferation, migration and differentiation [17]. CD326 is a well-established epithelial cell marker which is one of the most common expressed tumor-associated antigens found in various cancers including BC [18]. CD326 is involved in cell adhesion in a manner that is independent of Ca2 + and also regulates other adhesion molecules' functions [19]. CD326-expressing cells show low contact inhibition and polarization [19]. The influence of CD326 expression on prognosis depends on the type of tumor, and a correlation with poor prognosis in BC has been reported [20]. Currently, two available approaches for CD326 expressing tumor cells are immunotherapy and targeted drug delivery [19].

In this study, we hypothesized that estrogen treatment may affect expression of proteins described as tumor stem cell biomarkers in estrogen-sensitive BC. Therefore, the aim of this study is to analyze protein expression levels of CD44 and CD326 in MCF-7 and MDA-MB-231 cell lines treated with 17β-estradiol for different time periods.

Material And Methods

Materials

Breast cancer cell lines MCF-7 and MDA-MB-231, purchased from American Type Culture Collection (Manassas, USA) were obtained as a gift from Medical Genetics Department, Yeditepe University. Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin antibiotic solution, Trypsin-EDTA solution (0.25%) and Dulbecco's Phosphate Buffered Saline (DPBS) were bought from Gibco-Thermo Fisher Scientific (Massachusetts, USA). 17β-estradiol was obtained from Sigma Aldrich (St. Louis, Missouri, ABD). Dimethyl sulfoxide (DMSO) was obtained from Santa Cruz Biotechnology (Texas, USA). Mouse anti-human CD44-PE (clone BJ18) and mouse anti-human CD326-

PerCP (clone 1B7) antibodies were obtained from Biolegend (California, USA) and Thermo Fisher Scientific (Massachusetts, USA), respectively.

Cell culture

MCF-7 (passage 24) and MDA-MB-231 (passage 39) cell lines were cultured in high glucose DMEM supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin antibiotic solution. Cells were seeded in a 6-well culture plate as $1x10^5$ cells per well and incubated overnight to allow cell attachment in a humidified environment at 37°C and 5% CO_2. 17β-estradiol was dissolved in DMSO to obtain 5 mM stock solution. This solution was further diluted with medium to prepare complete cell culture media supplemented with 100 nM 17β-estradiol. Cells cultured without hormone addition were used as control. Protein expressions were evaluated on 6, 24, 48 and 72 hours of culture by flow cytometry.

Measurement of protein expression using flow cytometry

Cells were detached with Trypsin-EDTA solution at respective timepoints and washed twice with DPBS, followed with staining with mouse anti-human CD44-PE and mouse anti-human CD326-PerCP/eFluor710 antibodies by incubating at room temperature for 15 minutes in the dark. Analyses were performed with Navios flow cytometry system (Beckman Coulter, USA). Cells were gated according to Side Scatter/Forward Scatter Signals. Each analysis was performed as triplicates and $25x10^3$ events per tube were analyzed. Data acquisition was performed using CXP software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 8). Two-way ANOVA, followed by Tukey's multiple comparison tests was performed considering two independent variables: treatment (control and treated with 17β-estradiol) and time (6h, 24h, 48h and 72h). p values lower than 0.05 were considered as statistically significant.

Results

Figure 1 shows representative dot plots indicating CD44 and CD326 protein expressions on cells in a time dependent manner. The main findings are described below.

Expression of CD44 and CD326 on MCF-7 cell line

17β-estradiol treatment significantly reduced CD44 expression compared to control group on 6h ($p < 0.001$). However, no difference between cells analyzed on 24, 48 and 72h was observed. Yet, CD44 protein levels on 6h was found significantly lower compared to 24h ($p < 0.01$) and 72h ($p < 0.001$) (Fig. 2.a). CD326 were found to be expressed at different levels on untreated MCF-7 cells in a time dependent manner: Its expression was slightly decreased on 24h compared to 6 h ($p < 0.05$), and later significantly increased on 48h ($p < 0.0001$). On 72h, CD326 was decreased again compared to 48h ($p < 0.001$), which may indicate that CD326 expression dynamically changes in cell culture over time. When treated with

17β-estradiol, CD326 expression decreased slightly but not significantly within the first 24h. On 48h, 17β-estradiol significantly decreased CD326 expression compared to control ($p < 0.05$). Moreover, incubation with 17β-estradiol up to 72h further decreased CD326 compared to 48h ($p < 0.01$), yet no significant difference between control at respective timepoint was detected (Fig. 2.c).

Ratios of CD44 + CD326 + double positive cells were altered in a time dependent manner; slightly decreasing on 24h compared to 6h ($p < 0.05$), increasing significantly in 48h compared to 24h ($p < 0.001$) and further decreasing on 72h compared to 48h ($p < 0.01$). When comparing 17β-estradiol treated cells with their untreated counterparts, significant decreases in terms of double positive cells were detected on 6h ($p < 0.05$) and 48h ($p < 0.05$). Treatment decreased CD44-CD326- cells. In addition, there was a decrease in double-negative cells in the 72h incubation compared to 48h ($p < 0.05$) (Fig. 2.e). On the other hand, treatment with 17β-estradiol only significantly increased the double-negative population compared to the control group on 6h ($p < 0.001$). In comparison with 6h, treatment with 17β-estradiol significantly decreased double-negative cells on 24h ($p < 0.05$), 48h ($p < 0.05$) and 72h ($p < 0.001$), although it was not observed difference between treatments and their respective controls (Fig. 2.g)

Altogether, our data shows that 17β-estradiol exerts its effects on MCF-7 cells' CD44 expression within first 6h and loses its effects by 24h. On the contrary, 17β-estradiol does not affect CD326 expression until 48h, which may indicate that CD44 and CD326 expressions are controlled by distinct molecular mechanisms.

Expression of CD44 and CD326 on MDA-MB-231 cell line

17β-estradiol treatment significantly reduced MDA-MB-231 cells' CD44 expression on 48h compared to both 24h ($p < 0.05$) and 6h ($p < 0.01$) treatment groups, though no difference between treatments and their controls at respective timepoints was observed (Fig. 2.b). On the other hand, 17β-estradiol treatment did not affect CD326 expression on MDA-MB-231 cells ($p > 0.05$) (Fig. 2.d). In the untreated group, the CD44 + CD326 + population was higher at 48h compared to 6h ($p < 0.01$) and 24h ($p < 0.01$). In the group treated with 17β-estradiol, the double-negative cell population was also higher on 48h compared to 6h ($p < 0.05$) and 24h ($p < 0.05$), although no difference was observed between treatment and the respective controls (Fig. 2.f). Compared to 48h, ratio of double-negative cells was lower at 6h ($p < 0.001$), 24h ($p < 0.01$) and 72h ($p < 0.05$). In addition,17β-estradiol treatment increased the double-negative cell population on 48h ($p < 0.01$) (Fig. 2.h).

Discussion

With the emergence of molecular targeted therapy, the interest in studying the immunogenetic components that act in carcinogenesis has increased [21]. Hyaluronic acid receptor CD44 regulates cell-extracellular matrix and cell-cell interactions and is a well-known breast cancer stem cell (BCSC) marker [22]. CD44 can also upregulate regulate immune checkpoint protein Programmed death-ligand 1 expression to prevent tumor from eradication by the immune system [23]. BCSCs can exist in both epithelial-like and mesenchymal-like states reflecting their healthy counterparts and under the regulation

of tumor microenvironment, they undergo reversible epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions [24]. Moreover, BCSCs can modulate hyaluronic acid protein levels for promoting migration of tumor-associated macrophages in the CSC niches to maintain the proliferative capacity [25]. Epithelial adhesion molecule, CD326 is normally reside in the basolateral membrane while it translocates to the outer leaflet of the cell membrane during cancer progression [26]. Overexpression of CD326, one of the first biomarkers of epithelial cancers discovered in 1970s on the cell membrane [19], is detected in most of human epithelial carcinomas including BC, and attracted researchers' attention as a target for immunotherapy [27]. Moreover, as a signaling receptor modulating stem cell plasticity and involving in regulation of malignant transformation, CD326 is considered as a marker of tumor initiating cells [28]. In fact, Braun et al. revealed that administration of murine monoclonal antibody 17-1A (Edrecolomab) can contribute to disease-free survival in breast cancer [29].

The role of estrogen on initiation and progression of breast cancer is well documented in the literature [2, 6]. Here, we aimed to reveal if 17β-estradiol has a modulatory effect on CD44 and CD326 protein expression levels in MCF-7 (ER+) and MDA-MB-231 (ER-) cell lines in a time dependent manner. Our results indicate that 17β-estradiol may modulate CD44 and CD326 expression in ER + breast cancer cells, as well as having no significant effect on ER- cells.

CD44 protein expression in MCF-7 cells is previously shown to be associated with higher resistance to hormonal treatments and higher invasive capacity [30]. In a murine breast cancer xenograft model, CD44 targeting with a monoclonal antibody, P245, is shown to inhibit tumor growth, decrease chemotherapy resistance in addition to preventing reoccurrence [31]. Moreover, CD44 may contribute to the silencing of genes influenced by estrogen treatment through mechanisms that are independent of the ER [32]. In our experiments, the MCF-7 control group showed a high percentage of CD44 + cells, which is consistent with previous data [33]. 17β-estradiol treatment led to a small yet significant reduction in CD44 percentages. Thus, these data may suggest that estrogen treatment may decrease CD44 protein expression on ER + breast cancer cells to increase treatment effectivity.

It is well established that MCF-7 cell line has prominent CD326 expression, which has motivated further studies using this protein as a possible target for anticancer therapies [34, 35]. Our data indicates that untreated MCF-7 cells' CD326 expression levels are compatible with the literature findings [36]. In addition, 17β-estradiol treatment reduced CD326 protein levels significantly after 48h. Thus, it is possible that the signaling pathways triggered by the estrogen receptor in this cell line act by modulating CD326 expression.

Increased CD44 expression in MDA-MB-231 cells has been associated with elevated metastatic potential [13, 37]. In addition, recent studies have shown CD44 as a potential target for disabling immunosuppression mechanisms in triple-negative cancer cells [23]. In our experiments, both the 17β-estradiol treated and control groups showed high CD44 protein levels, which is consistent with previous data [33]. Since triple negative breast cancer cells lack estrogen receptors, 17β-estradiol treatment alone

may not be enough to alter CD44 expression when compared to the control. However, alteration of CD44 expression on control group may be associated with time rather than 17β-estradiol treatment.

In cell lines of mesenchymal origin such as MDA-MB-231, CD326 expression is low and tumor cells grow independently of the signaling of this membrane protein [38]. Previously, MDA-MB-231 cells that were genetically engineered to overexpress CD326 were used in a study that found decreased migration and tumor invasion in an animal model, as well as increased inflammation and innate immune responses [38]. In our experiment, MDA-MB-231 cells had CD326 expression which is consistent with previous studies [36, 39]. 17β-estradiol had no effect on CD326 expression, which was expected, since MDA-MB-231 cells lack ER. Therefore, even in the perspective of genetically engineered cells to overexpress CD326, 17β-estradiol is likely to have no influence on the expression of this membrane protein.

In the context of cancer stem cell development, the importance of CD44 and CD326 as biomarkers of cell subpopulations responsible for sustaining tumor growth is evident [10]. In fact, molecular therapeutic strategies targeting cells expressing these proteins may be the pathway to successful treatments [40]. Our findings confirm that CD44 and CD326 are relevantly expressed in certain breast cancer cell types and demonstrate that the expression of these biomarkers may be influenced by the hormonal microenvironment.

In conclusion, our results indicate that 17β-estradiol can modulate CD44 and CD326 expression in breast cancer cells that have functional estrogen receptors in a time dependent manner. To our knowledge, this is the first study to investigate the influence of 17β-estradiol on CD44 and CD326 expression in MCF-7 and MDA-MB-231 cell lines. Further investigations with primary patient samples and their cultures will enhance our knowledge on the effect of hormones on breast cancer.

Declarations

Funding: This study was conducted with self budget.

Conflicts of interest: The authors declare that they have no conficts of interest.

Ethics approval: Not applicable.

Consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: Not applicable.

Code availability: Not applicable.

Author's contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by DPMB, ANA, BA and TS. The first draft of the manuscript

was written by DPMB and ANA. BA and GYD commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

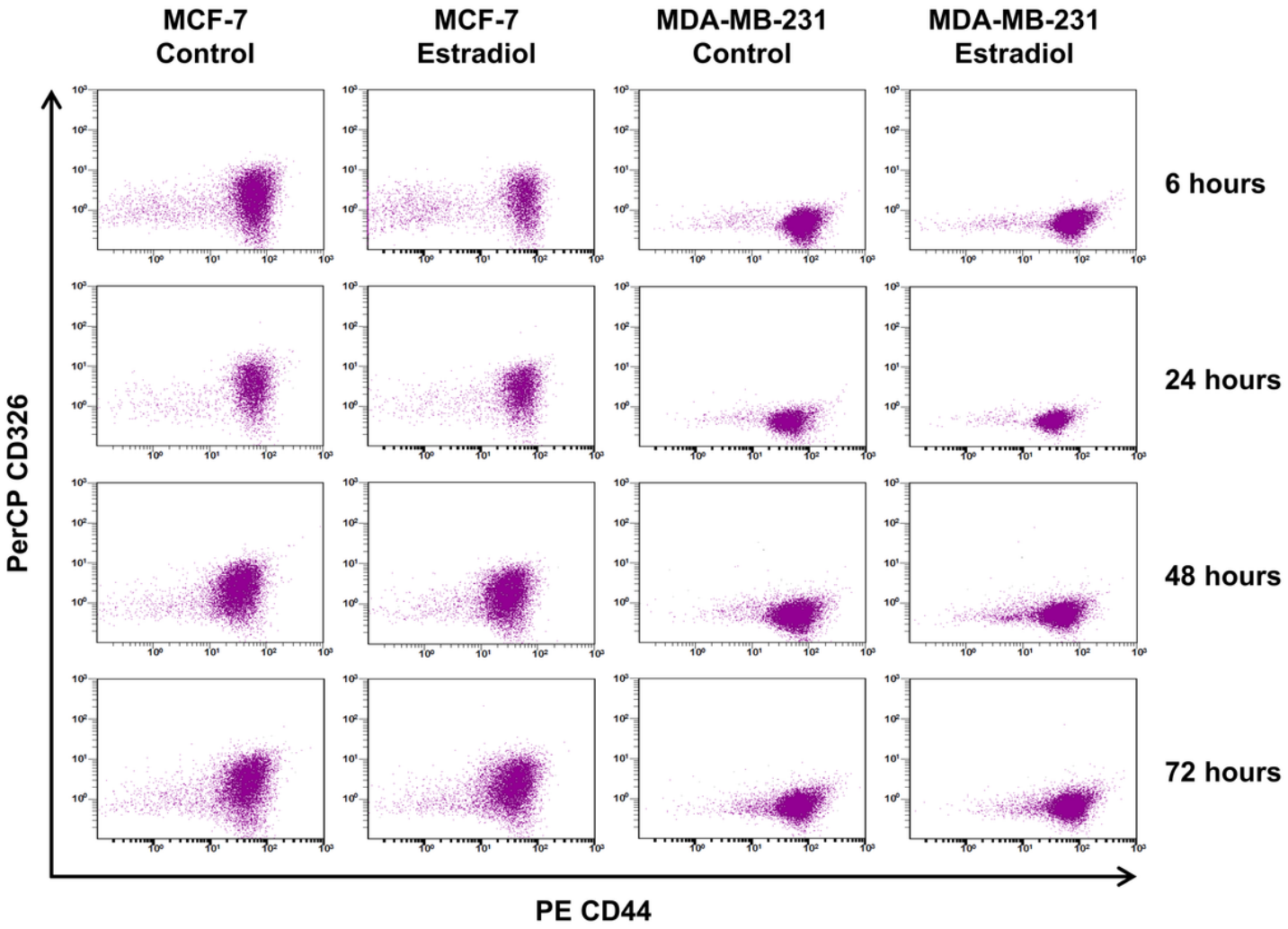

Figure 1

Flow cytometry quadrants of the MCF-7 and MDA-MB-231 cell lines stained with mouse anti-human CD44-PE (clone BJ18) and mouse anti-human CD326-PerCP/eFluor710 (clone 1B7) antibodies on different timepoints

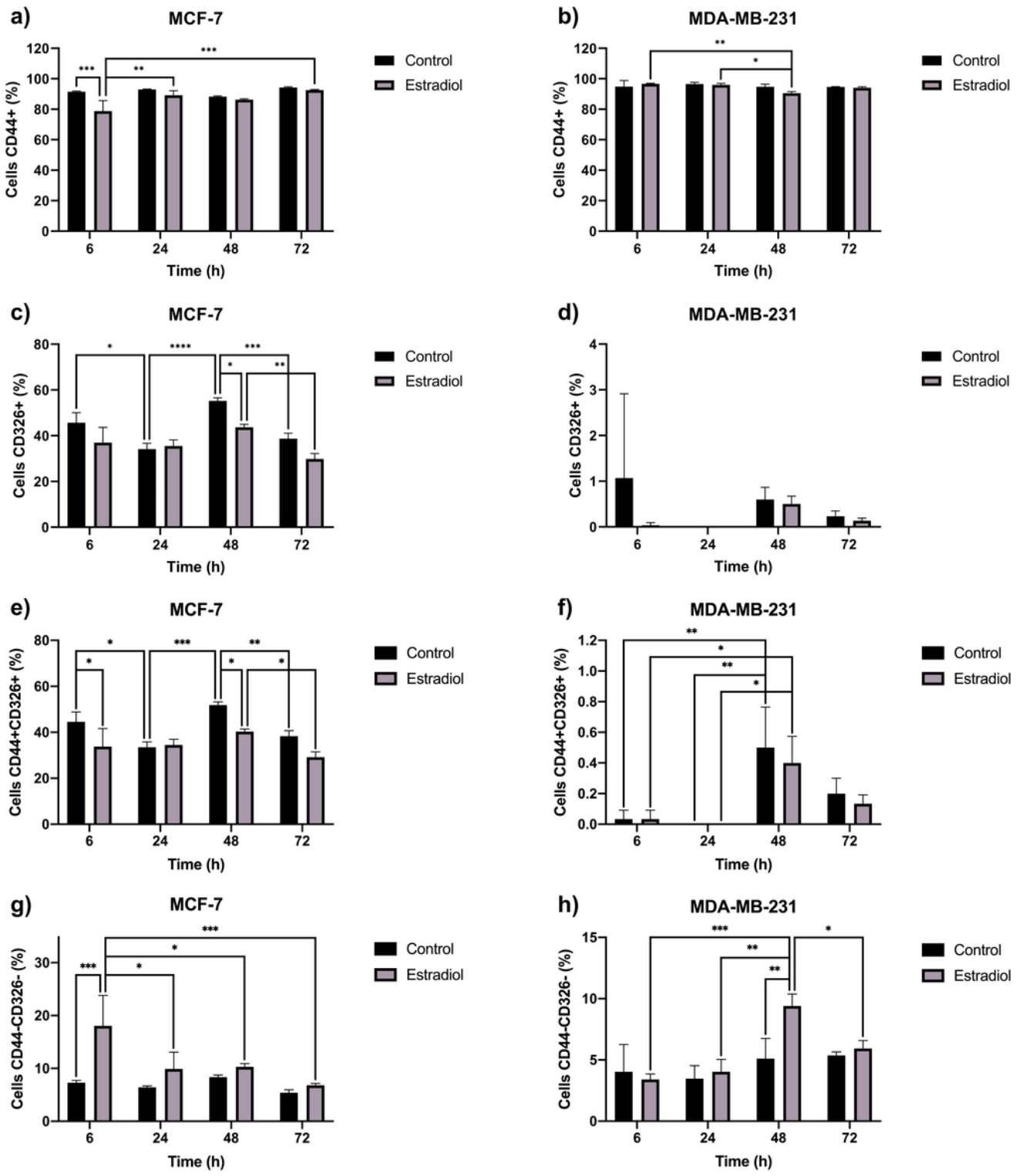

Figure 2

The effect of 17β-estradiol on CD44 and CD326 expression in MCF-7 and MDA-MB-231 cells. a) Percentage of MCF-7 cells positive for CD44; b) Percentage of MDA-MB-231 cells positive for CD44; c) Percentage of MCF-7 cells positive for CD326; d) Percentage of MDA-MB-231 cells positive for CD326; e) Percentage of MCF-7 cells double-positive; f) Percentage of MDA-MB-231 cells double-positive; g) Percentage of MCF-7 cells double-negative; h) Percentage of MDA-MB-231 cells double-negative. Each

sample was analyzed as triplicates. Data was represented by mean and standard deviation. Two-way ANOVA followed by Tukey's multiple comparison test was performed. Significance levels: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001