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Differential impacts of charcoal-stripped fetal bovine serum on c-Myc among distinct subtypes of breast cancer cell lines

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ABSTRACT

Charcoal-stripped fetal bovine serum (CS-FBS) is frequently used in studies on hormone-responsive cancers to provide hormone-free cell culture conditions. CS-FBS may influence the growth of cancer cells; however, the underlying mechanisms remain unclear. In this study, we aimed to clarify the effects of CS-FBS on distinct subtypes of breast cancer cells. We found that the crucial oncoprotein c-Myc was significantly inhibited in estrogen receptor alpha (ER-α)-positive breast cancer cells when cultured in CS-FBS-supplemented medium, but it was not suppressed in ER-α-negative cells. The addition of 17β-estradiol (E2) to CS-FBS-supplemented medium rescued the CS-FBS-induced inhibition of c-Myc, while treatment with 5α-dihydrotestosterone (DHT) suppressed c-Myc expression. Our data demonstrated that CS-FBS may impede the growth of ER-α-positive breast cancer cells via c-Myc inhibition, and this was possibly due to the removal of estrogen. These results highlighted that the core drivers of c-Myc expression were subtype-specific depending on the distinct cell context and special caution should be exercised when using CS-FBS in studies of hormone-responsive cancer cells.

1. Introduction

Fetal bovine serum (FBS) is frequently supplemented in cell culture medium to facilitate cell growth, proliferation and differentiation, and it is widely applicable for a majority of human and animal cell lines [1]. In general, essential serum factors include hormones (e.g. estrogen, androgen, and progesterone), growth factors, cytokines, transport proteins, attachment and spreading proteins, fatty acids and a variety of low-molecular-weight nutrients (e.g. vitamins, trace elements and carbohydrates) [2–4]. Charcoal stripping of FBS can nearly completely remove hormonal factors and remarkably reduce the concentration of certain growth factors, vitamins, and metabolites [2,4,5]. Thus, FBS is usually switched to charcoal-stripped fetal bovine serum (CS-FBS) in studies of hormone-responsive cells to mimic hormone-free conditions and avoid interference from a wide variety of serum factors [4,6–11].

Breast cancer is the most common malignant cancer in women worldwide [12–14]. Breast tumors are highly heterogeneous and can be divided into different subtypes [15–17]. CS-FBS is widely used in breast cancer research *in vitro* to eliminate interference from trace amounts of hormones (mainly estrogen and androgen) found in normal FBS [8,18,19]. However, it has been reported that growth was suppressed [20,21] and mitochondrial capacity was reduced [22] in MCF-7 breast cancer cells when cultured in CS-FBS-supplemented medium. Moreover, CS-FBS affected the endocrine response phenotypes and promoted endocrine resistance in MCF-7 cells [5]. These changes may be attributed to the removal of estrogen from FBS, but the underlying mechanisms remain to be fully elucidated. We hypothesize that the oncoprotein c-Myc may be involved in these processes for four reasons: (i) c-Myc is frequently aberrantly expressed in breast cancer [23–27]; (ii) c-Myc is a key oncogene that promotes proliferation and inhibits apoptosis in breast cancer [28–34]; (iii) c-Myc is a well-known target of both estrogen receptor alpha (ER-α) [35–38] and androgen receptor (AR) [39–42]; and (iv) overexpression of c-Myc is able to partially reverse the CS-FBS-mediated inhibition of cell growth [21].

Abbreviations: AI, aromatase inhibitor; AR, androgen receptor; CS-FBS, charcoal-stripped fetal bovine serum; DHT, 5α-dihydrotestosterone; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; EGF, epidermal growth factor; ER-α, estrogen receptor alpha; EtOH, ethyl alcohol; FBS, fetal bovine serum; HER2, human epidermal growth factor receptor 2; LAR, luminal AR; PR, progesterone receptor; TNBC, triple-negative breast cancer.

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In the current study, we focused on the effects of CS-FBS on c-Myc in two normal human mammary epithelial cells and nine different subtypes of breast cancer cells. We found that c-Myc expression was dramatically reduced in response to CS-FBS in ER-α-positive breast cancer cells, but it was not changed in either normal human mammary epithelial cells or ER-α negative breast cancer cells. Reconstitution of CS-FBS with 17β-estradiol (E2) rescued the CS-FBS-induced inhibition of c-Myc in ER-α-positive breast cancer cells, while supplementation with 5α-dihydrotestosterone (DHT) suppressed c-Myc expression. Therefore, CS-FBS needs to be utilized with appropriate caution in ER-α-positive breast cancer cells.

2. Materials and methods

2.1. Chemicals and reagents

E2, ethyl alcohol (EtOH), dimethyl sulfoxide (DMSO) and bovine insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). DHT and hydrocortisone were provided by Selleckchem (Shanghai, China). The cell culture media, FBS, sodium pyruvate, non-essential amino acids, l-glutamine and epidermal growth factor (EGF) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). CS-FBS was purchased from Biological Industries (Cromwell, CT, USA).

2.2. Cell culture and treatment

The normal human mammary epithelial cell line, MCF-10A, and six breast cancer cell lines, BT-474, SK-BR-3, HCC1937, MDA-MB-468, BT-549 and MDA-MB-453 cells, were purchased from China Infrastructure of Cell Line Resource. The immortalized human mammary epithelial cell line, HMLE, was kindly provided by Prof. Er-Wei Song (Sun Yat-sen University, Guangzhou, China). MCF-7, T47D and MDA-MB-231 cells were acquired from Dr. Jian-You Liao (Sun Yat-sen University, Guangzhou, China). HMLE cells were maintained in Dulbecco's Modified Eagle Medium/Ham's F12 medium supplemented with 10% FBS, 10 ng/ml EGF, 0.5 μg/ml hydrocortisone and 10 μg/ml bovine insulin as monolayers at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 and 95% air. MCF-10A and all the breast cancer cell lines used in our study were cultured as described previously [43]. All cells were switched to phenol red-free medium containing 8% CS-FBS for three days prior to stimulation with 10 nM E2 or 10 nM DHT for another two days in fresh medium supplemented with 10% FBS or 8% CS-FBS. EtOH (0.1%) or DMSO (0.1%) was used as a vehicle respectively.

2.3. RNA isolation and quantitative real time reverse transcription polymerase chain reaction (qRT-PCR)

RNA isolation and qRT-PCR assays were performed as described in our previous study [8]. The forward and reverse primers used for PCR amplification were listed in Supplementary Table 1.

2.4. Western blot analysis

Western blot analysis was operated as described in our previous study [44]. The primary antibodies used in this study were listed in Supplementary Table 2.

2.5. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of three biological replicates. Student's t-tests were used for comparisons of two samples between treatment and control groups. P-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Elimination of c-Myc in ER-α-positive breast cancer cells under CS-FBS culture conditions

To unravel the potential regulatory mechanisms of CS-FBS on cell growth and proliferation, we compared the expression of the pivotal proteins in two normal human mammary epithelial cells and nine breast cancer cell lines cultured in medium supplemented with normal FBS (Fig. 1A) or CS-FBS (Fig. 1B). Our results showed that the three conventional receptors, ER-α, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), were not affected by CS-FBS. AR was apparently upregulated in both T47D and BT-474 cells when cultured in CS-FBS-supplemented medium. Notably, the crucial oncoprotein c-Myc was dramatically eliminated in response to CS-FBS in ER-α-positive breast cancer cells, but not in either normal human mammary epithelial cells or ER-α-negative breast cancer cells. We also found that the expression of two proliferation markers (PCNA and Ki-67) and the epithelial marker E-cadherin were not changed by CS-FBS in any of the cell lines used in our study. PTEN and the mTOR pathway were not altered neither.

c-Myc is a key target of ER-α [35,38] and it is necessary for estrogen-induced proliferation through suppressing the expression of *p21* in MCF-7 cells [37]. c-Myc also plays an important role in promoting oncogenic growth stimulated by androgen in molecular apocrine breast cancers [40]. In this study, we found that c-Myc was eliminated in ER-α-positive breast cancer cells when cultured in CS-FBS-supplemented medium, but a similar effect was not observed in ER-α-negative cells. These results suggested that the CS-FBS-induced inhibition of c-Myc was most likely due to the removal of hormones (e.g. estrogen and androgen), and the growth inhibitory impacts mediated by CS-FBS may be achieved through inhibition of c-Myc expression in ER-α-positive breast cancer cells. Previous observations published by Venditti et al. revealed that the growth of MCF-7 cells was appreciably suppressed in medium supplemented with CS-FBS and overexpression of c-Myc could partially restore cell proliferation [21]. However, the influence of CS-FBS on c-Myc expression was not measured in this study. Our data extended the findings of Venditti et al. and confirmed that CS-FBS was able to drastically inhibit c-Myc expression, not only in MCF-7 cells but also in T47D and BT-474 cells.

3.2. Opposite effects of E2 and DHT on c-Myc in ER-α-positive breast cancer cells

To confirm the possible roles of estrogen and androgen on c-Myc expression under CS-FBS culture conditions, we treated three ER-α-positive breast cancer cells (MCF-7, T47D and BT-474) with E2 or DHT. ER-α was decreased by E2 and AR was increased by DHT (Fig. 2). These data were consistent with those reported previously by other labs [45,46]. The expression of c-Myc plunged sharply when the cells were cultured under CS-FBS conditions. Treatment with exogenous E2 rescued c-Myc expression in cells cultured in CS-FBS-supplemented medium, suggesting that the reduction of c-Myc was mainly due to the lack of E2 in CS-FBS and the E2/ER-α pathway was the core driver of c-Myc in ER-α-positive cells. However, DHT inhibited c-Myc expression, indicating an opposite role for AR in c-Myc regulation in these cells.

These findings implied the applications of aromatase inhibitors (AIs; such as anastrozole, letrozole and exemestane) in hormone-dependent breast cancer for postmenopausal women. Estrogen is mainly synthesized through the conversion of androstenedione and testosterone to estrone (E1) and estradiol (E2) respectively by aromatase in postmenopausal women. AIs effectively suppressed

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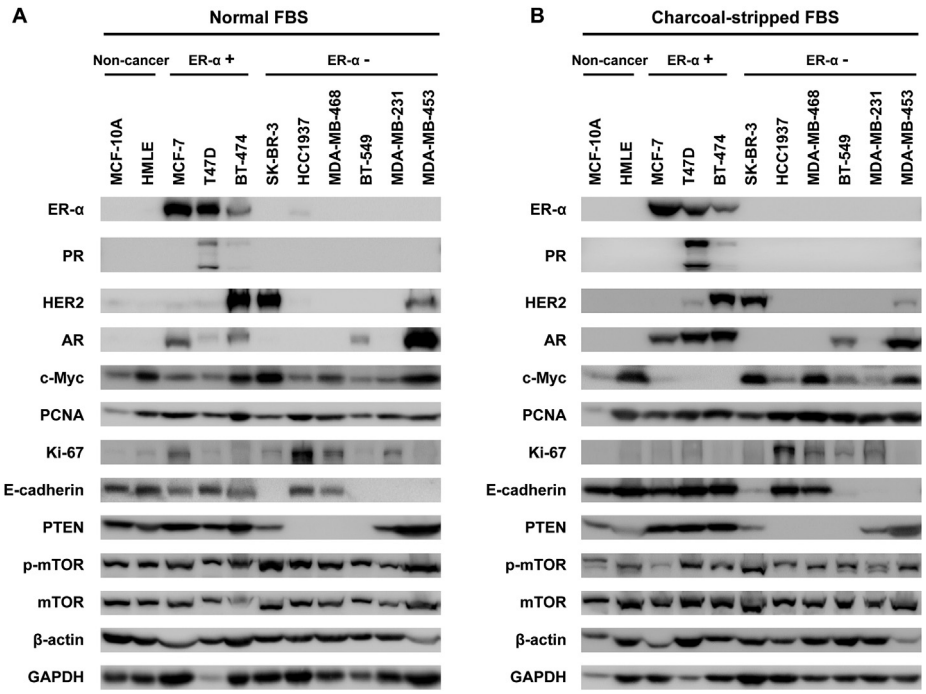

Fig. 1. The influence of CS-FBS on normal human mammary epithelial cells and breast cancer cells. Two normal human mammary epithelial cell lines and nine breast cancer cell lines were cultured in (A) FBS-supplemented medium or (B) CS-FBS-supplemented medium. Western blot analysis showed the changes in the designated proteins. β-actin and GAPDH acted as loading controls. FBS, fetal bovine serum; CS-FBS, charcoal-stripped FBS; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; AR, androgen receptor.

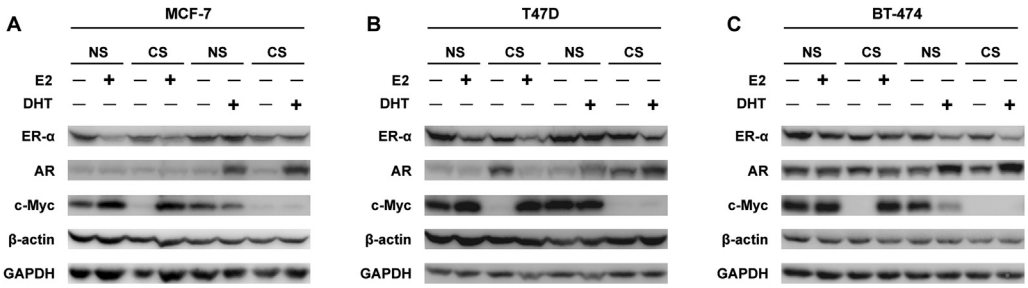

Fig. 2. Regulation of c-Myc by E2 and DHT in ER-α-positive breast cancer cells. The expression of c-Myc protein was markedly decreased in response to CS-FBS in (A) MCF-7, (B) T47D and (C) BT-474 cells. After treatment with 10 nM E2 or 10 nM DHT, we found that E2 promoted c-Myc expression, while DHT inhibited it. β-actin and GAPDH served as loading controls. NS, normal FBS; CS, charcoal-stripped FBS; E2, 17β-estradiol; DHT, 5α-dihydrotestosterone.

the transformation of androgen to estrogen, leading to the reduction of estrogen abundance and maintaining the antitumous effects of androgen [47–49]. Our data may indicate the superiority of AIs in c-Myc inhibition by simultaneously blocking the E2/ER-α pathway and activating the DHT/AR pathway.

3.3. No response of c-Myc to either E2 or DHT in ER-α-negative/AR-negative cells

We further evaluated the influence of E2 and DHT on two normal human mammary epithelial cell lines and six ER-α-negative breast cancer cell lines. Our data showed that c-Myc was hardly changed by exposure to either CS-FBS or hormones (E2 and DHT) in ER-α-negative/AR-negative cells (Fig. 3A–F). These results demonstrated that c-Myc was probably induced by other signaling pathways independent of either E2 or DHT in these cells. According to our data, endocrine therapy may have no effects on c-Myc in ER-α-negative/AR-negative breast cancer cells; thus, it is not suitable for ER-α-negative/AR-negative breast cancer treatment. By contrast, it is important to encourage strategies targeting c-Myc in ER-α-negative/AR-negative breast cancer by addressing other pathways or factors, such as inhibitors of bromodomain and extraterminal proteins [29,50], histone deacetylases [51] and cyclin-dependent kinases [52].

3.4. Upregulation of c-Myc by DHT in ER-α-negative/AR-positive breast cancer cells

AR was highly expressed in MDA-MB-453 cells as shown in Fig. 1. Thus, the MDA-MB-453 cell line was frequently applied as a valuable model for investigating the functions of androgen in breast cancer [53]. We found that DHT promoted c-Myc expression through activation of AR in MDA-MB-453 cells (Fig. 3H). AR became an oncogene in this breast cancer subtype, suggesting the potential

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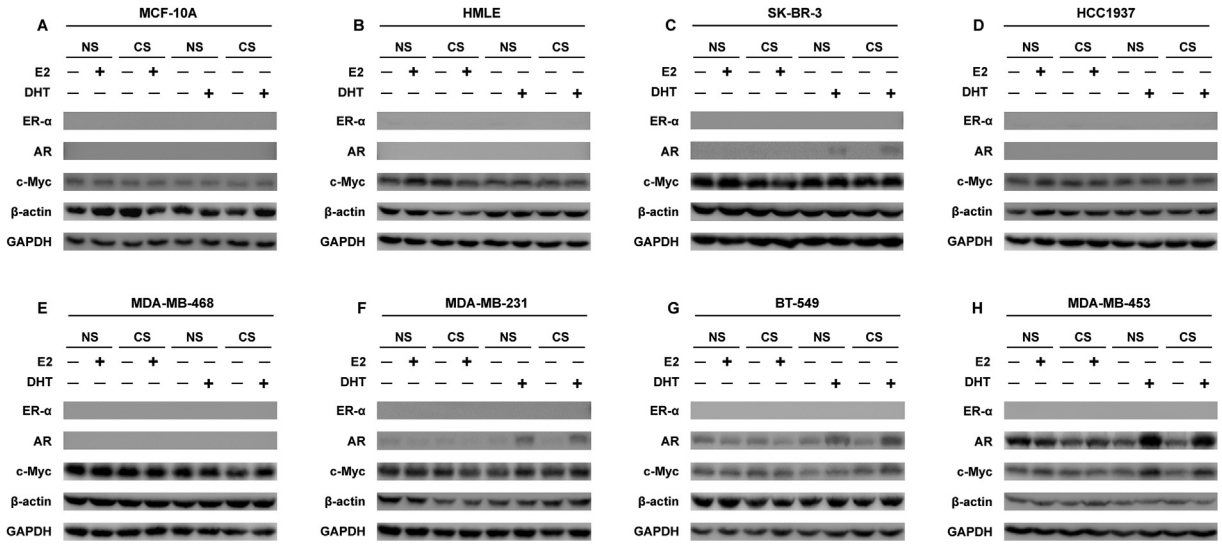

Fig. 3. Modification of c-Myc by E2 and DHT in ER-α-negative cells. The expression of c-Myc protein was unchanged in response to CS-FBS in two normal human mammary epithelial cell lines and six ER-α-negative breast cancer cell lines. Stimulation with 10 nM E2 did not alter the expression of c-Myc in all these cells. Treatment with 10 nM DHT increased c-Myc expression only in ER-α-negative/AR-positive breast cancer cells. β-actin and GAPDH served as loading controls. NS, normal FBS; CS, charcoal-stripped FBS; E2, 17β-estradiol; DHT, 5α-dihydrotestosterone.

applications of anti-androgen therapeutic strategies in AR-dependent TNBC. We also noticed that AR was moderately expressed in BT-549 cells (Fig. 1). However, c-Myc was not obviously activated, although DHT slightly increased the expression of AR in BT-549 cells (Fig. 3G). These results showed that the AR pathway may be the core oncogenic pathway in MDA-MB-453 cells, but it was probably not in BT-549 cells.

3.5. Transcriptional regulation of c-Myc mRNA and its target genes in response to CS-FBS

We next determined whether *c-Myc* gene was transcriptionally regulated in response to CS-FBS and hormones by qRT-PCR assays (Fig. 4). We found that the expression of *c-Myc* mRNA was inhibited by CS-FBS and was rescued by E2 (Fig. 4A), while it was suppressed

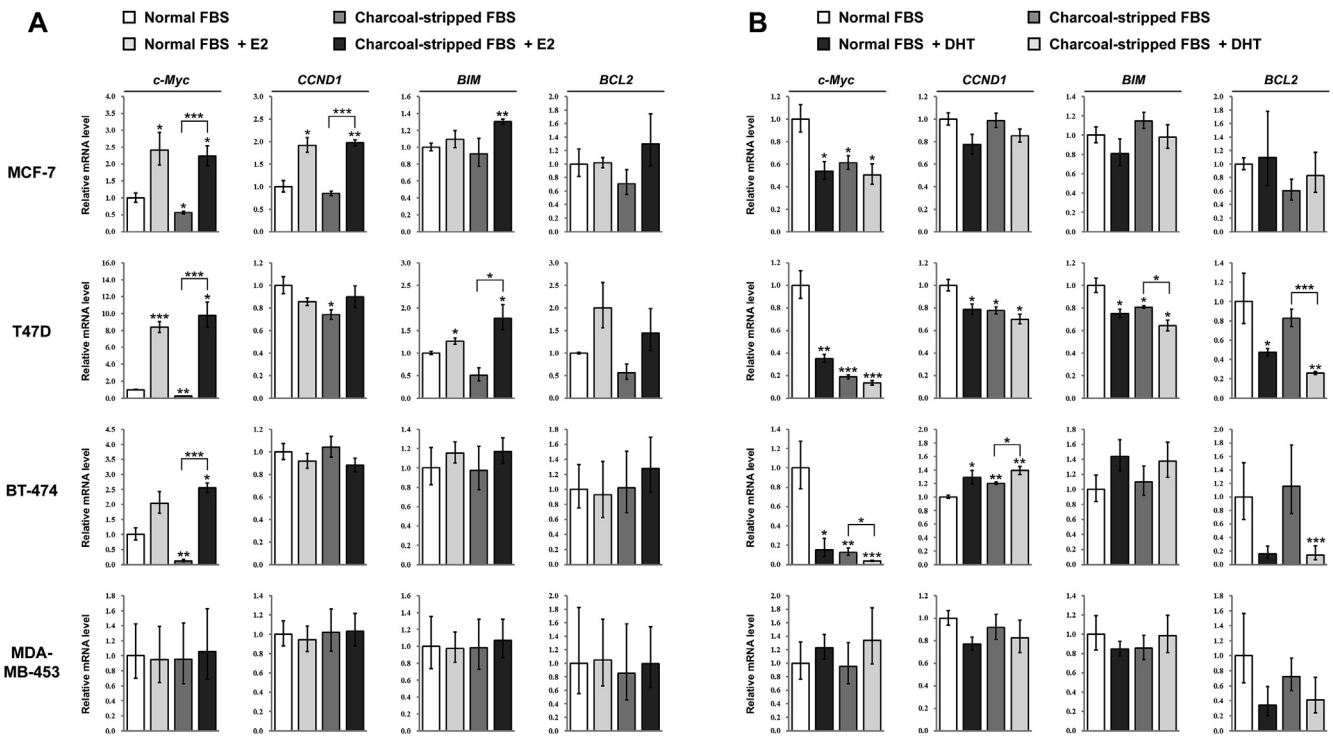

Fig. 4. Alteration of the transcription of *c-Myc* mRNA and its target genes by CS-FBS and hormone treatment. All cells were cultured in phenol red-free medium containing 8% CS-FBS for three days. Then, they were treated with (A)10 nM E2 or (B)10 nM DHT for another two days in fresh medium supplemented with 10% FBS or 8% CS-FBS. EtOH (0.1%) or DMSO (0.1%) was used as a vehicle respectively. The levels of mRNA transcripts were assessed by qRT-PCR analysis. *ACTB* was used as an internal control for normalization. Values are mean ± SEM (n = 3). Asterisks indicate statistically significant differences compared to the vehicles. *P < 0.05; **P < 0.01; ***P < 0.001.

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by DHT (Fig. 4B) in ER-α-positive breast cancer cells. These results indicated that E2 and DHT regulated the expression of *c-Myc* at the transcriptional level. *CCND1*, *BIM* and *BCL2* were target genes of c-Myc [54], and they were modified largely based on *c-Myc* expression. However, the changes in *c-Myc* and its target genes were not remarkable in MDA-MB-453 cells (Fig. 4A and B) and the other ER-α-negative cells used in our study (Supplementary Fig. 1).

In conclusion, our data demonstrated that the impacts of CS-FBS on ER-α-positive breast cancer cells were most likely due to the elimination of estrogen and the inactivation of the c-Myc pathway (as summarized in the graphical abstract). These results highlighted that the specific core drivers of c-Myc expression were dependent on the cell context and special attention should be paid to the usage of CS-FBS in ER-α-positive breast cancer research.

Declaration of competing interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.03.049.

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