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GPER mediated estradiol reduces miR-148a to promote HLA-G expression in breast cancer

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ABSTRACT

Breast cancer is the most common malignant diseases in women. miR-148a plays an important role in regulation of cancer cell proliferation and cancer invasion and down-regulation of miR-148a has been reported in both estrogen receptor (ER) positive and triple-negative (TN) breast cancer. However, the regulation mechanism of miR-148a is unclear. The role of estrogen signaling, a signaling pathway is important in development and progression of breast cancer. Therefore, we speculated that E2 may regulate miR-148a through G-protein-coupled estrogen receptor-1 (GPER). To test our hypothesis, we checked the effects of E2 on miR-148a expression in ER positive breast cancer cell MCF-7 and TN cancer cell MDA-MB-231. Then we used GPER inhibitor G15 to investigate whether GPER is involved in regulation of E2 on miR-148a. Furthermore, we analyzed whether E2 affects the expression of HLA-G, which is a miR-148a target gene through GPER. The results showed that E2 induces the level of miR-148a in MCF-7 and MDA-MB-231 cells, GPER mediates the E2-induced increase in miR-148a expression in MCF-7 and MDA-MB-231 cells and E2-GPER regulates the expression of HLA-G by miR-148a. In conclusion, our findings offer important new insights into the ability of estrogenic GPER signaling to trigger HLA-G expression through inhibiting miR-148a that supports immune evasion in breast cancer.

1. Introduction

Breast cancer is the most common malignant diseases in women. However, the molecular pathogenesis of breast cancer remains poorly defined due to its heterogeneity [1]. Approximately 70% of human breast cancer is estrogen receptor-α positive (ER+) and up to 20% of breast cancer is triple-negative breast cancer (TNBC) [2]. In postmenopausal women with early-stage ER positive breast cancer, anti-estrogen therapy is utilized as an effective adjuvant treatment [3]. However, TNBC is a highly aggressive breast cancer subtype that lacks effective targeted therapies [4]. Therefore, an improved understanding of the molecular basis of estrogen action and development of new strategies to improve the efficacy of anti-estrogens are required.

MicroRNAs (miRNAs) play an important role in carcinogenesis in various solid cancers including breast cancer. microRNA-148a (miR-148a) is an important miRNA which can regulate several different target genes and pathways involving tumor proliferation, invasion and metastasis [5–7]. It was reported to inhibit tumor angiogenesis through ERBB3 [9] and act as an EMT suppressor in NSCLC cells [10]. miR-148a is also involved in immune evasion by targeting HLA-G [11,12]. Down-regulation of miR-148a has been reported in certain cancer types [13–16] and is correlated with the outcome of certain malignancies [16,17]. In breast cancer, miR-148a was down-regulated in both triple-negative [18] and ER positive breast cancer cells [8]. However, the regulation mechanism of miR-148a is unclear.

The hormone estrogen (17β-estradiol, E2) has a key role in cell proliferation and differentiation through receptor binding and activation [19,20]. The effects of E2 have been widely analyzed in human mammary gland where it is responsible for normal epithelial growth and for the development of 70–80% of human breast cancer tumors [21]. TN breast cancer lacks a known signaling pathway amenable to targeted therapy. However, the G-protein-coupled estrogen receptor-1 (GPER, formerly known as GPR30) has attracted increasing interest, considering its ability to mediate estrogenic signaling in breast cancer [22]. GPER has also been proposed as a candidate biomarker in triple-negative breast cancer, opening a novel scenario for a more comprehensive assessment of breast tumor patients [23]. Since miR-148a decreases in both ER positive and TN breast cancer, we supposed that estrogen may regulate miR-148a expression through GPER.

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2. Materials and methods

2.1. Cell culture

MCF-7 and MDA-MB-231 BT549 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in a humidified incubator at 37 °C and 5% CO_2. For E2 stimulation experiments, cells were cultured for at least 3 days in phenol red-free RPMI1640 with 5% dextran-coated charcoal-treated serum before E2 treatment.

2.2. RNA oligonucleotides and transfection

miRNAs were synthesized by Ruibo Biotech (Guangzhou, China). miRNA mimics are synthetic duplexes representing mature miRNAs. miRNA inhibitors are synthetic duplexes inhibiting expression of mature miRNAs. miRNA transfection was performed using lipo2000 (QIAGEN). 20 nmol/L miRNA was used for transfection in serum free medium. Total RNA was prepared 24–48 h after transfection and further used for qPCR analysis.

2.3. Quantitative analysis of miRNAs and mRNAs

Total RNA and miRNA was extracted from cultured cells using miRVana miRNA Isolation Kit (*Ambion*) according to the manufacturer's protocols. The TaqMan stem-loop RT-PCR approach was used to assess the expression of miRNAs with kits from Applied Biosystems. For quantitative analysis of mRNA expression, 1 μg of total RNA was used for synthesis of random-primed single stranded cDNA using Primescript RT reagent kit (TaKaRa) and cDNA was subjected to quantitative PCR using SYBR green Master MIX (Applied Biosystem). The relative amount of gene transcripts was normalized to GAPDH and miRNA was normalized to U6 using comparative threshold cycle (DDCT) method.

2.4. Protein extraction and Western blot

Cells were lysed using cell lysis buffer with protein concentration determined with the BCA Protein Assay kit (*Pierce*). Equal amounts of total proteins were separated in 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes (*Bio-Rad*). Membranes were blocked for 1 h with 1% BSA in TBST containing 0.05% Tween 20, incubated overnight with primary antibody (GREB, *Abcam* and GAPDH, *Sigma*), washed and incubated with secondary antibody, and visualized by chemiluminescence.

2.5. Luciferase reporter assay

The full-length 3′ UTR of the HLA-G was amplified and cloned into downstream of PGL3-control vector (*Promega*). Cells plated on 24-well plates were transfected with 100 ng plasmid and 200 nmol/L of miR-148a mimics or negative control. After 48 h, cells were lysed and assayed with Dual Luciferase Assay (*Promega*) according to the manufacturer's instructions. Transfection efficiency was estimated by co-transfecting the cells with SV-40 Renilla luciferase. Luciferase activity was measured using the dual luciferase assay system (Promega) and a 96-well luminometer (Fluoroskan Ascent Fl, Labsystems). Three independent experiments were performed in triplicates.

2.6. Statistics

Statistical analysis was performed using prism 5.0. One-way analysis of variance (ANOVA) and Tukey post hoc tests were used for comparisons within a group. The Student *t* test was used for

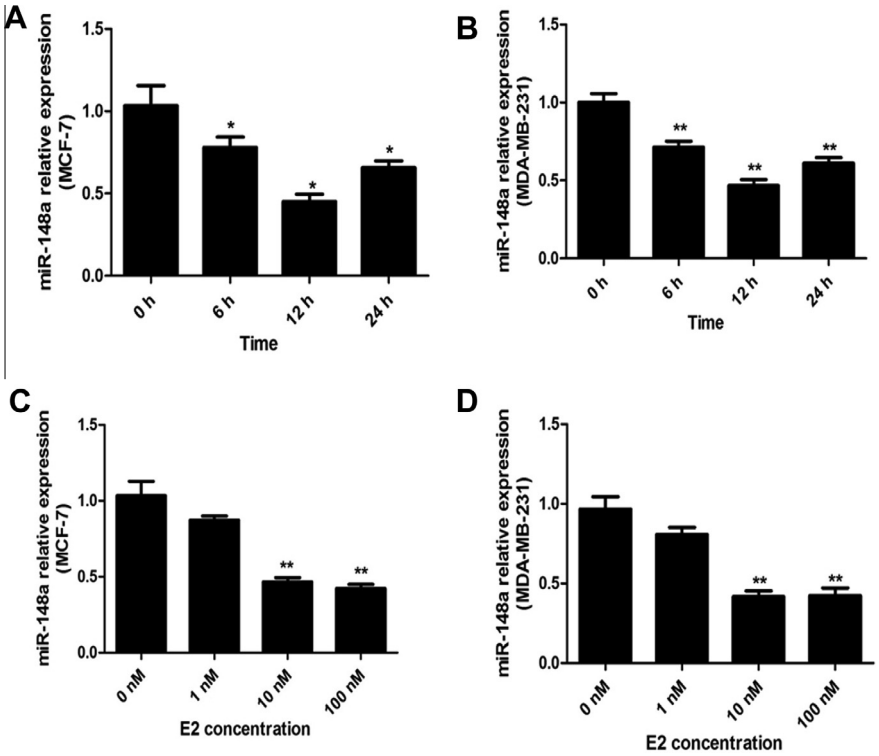

Fig. 1. E2 induces the level of miR-148a in MCF-7 and MDA-MB-231 cells. (A–D) miR-148a expression was determined by qRT-PCR as described in Section 2 (A) MCF-7 cells were treated with EtOH or 10 nM E2 for the time indicated. (B) MDA-MB-231 cells were treated with EtOH or 10 nM E2 for the time indicated. (C) MCF-7 cells were treated with EtOH or different concentration of E2 indicated for 12 h. (D) MDA-MB-231 cells were treated with EtOH or different concentration of E2 indicated for 12 h. The results are shown as mean ± S.E. from three representative independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control.

comparing two different treatments for one cell. All tests were two-sided and $p < 0.05$ was considered significant.

3. Results

3.1. E2 induces the level of miR-148a in MCF-7 and MDA-MB-231 cells

To determine whether miR-148a is a target gene of E2, ER-positive breast cancer cell, MCF-7 and triple-negative (TN) breast cancer cell, MDA-MB-231 were treated with E2, and miR-148a expression was measured by quantitative PCR. miR-148a expression was significantly down-regulated by E2 in both cell lines (Fig. 1A and B). Dose–response experiments revealed maximal miR-148a reduction with 10–7 M after 12 h of treatment in MCF-7 and MDA-MB-231 cells, about half of the control group (Fig. 2C and D). Because 10^{-8} M is close to physiological concentration, this concentration is used in the following study.

3.2. GPER mediates the E2-induced increase in miR-148a expression in MCF-7 and MDA-MB-231 cells

Because classical estrogen receptors were negative in TN breast cancer cells, we speculated that GPER may mediate the effects of E2. We first detected the expression of GPER in MCF-7 and MDA-MB-231 cells. Meanwhile, we chose OVCAR5 cells as positive control, which was reported as a GPER positive cell [24]. As shown in Fig. 2A, GPER was expressed in both MCF-7 and MDA-MB-231 cells. G15 is a well-established antagonist of GPER. To determine whether the E2-induced decrease in miR-148a is mediated directly by GPER, MCF-7 and MDA-MB-231 cells were pretreated with G15 for 6 h before E2 treatment. G15 blocked the E2-induced decrease in miR-148a mRNA, indicating that GPER mediated this response (Fig. 2B).

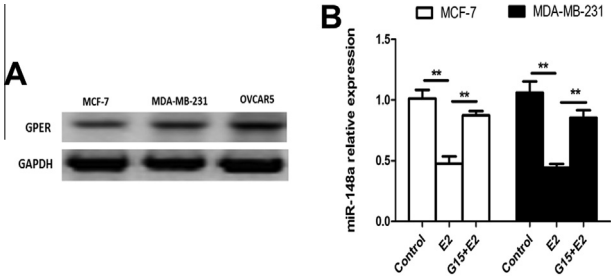

Fig. 2. GPER mediates the E2-induced increase in miR-148a expression in MCF-7 and MDA-MB-231 cells. (A) GPER protein expression was determined by Western blot in MCF-7, MDA-MB-231 and OVCAR5 cells. (B) MCF-7 and MDA-MB-231 cells were pretreated with or without 100 nM G15 for 6 h before addition of 10 nM E2 for 12 h. Then the expression of miR-148a was determined by qRT-PCR. The results are shown as mean ± S.E. from three representative independent experiments. $^{**}P < 0.01$ compared with control.

3.3. Characterization of HLA-G as direct target of miR-148a in breast cancer cells

It was reported that HLA-G was a target of miR-148a in the placenta [11]. To further identify that miR-148a target HLA-G in

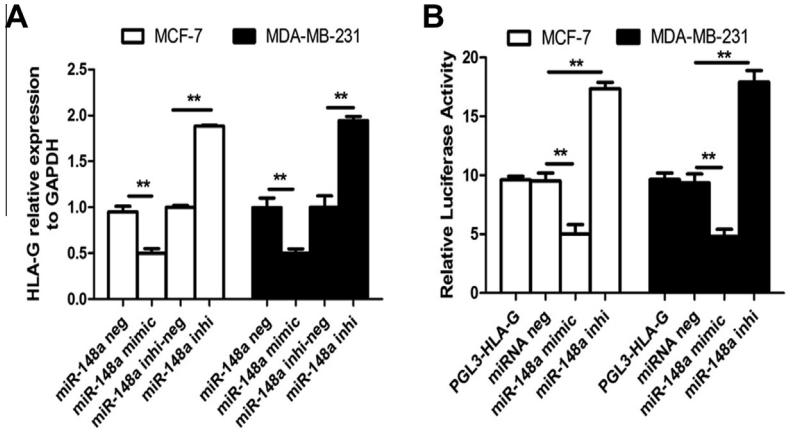

Fig. 3. miR-148a targets HLA-G. (A) MCF-7 and MDA-MB-231 cells were transfected with indicated miRNAs for a total of 24 h. HLA-G expression was checked by qPCR. (B) The luciferase activities of MCF-7 and MDA-MB-231 cells were measured after co-transfection with the indicated HLA-G 3′UTR constructs and miR-148a or its inhibitor for 24 h. The results are shown as mean ± S.E. from three representative independent experiments. $^{**}P < 0.01$ vs control.

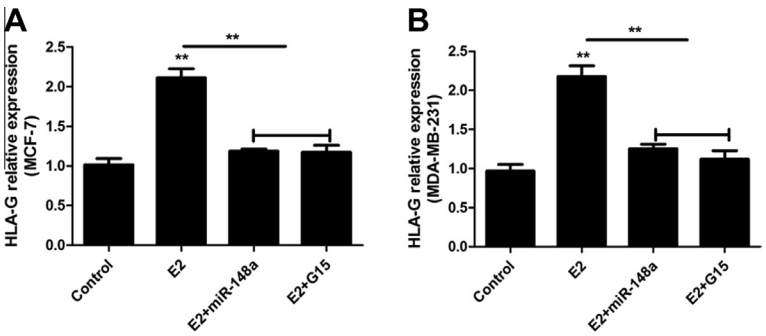

Fig. 4. E2-GPER regulates the expression of HLA-G by miR-148a. (A) MCF-7 cells were pretreated with 100 nM G15 for 6 h or pre-transfected with miR-148 mimic for 12 h before addition of 10 nM E2 for 12 h. Then the expression of miR-148a was determined by qRT-PCR. (B) MDA-MB-231 cells were pretreated with 100 nM G15 for 6 h or pre-transfected with miR-148 mimic for 12 h before addition of 10 nM E2 for 12 h. Then the expression of miR-148a was determined by qRT-PCR. The results are shown as mean ± S.E. from three representative independent experiments. $^{**}P < 0.01$ compared with control.

breast cancer cells, we transfected miR-148a mimics and inhibitors into MCF-7 and MDA-MB-231 cells. Control group was treated only with transfection regent-Lipofectamine 2000. The results showed that miR-148a down-regulated HLA-G expression, while its inhibitor could up-regulate HLA-G expression (Fig. 3A). Furthermore, luciferase reporter analysis results showed that miR-148a significantly suppressed the expression of a luciferase reporter gene fused to 3′UTR region of HLA-G, which could be reversed by further introduction of miR-148a inhibitor in MCF-7 and MDA-MB-231 cells (Fig. 3B). These results proved that miR-148a could suppress HLA-G expression through binding to its 3′UTR.

3.4. E2-GPER regulates the expression of HLA-G by miR-148a

To investigate whether HLA-G was regulated by E2-GPER signaling pathway via inhibiting miR-148a, we pretreated MCF-7 and MDA-MB-231 cells with 100 nM G15 for 2 h or pre-transfected with miR-148 mimic for 12 h before addition of 10 nM E2 for 12 h. Then the expression of HLA-G was checked by qRT-PCR. As shown in Fig. 4A and B, E2 increased 2-fold the expression of HLA in both cells. Whereas, after pretreatment with miR-148 mimic or G15, the promotion effects of E2 on HLA-G expression were blocked. These results indicate that E2-GPER regulates the expression of HLA-G by inhibiting the expression of miR-148a.

4. Discussion

miRNAs are of high interest as potential breast cancer therapeutics. However, their expression and function in breast cancer remain to be elucidated [18]. Estrogen signaling is important in development and progression of breast cancer [19]. The fact of miR-148a is specifically downregulated in triple-negative breast cancer cells [18] indicates that novel estrogen receptor may mediate the E2 affected miR-148a expression in breast cancer. In the present study, we found that estrogen inhibits miR-148a by its receptor GPER. Furthermore, we identified that E2-GPER promotes the expression of HLA-G through suppression of miR-148a.

MiR-148a functions as a tumor suppressor in cancer cells. It was reported that miR-148a functions as a tumor metastasis suppressor in gastric cancer and downregulation of miR-148a contributes to gastric cancer lymph node-metastasis and progression likely via SMAD2 [25,26]. Moreover, miR-148a acts as a tumor suppressor by targeting IGF-IR and IRS1 [27]. Therefore, it is important to investigate the expression regulation mechanism of miR-148a.

GPER is recently identified as a membrane-associated estrogen receptor that mediates non-genomic effects of estrogen. GPER has an important function in the proliferation of ovarian cancer cells lacking ERα [28,29]. Moreover, GPER is an initiator of tamoxifen resistance in hormone-dependent breast cancer [30]. GPER-mediated cancer-associated fibroblasts are likely to contribute to breast cancer progression, especially TAM resistance, via a positive feedback loop involving GPER/EGFR/ERK signaling and E2 production [31]. GPER overexpression and PM localization are critical events in breast cancer progression, and lack of GPER in the PM is associated with excellent long-term prognosis in ER-positive and PgR-positive tamoxifen-treated primary breast cancer [32,33]. Our results showed that GPER mediates E2 reduced miR-148a expression indicating inhibition of estrogen/GPER signaling represents a novel targeted therapy in breast cancer.

Human leukocyte antigen-G (HLA-G) is known to be implicated in a tumor-driven immune escape mechanism in malignancies. Several studies revealed that HLA-G was more frequently observed in advanced stages of the disease and tumor grade in breast cancer, indicating its considerable clinical relevance to breast cancer [34,35]. HLA-G expression at the tumor cell surface might allow it to escape T and natural killer (NK) cell immune surveillance. Surface HLA-E appears to confer protection from the NK cell-mediated lysis via the CD94/NKG2A receptor. Indeed, the role of HLA-G may be to interact with NK cell inhibitory receptors, such as ILT2 or ILT4 [12]. In the present study, we identified that HLA-G is a direct target gene of miR-148a in breast cancer. It is in line with the report that miR-148a regulates the expression of HLA-G in the placenta [11].

In conclusion, E2 reduces the level of miR-148a in breast cancer medicated by GPER. The downregulated miR-148a increases the expression of HLA-G which probably contributes to breast cancer immune evasion. Our findings offer important new insights into the ability of estrogenic GPER signaling to trigger HLA-G expression through inhibiting miR-148a that supports immune evasion in breast cancer.

Conflicts of interest and informed consent

None declared.

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