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Regulatory interplay between miR-21, *JAG1* and 17beta-estradiol (E2) in breast cancer cells

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ABSTRACT

Overexpression of the oncomir miR-21 is associated with many cancers, including breast cancer. Elevated levels of *Jagged-1* (*JAG1*), a predicted miR-21 target, are implicated in estrogen receptor negative (ER−) breast cancer. We demonstrate (by ablation of the miR-21 binding site in the *JAG1* 3′UTR) that miR-21 directly targets and represses *JAG1* levels in MCF-7 (ER+) breast cancer cells. MiR-21 targeting of *JAG1* in MDA-MB-231 (ER−) breast cancer cells is dependent on miR-21 dosage (levels). In both cell lines, miR-21 and *JAG1* expression levels were negatively correlated due to their regulatory relationship. In addition, 17beta-estradiol (E2) increases *JAG1* levels by limiting (via downregulating miR-21 levels) the repressive effects of miR-21 on the *JAG1* 3′UTR. Our results reveal a regulatory interplay between miR-21, *JAG1* and E2 that is important for advancing understanding of how the oncogenic potential of miR-21 and JAG1 manifests in different sub-types of breast cancer.

1. Introduction

MicroRNAs (miRs) are small non-coding RNAs regulating gene expression post-transcriptionally [1]. Numerous miRs are dysregulated in cancer [2,3] and can have tumor-suppressor [4,5] or oncogenic activity (oncomirs) [6]. For instance, miR-21 promotes cancer cell growth via regulating several genes through their 3′UTR complementary target sites [7].

The miR:mRNA interaction can depend on the cellular context, including whether miR/target are co-expressed, tissue type and cellular stage [8]. For instance, endogenous levels of JAG1 protein are reduced by miR-34a and miR-21 in human monocyte-derived dendritic cells whereas mRNA levels of *JAG1* were upregulated during differentiation [9]. A similar lack of association between JAG1 mRNA and protein levels has been previously described [10].

Previous studies have observed differential expression levels of *JAG1* expression in cancer cells. High levels of *JAG1* have been observed in oral squamous cell [11] and adrenocortical [12] carcinomas. Higher *JAG1* levels were associated with poor prognosis [13–15], high tumor grade, and ER/PR negativity [10] in breast cancer patients.

JAG1 encodes a ligand important for the Notch intercellular signaling pathway and for angiogenesis [16]. Notch signaling is important for development and tissue homeostasis and is activated in many human cancers [17]. Triple negative (ER/PR/Her−) breast cancer cell lines display higher expression levels of *JAG1* compared to ER+ cell lines, indicating that in the absence of ER/PR/Her stimulation, activation of Notch signaling by JAG1 promotes cell growth [18].

In a cDNA microarray study, MCF-7 cells stimulated with 17beta-estradiol (E2) showed upregulation of *JAG1* [19]. In contrast to *JAG1*, miR-21 is downregulated by E2 via promoter binding in MCF-7 [20] and also other breast cancer cell lines [20]. These studies indicate that *JAG1* and miR-21 are both downstream targets of estrogen signaling (via E2 stimulation). In addition, miR-21 [7] and *JAG1* [13,14] are independently associated with cancer, yet the possible regulatory interplay between miR-21, *JAG1* and estrogen has not been investigated.

In this study, we investigated the miRNA-mediated regulation of *JAG1* expression levels by miR-21 in MCF-7 and MDA-MB-231 (as ER+ and ER−) breast cancer cell lines and have elucidated the effects of 17beta-estradiol (E2) on miR-21:*JAG1* interaction. We demonstrate that *JAG1* is targeted and its expression levels suppressed by miR-21 in MCF-7 cells, but not in MDA-MB-231 cells. However, by changing the levels of miR-21 dosage ectopically we could control the *JAG1* suppression by miR-21 in both cell lines. We also demonstrate that miR-21 and *JAG1* levels display an inverse correlation in both breast cancer cell lines and that E2 interferes with the miR-21:*JAG1* interaction by decreasing the

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expression of miR-21 in estrogen responsive MCF-7 breast cancer cells.

2. Materials and methods

2.1. Tissue culture and reagents

Human breast cancer cell lines MCF-7 and MDA-MB-231 were grown in DMEM containing 10% fetal bovine serum (FBS), penicillin (100 U ml^{-1}), streptomycin (100 mg ml^{-1}) at 37 °C in 5% CO_2/95% air. For 17beta-estradiol (E2) (Sigma, USA, #E2758) treatments, cells were grown in DMEM without phenol-red, supplemented with 5% charcoal-stripped FBS (Sigma, UK, #F6765). 17beta-estradiol (resuspended in absolute ethanol) was used in final concentration of 10, 50 nM (E2+). Absolute ethanol was used as negative control (E2−).

2.2. Reporter constructs and luciferase assay

The *JAG1* 3′UTR region (632 bp) harboring the miR-21 binding site was PCR amplified from human genomic DNA, cloned into SpeI/HindIII restriction sites in the pMIR-REPORT luciferase vector (Invitrogen, USA), designated as pMIR/JAG1-UTR. A mutated construct lacking the miR-21 seed sequence was generated using Quikchange Lightning SDM kit (Agilent Technologies, USA), designated as pMIR/JAG1-UTRdel. Cells were co-transfected with 300 ng of each plasmid (pMIR/JAG1-UTR, pMIR/JAG1-UTRdel or pMIR) and 1 ng of phRL renilla vector (for normalization) (Promega, USA) in 24-well plates. Twenty-four hours post-transfections, dual luciferase assay was performed according to the manufacturer's protocol (Promega, USA, #E1910).

2.3. Transfections and RNA isolation

Cells were transfected with 50 nM anti-21 or anti-control oligonucleotides and 1 μg of pcDNA-21 or pcDNA empty vector in 6-well plates using the Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, USA). Forty-eight hours post-transfection cells were harvested and total RNA was isolated using the Nucleospin miRNA isolation kit according to manufacturer's instructions (Macherey-Nagel, Germany).

2.4. Quantitative RT-PCR

For qRT-PCR of miR-21, reverse transcription reaction was performed using the MicroRNA Reverse Transcription kit and qPCR was performed using the Taqman Assays (RT primers and probes specific for miR-21) according to manufacturer's protocol (ABI, UK). RNU6B (Taqman assay, ABI, UK) was used as endogenous control for normalizations. For qRT-PCR of *JAG1*, 1 μg of total RNA was reverse transcribed using the Revertaid H-minus first strand cDNA synthesis kit (Fermentas, USA). SYBR Green qPCR detection (ABI, UK) was performed. *TFF1* and *β-Actin* were used as E2 positive control and as endogenous control for normalizations, respectively. QPCR reaction was performed on the CFX96 system (Bio-Rad, USA). Relative quantification analysis was done automatically in the system software analysis based on $2^{-\Delta\Delta Ct}$ method.

2.5. Western blot analysis

Following treatments, cells were harvested and 30 μg of protein was separated on 10% SDS–PAGE, transferred onto PVDF membrane. Anti-Jagged-1 (rabbit, #sc-8303, Santa Cruz Biotechnology, USA) and anti-β-Actin (mouse, #A3854, Sigma, UK) and secondary antibodies anti-rabbit (#A6667, Sigma, UK) and anti-mouse (#A0168, Sigma, UK) were used according to manufacturer's instructions. Blots were visualized with enhanced chemiluminescence (ECL Plus Kit, Perkin Elmer, USA) using the G:Box Chemi imaging system (Syngene, UK).

3. Results and discussion

Overexpression of miR-21 promotes cell proliferation and invasiveness [21]. However, the oncogenic potential of miR-21 expression levels can differ between cell lines; e.g. anti-miR-21 has no effect on proliferation of MDA-MB-231 cells, whereas a significant decrease in cell proliferation occurs in MCF-7 cells [22]. Expression levels of some predicted target genes of miR-21 (such as *JAG1*) are implicated in cancer cell behavior. Higher levels of *JAG1* are associated with more aggressive breast tumors [10]. In addition, higher *JAG1* levels are associated with aggressiveness of the tumor triple negative state (TN; ER−/PR/Her2−), whereas less aggressive luminal (ER+/Her2−) or Her2+ cell lines (such as MCF-7) display lower *JAG1* levels [18]. Furthermore, *JAG1* is expressed at higher levels in lymph node-negative cancer cells displaying lymphatic invasion [10], and *JAG1* levels can also promote cancer cell invasion [23,24]. Expression level differences for both miR-21 and *JAG1* clearly affect cancer cell behavior, requiring a deeper understanding of possible regulatory relationships between miR-21 and *JAG1* in breast cancer.

3.1. The JAG1 3′UTR is targeted by miR-21 in MCF-7 breast cancer cells

JAG1 is identified as a predicted target of miR-21 using all three miRNA-targeting prediction tools TargetScan [25], PicTar [26] and MiRanda [27]. In the 3′UTR of *JAG1* there is a single predicted seed binding site (*AUAAGCUA*) for miR-21, which is highly conserved (Fig. 1). MiR-21 is expressed in both MCF-7 and MDA-MB-231 breast cancer cells, albeit with lower relative endogenous expression levels in MDA-MB-231, indicating an endogenous miR-21 dosage difference in the two cell lines (Fig. 2A). This is consistent with previous reports of lower expression of miR-21 in MDA-MB-231 cells compared to MCF-7 cells [21,22]. Also, in MCF-7 cells, miR-21 has the highest levels of expression compared to other microRNAs predicted to target *JAG1* 3′UTR based on the available expression data (www.microrna.org) [28].

While *JAG1* has been suggested as a candidate target of miR-21, this has not been demonstrated through ablation of the miR-21 binding site in the 3′UTR of *JAG1* [9]. To determine if the *JAG1* 3′UTR is directly targeted by miR-21 via its putative binding site in breast cancer cells, the luciferase activity (in MCF-7 and MDA-MB-231) of a reporter construct containing part (632 bp) of the ~1800 bp *JAG1* 3′UTR (pMIR/JAG1-UTR) was compared with that of a mutant construct (pMIR/JAG1-UTRdel) lacking the miR-21 seed region (Fig. 2B). The 632 bp 3′UTR region was chosen to minimize the number of predicted binding sites of other miRs (Fig. 2B), so that the individual effect of miR-21 binding to the *JAG1* 3′UTR could be more easily analyzed. A significant decrease in luciferase activity was observed in pMIR/JAG1-UTR transfected MCF-7 cells relative to the empty vector pMIR control (Fig. 2C), indicating that the JAG1-UTR region is negatively regulated. To determine if miR-21 was involved in the downregulation of *JAG1* 3′UTR, the miR-21 pMIR/JAG1-UTRdel construct lacking the miR-21 binding site in the 3′UTR was used. In MCF-7 cells, there was a significant restoration of luciferase activity when the pMIR/JAG1-UTRdel construct (lacking the miR-21 binding site) was transfected (Fig. 2C). However, in contrast to MCF-7 cells, there was no repression effect observed on luciferase activity in MDA-MB-231 cells transfected with pMIR/JAG1-UTR (Fig. 2C).

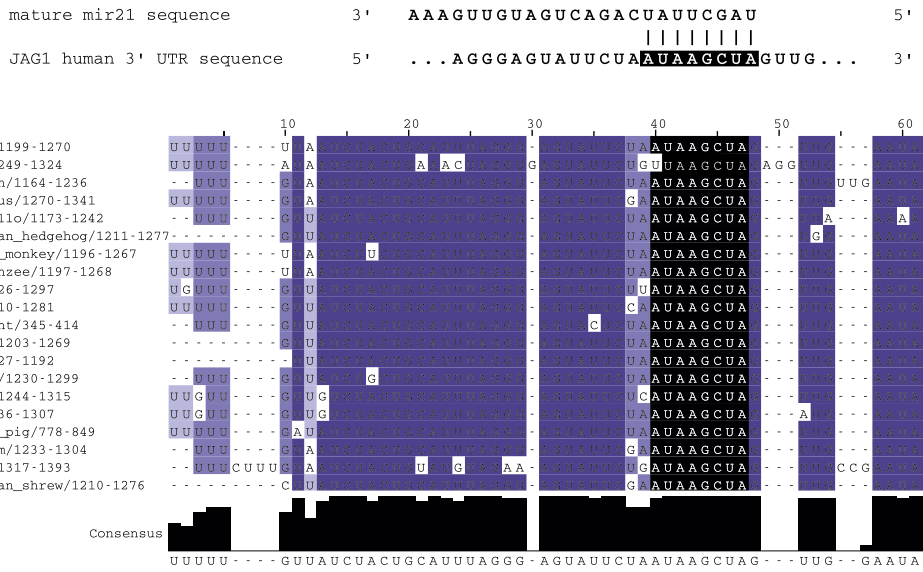

Fig. 1. Multiple sequence alignment of *JAG1* 3′UTR in 20 species. MiR-21 target site (highlighted) on *JAG1* 3′UTR contains a highly conserved 8mer '*AUAAGCUA*' complementary to the miR-21 seed region.

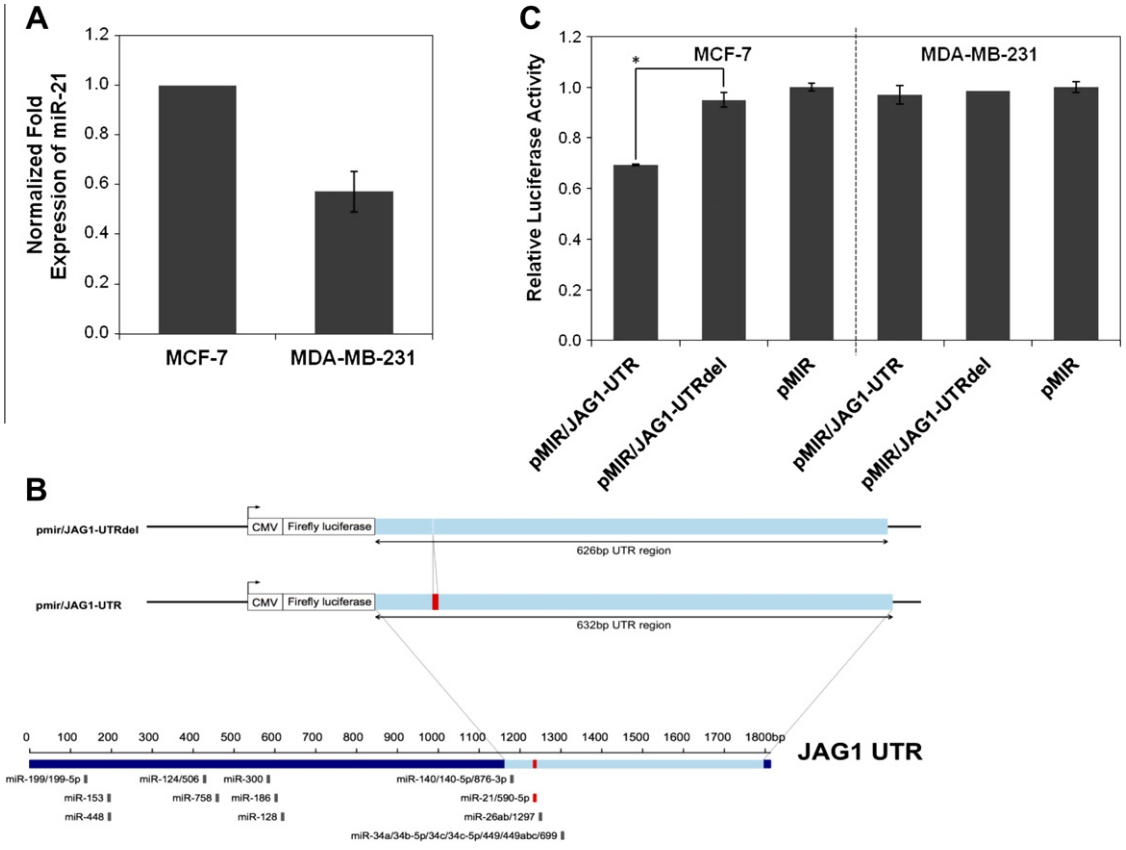

Fig. 2. Luciferase Reporter Assay of *JAG1* 3′UTR. (A) Schematic representation of *JAG1* 3′UTR, putative microRNA binding sites (TargetScan v6.1) and linear representation of pMIR/JAG1-UTR and -UTRdel luciferase reporter constructs. (B) Relative luciferase activity of pMIR/JAG1 constructs in MCF-7 and MDA-MB-231 cells, displaying the mean of triplicate experiments with +/− SEM (standard error of mean), $^{*}p < 0.05$ (Student's *t*-test). (C) Normalized fold expression (Taqman) of miR-21 in MCF-7 and MDA-MB-231. RNU6B was used as endogenous control in normalizations.

Our results indicate that miR-21 can target the conserved binding site in the *JAG1* 3′UTR and lead to reduced expression levels in MCF-7 breast cancer cells. The endogenous levels of miR-21 were lower in MDA-MB-231 cells compared to MCF-7 cells. The comparison of miR-21 targeting effects in MCF-7 with MDA-MB-231 indicates that such targeting can be cell-line

specific, possibly due to endogenous miR-21 levels and/or other factors.

3.2. MiR-21 and JAG1 expression levels are negatively correlated in breast cancer cells

To test whether miR-21 expression levels are correlated with *JAG1* expression levels, the effective levels of miR-21 in MCF-7 were decreased (using anti-21 oligonucleotides), while the effective levels of miR-21 in MDA-MB-231 cells were increased (using the pc-21 overexpression construct). It was observed that decreased miR-21 expression levels (anti-21) led to increased *JAG1* levels compared to control (anti-ctr), in MCF-7 cells (Fig. 3A). Western blot analysis of JAG1 protein in MCF-7 cells treated with anti-21 showed significant upregulation compared to anti-control or untreated samples, indicating that miR-21 levels are negatively correlated with JAG1 protein levels (Fig. 3B). Supporting this correlation, MDA-MB-231 cells overexpressing miR-21 (via pc-21) exhibited a significant decrease in *JAG1* expression levels compared to control (pcDNA empty vector) (Fig. 3C). Furthermore, comparative analysis of pMIR/JAG1-UTR and pMIR/-UTRdel constructs with the miR-21 knock-down (via anti-21) in MCF-7 cells showed increased luciferase activity of pMIR/JAG1-UTR when miR-21 was knocked-down (Fig. 3D). In addition, increasing the levels of miR-21 (via pc-21 construct) caused a significant decrease in the luciferase activity of pMIR/JAG1-UTR in MDA-MB-231 cells (Fig. 3D), suggesting that increased miR-21 levels induce miR-21:*JAG1* targeting and that the low endogenous levels of miR-21 in MDA-MB-231 cells allow higher *JAG1* expression levels.

Overall, our results indicate that miR-21 dosage (i.e. expression levels) is important in determination of the extent of the miR-21:*JAG1* interaction. Although, endogenous levels of miR-21 in MDA-MB-231 may not be sufficient for effective *JAG1* suppression via the 3′UTR miR-21 target site, miR-21 targeting of *JAG1* could be induced by ectopically increasing miR-21 levels. Conversely, *JAG1* 3′UTR targeting by miR-21 in MCF-7 cells can be limited by depleting miR-21 availability (anti-21). Similar dosage-dependent complexity in miR targeting effects has been previously reported, mostly in the context of tissue-specific expression of miRs [29,30].

3.3. MiR-21 and JAG1 behave oppositely in response to 17beta-estradiol (E2)

Estrogen signaling is important for determining cell fate, particularly in the context of breast cancer progression and therapies [31]. Both tumor-promoting [32,33] and tumor-preventing (pro-apoptotic) [34,35] effects of estrogen have been reported. Furthermore, estrogen-directed regulation of microRNA expression levels in breast cancer [36–38], and a link between estrogen receptor status and microRNA regulation [37,39,40] have been described. Expression of miR-21 is downregulated by 17beta-estradiol (E2) through a regulatory site in the miR-21 producing gene (*MIRN21*) promoter region. When E2 is applied this leads to a reduction in miR-21 levels, and a concomitant increase in the expression levels of downstream miR-21 target genes in MCF-7 cells [20]. Conversely, it has been observed that 17beta-estradiol (E2) promotes expression of *JAG1* up to six-fold in MCF-7 cells [19]. To investigate whether the E2 effects on *JAG1* levels are due to E2 perturbation of

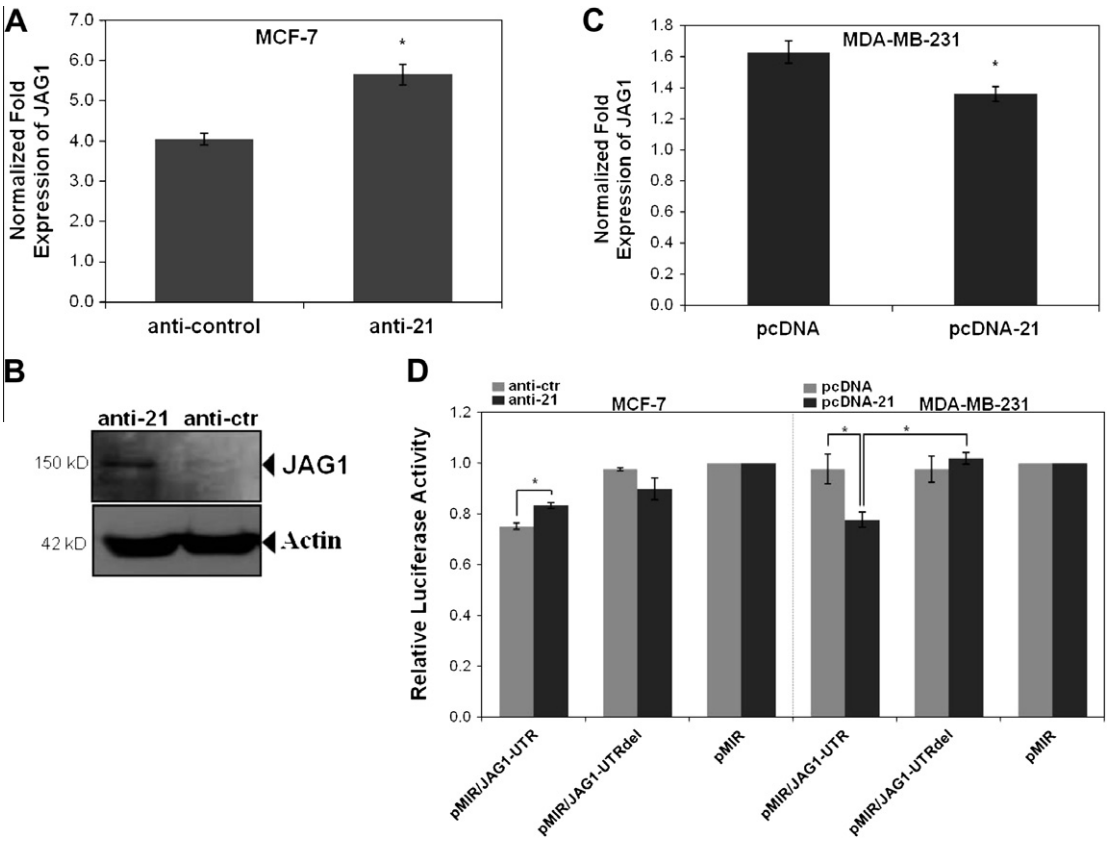

Fig. 3. Inverse correlation of miR-21 and *JAG1* levels. (A) Normalized fold expression (qRT-PCR analysis) of *JAG1* in MCF-7 treated with anti-control or anti-21 oligonucleotides. (B) Western blot analysis of JAG1 protein in MCF-7 transfected with anti-21 or anti-control oligonucleotides; β-Actin detected as loading control. (C) Normalized fold expression (qRT-PCR analysis) of *JAG1* in MDA-MB-231 transfected with pc-DNA empty vector or pc-21 overexpression construct. GAPDH was used as endogenous control in normalizations. (D) Relative luciferase activity of pMIR/JAG1 constructs in MCF-7 and MDA-MB-231 transfected with anti-control or anti-21, pcDNA or pc-21, respectively. Graphs represent the mean of triplicate experiments with +/− SEM (standard error of mean), $^{*}p < 0.05$ (Student's *t*-test).

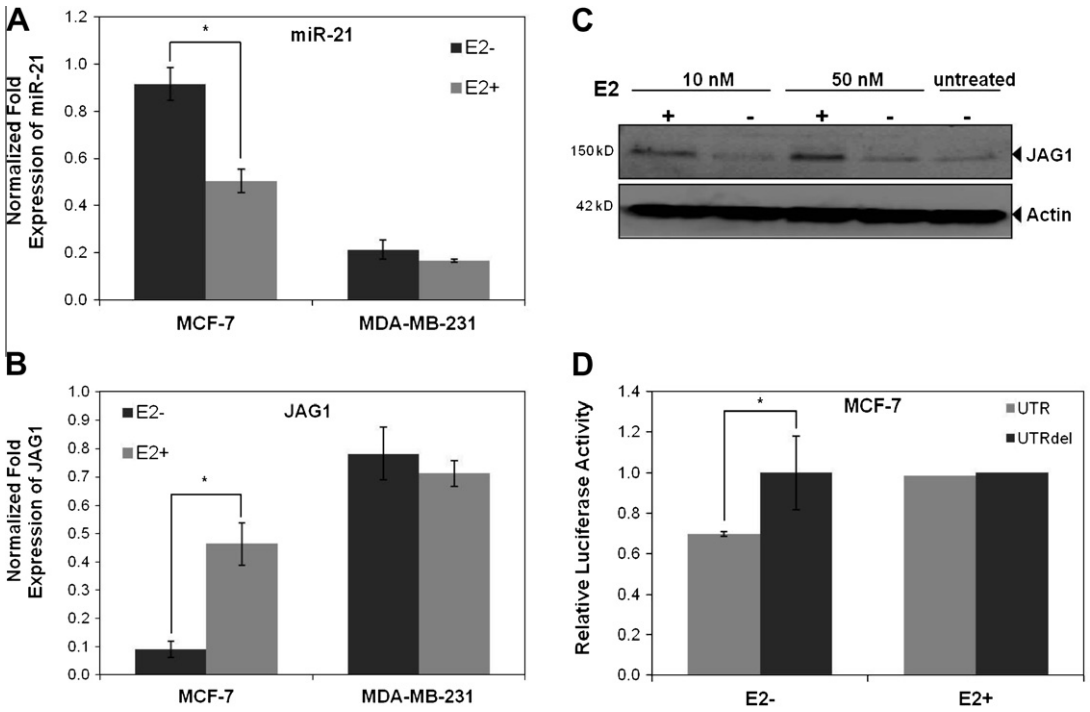

Fig. 4. 17beta-estradiol effects on miR-21 and JAG1. Normalized fold expression of (A) miR-21 (Taqman) and (B) *JAG1* (qRT-PCR analysis) in MCF-7 and MDA-MB-231 treated with 10 μM of 17beta-estradiol (E2+) or absolute ethanol as negative control (E2−). (C) Western blot analysis of JAG1 protein in MCF-7 treated with 10 and 50 μM of 17beta-estradiol (E2+) or absolute ethanol as negative control (E2−) or untreated. β-Actin was detected as loading control. (D) Relative luciferase activity of pMIR/JAG1 constructs in MCF-7 treated with 10 μM of 17beta-estradiol (E2+) (*$p = 0.043$) compared to absolute ethanol as negative control (E2−).

miR-21:*JAG1* targeting, the levels of miR-21 and *JAG1* in estrogen receptor positive (ER+) MCF-7 and estrogen receptor negative (ER−) MDA-MB-231 cells were determined by qRT-PCR, both before and after E2 treatment. The activity of E2 was confirmed by qRT-PCR analysis using the E2 responsive biomarker *TFF1* as a positive control. *TFF1* was expressed in untreated MCF-7 cells and was undetectable in MDA-MB-231 cells (Fig. S1). E2 treatment significantly increased *TFF1* expression in MCF-7 cells as a demonstration of E2 response, whereas *TFF1* was non-responsive to E2 in MDA-MB-231 cells (Fig. S1). E2 treatment (E2+) of MCF-7 cells displayed a significant decrease in miR-21 levels, whereas in MDA-MB-231 cells miR-21 expression levels did not change (Fig. 4A). In contrast to the repressive effect on miR-21 expression levels, E2 treatment (E2+) increased *JAG1* levels significantly in MCF-7 cells (Fig. 4B). However, MDA-MB-231 cells were non-responsive to E2 and showed no change in *JAG1* levels in E2+ or E2− treatments (Fig. 4B). The response of *JAG1* expression induced by E2 treatment in MCF-7 cells was also confirmed to be E2 dosage-dependent at the protein level (Fig. 4C). These results are consistent with the previous findings that highly invasive MDA-MB-231 (ER−) cells showed higher *JAG1* expression compared to non-invasive MCF-7 (ER+) cells. It is possible that the higher expression levels of *JAG1* (due to insufficient targeting by miR-21) could contribute to the highly invasive nature of MDA-MB-231 cells.

To test whether 17beta-estradiol (E2) could enhance *JAG1* expression in MCF-7 cells via decreasing the miR-21 expression levels therefore limiting the access of miR-21 binding to *JAG1* 3′UTR, luciferase assays on pMIR/JAG1 reporter constructs were performed in E2+ and E2− treated cells. In the absence of E2 (E2−) luciferase activity of pMIR/JAG1-UTR was suppressed compared to pMIR/JAG1-UTRdel in MCF-7 cells (Fig. 4D, also Fig. 2B). Furthermore, addition of E2 (E2+) in MCF-7 cells restored the luciferase activity of the pMIR/JAG1-UTR (Fig. 4D), illustrating that miR-21 and *JAG1* interaction in MCF-7 cells can be perturbed by 17beta-estradiol (E2) treatment. Our results demonstrate that the E2 treatment of MCF-7 cells restores *JAG1* levels via decreasing the miR-21 expression levels i.e. that the E2 treatment positively affects *JAG1* levels via its repressive effect on miR-21 levels. Consistent with this, expression levels of miR-21 are not affected in the MDA-MB-231 cells treated with E2, with no effects evident on *JAG1* 3′UTR targeting by miR-21 (Fig. S2).

Overall, our results indicate that the E2 effect that increases *JAG1* levels in ER+ breast cancer cells results from E2 reducing the levels of miR-21 that are subsequently available for repression of *JAG1* expression levels via the binding site in the *JAG1* 3′UTR. Hence, the association between estrogen receptor status and therefore miR-21 expression levels contributes to modulating *JAG1* levels in estrogen responsive MCF-7 cells. Whilst this E2 effect on miR-21 & JAG1 levels is not observed in the ER− MDA-MB-231 cells, we have demonstrated that miR-21:*JAG1* interaction can be elicited by ectopically over-expressing miR-21 in MDA-MB-231 cells. The E2 induced regulatory relationship we have established between miR-21 and *JAG1* in breast cancer cells should be investigated further in clinical cancer samples in relation to estrogen receptor status (ER+ versus ER−) and estrogen treatments, in order to both inform and better understand breast cancer diagnostics and therapeutics.

Authors' contributions

S.D.S. performed all experiments. M.T.A.D. did bioinformatic analysis. S.D.S., M.T.A.D., M.J.K. and C.S. drafted the manuscript. C.S. managed the research and finalized the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.074.

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