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A Truncated-Flt1 Isoform of Breast Cancer Cells Is Upregulated by Notch and Downregulated by Retinoic Acid

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ABSTRACT

We have previously reported that the major isoform of Flt1/VEGFR-1 expressed in MDA-MB-231 breast cancer cells was a truncated intracellular isoform transcribed from intron 21 (i_{21}Flt1). This isoform upregulated the active form of Src and increased breast cancer cell invasiveness. Since expression of the transmembrane and soluble Flt1 isoforms of HUVEC is activated by Notch signaling, we wondered whether the expression of the intracellular isoform i_{21}Flt1 was also dependent on Notch activation. We report here that the expression of i_{21}Flt1 in HUVEC and MDA-MB-231 cells is downregulated by the γ-secretase inhibitor DAPT. In addition, treatment of MDA-MB-231 cells with siRNA specific for Notch-1 and Notch-3 downregulates the expression of i_{21}Flt1. In agreement with these findings, HUVEC and MDA-MB-231 breast cancer cells, cultured on dishes coated with recombinant human Dll4 extracellular domain, express higher levels of i_{21}Flt1. In cancer cells, Flt1 is a target of the micro RNA family miR-200. In MDA-MB-231 breast cancer cells, the truncated intracellular isoform i_{21}Flt1 is also negatively regulated by miR-200c. Retinoic acid interferes i_{21}Flt1 expression by downregulating Notch-3 and upregulating miR-200 expression. Treatment of MDA-MB-231 breast cancer cells with both a γ-secretase inhibitor and retinoic acid suppresses the expression of i_{21}Flt1, providing a new mechanism to explain the effectiveness of this therapeutic approach. J. Cell. Biochem. 115: 52–61, 2014.

KEY WORDS: VEGFR1; Truncated Flt1; NOTCH-1; NOTCH-3; miR-200; BREAST CANCER; CELL INVASION

Metastasis is the primary cause of death from cancer. The lack of curative treatment options for patients with metastatic disease emphasizes the need for a better understanding of the molecular mechanisms involved in metastasis. Hiratsuka et al. [2002] originally reported that $Flt1^{TK-/-}$ mice had impaired metastatic progression. Several reports demonstrated that inhibition of Flt1 by anti-Flt1 peptide blocked micro- and macrometastasis, while over-expression of placental growth factor (PlGF), which signals exclusively through Flt1, increased metastatic spread [Marcellini et al., 2006; Taylor and Goldenberg, 2007]. Flt1 is required for lung adenocarcinoma cell invasion and metastasis [Roybal et al., 2011]. Knocking down Flt1 in lung cancer cells decreased proliferation in monolayer culture, colony formation in soft agar, invasion in coculture with cancer associated fibroblasts, and metastatic potential following subcutaneous injection into syngeneic mice [Roybal et al., 2011]. Similarly, Flt1 maintained cell survival in colorectal and pancreatic cancer cells, and was required for tumor cell migration and invasion [Fan et al., 2005; Wey et al., 2005]. Flt1 activation

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induced tumor cell epithelial–mesenchymal transition and increased cell invasion through phosphorylation of Src family members [Bates and Mercurio, 2005; Lesslie et al., 2006; Yang et al., 2006].

In addition to expression of Flt1 in cancer cells, expression of Flt1 in cells of the tumor microenvironment is also important for metastasis. VEGFR1-positive hematopoietic bone marrow progenitors initiate the pre-metastatic niche [Kaplan et al., 2005]. Knockdown of Flt1 in myelomonocytic cells eradicates micro- and macrometastases (see Kaplan et al. reply to [Dawson et al., 2009]). Chemotherapy-induced expression of Flt1 on endothelial cells can create an environment favorable to tumor cell homing [Daenen et al., 2011].

Although Flt1 is involved in cancer metastasis, Flt1 is not always expressed in cancer cells. For instance, highly invasive MDA-MB-231 breast cancer cells show epigenetic gene silencing of Flt1 as a consequence of promoter hypermetylation [Kim et al., 2009]. No expression of full-length Flt1 or soluble Flt1 can be detected in MDA-MB-231 cells [Mezquita et al., 2010]. Aberrant promoter methylation of Flt1 was also reported in prostatic cancer [Yamada et al., 2003] and in 15 cancer cell lines studied [Kim et al., 2009]. Interestingly, we have shown that highly invasive MDA-MB-231 breast cancer cells that do not express the full length or soluble Flt1 expressed intracellular truncated isoforms transcribed from intronic sequences [Mezquita et al., 2010]. The major isoform, transcribed from intron 21 (i_{21}Flt1), codified for the phosphotransferase domain and the C-terminal fragment of Flt1. This isoform increased invasiveness of MDA-MB-231 cells through activation of Src [Mezquita et al., 2010].

Due to the relevance of Flt1 expression in cancer invasion and metastasis, it is essential to know the mechanisms involved in transcriptional and postranscriptional regulation of Flt1. It has been reported that the expression of the transmembrane and soluble Flt1 isoforms of human umbilical vein endothelial cells (HUVEC) are activated by Notch signaling [Harrington et al., 2008]. Notch signaling is an evolutionarily conserved intercellular signaling pathway mediated by membrane-tethered receptor–ligand interactions between adjacent cells [Artavanis-Tsakonas and Muskavitch, 2010; Guruharsha et al., 2012]. Receptor–ligand binding induces sequential cleavages of the Notch receptor, the last of which is performed by the γ-secretase complex, releasing the Notch intracellular domain (NICD), which translocates to the nucleus. In the nucleus, NICD typically interacts with RBP-Jκ (recombination signal binding protein Jκ) leading to the transcription of Notch target genes. There are four Notch receptors (Notch1–4) and five Notch ligands in mammals: Jagged1, Jagged2, Dll1, Dll3, and Dll4. It has been reported that colon cancer metastasis can be suppressed by inhibiting the Notch signaling pathway [Sonoshita et al., 2011]. Aberrant activation of Notch signaling is an early event in breast cancer and high expression of Notch-1 intracellular domain (NICD) in ductal carcinoma in situ also predicted a reduced time to recurrence 5 years after surgery [Farnie and Clarke, 2007; Farnie et al., 2007]. Decreasing Notch-1 expression by anti-γ-secretase antibodies reduced the invasive capacity of MDA-MB-231 breast cancer cells in vitro [Filipović et al., 2011]. Notch-1 and Notch-3 receptors are involved in the development of triple negative breast cancer [Touplikioti et al., 2012] and highly invasive inflammatory breast cancer exhibits addiction to Notch-3 [Xiao et al., 2011].

In addition to regulation by the Notch pathway, Flt1 expression is post-transcriptionally controlled by the microRNA-200 (miR-200) family. This family of micro RNAs inhibits lung adenocarcinoma cell invasion and metastasis by targeting Flt1 [Roybal et al., 2011]. Decreased expression of miRNAs of the miR-200 family has been implicated in the invasion and metastasis of breast cancer cells [Ahmad et al., 2011]. Reexpression of miR-200 suppressed pulmonary metastases of MDA-MB-231 cells in vivo and anti-miR-200 treatment in vivo resulted in increased metastasis [Ahmad et al., 2011].

We wondered whether the expression of the intracellular truncated isoform i_{21}Flt1, preferentially expressed in MDA-MB-231 highly invasive breast cancer cells, was controlled by the same mechanisms than the full-length receptor. We show here that the expression of i_{21}Flt1 is downregulated by the γ-secretase inhibitor DAPT in both HUVEC and MDA-MB-231 cells. In addition, treatment of MDA-MB-231 cells with siRNA specific for Notch-1 and Notch-3 downregulates the expression of i_{21}Flt1, while activation of Notch signaling in HUVEC and MDA-MB-231 by recombinant human Dll4 extracellular domain upregulates the expression of i21Flt1. Transfection of miR-200c precursors to MDA-MB-231 cells resulted in reduced i21Flt1 expression. Moreover, we show that inhibition of expression of i_{21}Flt1 by retinoic acid is accompanied by downregulation of Notch-3 expression and an increase of expression of the miR-200 family of micro RNAs.

MATERIALS AND METHODS

REAGENTS

All-trans-retinoic acid (RA) (Sigma) was dissolved in ethanol (stock solution 5 mM). The reagent was diluted to its final concentration (5 μM) using cell culture medium.

CELL CULTURE

MDA-MB-231 cells, obtained from the American Type Culture Collection (ATTC) were maintained in Dulbecco's modified Eagle medium/Ham's F-12 (1:1) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin G, and 50 mg/ml streptomycin sulphate. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (cc-2516 and cc-2517) and cultured following the provider's recommendations and media. Cells were maintained in a 5% CO_2 atmosphere at 37°C. All cell lines were used at low passage (<20) and regularly tested against mycoplasma.

PREPARATION OF RNA, NORTHERN, AND RT-PCR

RNA was obtained using RNeasy Mini kit from Qiagen. For microRNA preparation, the mirVana miRNA isolation kit (Lifetechnologies) was used. Northern blot analysis, preparation of DNA probes and RT-PCR have been previously described [Mezquita et al., 2010].

WESTERN BLOTTING

Cells were lysed on ice in NP40 lysis buffer: 150 mM NaCl, 20 mM HEPES (pH 7.5), 0.5% NP-40, a cocktail of protease inhibitors (Complete, Roche) and phosphatase inhibitors (Calbiochem). Cell extracts were separated on 10% SDS–PAGE, transferred to PVDF

membranes, and probed with antibodies. Antibodies for Flt1, cleaved Notch-1 (Val 1744) (D3B8) and Notch-3 (D11B8), were obtained from Cell Signaling Technology.

Γ-SECRETASE INHIBITION

The γ-secretase inhibitor DAPT (Calbiochem) dissolved in DMSO was used at a final concentration of 10 μM. Cells were seeded in the presence of DAPT or an equivalent amount of DMSO vehicle.

TRANSFECTION OF SIRNA

Dharmacon ON-TARGET plus SMART pool siRNAs from Thermo Scientific were used for the interference of i_{21}Flt1 and Flt1. ON-TARGET plus SMART pool siRNAs sequences were (sense) 1 (GGAAATAGTGGGTTT ACAT), 2 (CGTGTGG TCTTACGGAGTA), 3 (TTGAAGAACTTTTACCGAA), and 4 (GAGAAAA GGAGATACT CGA). Interference of Notch-1 and Notch-3 was accomplished using a pre-designed ON-TARGET SMART pool also from ThermoScientific (Notch-1:L-007771 and Notch-3: L-011093). ON TARGET plus non-targeting pool was used as a negative control. Transfections were carried using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The transfection efficiency, between 80% and 90% was assessed using the BLOCK-iT Fluorescent Oligo (Invitrogen).

ACTIVATION OF THE NOTCH SIGNALING PATHWAY BY RECOMBINANT DLL4

Recombinant human Dll4 extracellular domain (rDll4) was purchased from R&D Systems. Tissue culture plates were coated with 0.2% gelatin (w/v) in PBS containing 1 μg/ml rDll4 or BSA as a control [Williams et al., 2006] and incubated at 4°C for 24 h before use. Plates were warmed to 37°C and the coating solution aspirated prior to seeding the cells.

TRANSFECTION OF MIR-200C PRECURSOR MOLECULES

In order to induce miR-200c expression, cells were transfected with hsa-miR-200c precursor (Lifetechnologies) using Lipofectamine 2000 (Invitrogen). To verify the transfection effect of hsa-miR-200c precursor, Pre-miR miRNA Precursor Negative Control (Applied Biosystems) containing a random sequence with no identifiable effects on known miRNA function was included in each transfection experiment.

ANALYSIS OF MIRNA EXPRESSION

Expression of miR-200a, miR-200b, and miR-200c was analyzed by qRT-PCR using TaqMan microRNA assays with the endogenous control RNU6B (Lifetechnologies). Reverse transcription was carried out from 10 ng of total RNA, specific looped-primer RT-PCR (TaqMan microRNA RT-transcription kit, Lifetechnologies). Due to the low miRNA levels in the MDA-MB-231 cells samples, pre-amplification was required. Ten cycles of preamplification were done with the TaqMan mastermix and miRNA-specific forward and reverse PCR primers (Lifetechnologies). One tenth of first strand cDNA or preamplificated samples were analyzed in triplicates by real time qPCR (Applied Biosystems) in 20 μl volumes and 96 well plate format. Real-time PCR amplification was performed on Applied Biosystems 7500 Fast Real-Time PCR system, using miRNA-specific forward and reverse PCR primers and miRNA-specific TaqMan MGB probe. Cycling conditions were as follow: a holding step cycle of 10 min at 95°C for AmpliTaq Enzyme Activation, and 40 cycles of 15 s at 95°C and 60 s at 60°C. The expression levels of miRNA were normalized by the relative delta-delta Ct (ΔΔCt) quantification method.

STATISTICAL ANALYSIS

Data are expressed as means ± standard error. Comparisons between the groups were determined using unpaired *t*-test. *P*-value of <0.05 was considered statistically significant.

RESULTS

EXPRESSION OF I21FLT1 IS DOWNREGULATED BY THE Γ-SECRETASE INHIBITOR DAPT BOTH IN HUVEC AND MDA-MB-231 CELLS

We have previously shown that the i_{21}Flt1 isoform upregulated the active form of Src and increased the invasiveness of MDA-MB-231 breast cancer cells [Mezquita et al., 2010]. Decreasing Notch-1 expression by anti-γ-secretase antibodies reduced the invasive capacity of MDA-MB-231 breast cancer cells in vitro [Filipović et al., 2011]. Since Flt1 expression is upregulated in HUVECs by Notch signaling, we wondered whether or not the intracellular isoform i_{21}Flt1 was also dependent on the Notch pathway in HUVECs and MDA-MB-231 highly invasive breast cancer cells.

We have used the γ-secretase inhibitor DAPT to prevent Notch signaling. DAPT treatment reduced the protein expression of the full-length transmembrane receptor Flt1 and the intracellular i_{21}Flt1 isoform, both in HUVECs and MDA-MB-231 cells (Fig. 1A,B). To confirm the specificity of the bands detected by the anti-Flt1 antibody, we inhibited the expression of Flt1 and i_{21}Flt1 by RNA interference. Bands of 170 and 39 kD corresponding to the full-length transmembrane Flt1 and the truncated intracellular isoform respectively disappear after RNA interference in HUVECs. Furthermore, the band of 39 kD, corresponding to i_{21}Flt1, is no longer detected after RNA interference of i_{21}Flt1 in MDA-MB-231 cells (Fig. 1C). Inhibition of Notch signaling by the γ-secretase inhibitor DAPT decreases the expression of i21Flt1 not only at the protein level, but also at the transcriptional level (Fig. 2).

TREATMENT OF MDA-MB-231 CELLS WITH siRNA SPECIFIC FOR NOTCH-1 AND NOTCH-3 DOWNREGULATES THE EXPRESSION OF I_{21}FLT1

In the Notch signaling pathway, intramembrane cleavage by γ-secretase serves to release the intracellular domain of Notch that translocates to the nucleus. In addition to Notch, many types of transmembrane proteins have been shown to be substrates of γ-secretase [Nakayama et al., 2011]. To confirm whether Notch-1 is involved in the expression of Flt1 and i_{21}Flt1, we transfected HUVEC and MDA-MB-231 cells with anti-Notch-1 siRNA. The results showed that, after siRNA interference of Notch-1, the nuclear fragment NICD decreased in both HUVEC and MDA-MB-231 cells, as expected, and Flt-1 and i_{21}Flt1 were downregulated (Fig. 3).

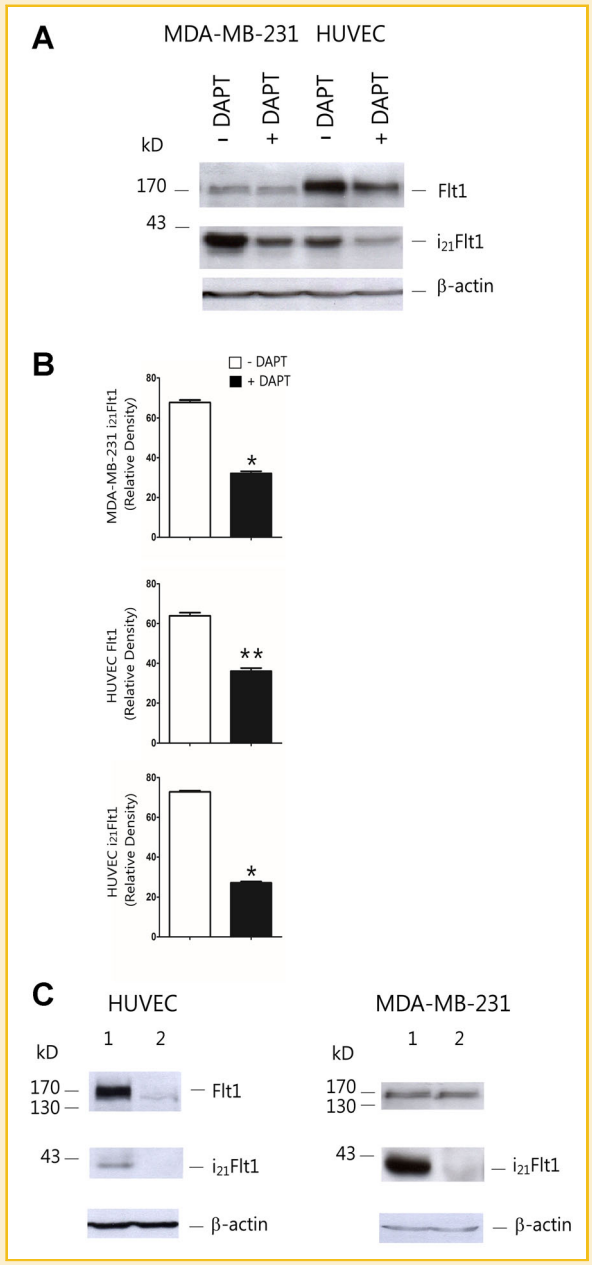

Fig. 1. Inhibition of γ-secretase by DAPT downregulates the expression of Flt1 full-length transmembrane receptor (170 kD) and the truncated intracellular isoform i_{21}Flt1 (39 kD). A: Western blot analysis of Flt1 and i_{21}Flt1 expression in MDA-MB-231 cells and HUVEC. B: The bar graphs show quantification of data of three independent experiments. *, $P < 0.0001$. **, $P = 0.0002$. C: Western blotting showing the effect of RNA interference on the expression of Flt1 full length transmembrane receptor (170 kD) and i_{21}Flt1 truncated intracellular isoform (39 kD) in HUVEC and MDA-MB-231 cells. Expression of Flt1 and i_{21}Flt1 proteins (1) was suppressed by specific siRNA (2) showing the specificity of the antibody used for detection of these proteins. β-actin has been used for equalization of protein samples.

Interference of Notch-3 with siRNA also decreases i_{21}Flt1 in MDA-MB-231 cells (Fig. 4). Downregulation of i_{21}Flt1 was more pronounced in MDA-MB-231 cells when both Notch-1 and Notch-3 were interferred by siRNA (Fig. 4).

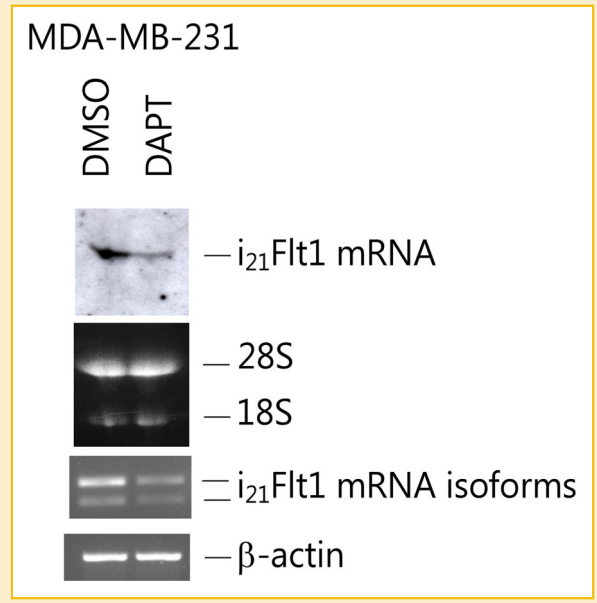

Fig. 2. Expression of i_{21}Flt1 in MDA-MB-231 breast cancer cells after γ-secretase inhibition by DAPT. Top: Northern blot analysis of total RNA from MDA-MB-231 cells. Total RNA was obtained as described under the Materials and Methods Section. Ribosomal RNAs were used as a loading control and control of integrity. Bottom: RT-PCR analysis of i_{21}Flt1 isoform expression in MDA-MB-231 cells. β-Actin amplification has been used for equalization of RNA samples.

NOTCH LIGAND DLL4 UPREGULATES THE EXPRESSION OF I_{21}FLT1 IN HUVEC AND MDA-MB-231 CELLS

To further confirm that the Notch-1 signaling pathway is involved in Flt1 and i_{21}Flt1 expression, HUVECs and MDA-MB-231 cells were cultured on dishes coated with recombinant human Dll4 extracellular domain (rDll4) or with BSA as a control. Immobilization of the Notch ligand is thought to mimic the tethering of the ligand on the cell surface [Varnum-Finney et al., 2000]. This approach activated Notch signaling as demonstrated by the accumulation of the cleaved active NICD in HUVECs and MDA-MB-231 cells cultured on rDll4 for 48 h (Fig. 5). Western blotting analyses showed that, upon induction with DLL4, both the levels of transmembrane Flt1 and intracellular i_{21}Flt1 increased compared to control (Fig. 5).

EXPRESSION OF I_{21}FLT-1 IS NEGATIVELY REGULATED BY miR-200C IN MDA-MB-231 CELLS

It has been reported that miR-200s can inhibit cell invasion and metastasis in lung adenocarcinoma by targeting Flt1 [Roybal et al., 2011]. In addition, reexpression of miR-200s in highly invasive MDA-MB-231 breast cancer cells decreased motility and invasion in vitro and suppressed pulmonary metastasis in vivo [Ahmad et al., 2011]. For these reasons, we were interested to know if i21Flt-1 expression was negatively regulated by miR-200s in MDA-MB-231 cells. When MDA-MB-231 cells were cultured during 6 days without changing the culture medium, miR-200c decreased, while i21Flt-1 increased (Fig. 6). Additionally, expression of i_{21}Flt-1 was markedly reduced in MDA-MB-231 cells transfected with pre-miR-200c (Fig. 7).

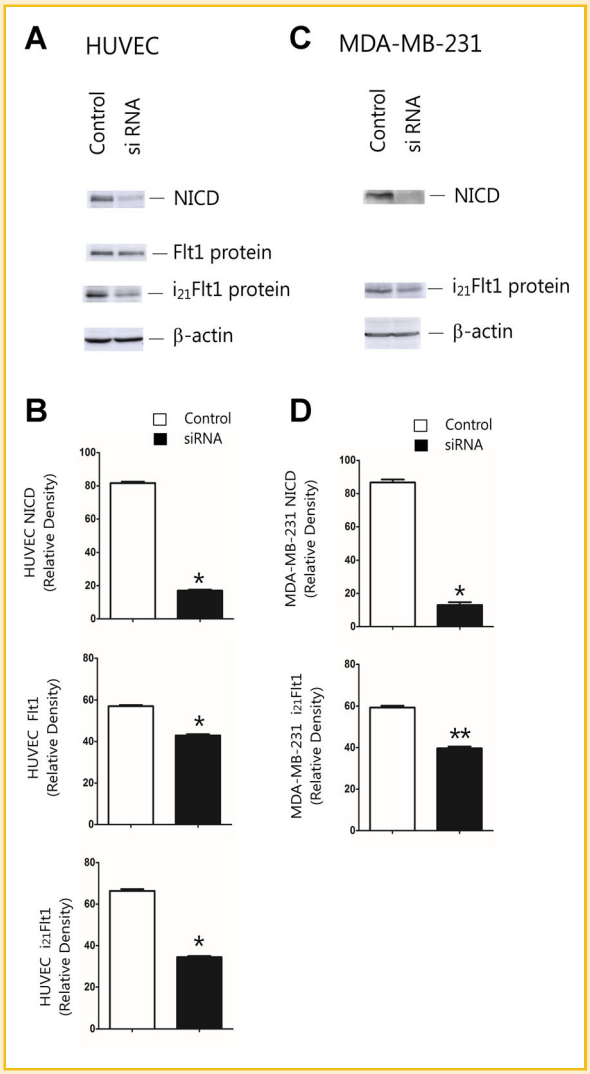

Fig. 3. Anti-Notch-1 siRNA downregulates the expression of Flt1 full-length transmembrane receptor (170 kD) and the truncated intracellular isoform i_{21}Flt1 (39 kD). A: Western blot analysis of NICD, Flt1, and i_{21}Flt1 expression in HUVEC. B: The bar graphs show quantification of data of three independent experiments. *, $P < 0.0001$. C: Western blot analysis of NICD and i_{21}Flt1 expression in MDA-MB-231. D: The bar graphs show quantification of data of three independent experiments. *, $P = 0.0002$. **, $P = 0.0001$. β-actin has been used for equalization of protein samples.

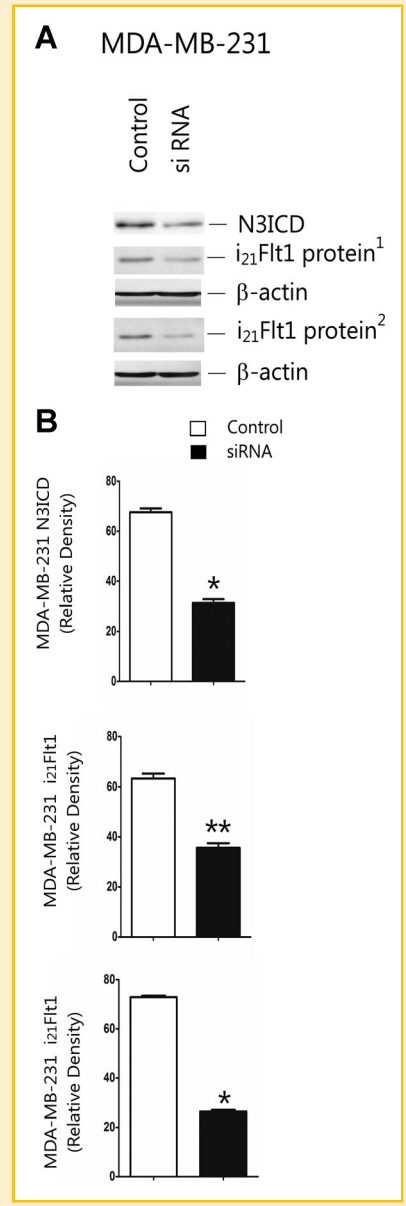

Fig. 4. Anti-Notch-3 siRNA downregulates the expression of the truncated intracellular isoform i_{21}Flt1 (39 kD) of MDA-MB-231 cells. A: Western blot analysis of N3ICD and i_{21}Flt1 expression in MDA-MB-231 cells. i_{21}Flt1 protein1, RNA silencing of Notch-3. i_{21}Flt1 protein2 RNA silencing of Notch-1 and Notch-3. B: The bar graphs show quantification of data of densitometer analyses of three independent experiments. *, $P = 0.0001$. **, $P = 0.0005$. β-actin has been used for equalization of protein samples.

RETINOIC ACID DOWNREGULATES NOTCH-3, UPREGULATES miR-200S AND DECREASES THE EXPRESSION OF I_{21}FLT1 IN MDA-MB-231 CELLS

Previously, we have shown that when MDA-MB-231 breast cancer cells were cultured for 2, 4, and 6 days in the absence of retinoic acid, the expression of the intracellular isoform i_{21}Flt1, assayed by RT-PCR, increased markedly with time. However, no increase of expression was observed in the presence of 5 μM retinoic acid [Mezquita et al., 2010]. Retinoic acid reduces the expression of the protein i_{21}Flt1 in MDA-MB-231 breast cancer cells (Fig. 8). The effect of retinoic acid is more pronounced than the decrease of expression produced by the γ-secretase inhibitor DAPT. The combined effect of DAPT and retinoic acid suppresses the expression of i_{21}Flt1 almost entirely (Fig. 8).

Addition of retinoic acid to the culture medium of MDA-MB-231 breast cancer cells does not change the expression of Notch-1, however the expression of Notch-3 decreases markedly upon treatment with retinoic acid (Fig. 9). Since Notch-3 increases the expression of i_{21}Flt1, downregulation of the truncated intracellular isoform may occur, at least in part, as a consequence of Notch-3

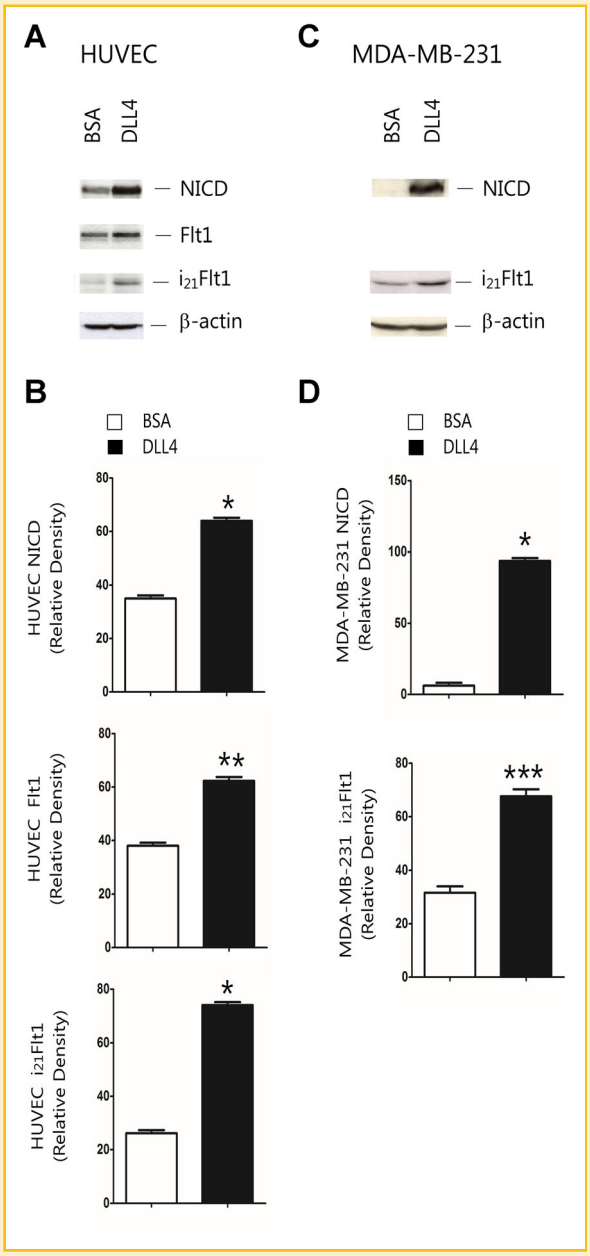

Fig. 5. Dll4 upregulates NICD, Flt1 full-length transmembrane receptor (170 kD) and the truncated intracellular isoform i_{21}Flt1 (39 kD). A: Western blot analysis of NICD, Flt1 and i_{21}Flt1 expression in HUVEC. B: The bar graphs show quantification of data of three independent experiments. *, $P < 0.0001$. **, $P = 0.0002$. C: Western blot analysis of NICD and i_{21}Flt1 expression in MDA-MB-231. D: The bar graphs show quantification of data of three independent experiments. *, $P < 0.0001$. ***, $P = 0.0005$. β-actin has been used for equalization of protein samples.

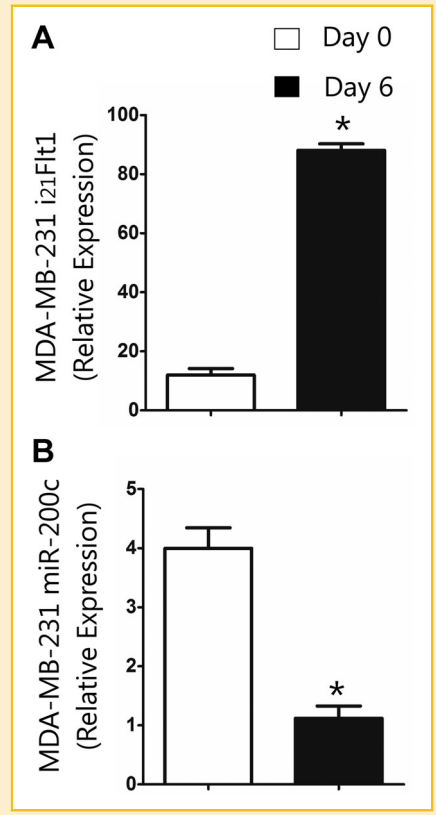

Fig. 6. Expression of miR-200c is inversely correlated with the expression of the protein i_{21}Flt1 in MDA-MB-231 cells. Expression of the protein i_{21}Flt1 (A) and miR-200c (B) at days 0 and 6 of incubation. The bar graphs show quantification of data of three independent experiments. *, $P < 0.0001$.

inhibition by retinoic acid. In addition, Flt1 has been validated as a miR-200s target and overexpression of miR-200s reduced significantly the expression of Flt1 in both lung adenocarcinoma cells [Roybal et al., 2011] and colon cancer cells [Hur et al., 2012]. For these reasons we wondered if the marked decrease in the expression of the intracellular isoform produced by retinoic acid could be also the consequence of upregulation of miR-200s. Both the full length receptor and the intracellular i_{21}Flt1 possess the same 3′UTR with the target sequences for miR-200s. To determine whether the effect of retinoic acid could be mediated by changes in the expression of miR-200, MDA-MB-231 cells were incubated with 5 μM retinoic acid and the expression of the miR-200 family of micro RNAs was compared with the low levels normally expressed in these cells [Ahmad et al., 2011]. MDA-MB-231 breast cancer cells treated with retinoic acid showed an increase in the expression of miR-200a, miR-200b, and miR-200c (Fig. 10).

DISCUSSION

Flt1 is upregulated in different tumor cells, and its expression has been related to cell motility and invasion [Hiratsuka et al., 2002; Kaplan et al., 2005; Wey et al., 2005; Lesslie et al., 2006; Roybal et al., 2011], epithelial–mesenchymal transition [Bates and Mercurio, 2005; Yang et al., 2006], cell resistance to therapy [Bianco et al., 2008; Fan et al., 2011] and also with the phenotype of stem cells [Boscolo et al., 2011; Frank et al., 2011; Ferlosio et al., 2012]. However, not all tumor cells express Flt1. For instance, in highly invasive MDA-MB-231 breast cancer cells the Flt1 gene promoter is hypermethylated [Kim et al., 2009] and the expression of the transmembrane and soluble Flt1 are barely detectable [Mezquita et al., 2010]. Interestingly,

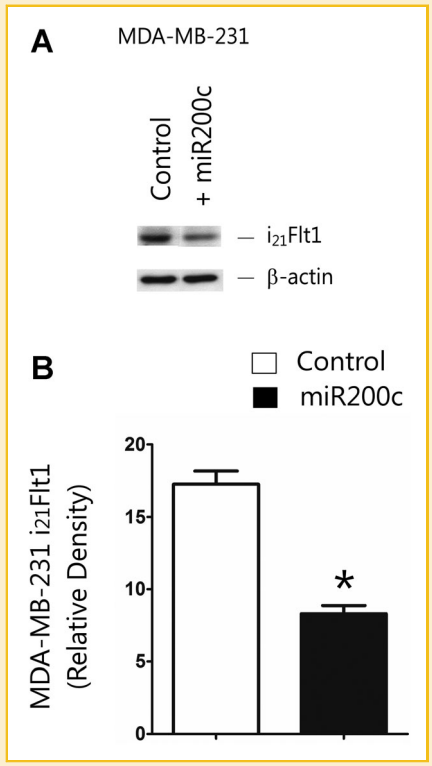

Fig. 7. miR-200c downregulates the expression of the truncated intracellular isoform i_{21}Flt1in MDA-MB-231 cells. A: Western blot analysis of i_{21}Flt1 expression in MDA-MB-231 cells transfected with a negative control and pre-miR-200c. B: The bar graphs show quantification of data of three independent experiments. *, $P = 0.0015$. β-actin has been used for equalization of protein samples.

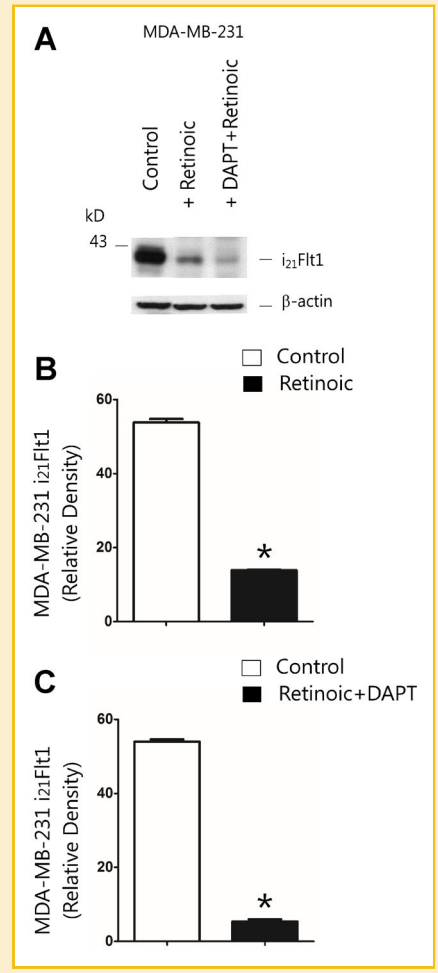

Fig. 8. Retinoic acid downregulates the expression of the truncated intracellular isoform i_{21}Flt1 in MDA-MB-231 cells. Addition of the γ-secretase inhibitor DAPT further decreases the expression of i_{21}Flt1. A: Western blot analysis of i_{21}Flt1 expression in MDA-MB-231 cells treated for 6 days with 5 μM retinoic acid, 10 μM DAPT, and 5 μM retinoic acid plus 10 μM DAPT. B,C: the bar graphs show quantification of data of three independent experiments. *, $P = 0.0001$. β-actin has been used for equalization of protein samples.

these cells express a truncated isoform consisting of the phospho-transferase domain and the C-terminal domain of the molecule. In the absence of the transmembrane receptor, the intracellular isoform still can activate Src and increase the invasiveness of MDA-MB-231 cells [Mezquita et al., 2010].

The intracellular isoform transcribed from intronic sequences i_{21}Flt1 could represent an alternative signaling pathway to the canonical activation of Flt1 by VEGF-A, VEGF-B and PlGF. The aim of this work was to investigate what kind of mechanisms are involved in the expression of the new intracellular isoform in MDA-MB-231 highly invasive breast cancer cells. Interference of the Notch signaling pathway by the inhibitor of γ-secretase DAPT decreases the expression of both the transmembrane receptor and the intracellular i_{21}Flt1 in endothelial cells and i_{21}Flt1 in MDA-MB-231 cells. Interference of the Notch-1 and Notch-3 signaling pathways by siRNA downregulates i_{21}Flt1 in MDA-MB-231 cells. By contrast, activation of Notch signaling pathway in vitro by the ligand Dll4 activates the expression of both the transmembrane receptor and the i_{21}Flt1 in endothelial cells and of i_{21}Flt1 in MDA-MB-231 cells. Taken together, our results show that the Notch signaling pathway is involved in the expression of the i_{21}Flt1 intracellular isoform.

Aberrant activation of Notch signaling has been reported as an early event in breast cancer and leads to poor prognosis [Farnie and Clarke, 2007; Farnie et al., 2007]. Notch-1 and Notch-3 receptors are involved in the development of triple negative and highly invasive inflammatory breast cancer [Xiao et al., 2011; Touplikioti et al., 2012]. A role for the Notch pathway in tumor metastasis has been proposed [Nam et al., 2008; Hughes, 2009; Chen et al., 2010; McGowan et al., 2011; Sonoshita et al., 2011]. Since we have previously reported that i_{21}Flt1 can activate Src and increase the invasiveness of MDA-MB-231 cells, there is the possibility that upregulation of i_{21}Flt1 by Notch-1 and Notch-3 signaling pathways may contribute to the invasive phenotype of MDA-MB-231 breast cancer cells.

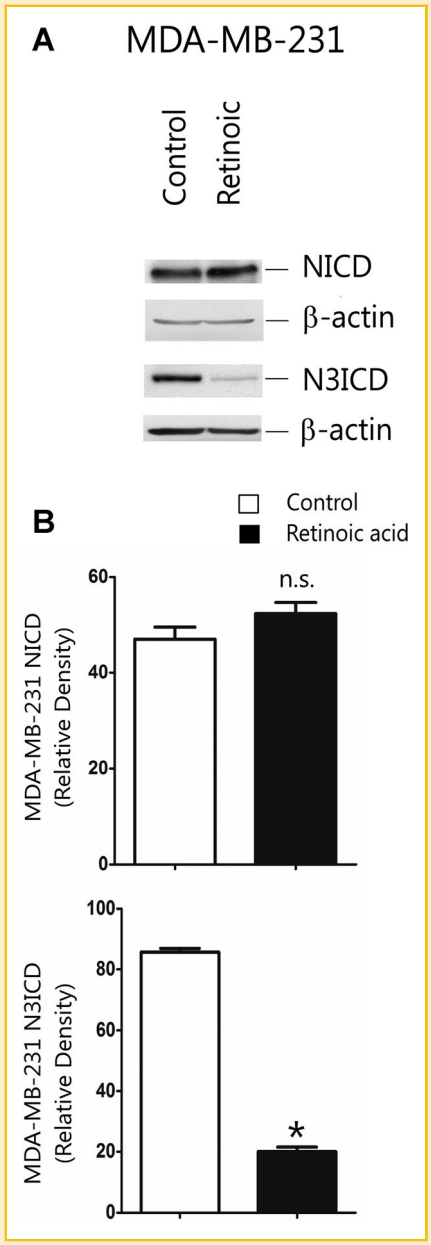

Fig. 9. Retinoic acid downregulates the expression of Notch-3 in MDA-MB-231 cells. A: Western blot analysis of Notch-1 and Notch-3 expression in MDA-MB-231 cells treated for 6 days with 5 μM retinoic acid. B: The bar graphs show quantification of data of three independent experiments. n.s., not significant *, $P = 0.0001$. Densitometric values were corrected for the amount of β-actin.

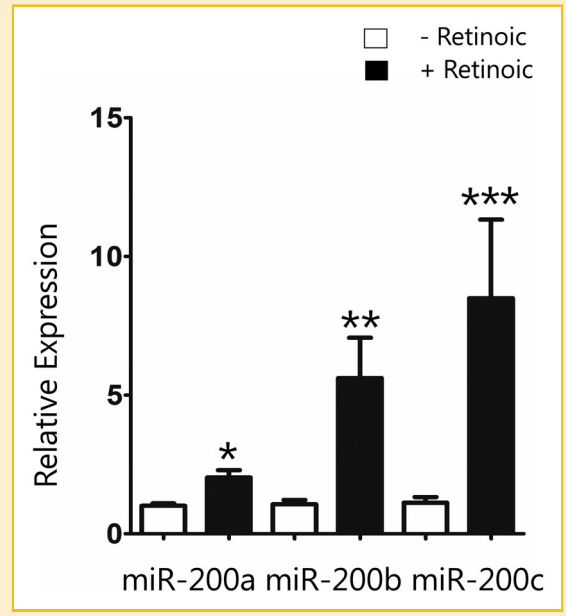

Fig. 10. Retinoic acid upregulates the expression of miR-200a, miR-200b, and miR-200c in MDA-MB-231 cells. Addition of 5 μM retinoic acid for 6 days increases the expression of the miR-200 family. The bar graphs show quantification of data of three independent experiments. *, $P = 0.0092$. **, $P = 0.0260$. ***, $P = 0.0180$.

We have previously observed that the addition of retinoic acid to the culture medium of MDA-MB-231 inhibits i_{21}Flt1 expression. We wondered if retinoic acid could inhibit the Notch signaling pathway and downregulate i_{21}Flt1 through this mechanism. Retinoic acid does not change the expression of Notch-1 in MDA-MB-231 cells. However, the expression of Notch-3 decreases markedly. This observation is in agreement with a previous finding of down-regulation of Notch-3 expression by retinoic acid in MCF7 breast cancer cells [Papi et al., 2012].

In addition, retinoic acid can operate at a post-transcriptional level by upregulating the miR200 family and decreasing translation of i_{21}Flt1. The 3′ end UTR of the mRNA of Flt1 contains target sequences of miR-200 that have been validated [Roybal et al., 2011]. Both the transmembrane full-length receptor and the intracellular isoform i_{21}Flt1 contain these sequences. We show here that retinoic acid increases the expression of miR-200a, miR-200b, and miR-200c when added to the culture medium of MDA-MB-231 cells. These mechanisms can contribute to the downregulation of i_{21}Flt1 we have observed in MDA-MB-231 cells in the presence of retinoic acid. Downregulation of miR-200s has been reported in highly invasive MDA-MB-231 cells [Ahmad et al., 2011]. Reexpression of miR-200 in these cells decreased motility and invasion in vitro and suppressed pulmonary metastasis in vivo, while anti-mir-200 treatment resulted in increased metastasis [Ahmad et al., 2011].

In summary, our results show that the expression of the truncated intracellular i_{21}Flt1 isoform, which is involved in the invasiveness of MDA-MB-231 breast cancer cells, decreases when the Notch signaling pathway is interfered with γ-secretase inhibitors, or when Notch-3 expression is inhibited with retinoic acid. In addition, retinoic acid can decrease i_{21}Flt1 expression through upregulation of miR-200. This family of micro RNAs may suppress i_{21}Flt1 expression both directly, by targeting the i_{21}Flt1 mRNA 3′-end, and indirectly, by inhibiting the Notch signaling pathway. An inverse relationship between miR200 expression and Notch activity has been previously reported in MDA-MB-231 breast cancer cells [Brabletz et al., 2011]. The proposed model for links between γ-secretase inhibitors, retinoic acid, Notch pathway, miR-200 an i_{21}Flt1 is shown in (Fig. 11). Both,

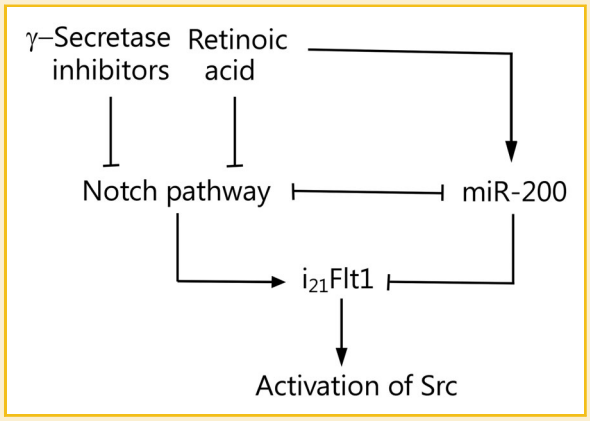

Fig. 11. Proposed model for links between γ-secretase inhibitors, retinoic acid, Notch pathway, miR-200 and i_{21}Flt1 (for explanation see Discussion Section).

γ-secretase inhibitors and retinoic acid have been proposed as potential therapies for invasive breast cancer.

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