Downregulation of Fibronectin 1 Attenuates ATRA-induced Inhibition of Cell Migration and Invasion in Neuroblastoma Cells

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Original Article

Keywords: neuroblastoma, all-trans retinoic acid, fibronectin 1, cell migration, cell invasion

Posted Date: February 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-189238/v1>

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Version of Record: A version of this preprint was published at Molecular and Cellular Biochemistry on May 22nd, 2021. See the published version at <https://doi.org/10.1007/s11010-021-04113-5>.

Abstract

Neuroblastoma (NB) is the most common malignant extra cranial solid tumors in children. It has been well established that retinoic acid (RA) inhibits proliferation of neuroblastoma (NB) by blocking cells at G1 phase of the cell cycle. Clinically, RA has been successfully used to treat NB patients. However, the precise mechanism underlying the potent action of RA-treated NB is not fully explored. In this work, we carried out a gene expression profiling by RNA sequencing on all-trans retinoic acid (ATRA)-treated NB cells. Cancer-related pathway enrichment and subsequent protein-protein interaction (PPI) network analysis identified Fibronectin 1 (FN1) as one of the central molecules in the network, which was significantly upregulated during ATRA treatment. In addition, we found that although downregulation of FN1 had no significant effects on either cell proliferation or cell cycle distributions in the presence or absence of ATRA, it increased cell migration and invasion in NB cells and partially blocked ATRA-induced inhibition of cell migration and invasion in SY5Y NB cells. Consistent with this finding, FN1 expression levels in NB patients positively correlate with their overall survivals. Taken together, our data suggest that FN1 is a potential target for effective ATRA treatment on NB patients, likely by facilitating ATRA-induced inhibition of cell migration and invasion.

Introduction

Neuroblastoma (NB) is one of the most common malignant solid tumors in children. Although its morbidity accounts for only 8-10% of all pediatric cancers, ranking the third, with the cases distribution reaching 6% in the U.S.[1], its mortality rate accounts for 15% of all pediatric cancer deaths and ranks the first[2,3]. Even after comprehensive treatment, the survival rate of NB patients only reaches 40-50%, and nearly 90% of cases occur in children under 10 years old[4]. Large-scale epidemiological studies showed that some pediatric NB patients had rare spontaneous regression, a phenomena that was suggested to be closely related to differentiation of NB [5]. In addition, while the overall survival rate of patients with undifferentiated or poorly differentiated NB is only about 65%, it can reach 84% for patients with well-differentiated NB[6]. These data suggest that inducing differentiation of NB is a promising therapeutic approach for NB patients. Indeed, as a potent inducer of NB cell differentiation, retinoic acid (RA) has been successfully used in clinics to treat NB patients by its ability to induce differentiation of NB[7]. Unfortunately, multiple fatal adverse effects of RA treatment in NB patients have been reported[8], making it imperative to decipher critical downstream targets mediating RA-induced differentiation. In the present study, we utilized RNA-seq to identify important differentially expressed genes (DEGs) in NB cells treated with ATRA. We found that fibronectin 1 (FN1), a critical component of the extracellular matrix (ECM), implicated in various cellular processes, was significantly upregulated during the ATRA-treatment. FN1 is a relatively large glycoprotein with a molecular weight of ~440 kDa. As an abundant and important component of the ECM, FN1 plays important roles in promoting cell adhesion, migration and invasion[9,10,11]. Therefore, the roles of FN1 in cell proliferation, cell cycle distributions, cell migration and invasion were investigated in NB cells in the presence or absence of ATRA in this study.

Materials And Methods

Cell culture and ATRA treatment

NB cell lines NGP, SH-SY5Y (SY5Y) and SK-N-BE2 (BE2) were kindly provided by Dr. Carol J. Thiele from the National Institute of Health (NIH), and cultured in RPMI-1640 medium (Pierce, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2mM L-glutamine, and 100U/ml of penicillin/streptomycin, and maintained in a humidified incubator at 37°C with 5% CO₂. ATRA (Sigma, USA) was dissolved in dimethyl sulfoxide (DMSO), and used at a concentration of 5µM.

mRNA sequencing and bioinformatic analysis

ATRA was used to treat NGP cells for 48hrs with biological triplicates. Cells treated with DMSO served as negative controls. Total RNA was extracted by using TRIzol (Sigma, USA). High quality RNA was processed for mRNA-seq using standard procedures. FDR and log2FC were used to screen for DEGs with a cut-off of P-value < 0.05, FDR < 0.05 and |log2FC| > 2 using DAVID (david.abcc.ncifcrf.gov/) and R language. DEGs were used to create heatmaps and volcano plots by R language through Gene Ontology (GO) analysis, which was also used to perform pathway enrichment analysis. String (<http://www.string-db.org/>) was used for PPI network analysis. In addition, we downloaded a gene expression profile data from GEO database (GSE45587; <http://www.ncbi.nlm.nih.gov/geo/>) as a reference to compare with our RNA-seq results.

Reverse transcription quantitative PCR

We used the GoScript™ reverse transcription system kit (Promega, UK) to convert mRNA into cDNA, which was used to carry out FN1 reverse transcription quantitative PCR (RT-qPCR) by using SYBR premix Ex Taq™II (TaKaRa Clontech, USA). GAPDH was used as an internal control. The following primer sequences were designed using “pubmed” (www.pubmed.com): GAPDH sense 5'-GCACCGTCAAGGCTGAGAAC-3', anti-sense 5'-TGGTGAAGACGCCAGTGGA-3'; FN1 sense 5'-ACAACACCGAGGTGACTGAGA-3', anti-sense 5'-GGACACAACGATGCTTCCTGAG-3'.

Western blot

Whole cell lysates prepared with a standard protocol were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by PVDF membrane (Millipore, USA) transfers. Membranes were incubated with antibodies against GAPDH (Proteintech, China, 1:10,000) or FN1 (Abcam, UK, 1:1000), and then processed with enhanced chemiluminescent reagents (Thermo-Fisher Scientific, USA) and a ChemiDoc Molecular Imager (BIO-RAD, USA).

Transfection

FN1-siRNA or empty vector was transiently transfected into NGP and SY5Y cells using JetPRIME buffer. Target sequences of FN1-siRNAs (Tongyong, China) were as the following: #1:

GGAAAACACTATCAGATAA; #2: GCCAACCTTTACAGACCTA. After 48hrs, cells were collected to extract RNA for RT-qPCR and protein lysates for Western blot to evaluate FN1 expressions.

Cell proliferation and cell confluency

NGP and SY5Y cells were transiently transfected with FN1-siRNA. Cells were seeded into 96-well plates 16hrs after transfection, and were immediately placed in an IncuCyte ZOOM live cell imaging station (Essen, USA) for dynamically monitoring their proliferation. Live cell images were automatically collected with a 40×objective for a total of either 32hrs (from 16hrs to 48hrs) (Supplementary Fig. 1) or 56hrs (from 16hrs to 72hrs) when ATRA (5μM) was added 8hrs after seeding (Supplementary Fig. 2). Cell confluencies were assessed simultaneously. In addition, an absorbance-based cell counting kit-8 (CCK-8) assay was used to assess cell proliferation by a microplate reader at the experimental end point.

Cell cycle analysis

Transient FN1-siRNA transfection and ATRA treatment on NGP and SH-SY5Y cells were done in the same way as “Cell proliferation” as described above. Live cells were fixed with 70% ethanol at 4°C for 12hrs, followed by propidium iodide (PI) staining for 30min. Cells were processed for cell cycle profiling using a C6 Plus flow cytometer (BD, USA) to assess the percentage of cells in each phase of the cell cycle.

Cell migration assays

NGP and SY5Y cells were seeded into 96-well plates with biological duplicates in medium with only 5% fetal bovine serum to minimize the influence of cell proliferation. Scratches were automatically created by a wound maker (Essen, USA) 8hrs after seeding, and images were collected every 4hrs for a total of 24hrs in the IncuCyte Zoom live imaging station. Cells were automatically quantified as pixels, and relative wound densities were automatically calculated with the station as (wound area of 0hr - wound area of 24hr) / wound area of 0hr × 100%.

Transwell invasion assays

Cell invasion were assessed by using matrigel (0.7 mg/ml, Corning, USA)-coated transwell inserts. FN1-siRNA was transfected into NB cells in the presence or absence of ATRA and seeded to the upper chamber of transwell inserts in medium with 5% serum. Medium with 15% serum was added to the lower chamber. After 24hrs incubation, cells tranversed to the lower surface of the upper chamber were stained with a Giemsa staining system after removal of nonmigrated cells.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). For comparisons between two groups, 2-tailed T tests were used. For multiple tests, one-way ANOVA was utilized. Values of $P \leq 0.05$ were considered as statistically significant.

Results

Fibronectin 1 was significantly up-regulated by ATRA treatment in NB

cells

To better understand the molecular mechanism underlying the critical role of ATRA in promoting differentiation of NB cells, we used RNA-seq to carry out a global gene expression profiling on NGP cells treated with ATRA or its solvent DMSO for 48hrs. Differential gene expression analysis identified 635 up-regulated genes and 273 downregulated genes in ATRA-treated cells compared with control cells (Fig. 1A & B). To understand the biological relevance of DEGs in ATRA-treated cells, we utilized GO to perform a gene pathway enrichment analysis. Among the top 20 enriched pathways, 12 were related to cancer and 5 to neurogenesis (Fig. 1C), two important biological properties that are associated with NB. Interestingly, the most highly enriched pathway was “ECM-receptor interaction” (Fig. 1C), which controls multiple cellular activities related to cancer metastasis, such as cell migration and adhesion. To gain additional insights into critical molecular events associated with ATRA-treated NB, we used GO pathway enrichment analysis to focus on cancer-related pathways. We found that the top 3 enriched pathways were “Cell proliferation” and two pathways implicated in cancer metastasis, “Cell motility” and “Cell adhesion”(Fig. 1D). PPI network of DEGs in enriched cancer-related pathways revealed FN1 as one of the hub upregulated genes (Fig.2A), which encodes for a ubiquitous large protein of extracellular matrix (ECM) that is critical for wound healing, cell migration and adhesion[12] . In addition, PPI network of a previously published gene expression microarray data on another NB cell line BE2C (a clone of BE2) treated with ATRA[13] also identified FN1 as one of the key hub upregulated genes (Fig. 2B).

FN1 expression levels were positively correlated with overall survivals of NB patients

Considering the upregulation of FN1 during ATRA-treatment in NB cells and its critical position on the PPI network of enriched cancer-related pathways, we determined whether there was any correlation between FN1 expression levels in NB patients and their clinical outcomes. We chose the largest NB patient dataset with 649 patients in the R2 database (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>). We found that patients with low levels of FN1 had lower overall survivals than those with higher levels of FN1 (rbonf $p = 6.1 \times 10^{-3}$, Fig.3A). In addition, we verified increased expressions of FN1 after ATRA treatment in BE2 NB cell line and the two other NB cell lines (i.e.SY5Y and NGP) at both the mRNA level (Fig. 3B) and the protein level (Fig. 3C). Taken together, these data suggest that FN1 is a potential target during ATRA treatment in NB cells.

Downregulation of FN1 had no significant influence on cell proliferation in NB cells

It has been well established that ATRA-induced differentiation of NB involves inhibition of cell proliferation by G1 cell cycle arrest[14]. To investigate the effect of FN1 on proliferation in NB cells, we downregulated FN1 by transiently transfecting FN1-siRNA (#1 and #2) into NB cells following the time line in Supplementary Fig. 1. Figure 4A showed that transfection of these two FN1-siRNAs decreased FN1

expressions at both the mRNA level (left) and the protein level (right) in NGP and SY5Y cells. We chose FN1-siRNA #2 for further experiments.

To study the role of FN1 on cell proliferation, we transfected FN1-siRNA into NGP and SY5Y cells. Cell proliferation was evaluated by a CCK8 assay 48hrs after transfection (Figure 4B-left), and the cell confluency was dynamically monitored by an IncuCyte Zoom machine for 32hrs (Fig. 4B-middle). Figure 4B (left and middle) showed that there was no significant difference in cell counting or cell confluency in both NGP and SY5Y cells when FN1 was downregulated. Figure 4B (right) showed the NB cells under a microscope, and no morphological changes were detected when FN1 expression decreased.

To investigate the role of FN1 on cell cycle, we transfected FN1-siRNA into NGP and SY5Y cells, and stained them with PI 48hrs after transfection for cell cycle analysis by flow cytometry. Figure 4C showed no significant differences for the percent of cells at different phases (sub-G1, G1, S, G2-M) between FN1-siRNA-transfected cells and control-siRNA-transfected cells in both cell lines. These data indicated that downregulation of FN1 had no effect on cell proliferation or cell cycle distributions in NB cells.

Downregulation of FN1 increased migration and invasion of NB cells

To evaluate the effects of FN1 on cell migration and invasion, we downregulated FN1 by transfecting FN1-siRNA into NB cells, and then performed a scratch wound healing assay (Fig.5A) and a transwell invasion assay (Fig.5B). Fig. 5A (left) showed that FN1-siRNA-transfected NB cells had smaller wounds than control-siRNA-transfected cells. Figure 5A (middle) showed the dynamic changes of wound densities in the transfected cells. At the end point of the experiment, wound densities in the FN1-siRNA-transfected cells were 44.4% (in NGP cells) and 27.0% (in SY5Y cells) higher than those in the control-siRNA-transfected cells (Fig.5A, right). Fig. 5B showed significantly higher numbers of invasive cells in the FN1-siRNA-transfected groups (69.2% higher in NGP cells and 23.6% higher in SY5Y cells) compared to the control-siRNA-transfected groups. These data suggest that downregulation of FN1 increased cell migration and invasion in NB cells.

Downregulation of FN1 had no significant influence on the inhibitive effect of ATRA on NB cell proliferation

To investigate whether FN1 mediates the inhibitive effect of ATRA on cell proliferation, we treated FN1-siRNA-transfected NB cells with ATRA following the timeline shown in Supplementary Figure 2. Figure 6A showed that although ATRA-treatment reduced cell numbers by 24.2% in NGP cells, and by 36.7% in SY5Y cells as compared to their control cells, downregulation of FN1 in the presence of ATRA did not change cell numbers significantly in either cell line as compared to the ATRA-treated, control-siRNA-transfected cells. Cell cycle analysis by flow cytometry showed that although ATRA-treatment increased G1 phase cells by 21.8% in NGP cells and by 28.7% in SY5Y cells as compared to their control cells, in both cell lines ATRA-treated cells transfected with FN1-siRNA had similar proportions of G1 phase cells as those transfected with control-siRNA (Fig.6B). Taken together, our data indicated that ATRA-induced inhibition of cell proliferation and cell cycle arrest at G₁ phase were not blocked by downregulation of FN1.

FN1 knockdown partially blocked ATRA-mediated inhibition of cell migration and invasion in SY5Y NB cells

We next determined whether ATRA had any impacts on cell migration and invasion potentials in NGP and SY5Y cells by a wound healing assay and a transwell invasion assay with matrigel. We found that in NGP cells, ATRA had no significant impacts on either migration (Fig.7A, top) or invasion (Fig.7B). However, in SY5Y cells, ATRA treatment inhibited cell migration by 20.5% (Fig.7A, bottom) and invasion by 14.2% (Fig.7B) as compared to their control cells.

Given that downregulation of FN1 promoted cell migration and invasion of SY5Y cells, we then investigated whether FN1 downregulation had any effects on ATRA-mediated inhibition of cell migration and invasion in those cells. As shown in Figure 7C and 7D, FN1 knockdown attenuated the effects of ATRA-dependent inhibition of cell migration and invasion. Specifically, relative wound densities in ATRA-treated cells transfected with control-siRNA or transfected with FN1-siRNA were 35.7% and 23.5% lower than those in control cells, respectively (Fig.7C). Similarly, there were 17.8% fewer ATRA-treated cells transfected with control-siRNA passed through the matrigel than control cells, but there were only 3.2% fewer ATRA-treated cells transfected with FN1-siRNA passed through the matrigel than control cells (Fig.7D). Taken together, our data suggest that downregulation of FN1 partially blocked ATRA-mediated inhibition of cell migration and invasion in SY5Y cells.

Discussion

In the present study we uncovered FN1 as a key hub gene that connects a PPI network of enriched cancer-related pathways in NB cells treated with ATRA (Fig.2A). Analysis of a previously published microarray data from another NB cell line (i.e. BE2C) treated with ATRA also identified FN1 as a central molecule connecting a similar PPI network (Fig.2B), further supporting an important role of FN1 in ATRA-induced differentiation of NB cells.

GO pathway enrichment analysis of our RNA-seq data and the published microarray data (GSE45587) derived from ATRA-treated NB cells both revealed “Proteoglycans in cancer” and “ECM-receptor interaction” as top enriched signaling pathways. Both pathways include FN1 and one of its key receptors, the $\alpha 5\beta 1$ integrin, which is a cell adhesion glycoprotein involved in cell-ECM interaction, cell migration and metastasis [15]. Thus, it is plausible that in ATRA-treated NB cells, the aforementioned enriched signaling pathways regulate FN1 and its associated ligand/receptor complex (i.e. the FN1/ $\alpha 5\beta 1$ complex) to mediate cell migration and invasion.

In a variety of human cancers such as gastric cancer[16], bladder cancer[17], esophageal squamous cell carcinoma[9], breast cancer[18] and thyroid cancer[10], FN1 was up-regulated, and high levels of FN1 correlated with poorer clinical outcomes. However, in other human cancers or in different contexts, FN1 appeared to play different or even opposite roles. For example, although FN1 was overexpressed in sporadic medullary thyroid cancers, there was an inverse correlation between FN1 levels and progression-free survivals [19, 20,21]. Similarly, in a murine breast cancer model, FN1 levels were lower in tumors with

high metastatic potentials than those with lower metastatic potentials[20]. Moreover, polypeptides derived from FN1 inhibited adhesion and invasion of liver cancer cells[21]. In the present study, we found that during ATRA-mediated differentiation of NB cells, FN1 was significantly up-regulated (Fig. 3B & C), and that downregulation of FN1 promoted migration and invasion of NB cells (Fig. 5A&B), suggesting for the first time that in ATRA-treated NB, FN1 may inhibit cell invasion and cancer metastasis. Consistent with an inhibitive role of FN1 on invasion of NB cells, NB patients with higher levels of FN1 had better overall survival (Fig.3A), implying that FN1 may improve survival of NB patients by inhibiting cancer metastasis. As in the present study we met technical challenges to overexpress full-length FN1 in NB cells due to its large size, further efforts need to be dedicated to overexpress FN1 in NB cells to investigate its effects in NB in the future.

NB originates from neural crest, which is characterized by extensive migration and pluripotency[22]. Epidemiological studies on NB patients uncovered unique spontaneous regressions, which were thought to be related to the high heterogeneity of NB[5,22]. The high heterogeneity was further appreciated by recent findings that NB contained two types of tumor cells (i.e. undifferentiated mesenchymal cells and committed adrenergic cells) that had different gene expression profiles but were interchangeable[23,24,25]. This heterogeneity and interchangeable nature of the two types of cells present therapeutic challenges to NB patients. Considering that migration is a critical factor for cell fate determination of NB-originating cells in neural crest[22], and that FN1 inhibits migration and invasion of NB cells, whether and how FN1 mediates cell fate determinations and invasion of NB cells warrant future investigations.

Conclusions

In summary, we found that FN1 was significantly upregulated during ATRA-induced differentiation of NB cells. In addition, downregulation of FN1 increased cell migration and invasion, and attenuated ATRA-induced inhibition of cell migration and invasion in NB cells. These data, combined with that FN1 expression levels positively correlated with overall survivals of NB patients suggest that FN1 is a potential therapeutic target for NB patients, especially in combination with ATRA treatment.

Declarations

Funding

This work was supported by the National Natural Science Foundation of China [No. 81472359], Key Research and Development Foundation of Liaoning Province [2019JH8/10300024,2019], Liaoning Climbing Scholar Foundation [2013], and 345 Talent Project of Shengjing Hospital of China Medical University.

Competing interests

The authors declare no competing interests.

Availability of data and material

The RNA-sequence data will be available upon reasonable request.

Code availability

Not applicable.

Author's contributions

Xiaolin Tan contributed to study design, collection and interpretation of data, writing of the manuscript; Wei Gong, Bo Chen, Baocheng Gong, Zhongyan Hua, Simeng Zhang, Yang Chen, Qi Li contributed to collection of data; Zhijie Li contributed to study design, interpretation of data, writing and submission of the manuscript for publication.

Acknowledgments

We thank Dr. Carol J. Thiele from the National Institute of Health (NIH) for providing cell lines.

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Figures

Figure 1

Figure 1

An overview of DEGs in NB cells treated with ATRA (A) A heatmap of 908 DEGs during ATRA-induced differentiation of NGP cells. Each column represents a sample, and each row represents a DEG. The color scale represents the expression of DEGs from low (blue) to high (red). (B) A volcanic plot of the DEGs. Each dot represents a DEG, with red dots represent highly expression genes while blue ones represent

lowly expression genes. (C and D) Top 20 enriched pathways (C) and enriched cancer-related pathways (D) from GO pathway enrichment analysis.

Figure 2

Figure 2

Fibronectin 1 (FN1) is one of the central molecules connecting PPI network of DEGs in ATRA-treated NB cells (A) PPI network of DEGs in enriched cancer-related pathways. (B) PPI network of DEGs in gene microarray dataset GSE45587 showing one of the hub genes.

Figure 3

Figure 3

Low expression of FN1 correlated with poor overall survivals in NB patients (A) A Kaplan-Meier survival curve for overall survival of NB patients based on FN1 expression levels from an NB patient dataset. (B and C) The relative mRNA levels (B) and protein levels (C) of FN1 in BE2, NGP and SY5Y cells treated with ATRA. *** $P < 0.001$.

Figure 4

Figure 4

Downregulation of FN1 did not have significant impacts on proliferation of NB cells (A) Relative mRNA levels (left) and protein levels (right) of FN1 in NGP and SY5Y cells transfected with FN1-siRNA ("#1 and #2"). (B) FN1-siRNAs were transfected into NGP and SY5Y cells. Cell proliferation was monitored by CCK8 assay (Left) 48hr after transfection, whereas cell confluency was dynamically measured by an IncuCyte ZOOM from 16hr to 48hr (total 32hr) after transfection (Middle). (C) NGP and SY5Y cells were

transfected with FN1-siRNA, and stained with PI 48hr after transfection for cell cycle analysis by flow cytometry. Data were presented as representative histograms (Left) and percentage of cells at different cell cycle phases (Right).

Figure 5

Figure 5

Downregulation of FN1 increased migration and invasion of NB cells (A) A wound healing assay for two NB cell lines transfected with FN1-siRNA. Data were presented as representative microscopic images

taken with an IncuCyte ZOOM (Left), relative wound densities (Middle), and relative wound densities at the 24hr time point (Right). (B) A transwell invasion assay for two NB cell lines transfected with FN1-siRNA. Data were presented as representative microscopic images (Left) and relative numbers of invasive cells (Right). * $P < 0.05$; ** $P < 0.01$. Scale bar = 200 μ m.

Figure 6

Left

ATRA	FN1-siRNA	NGP Flow Cytometry	SY5Y Flow Cytometry
-	-		
+	-		
+	+		

Right

Cell Line	ATRA	FN1-siRNA	Cells at G1 phase (%)
NGP	-	-	~65
	+	-	~80 (**)
	+	+	~80 (NS)
SY5Y	-	-	~65
	+	-	~85 (**)
	+	+	~85 (NS)

Figure 6

Downregulation of FN1 had no significant impacts on the inhibitive effect of ATRA on NB cell proliferation (A) Data from cell proliferation analysis for two NB cell lines, showing CCK-8 assay data (Left), cell confluency curves (Middle), and microscopic images (Right). (B) Flow cytometric cell cycle analysis with PI showing representative histograms (Left) and percentages of cells at G1 phase (Right).

Figure 7

Figure 7

Downregulation of FN1 partially blocked ATRA-mediated inhibition of cell migration and invasion in SY5Y cells (A) Data from a wound healing assay, showing representative microscopic images (Left), relative wound densities (Middle), and relative wound densities at the 24hr time point (Right). (B) Data from a transwell invasion assay, showing representative microscopic images (Left) and relative numbers of invasive cells (Right). (C) Data from a wound healing assay for SY5Y cells, showing representative microscopic images (Left), dynamic changes of relative wound densities (Middle), and relative wound densities at the 24hr time point (Right). (D) Data from a transwell invasion assay for SY5Y cells, showing representative microscopic images (Left) and relative numbers of invasive cells (Right). NS: no statistical significance. Scale bar = 200 μ m.

Supplementary Files

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