Reactive astrocytes promote the metastatic growth of breast cancer stem-like cells by activating Notch signaling in brain


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Supplementary Figure Legend

Supplementary Fig. S1. Expression of Notch ligand in astrocytes and microglia.

(A) Primary rat astrocytes were cultured in the presence of conditioned medium prepared from MDA231 and 231BrM for 48 hrs, and the expressions of JAG1, JAG2, DLL1, DLL3 and DLL4 were measured by qRT-PCR. (B) Primary human microglial cells were treated with conditioned medium prepared from MDA231 and 231BrM for 48hrs, and the expression level of JAG1 was measured by immunocytochemical staining (left panel) and qRT-PCR (right panel) Bar, 100 μm.

Supplementary Fig. S2. IL-1β is highly expressed in 231BrM cells.

(A) CM of MDA231 and 231BrM cells were subjected to cytokine array (RayBiotech, Cat#AAH-CYT-2000). There are three sets of panels and the panels B and C were shown (see Fig. 2A for Panel A). (B) Heat map analysis of the expression of cancer associated cytokines using the breast cancer cohort data (GSE12273). (C) Representative pictures of immunohistochemical analysis for IL-1β in primary breast tumors that derived from patients with brain metastasis or without metastasis. Bar, 50μm

Supplementary Fig. S3. JAG1 is up-regulated by IL-1β.

(A) The immortalized human astrocyte UC1 cells (left panel) and immortalized rat astrocytes cells NRA, (right panel) were cultured in the presence of various concentrations of IL-1β, and the expression of mRNA and protein of JAG1 were measured by qRT-PCR and Western blot. (B) Primary rat astrocytes were treated with IL-1α or IL-1β for 24hrs, JAG1 was measured by Western blot. (C) Primary rat astrocytes were treated with the indicated cytokines for 24hrs, and JAG1 expression was measured by Western blot. (D) Primary rat astrocytes were treated with
recombinant IL-1β for 24hrs, and GFAP expression was examined by Western blot. (E) Primary rat astrocytes were cultured with 231BrM conditioned medium in the presence of various doses of anti-IL1α for 24hrs, and the JAG1 expression was examined by Western blot. (F) The expression of various Notch ligands in primary rat astrocytes after treatment with IL-1β was measured by qRT-PCR. (G) Primary rat astrocytes were cultured with 231BrM conditioned medium in the presence of various doses of Ro 106-9920 for 24hrs, and the JAG1 expression was examined by Western blot.

**Supplementary Fig. S4. Identification of CSCs and induction of Notch signaling.**

(A) Primary rat astrocytes were infected with lentivirus carrying sh-JAG1 or sh-scramble for 48hrs, and JAG1 expression was examined by Western blot. (B) Representative FACS analysis data of 231BrM cells after co-culturing with rat astrocytes. (C) MDA-MB-231 cells were treated with IL-1β (50ng/ml) and co-cultured with rat primary astrocytes for various time intervals. The population of CSCs (CD24+, CD44+, ESA+) was then measured by FACS. (D) 231BrM cells were treated with various doses of anti-IL1β or anti-IL1α and they were co-cultured with rat primary astrocytes for 48hrs. The population of CSCs (CD24+, CD44+, ESA+) was then measured by FACS. (E) The CSCs populations in 231BrM cells after MACS sorting were verified by FACS using antibodies to CD24, CD44 and ESA. (F) CSCs isolated from 231BrM cells were co-cultured with rat astrocytes in the presence or absence of DAPT for 72 hrs followed by FACS analysis using the anti-CK18 antibody. (G) The expression of HES1, HEY1 and HES5 in 231BrM/Tet-NICD cells was examined by qRT-PCR 48hrs after tetracycline induction.
Supplementary Fig. S5. Knockdown of JAG1 in astrocytes suppresses HES5 expression

(A) Rat primary astrocytes with or without knockdown of JAG1 were cultured to monolayer, and CN34BrM-GFP cells were cultured alone or on top of the astrocytes for 48 hrs. GFP+ cells were then isolated by FACS, and the expression of Hes5 in CN34BrM-GFP cells was measured by qRT-PCR. (B) Knockdown of HES5 expression by lentivirus carrying sh-HES5 in 231BrM cells was confirmed by Western blot. (C) 231BrM cells were infected with lentivirus carrying sh-scramble, sh-HES1 or sh-HEY1, and each cell line was established followed by measuring the population of CSCs (CD24−, CD44+, ESA+) by FACS. (D) Representative FACS analysis data of CSCs in primary breast tumor cells.

Supplementary Fig. S6. Expression of IL-1β in CSCs and the effect of Compound E on brain metastasis.

(A) CSCs were isolated from MDA231 and 231BrM and their tumor initiating abilities in nude mice were examined by a limiting dilution assay. P<0.0001(MDA231Vs MDA231stem), P=0.0182(231BrM Vs 231BrM stem). (B) RNAs were extracted from CSCs of MDA231, 231BrM and 231BoM and they were subjected to microarray analysis using Affymetrix expression array (Human gene 1.0ST chip). Heat map analysis was then performed for genes that were up- or down-regulated in these CSCs. (C) Expressions of IL-1β in the CSCs isolated from MDA231 and 231BrM cells was measured by qRT-PCR. (D) CSCs were prepared from 231BrM carrying both luciferase and GFP genes and they were transplanted into nude mice by intracardiac injection. The luciferase activity for the whole body was visualized by Xenogen Bioimager at time of injection and after 5 weeks (upper panel). At the end point, mice were sacrificed and the brains were removed. They were then examined for luciferase activity by Xenogen Bioimager (lower panel). (E) The brain tumor sample was sectioned and subjected to
immunohistochemical analysis using antibodies to CD44 (green, FITC), ESA (red, Alexa 555) and DAPI (blue). Arrow heads indicate the CD44$^+$/ESA$^+$ cells in the invasion front of the tumor. (F) MDA-MB-231, MDA231-IL1β and 231BrM were labeled with GFP, and $10^5$ cells were seeded on top of mouse brain endothelial cells layer in the transwell culture plate. After 24hrs, cells transmigrated to the lower chamber were counted. (G) 231BrM cells were treated with various doses of compound E, and the HES5 expression was examined by Western blot. (H) CSCs were prepared from 231BrM cells, and they were intracardially injected to mice followed by treatment with Compound E or vehicle for 32 days. BLI images of brain metastatic lesions of three representative mice from each experimental group on day 0 and day 32 were shown (See Fig. 6E).
A

mRNA expression (relative units)

CM 231 231BrM 231 231BrM 231 231BrM 231 231BrM 231 231BrM

JAG1 p=0.001

NS

JAG2

DLL1

NS

DLL3

NS

DLL4

NS

B

JAG1

231BrM CM

MDA231 CM

JAG1 mRNA expression (relative units)

CM MDA231 231BrM

NS

Xing_FigS1
**Figure 3**

**A**

Bar graphs showing JAG1 mRNA expression levels in cells treated with IL-1\(\beta\) at various concentrations (0, 10, 50 ng/ml). The mRNA expression is quantified in relative units. Error bars indicate standard error of the mean (SEM), and statistical significance is indicated by P-values (P=0.017, P=0.024).

**B**

Western blots comparing JAG1 and Tubulin expression levels in control and IL-1\(\alpha\) (50 ng/ml) treated cells.

**C**

Western blots showing the expression levels of JAG1 and Tubulin in cells treated with IL-1\(\beta\) (50 ng/ml) in the presence or absence of other cytokines: MCP-3, IGFBP-4, IL-11, IL-16, MCP-2, and Eotaxin-3.

**D**

Western blots for GFAP and Tubulin showing the expression levels in control and IL-1\(\beta\) (50 ng/ml) treated cells.

**E**

Western blots for JAG1 and Tubulin comparing anti-IL1\(\alpha\) (μg/ml) treatment in 231BrM CM. Anti-IL1\(\alpha\) concentrations are 0, 1, 5, 20 μg/ml.

**F**

Bar graph showing JAG1, JAG2, DLL1, DLL3, and DLL4 mRNA expression levels in cells treated with IL-1\(\beta\) and anti-IL1\(\alpha\). Statistical significance is indicated by P-values (P=0.03, NS = not significant).

**G**

Western blots for JAG1 and Tubulin comparing 231BrM CM with Ro 106-9920 (μM) treatment. Ro 106-9920 concentrations are 0, 5, 10, 20, 50 μM.
**A** Rat astrocyte

<table>
<thead>
<tr>
<th>sh-JAG1</th>
<th>+</th>
<th>-</th>
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<tr>
<td>JAG1</td>
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**B**

**C**

![Graph showing CD44^+ESA^+ population over time](image)

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**D**

![Bar graph showing CD24^+CD44^+ESA^+ population](image)

**E**

231BrM unsorted cells

**F**

![FACS analysis](image)

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**G**

![Graph showing mRNA expression](image)

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<tr>
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<td>HES5</td>
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**Xing_FigS4**
A

Hes5 mRNA (relative units)

Rat astrocyte

sh-JAG1

- - + +

P = 0.015

P = 0.016

B

Hes5

sh-scramble #1 #2 #3

sh-JAG1

C

CD24^CD44^ESA^ population (relative units)

sh-scramble sh-HEY1

n.s

D

GATE [No Gating]

P1 48.1%

FSC-A

SSC-A

GATE P1

Q1-UL 27.5%

Q1-UR 13.4%

Q1-LR 3.6%

Q1-LL 55.5%

GATE (R1 in P1)

V1-L 98.7%

V1-R 1.3%
**A** Tumor initiating ability of 24+, 44+, ESA+ cells

<table>
<thead>
<tr>
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**B** Diagram showing cell lineage.

**C** IL-1β mRNA expression.

**D** Ex vivo imaging.

**E** Confocal microscopy showing CD44, ESA, DAPI.

**F** Graph showing cell number per field.

**G** Western blot showing Hes5 and Tub expression.

**H** Imaging of tumor growth over time.

*P*=0.002, *P*=0.032, *P*=0.001

**Xing_FigS6**