Stroma-induced Jagged1 expression drives PC3 prostate cancer cell migration; disparate effects of RIP-generated proteolytic fragments on cell behaviour and Notch signaling

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Abbreviations

ADAM, A disintegrin and metalloproteinase; BMECs, bone marrow endothelial cells; BMS, bone marrow stroma; Dll, Delta-like ligand; FL-Jag, full-length Jagged; JCTF, Jagged1 C-terminal fragment; JICD, Jagged1 intracellular domain; NICD, Notch intracellular domain; nls, nuclear localisation sequence; PCa, prostate cancer; RIP, regulated intramembrane proteolysis; sJag, soluble Jagged1 ectodomain; TEM, transendothelial migration

Abstract

The Notch ligand Jagged1 is subject to regulated intramembrane proteolysis (RIP) which yields a soluble ectodomain (sJag) and a soluble Jagged1 intracellular domain (JICD). The full-length Jagged1 protein enhances prostate cancer (PCa) cell proliferation and is highly expressed in metastatic cells. However, little is known regarding the mechanisms by which Jagged1 or its RIP-generated fragments might promote PCa bone metastasis. In the current study we show that bone marrow stroma (BMS) induces Jagged1 expression in bone metastatic prostate cancer PC3 cells and that this enhanced expression is mechanistically linked to the promotion of cell migration. We also show that RIP-generated Jagged1 fragments exert disparate effects on PC3 cell behaviour and Notch signaling. In conclusion, the expression of both the full-length ligand and its RIP-generated fragments must be considered in tandem when attempting to regulate Jagged1 as a possible PCa therapy.

Key words

Invasion, Jagged1, metastasis, migration, Notch signaling, prostate cancer

1. Introduction

The Notch signaling pathway has been linked to tumour development/progression with aberrations being tumour-suppressive or -oncogenic in a wide range of cancers [1]. The mammalian pathway is activated when a Notch receptor and ligand (Jagged1, Jagged2, Delta-like ligand 1(Dll1), Dll3, or Dll4 [1]) on the surface of neighbouring cells interact, initiating regulated intramembrane proteolysis (RIP) of the receptor. This, ultimately, leads to the formation of a soluble Notch intracellular domain (NICD), a transcriptional regulator which translocates to the nucleus [1].

The Notch ligand Jagged1 is a 185 kDa membrane protein which, like its receptor, is subject to RIP [2,3]. The full-length protein is cleaved between E1054 and V1055 [4,5] by one or more members of the A Disintegrin And Metalloproteinase (ADAM) family of zinc metalloproteinases to liberate a soluble ectodomain (sJag) and a membrane-associated Jagged1 C-terminal fragment (JCTF). This latter fragment is subsequently cleaved by a γ-secretase activity to yield the soluble Jagged1 intracellular domain (JICD) [2]. The JICD is a functional transcriptional regulator like the NICD [2] and contains a putative nuclear localisation signal (nls) 'RKRRK' between R1094 and K1093.

A considerable body of work indicates an important role for Jagged1 in prostate cancer (PCa). Down-regulation of the protein in a range of PCa cells inhibits proliferation and induces growth arrest in the S phase of the cell cycle, possibly due to the reduced expression or activity of S phase-associated cyclins and cyclin-dependent kinases [6]. Jagged1 expression in PCa cells also increases the expression of anti-apoptotic proteins [7]. Expression of the ligand is significantly higher in metastatic PCa compared to benign or localized disease, further indicating that the protein may be linked to the growth, metastasis

and progression of prostate tumours [8]. However, despite an unarguable role for Jagged1 in PCa cell proliferation and its circumstantial link with metastatic disease, nothing is known of the mechanisms by which the ligand might promote disease metastasis. Furthermore, the role played by RIP-generated Jagged1 fragments in PCa has never been investigated.

Prostate cancer has a predilection to metastasize to the bone marrow stroma (BMS) and tumour-derived Jagged1 has previously been linked to the bone metastasis of breast cancer [9]. In the current study, we show that BMS can drive the expression of endogenous Jagged1 in the bone metastatic prostate cancer cell line PC3, an event which is mechanistically linked to their enhanced migration. Furthermore, the over-expression of full-length Jagged1 in PC3 cells promoted cell migration and invasion along with Notch signaling but did not promote cell proliferation. In contrast, sJag had no effect on migration, proliferation or Notch signaling but did inhibit invasion. Finally, the JICD also had no effect on migration but impaired invasion and Notch signaling whilst stimulating cell proliferation. Notably, the JICD nuclear localisation sequence was not required for any of these effects. Thus, whilst the BMS-induced expression of full-length Jagged1 can promote PC3 cell migration, RIP-generated Jagged1 fragments can have very different effects on cell behavior and Notch signaling. Therefore, the expression of the full-length ligand and production of its RIP fragments must be considered in tandem when attempting to regulate Jagged1 as a possible PCa therapy.

2. Materials and Methods

2.1. Materials

The human full-length Jagged1 plasmid pIREShyg-FL-Jag and the pIREShyg-sJag, pIRESneo-JICD and pIRESneo-JICDΔnls plasmids were synthesized and sequenced by Epoch Biolabs (Missouri City, U.S.A.). The anti-Jagged1 N-terminal (NT) polyclonal antibody was from R&D Systems Europe Ltd. (Abingdon, U.K.) and the anti-Jagged1 C-terminal (CT) polyclonal antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, U.S.A.). The anti-actin monoclonal antibody was from Sigma-Aldrich Company Ltd. (Gillingham, U.K.). GAPDH antibody was from Abcam (Cambridge, U.K.). All other materials, unless otherwise stated, were purchased from Sigma-Aldrich Company Ltd. (Gillingham, U.K.)

2.2. Cell culture

PC3 cells were purchased from the European Collection of Cell Cultures (ECACC) and used within six months of identity verification by the Cancer Research U.K. Manchester Institute tissue typing service. All cell culture reagents were purchased from Lonza Ltd. (Basel, Switzerland); cells were cultured in Ham's F12 medium supplemented with 7%(v/v) FBS, 2mM l-glutamine, penicillin (50 U ml^{-1}) and streptomycin (50 mg ml^{-1}) and maintained at 37°C in 5% CO_2 in air.

Human bone marrow stroma (BMS) was obtained from volunteers undergoing surgery for benign disease and cultured as described previously [10]. Briefly, cells were grown at a density of $2x10^6$ ml^{-1} in long-term bone marrow culture medium (LTBCM; Iscove's modified Dulbecco's medium at 350 mOsm, 10%(v/v) FBS, 10%(v/v) horse serum and 0.5μM hydrocortisone) at 33°C in 5% CO_2 in air for 4-5 weeks until haematopoietically active areas were evident.

Bone marrow endothelial cells (BMECs) used in the invasion assays were a gift from Dr. Gracia Almeida-Porada (University of Nevada, Reno NV, U.S.A.). Cell stocks were

cultured in LTBCM conditioned on human bone marrow stroma in tissue culture flasks pre-coated with 50 mg ml^{-1} fibronectin in phosphate-buffered saline (PBS; 20mM Na_2HPO_4, 2mM NaH_2PO_4, 0.15M NaCl, pH 7.4). Cultures were grown at 37^{o}C in a humidified atmosphere of 5% CO_2 in air and used up to passage 20.

2.3. Generation of PC3 cell stable transfectants

Lentiviral vectors expressing full-length Jagged1 and its fragments were constructed as described previously [11,12]. Briefly, coding DNA sequences were cloned into the PRRLsin backbone and transfected into HEK293T cells along with pMDLg/pRRE, rsv-REV and pMDG.2 plasmids. Lentiviral constructs were harvested on days 2-4, pooled and stored at -80^{o}C. Sub-confluent PC3 cells were transduced with viral supernatant containing DEAE-Dextran (10 μg ml^{-1}) for 4h prior to refreshing with PC3 medium. At day 5, transduced cells were FACS sorted based on green fluorescent protein (GFP) expression and expanded as stable cell lines.

2.4. Wound closure migration assays

PC3 cells were grown to confluence, the monolayer was wounded with a pipette tip and the medium removed and replaced with fresh medium containing 10 μg ml^{-1} mitomycin C. The wound was measured immediately after wounding and again at 72h in order to permit determination of percent wound closure. In the experiments using medium conditioned on BMS (Fig. 1) the wound closure was monitored over a 17h period as closure progressed more quickly under these conditions.

2.5. Cell viability assay

Cells were incubated with CellTiter 96® AQueous One Cell Proliferation Assay (methanethiosulfonate; MTS) solution (Promega, Wisconsin, U.S.A.) for 2h at 37^{o}C. Absorbance readings at 490nm were then taken using a Victor^{2} 1420 microplate reader (Perkin Elmer, Waltham, U.S.A.).

2.6. Invasion assays

PC3 cell invasion assays were performed using FlouroBlok cell culture inserts (8µm) coated with Matrigel™ (BD Biosciences, Oxford, U.K.) and placed in a 24-well plate containing 1ml of Dulbecco's modified Eagle's medium (DMEM)/0.1%(w/v) bovine serum albumin (BSA). In some instances an endothelial barrier was formed by culturing BMECs to confluence on top of the Matrigel™ as described previously [13]. Inserts were then transferred to new plates containing growth medium and either tissue culture plastic (TCP) alone or human BMS. PC3 cells stably expressing the various Jagged1 constructs and stained with DIL were seeded into the inserts on the surface of the Matrigel^{TM}/BMEC barrier. The invasion of cells through the barrier was assessed at hourly intervals or at an end point of 24h by bottom reading of fluorescence on a BMG Labtech FLUOstar OPTIMA plate reader at 544/590nm (excitation/emission filter). This plate reader is a self-contained incubator which maintains temperature and CO_2 levels at 37^{o}C and 5%, respectively.

2.7. Small interfering RNA transfection

Small interfering RNA (siRNA) was purchased from Thermo Scientific Dharmacon (St Leon-Rot, Germany). Cells (30-50% confluence) were treated with siRNA (25nM) which had been pre-complexed with Dharmafect 2 reagent (St Leon-Rot, Germany) for 72h.

2.8. Protein assay, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Cell lysates and conditioned medium samples were prepared as previously described [3] and protein levels were subsequently quantified using bicinchoninic acid [14] in a microtitre plate with BSA as a standard. Proteins were resolved by SDS-PAGE using 7-17% or 5-20% polyacrylamide gradient gels and transferred to Immobilon P polyvinylidene difluoride (PVDF) membranes [15] before incubating with primary antibody. Bound antibody was detected using peroxidase-conjugated secondary antibodies (Sigma-Aldrich, Poole, U.K. and R&D Systems Europe Ltd., Abingdon, U.K.) in conjunction with enhanced chemiluminescence detection reagents (Perbio Science Ltd, Cramlington, U.K.).

2.9. Notch luciferase reporter assay

The various PC3 Jagged1 stable transfectants were seeded in 6-well plates at 5 x10^4 cells per well and cultured overnight. The cells were then transiently transfected with the Notch luciferase reporter plasmid, pGa981-6 [16] using Lipofectamine 2000 (Invitrogen, Paisley, U.K.). After 72h the cells were then harvested and luciferase activities were measured using a dual-luciferase® reporter assay kit (Promega, Wisconsin, U.S.A) according to the manufacturer's instructions.

2.10. Statistical analysis

All data are presented as the means ±S.D. Data were subjected to statistical analysis via Student's t-test. Levels of significance are indicated in figure legends.

2.11. Ethics statement

The study complies with the Declaration of Helsinki. Ethics were approved under Trent MREC 01/4/061. Consent from patients donating BMS was not required as the material was classified as surgical waste which, under the HTA act of 1996, does not require patient consent.

3. Results and discussion

3.1. Bone marrow stroma-induced Jagged1 expression drives PC3 cell migration

There is circumstantial evidence to suggest a role for Jagged1 in PCa metastasis in that expression of the ligand is significantly higher in metastatic PCa compared to benign or localized disease [8]. Given the predilection of the disease to metastasize to bone, we initially examined whether soluble factors released by bone marrow stroma (BMS) might regulate Jagged1 expression and/or migration in PC3 cells. PC3 cells were incubated for 48h in the presence of unconditioned medium or medium that had been previously conditioned on BMS for 48h. Cell lysates were then prepared and immunoblotted with the anti-Jagged1 CT and anti-actin antibodies. The results (Fig. 1A) revealed a 343±32% increase in full-length Jagged1 expression in the PC3 cells incubated with BMS-conditioned medium.

Next, we examined whether BMS-conditioned medium could promote PC3 cell migration and whether the enhanced expression of Jagged1 was a pre-requisite for this event (Fig. 1B). PC3 cells stably expressing either vector alone (Mock PC3) or full-length Jagged1 (FL-Jag PC3) were treated with siRNA that specifically targeted the endogenous form of Jagged1 or

a scrambled siRNA control. Once confluent, the cells were then subjected to a wound closure assay over 17h in the presence of unconditioned medium or BMS-conditioned medium. The results (Fig. 1B) show that the BMS-conditioned medium enhanced wound closure by 1.45±0.30- and 1.55±0.35-fold, respectively, in the Mock and FL-Jag PC3 cells (compared to control medium). However, the endogenous Jagged1-targetted siRNA only ablated this increase in the Mock PC3 cells and not the FL-Jag PC3 cells. That is to say that the stable over expression of Jagged1 in the latter cells compensated for the depletion of the endogenous protein by siRNA treatment. Finally, the specificity of the Jagged1 siRNA for the endogenous protein was confirmed by immunoblotting cell lysates with the anti-Jagged1 CT and anti-GAPDH antibodies (Fig. 1C).

Collectively, these data indicate that BMS releases a soluble factor into conditioned medium that promotes the migration of PC3 cells and that BMS-induced expression of Jagged1 in these cells is mechanistically linked to this enhanced migration.

3.2. Full-length Jagged1 but not its RIP-generated fragments promotes PC3 cell migration

Having established that BMS could promote Jagged1 expression and subsequent migration in PC3 cells, we then investigated which forms of the protein (i.e. full-length or RIP-generated fragments) could promote migration. DNA constructs were generated encoding proteins analogous to sJag, JICD and a form of JICD lacking a putative nuclear localisation sequence (nls) between R1094 and K1098 (JICDΔnls) [2] (Fig. 2A). Following stable expression in PC3 cells, immunoblotting of cell lysates with the anti-Jagged1 CT antibody confirmed over-expression of FL-Jag (Fig. 2B, top panel) as an intense 185 kDa band consistent with previous reports [2,17]. The same antibody also detected elevated JCTF in lysates from the FL-Jag-

transfected cells (Fig. 2B, second panel). The JICD and JICDΔnls constructs were detected as intense bands at 22 kDa and 20 kDa, respectively in stably-transfected cell lysates immunoblotted with the anti-Jagged1 CT antibody (Fig. 2B, second panel). Finally, when conditioned medium samples were immunoblotted with the anti-Jagged1 NT antibody, elevated levels of soluble Jagged1 ectodomain were detected in medium from both the FL-Jag and sJag-transfected cells with the latter, predictably, containing more of this fragment (Fig. 2B, bottom panel).

The various PC3 cell stable transfectants were then subjected to a wound closure assay over 72h in the presence of unconditioned medium (the time here was longer than for the experiments in Fig. 1 as wound closure was slower in the absence of BMS-conditioned medium). The results (Fig. 3A) show that wound closure was enhanced by 194±5% in FL-Jag PC3 cells compared to Mock PC3 cells. However, sJag, JICD and JICDΔnls all had no effect on PC3 cell migration. These data indicate that it is the level of full-length Jagged1 as opposed to its RIP-generated fragments that is pivotal in mediating PC3 cell migration.

3.3. JICD enhances PC3 cell proliferation

We next examined the effects of full-length Jagged1 and its RIP-generated fragments on the proliferation of PC3 cells over a five day time course (after which the cells entered stationary phase). The results (Fig. 3B) show that both JICD and JICDΔnls significantly enhanced proliferation whereas the stable over-expression of FL-Jag and sJag had no effect. The FL-Jag result was unexpected given that previous studies have demonstrated that down-regulation of endogenous Jagged1 inhibits the growth of a range of prostate cancer cells [6,18]. In order to confirm these previous reports we attempted to replicate them in our PC3 cells. As expected,

the results show that the depletion of endogenous Jagged1 expression using siRNA effected a 32±3% reduction in cell proliferation 72h after the commencement of the siRNA treatment (Supplementary Fig. S1). It is notable that all bar one previous study have focused on the depletion of endogenous Jagged1 in PCa cells and the subsequent growth inhibitory effect [6,18,19]. The one other study that investigated the effect of over expressing Jagged1 in PC3 cells concluded that the ligand only promoted proliferation in the presence of transfected androgen receptor expression [20].

3.4. Full-length Jagged1 and its RIP-generated fragments exhibit reciprocal effects on PC3 cell invasion

Next we examined the effects of full-length Jagged1 and its RIP-generated fragments on PC cell invasion. The various PC3 stable transfectants were seeded into Matrigel™-coated invasion chambers and invasion was monitored as described in the Methods section. The results (Fig. 3C) show a small but significant enhancement of invasion by full-length Jagged1 and, conversely, significant reductions effected by sJag, JICD and JICDΔnls.

We also examined the effect of Jagged1 on PC3 cell invasion in a model co-culture system designed to mimic the blood/BMS boundary [13]. Here, Mock- and FL-Jag-transfected PC3 cells were stained with DIL prior to seeding them into a cell culture insert containing a barrier of either Matrigel™ or confluent human bone marrow endothelial cells (BMECs) on Matrigel™. PC3 cell invasion towards BMS in the lower chamber was monitored by bottom reading of fluorescence. The results (Fig. 3D) show that Jagged1 only stimulated PC3 cell invasion through Matrigel™ and not when the Matrigel™ was combined with BMECs. That is to say that BMECs ablated the Jagged1-induced invasion of PC3 cells indicating that the

protein stimulates mesenchymal as opposed to amoeboid transendothelial migration (TEM) [21].

3.5. Jagged1 fragment-mediated changes in PC3 cell behaviour do not correlate with changes in Notch signaling

Having established that full-length Jagged1 and its RIP-generated fragments exhibit differential effects on cell behaviour, we attempted to determine whether these changes correlated with changes in Notch signaling. The Jagged1 construct stable PC3 transfectants were transiently transfected with the Notch luciferase reporter plasmid pGa981-6 [16] and luciferase activities were determined in subsequently prepared cell lysates. The results (Fig. 4) demonstrate a 1.59±0.01-fold stimulation of Notch activity in the FL-Jag-transfected PC3 cells relative to mock-transfected cells. In contrast, sJag did not affect Notch activity whilst JICD and JICDΔnls decreased activity by 53±0.4% and 45±0.2%, respectively. Thus, it is apparent that FL-Jag and its proteolytic fragments have very different effects on Notch signaling that are distinct from their effects on PC3 cell behaviour.

3.6. Summary

In the current study, we have shown that BMS can drive Jagged1 expression and migration in prostate cancer PC3 cells. It is likely to be the full-length form of the ligand and not its RIP-generated fragments that promote this migration through an activation of Notch signaling. However, the situation is more complex in relation to other aspects of PCa cell behaviour and Notch signaling with the full-length ligand, sJag and JICD displaying disparate effects on these events. Thus, the altered expression of Jagged1 and production of its RIP

fragments must be considered in tandem when attempting to modulate levels of the ligand as a possible PCa therapy.

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Figure 1. Bone marrow stroma-induced Jagged1 expression drives PC3 cell migration. A. Untransfected PC3 cells were incubated for 48h in the presence of unconditioned medium or medium that had been conditioned on bone marrow stroma (BMS) for 48h. Cell lysates were then immunoblotted using the anti-Jagged1 CT and anti-actin antibodies. The results are expressed relative to the amount of the protein detected in the cells incubated with control (unconditioned) medium (means ±S.D., n=3). **B.** PC3 cells stably expressing either vector alone (Mock PC3) or full-length Jagged1 (FL-Jag PC3) were treated with siRNA that specifically targeted the endogenous form of Jagged1 (J) or a scrambled siRNA control (S). The cells were then subjected to a wound closure assay over 17h in the presence of unconditioned medium or BMS-conditioned medium. The results are expressed relative to wound closure in the Mock PC3 cells incubated with control (unconditioned) medium (means ±S.D., n=3 except for column 2 where n=2, * denotes significance at P=0.05). **C.** Mock PC3 and FL-Jag PC3 cells were treated with Jagged1 (J) or scrambled (S) siRNA and the subsequently prepared cell lysates were immunoblotted using the anti-Jagged1 CT and anti-GAPDH antibodies. The dashed lines on blots indicate where the lanes on the same immunoblot have been re-ordered for presentation purposes.

Figure 2. Design and expression of Jagged1 constructs. A. Human full-length Jagged1 (FL-Jag) consists of a 33 residue signal peptide (black) followed by a large ectodomain, a membrane spanning region between residues 1067 and 1093 (diagonal lines) and a cytosolic domain between residues 1093 and 1218 (dots). The soluble Jagged1 construct (sJag) consisted of FL-Jag truncated on the C-terminal side of D1067. Note that this construct was generated prior to the publication of reports demonstrating ADAM-mediated cleavage of Jagged1 between E1054 and V1055 [4,5]. However, the inclusion, in our construct, of an additional 13 amino acid

residues at a site distal to any relevant Jagged1 functional domains is unlikely to affect the functional capacity of the soluble protein. The Jagged1 intracellular domain (JICD) construct was analogous to the intracellular fragment generated by γ-secretase-mediated cleavage of the Jagged1 C-terminal fragment (JCTF). The construct lacked a secretory pathway signal peptide and consisted of residues 1086 to 1218 of FL-Jag. The rationale for choosing V1086 as the first residue of this construct was based on the facts that γ-secretase cleaves N-terminally to an analogous residue in both the amyloid precursor protein and Notch [22,23] and a similar construct has previously been shown to target effectively to the nucleus [2]. Finally, our JICDΔnls construct lacked the putative nuclear localisation sequence (nls) between R1094 and K1098. **B.** The DNA constructs were stably expressed in PC3 cells and lysates from the transfectants were immunoblotted with the anti-Jagged1 CT and anti-actin antibodies (top three panels). Conditioned medium from the transfectants was immunoblotted with the anti-Jagged1 NT antibody (bottom panel). The dashed lines on blots indicate where the lanes on the same immunoblot have been re-ordered for presentation purposes

Figure 3. The effects of full-length Jagged1 and its RIP-generated fragments on PC3 cell behaviour. A. *Migration;* the various Jagged1 construct PC3 stable transfectants were subjected to a wound closure assay over 72h in the presence of control (unconditioned) medium. Results are expressed relative to the wound closure in the Mock PC3 cells (means ±S.D., n=3). **B.** *Proliferation;* the proliferation of the PC3 stable transfectants was monitored over 5 days. Results are means ±S.D., n=3. **C.** *Invasion;* PC3 stable transfectants were seeded into Matrigel™-coated invasion chambers and invasion was monitored as described in the Methods section. Results are expressed relative to the invasion exhibited by the Mock PC3 cells (means ±S.D., n=3). **D.** *Invasion in a model co-culture system;* Mock- and FL-Jag-transfected

PC3 cells were stained with DIL prior to seeding into cell culture inserts containing a barrier of either Matrigel™ or confluent human bone marrow endothelial cells (BMECs) on Matrigel™. PC3 cell invasion towards bone marrow stroma (BMS) in the lower chamber was monitored by bottom reading of fluorescence. The results are expressed relative to the invasion exhibited by the Mock-PC3 cells (means ±S.D., n=3). **, ***, **** and ***** denote significance at *P*=0.01, 0.005, 0.001 and 0.0005, respectively.

Figure 4. The effect of Jagged1 and its RIP-generated fragments on Notch signaling in PC3 cells. The Jagged1 construct stable PC3 transfectants were transiently transfected with the Notch luciferase reporter plasmid pGa981-6 and luciferase activities were determined in subsequently prepared cell lysates. Results are expressed relative to the Notch activity exhibited by the Mock PC3 cells in the absence of transfected reporter plasmids and are means ±S.D., n=3. *** denotes significance at *P*=0.005.

Figure 1.

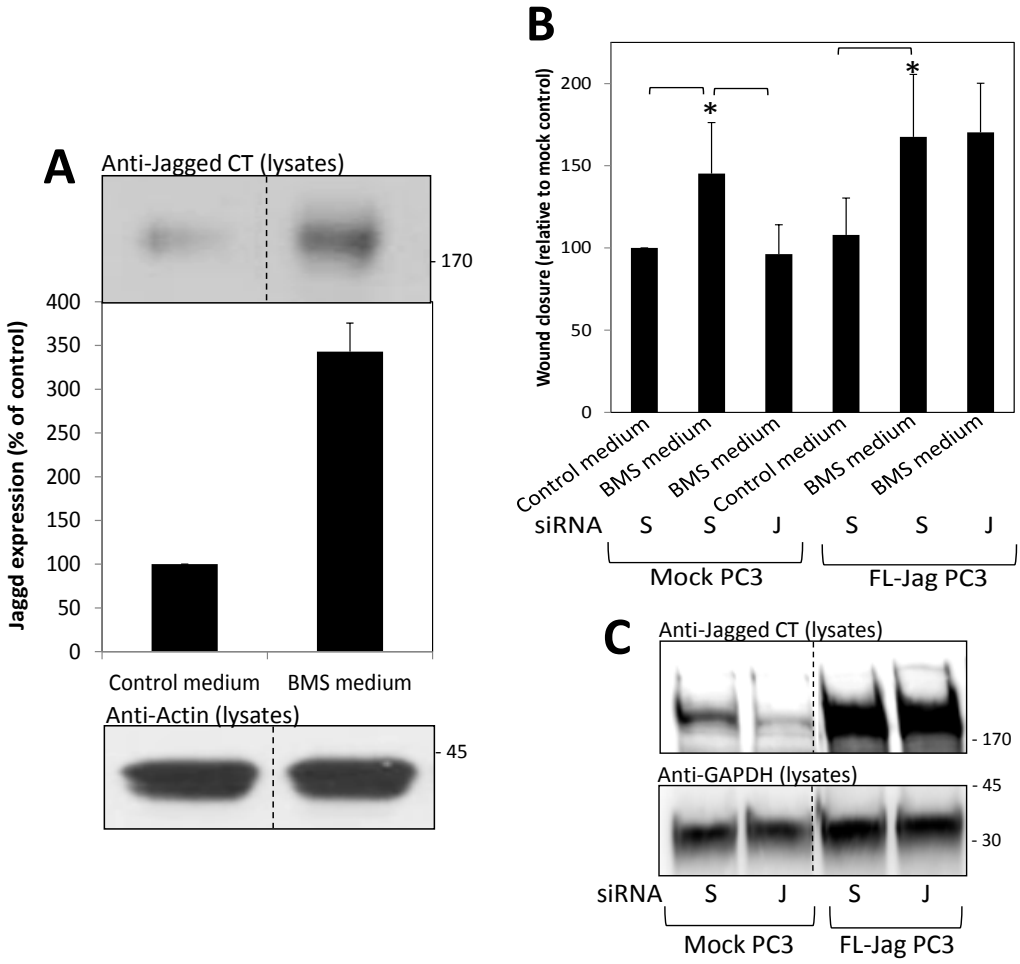
A
Anti-Jagged CT (lysates)
170
Jagd expression (% of control)
400
350
300
250
200
150
100
50
0
Control medium
BMS medium
Anti-Actin (lysates)
45
B
Wound closure (relative to mock control)
200
150
100
50
0
*
*
Control medium
BMS medium
BMS medium
Control medium
BMS medium
BMS medium
siRNA S S J S S J
Mock PC3
FL-Jag PC3
C
Anti-Jagged CT (lysates)
170
Anti-GAPDH (lysates)
45
30
siRNA S J S J
Mock PC3
FL-Jag PC3

Figure 2.

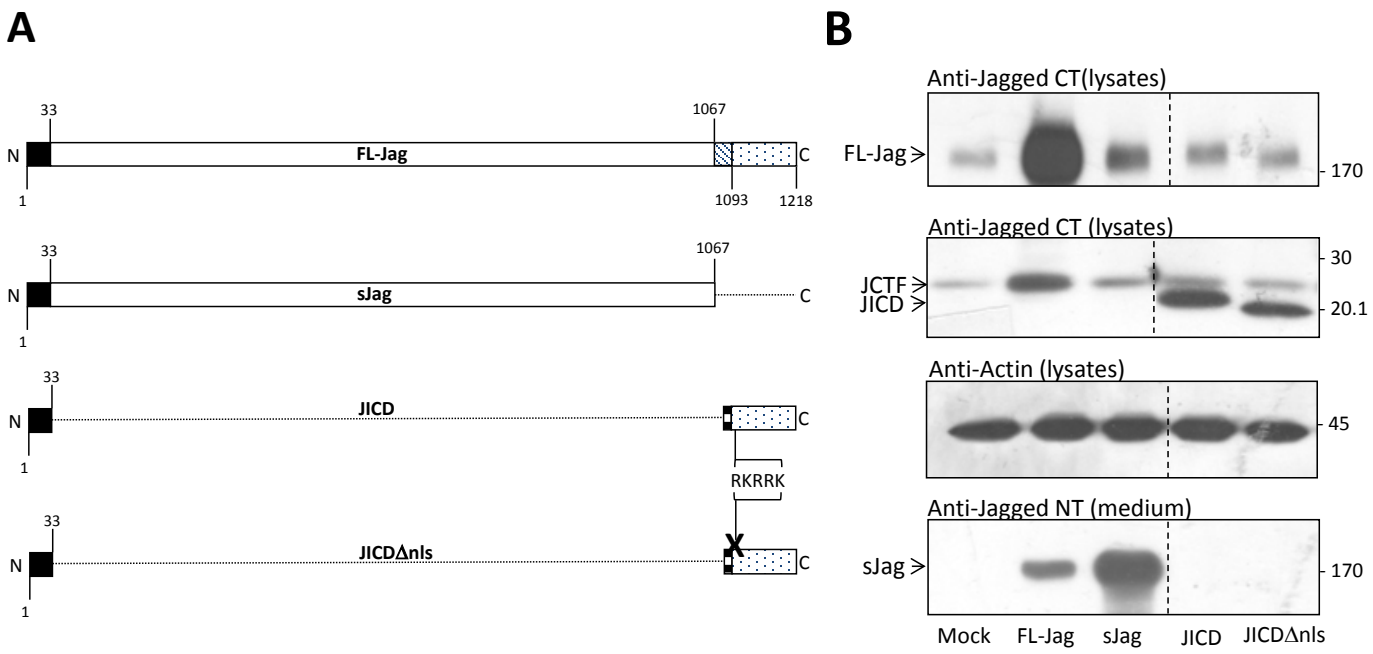
A
33
1067
N
FL-Jag
C
1
1093
1218
33
1067
N
sJag
C
1
33
N
JICD
C
1
RKRRK
33
N
JICDΔnls
C
1
B
Anti-Jagged CT(lysates)
FL-Jag
170
Anti-Jagged CT (lysates)
30
JCTF
JICD
20.1
Anti-Actin (lysates)
45
Anti-Jagged NT (medium)
sJag
170
Mock
FL-Jag
sJag
JICD
JICDΔnls

Figure 3.

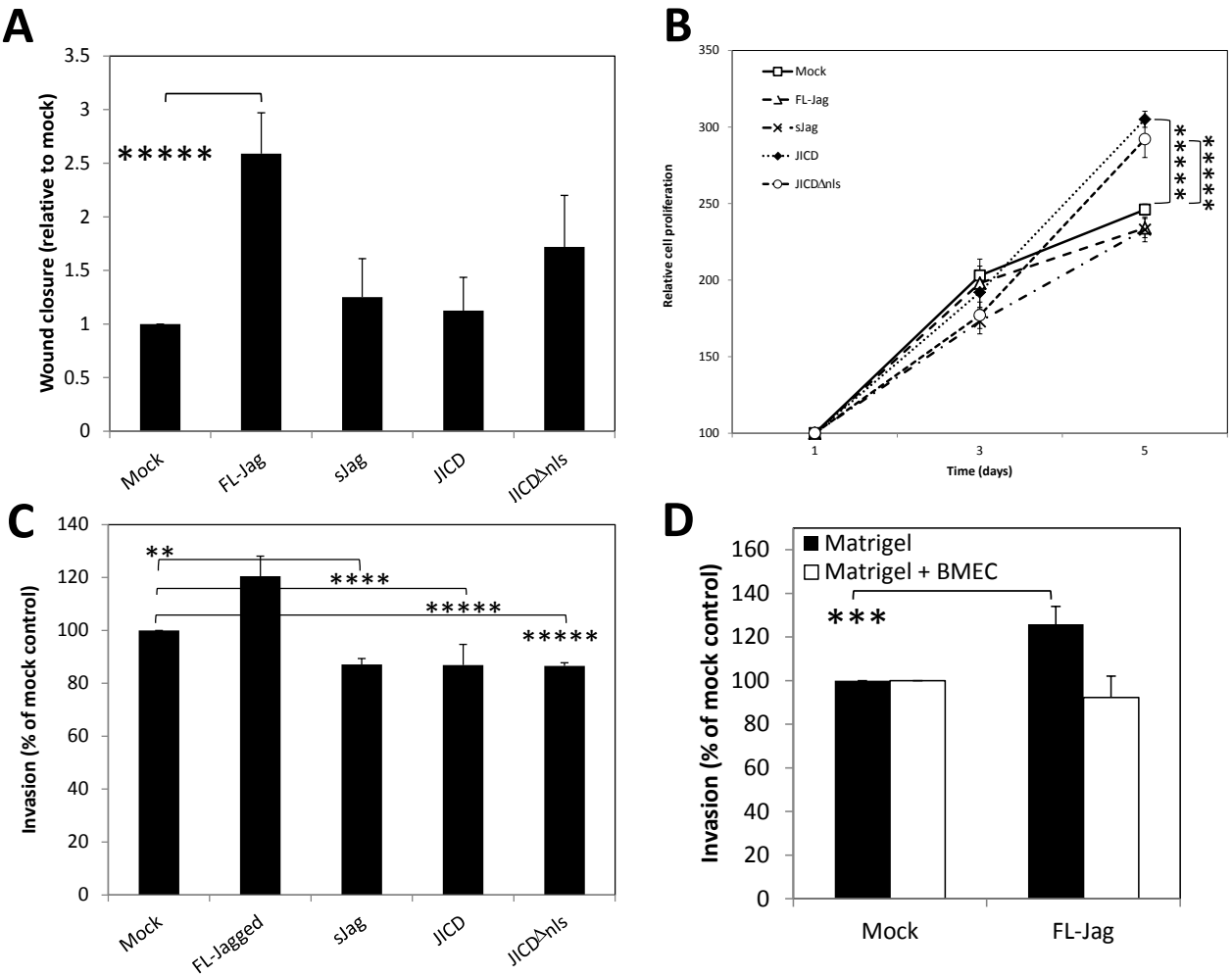
A
Wound closure (relative to mock)
3.5
3
2.5
2
1.5
1
0.5
0
*****
Mock
FL-Jag
sJag
JICD
JICDΔnls
B
Relative cell proliferation
350
300
250
200
150
100
Mock
FL-Jag
sJag
JICD
JICDΔnls
*****
*****
1
3
5
Time (days)
C
Invasion (% of mock control)
140
120
100
80
60
40
20
0
**
****
*****
*****
Mock
FL-Jagged
sJag
JICD
JICDΔnls
D
Invasion (% of mock control)
160
140
120
100
80
60
40
20
0
Matrigel
Matrigel + BMEC
***
Mock
FL-Jag

Figure 4.

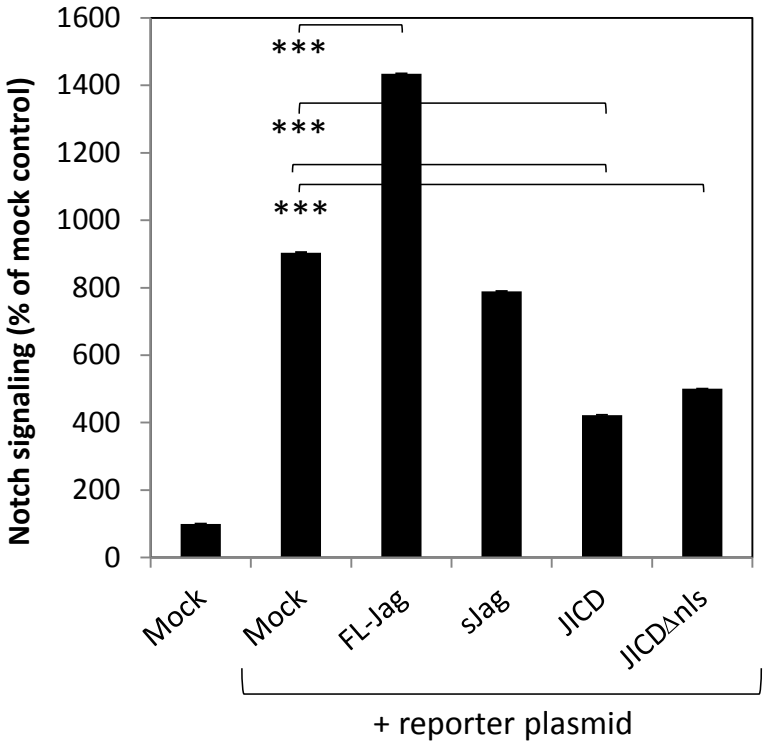
Notch signaling (% of mock control)
1600
1400
1200
1000
800
600
400
200
0
***
***
***
Mock
Mock
FL-Jag
sJag
JICD
JICDΔnls
+ reporter plasmid

Figure S1.

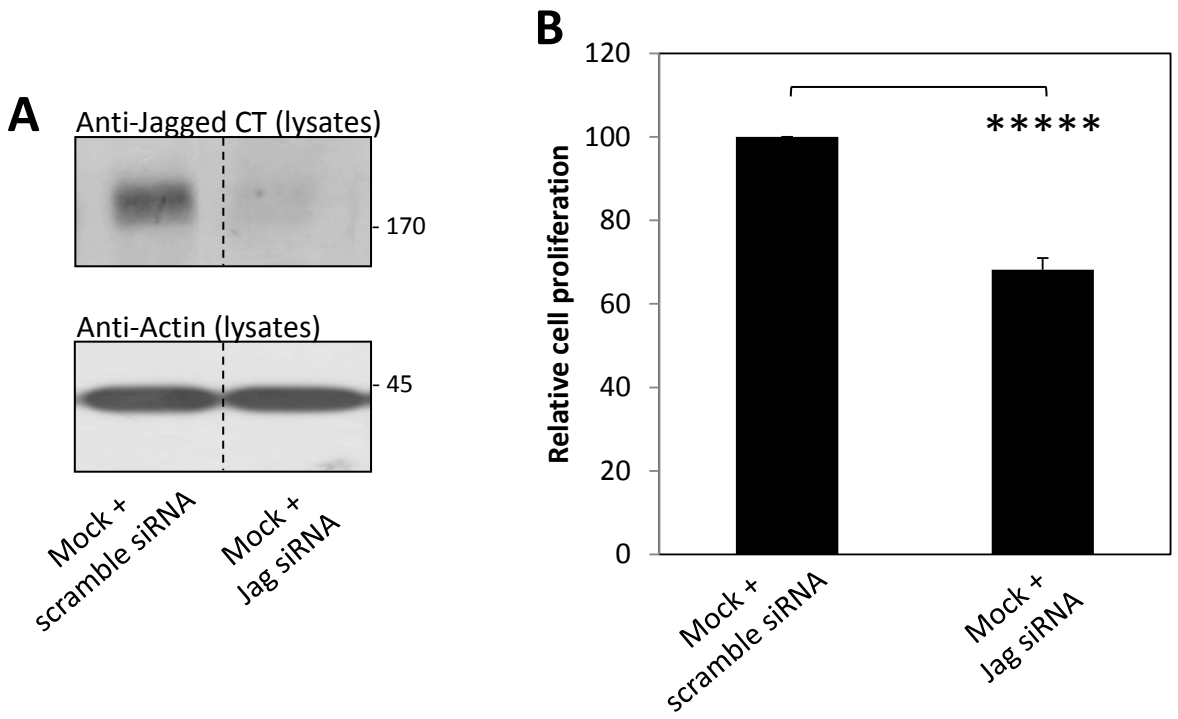

Figure S1. The effect of Jagged1 depletion on PC3 cell proliferation. Untransfected PC3 cells were transfected with scramble or Jagged1 siRNA and, after 72h, cell lysates were prepared and immunoblotted with the anti-Jagged1 CT and anti-actin antibodies (**A**) and cell proliferation was determined as described in the Methods section (**B**). The results are expressed relative to the proliferation exhibited by scramble siRNA-treated cells (means ±S.D., n=3). **** denotes significance at *P*=0.001. The dashed lines on blots indicate where the lanes on the same immunoblot have been re-ordered for presentation purposes