Differential Regulation of E-Cadherin Expression by the Soluble Ectodomain and Intracellular Domain of Jagged1

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Authors' contributions

This work was carried out in collaboration between all authors. Author EP designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. All authors managed the analyses of the study and read and approved the final manuscript.

Research Article

ABSTRACT

Aberrant Jagged1-mediated Notch activation is linked to cancer and induces epithelial-to-mesenchymal transition through the repression of E-cadherin transcription. All three proteins are subject to sequential proteolytic events referred to as regulated intramembrane proteolysis. This process releases soluble protein ectodomains from the cell and, concomitantly, generates intracellular domains capable of nuclear translocation and transcriptional regulation.
Aim: To determine the cognate roles of the Jagged1 ectodomain and intracellular domain fragments in the regulation of E-cadherin expression.
Methodology: Human embryonic kidney cells were stably transfected with coding DNA constructs analogous to full-length Jagged1, the soluble Jagged1 ectodomain, or the intracellular domain fragment of the protein. Correct construct expression and processing were confirmed by immunoblot analysis of transfectant cell lysates and conditioned culture medium. The effects of the various Jagged1 constructs on endogenous E-cadherin expression and processing were subsequently monitored by immunoblot and RT-qPCR

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analyses.
Results: Both full-length Jagged1 and the soluble Jagged1 ectodomain construct down-regulated E-cadherin expression at the protein and RNA level. In contrast, the Jagged1 intracellular domain fragment construct enhanced E-cadherin expression but only at the RNA level.
Conclusion: The soluble Jagged1 ectodomain is sufficient for the down-regulation of E-cadherin expression whereas the intracellular domain of the protein does not exhibit such an effect and actually increases E-cadherin RNA expression. These results raise the interesting possibility of E-cadherin regulation in cells distal to the site of soluble Jagged1 ligand generation.

ABBREVIATIONS

ADAM, a disintegrin and metalloproteinase; Dll, Delta-like ligand; EMT, epithelial-to-mesenchymal transition; HEK, human embryonic kidney; MMP, matrix metalloproteinase; NCTF, Notch C-terminal fragment; NICD, Notch intracellular domain; RIP, regulated intramembrane proteolysis; sJAG1, soluble Jagged1 ectodomain; JCTF, Jagged1 C-terminal fragment; JICD, Jagged1 intracellular domain; RT-qPCR, real-time quantitative polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; sE-CAD, soluble E-cadherin; WT-JAG1, wild-type Jagged1.

1. INTRODUCTION

Regulated intramembrane proteolysis (RIP) [1] constitutes the basic mechanism for a wide range of signaling pathways the original paradigm for which was Notch signaling [2].

The Notch pathway is an ancient and evolutionarily conserved signaling mechanism which influences, *inter alia*, cell proliferation, apoptosis and the organisation of tissue boundaries [3]. Binding of the Notch receptor to one of five known mammalian ligands (Dll(Delta-like ligand)1, Dll3, Dll4, Jagged1 or Jagged2 [4]) facilitates proteolytic cleavage within the juxtamembrane region of the receptor - a process referred to as 'ectodomain shedding' and catalysed by members of the a disintegrin and metalloproteinase (ADAM) family of zinc metalloproteinases [5]. This initial proteolytic event constitutes the rate-limiting step of Notch RIP generating, in addition to the soluble ectodomain, a residual membrane-associated Notch C-terminal fragment (NCTF) which can be further processed by a presenilin-dependent γ-secretase complex [6]. This second proteolytic event liberates a soluble Notch intracellular domain (NICD) which is capable of nuclear translocation and subsequent transcriptional regulation [7].

Whilst RIP of the Notch receptor is a well-established concept, more recently, it has become clear that various Notch ligands are themselves subject to RIP, raising the possibility of 'bi-directional' RIP-mediated intercellular signaling [8-10]. In particular, the ligand Jagged1 is known to be cleaved by an ADAM-like activity liberating a soluble ectodomain (sJAG1) and a residual membrane-associated Jagged1 C-terminal fragment (JCTF) which can be cleaved by a γ-secretase activity generating a transcriptionally active Jagged1 intracellular domain (JICD) [11]. We have reported previously that ADAM17 is the enzyme responsible for the

ectodomain shedding of Jagged1 [12] and that additional zinc metalloproteinases may be capable of cleaving the ligand in the presence of elevated copper [13].

Given the physiological roles of Notch signaling it is perhaps not surprising that deregulation of Jagged1-mediated Notch signaling has been implicated in the development and metastasis of a range of cancers [14]. Most relevantly, in the context of the current study, Jagged1-mediated Notch activation has been shown to induce epithelial-to-mesenchymal transition (EMT) [15]; an event which has a well-documented role in promoting the invasion and dissemination of malignant cells [16].

EMT is associated with decreased cell surface levels of the adhesion protein, E-cadherin causing loss of cell-cell adhesion and promoting cell migration [17]. E-cadherin can be lost from the cell surface by promoter hypermethylation, gene deletion/mutation or proteolytic cleavage [18,19]. In fact, E-cadherin is subject to ectodomain shedding by several proteinases including matrix metalloproteinase (MMP) 3, MMP7, ADAM10 and ADAM15 [20]. Cleavage of mature E-cadherin results in the release of soluble fragments of the extracellular domain (sE-CAD) and increased invasive behaviour of cells [20]. The residual membrane-associated E-cadherin fragment generated by metalloproteinase cleavage can be further processed by a γ-secretase activity generating a soluble ICD capable of nuclear translocation and transcriptional regulation [21].

Jagged1-mediated Notch activation is thought to induce EMT via induction of the transcriptional repressor, Slug, which, in turn, attenuates E-cadherin expression leading to loss of cell adhesion [15]. Whilst it is clear that the expression of Jagged1, *per se,* is linked to the down-regulation of E-cadherin expression, the effects of sJAG1 and JICD, generated following RIP of the full-length protein, have not been investigated in this respect. Certainly it would seem that sJAG1 does have a role to play in the regulation of Notch signaling. On the one hand, repression of transmembrane Jagged1-mediated Notch signalling by sJAG1 has been suggested [22] whilst, on the other hand, soluble forms of the protein have been shown to induce keratinocyte differentiation consistent with Notch activation akin to that by the full-length ligand [23,24]. Furthermore, the JICD contains a putative nuclear targeting signal sequence [11] raising the possibility that this fragment might also contribute to the transcriptional regulation of E-cadherin expression.

In the current study, we have stably over-expressed both full-length Jagged1 and truncated variants of the protein, analogous to the sJAG1 and JICD fragments, in human embryonic kidney (HEK) cells and subsequently monitored the effects of these constructs on the expression and proteolysis of endogenous E-cadherin. Full-length Jagged1 decreased E-cadherin protein and RNA levels and proteolytic shedding of the protein from the cell surface. Furthermore, the soluble fragment, sJAG1, also reduced E-cadherin expression and proteolysis demonstrating that membrane anchorage of the ligand is not a prerequisite in this respect. Conversely, we show that the JICD actually enhanced E-cadherin RNA levels although no significant changes in cell-associated E-cadherin protein levels could be determined in this respect. These results raise the possibility that the soluble form of Jagged1 might be able to induce EMT in cells distal to its site of generation.

2. MATERIALS AND METHODS

2.1 Materials

The human full-length Jagged1 plasmid, pIREShyg-Jagged1, was synthesized by Epoch Biolabs (Missouri City, U.S.A.) and its stable expression and proteolysis in HEK cells have been fully characterized previously [25]. The pIRESneo-JICD construct, consisting of residues 1086-1218 of human Jagged1, was also synthesized by Epoch Biolabs. The pIREShyg-sJAG1 construct was generated by site-directed mutagenesis using the original pIREShyg-Jagged1 plasmid as a template. The codon encoding F1068 was mutated from TTC to TGA. Anti-Jagged1 N-terminal (ectodomain) and anti-E-cadherin (ectodomain) polyclonal antibodies were from R&D Systems Europe Ltd. (Abingdon, U.K.) and anti-Jagged1 C-terminal polyclonal antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, U.S.A.). Anti-actin monoclonal antibody was from Sigma-Aldrich Company Ltd. (Gillingham, U.K.). All other materials, unless otherwise stated, were also purchased from Sigma-Aldrich Company Ltd.

2.2 Cell Culture and Stable Transfection

Cell culture reagents were purchased from Lonza Ltd. (Basel, Switzerland). HEK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 25 mM glucose, 4 mM L-glutamine and 10% (v/v) foetal bovine serum and were maintained at 37ºC in 5% CO_2 in air. Stable transfection was performed using Lipofectamine®: DNA complexes and subsequent antibiotic selection according to the manufacturer's instructions (Invitrogen, Paisley, U.K.).

2.3 Preparation of Cell Lysates and Conditioned Medium

For the study of protein shedding, cells were grown to confluence in 75 cm^3 flasks and rinsed twice *in situ* with reduced serum medium (10 ml) (OptiMEM; Invitrogen, Paisley, U.K.). A fresh 10 ml of OptiMEM was then conditioned on cells for 5 h. Media for immunoblot analyses were harvested, centrifuged at 10,000 *g* for 10 min to remove cell debris, and concentrated 50-fold using Vivaspin 6 centrifugal concentrators (Sartorius, Epsom, U.K.). For analysis of cell-associated proteins, cells were washed with phosphate-buffered saline (PBS; 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.15 M NaCl, pH 7.4) and scraped from the flasks into fresh PBS (10 ml). Following centrifugation at 500 *g* for 5 min, cell pellets were lysed in 100 mM Tris, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) Nonidet P40, 10 mM 1,10-phenanthroline, pH 7.4.

2.4 Protein Assay

Protein was quantified using bicinchoninic acid [26] in a microtitre plate with bovine serum albumin (BSA) as a standard.

2.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoelectrophoretic Blot Analysis

Samples were mixed with a half volume of reducing electrophoresis sample buffer and boiled for 3 min. Proteins were resolved by SDS-PAGE using 5-15%, 7-17% or 5-20% polyacrylamide gradient gels and transferred to Immobilon P polyvinylidene difluoride

membranes as previously described [27]. Anti-Jagged1 C-terminal and anti-Jagged1 ectodomain antibodies were used at dilutions of 1:2000 and 1:500, respectively. Anti-actin antibody was used at a dilution of 1:5000 and the anti-E-cadherin antibody was used at 1:1000. Bound antibody was detected using peroxidase-conjugated secondary antibodies (Sigma-Aldrich Company Ltd., Gillingham, U.K. and R & D Systems Europe Ltd., Abingdon, U.K.) in conjunction with enhanced chemiluminescence detection reagents (Perbio Science Ltd, Cramlington, U.K.).

2.6 RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

To monitor endogenous E-cadherin expression, RNA from cell samples was extracted using TRIzol (Invitrogen, Paisley, U.K.). cDNA for RT-qPCR was synthesized using SuperScript III reverse transcriptase (Invitrogen, Paisley, U.K.) according to the manufacturer's instructions. RT-qPCR was performed using SYBR green Jumpstart Taq ready mix (Sigma-Aldrich Company Ltd., Gillingham, U.K.) and validated QuantiTect primers for E-cadherin and Human Large Ribosomal Protein P0 (RPLP0) from Qiagen (Manchester, U.K.). Reactions were run in a CFX96 thermal cycler (Bio-Rad Laboratories Inc., Hemel Hempstead, U.K.) under the following conditions: initial denaturation at 94ºC for 2 min followed by 40 cycles of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min. Relative expression was based on ΔΔCt methodology analysed in CFX Manager Software Version 3.0 (Bio-Rad Laboratories Inc.).

2.7 Statistical Analysis

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

3. RESULTS

As human Jagged1 can be cleaved sequentially by ADAM and γ-secretase activities yielding soluble extracellular and intracellular domains, we designed constructs analogous to these fragments in order to examine their effects on E-cadherin expression and proteolysis (Fig. 1). Our sJAG1 construct consisted of the first 1067 amino acid residues (including the signal peptide) of wild-type Jagged1 (WT-JAG1) but was truncated by the introduction of a stop codon on the C-terminal side of D1067. Note that, since the original submission of the current manuscript, ADAM17 has been shown to cleave Jagged1 between E1054 and V1055 [28,29]. 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. Thus, inserting the stop codon immediately upstream of the first amino acid in the putative transmembrane region of the protein (F1068) would ensure that our soluble construct was similar to, and retained all the functional domains of, the shed form of the full-length protein. In contrast, our JICD construct consisted of residues 1086 to 1218 of the full-length protein and lacked the signal peptide thereby, theoretically, preventing entry into the secretory pathway. 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 [30,31] and a similar construct has previously been shown to target effectively to the nucleus [11].
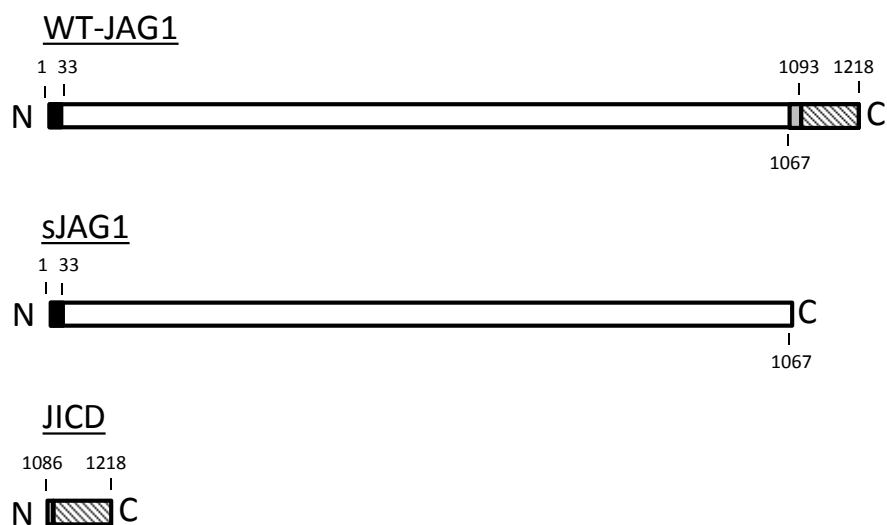
Fig. 1. Jagged1 constructs used in the study

Human wild-type Jagged1 (WT-JAG1) consists of a 33 residue signal peptide (black) followed by a large ectodomain (white), a membrane spanning region (grey) between residues 1067 and 1039 and a cytosolic domain (diagonal lines) between residues 1093 and 1218. The soluble Jagged1 construct (sJAG1) consisted of WT-JAG1 truncated on the C-terminal side of D1067. The Jagged1 intracellular domain construct (JICD) lacked a secretory pathway signal peptide and consisted of the amino acid residues from the putative γ-secretase cleavage site to the C-terminus of human Jagged1 (residues 1086-1218).

The three Jagged1 constructs were then stably transfected into HEK cells and their expression and proteolysis subsequently monitored by immunoblotting (Fig. 2). Using both C- and N-terminal-Jagged1 antibodies (Figs. 2A and 2B), full-length Jagged1 was detected as an intense 185 kDa band in the WT-JAG1-transfected cells consistent with previous reports [11,32]. The full-length endogenous protein was only within the limits of detection using the C-terminal-Jagged1 antibody (Fig. 2A). The JCTF was detected in all the cell lines as a 25 kDa band that was clearly more intense in the WT-JAG1-transfected cells (Fig. 2A). Under basal conditions, the amount of JICD generated in WT-JAG1-transfected cells was at the limits of detection (Fig. 2A) although we have previously shown that the generation of this fragment can be stimulated under enhanced copper concentrations in the same cell line [13]. It is, therefore, apparent that HEK cells possess the necessary cellular machinery for the generation of this fragment. In contrast, JICD-transfected cells exhibited a clearly enhanced band at 20 kDa consistent with the size of the fragment generated from the full-length protein reported previously [13]. Despite being a soluble protein containing a secretory pathway targeting signal peptide, the sJAG1 construct was detected in the lysates of transfected cells using the N-terminal Jagged1 antibody (Fig. 2B). However, as would be expected, the size of this fragment was notably smaller than the full-length protein detected in WT-JAG1-transfected cell lysates using the same antibody.
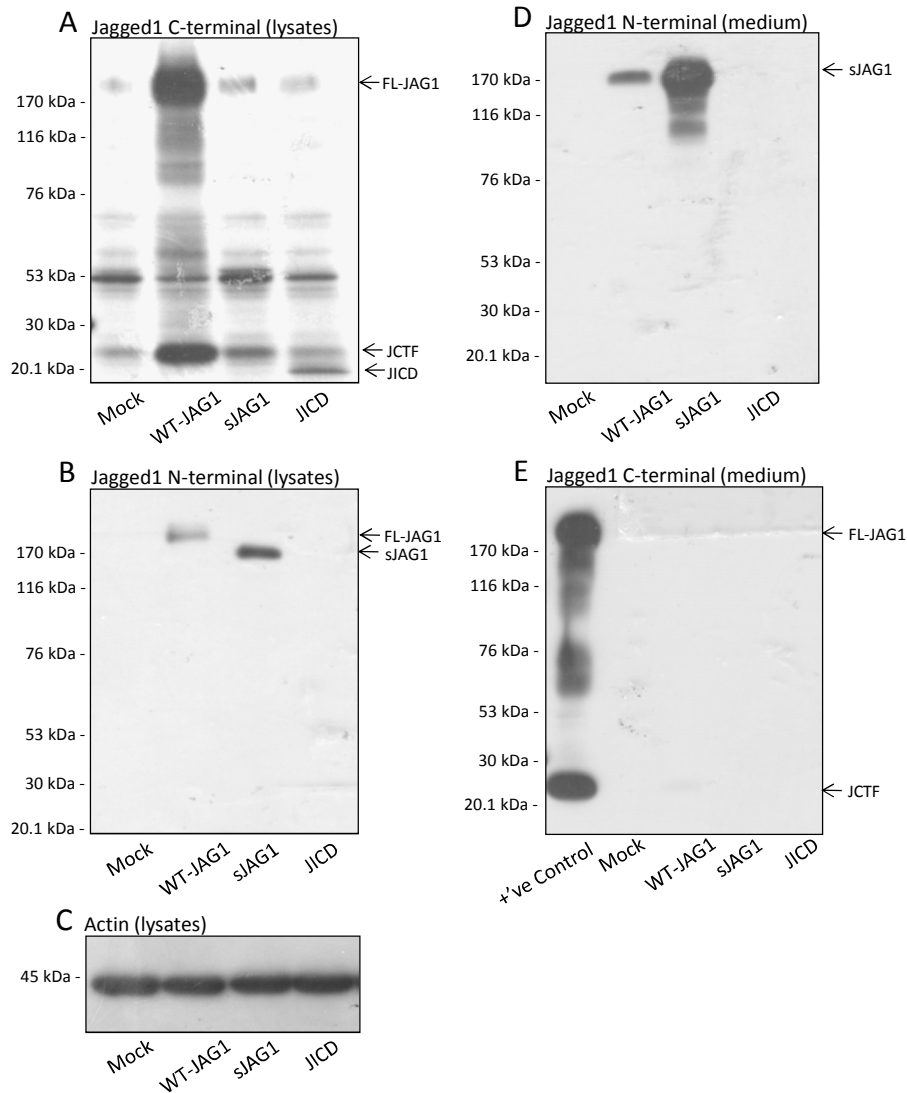
Fig. 2. Jagged1 construct expression and proteolysis in HEK cells

Plasmids encoding the Jagged1 constructs were stably expressed, along with the empty vector (Mock) in HEK cells. The transfectants were incubated for 5 h in reduced serum medium and lysates and conditioned medium were subsequently processed as described in the Materials and Methods section. A-C, lysates were immunoblotted with anti-Jagged1 C-terminal, anti-Jagged1 N-terminal and anti-actin antibodies, respectively. D and E, conditioned medium was immunoblotted with anti-Jagged1 N- and C-terminal antibodies, respectively (the positive control in panel E was lysate prepared from WT-JAG1-transfected cells). FL-JAG1, full-length Jagged1; JCTF, Jagged1 C-terminal fragment; JICD, Jagged1 intracellular domain; sJAG1, soluble Jagged1.

Soluble Jagged1 was detected in conditioned medium from both WT-JAG1- and sJAG1-transfected cells as a 170 kDa band using the anti-N-terminal-Jagged1 antibody (Fig. 2D). Notably, the sJAG1 construct was present at much higher levels than the fragment derived from the full-length protein indicating that it had been successfully targeted to the secretory pathway. The shed form of the endogenous protein was also visible at an identical size when

the same blot was over-exposed (data not shown). Thus, it was apparent that our synthetic sJAG1 construct approximated to the same fragment as that generated by shedding of the full-length protein. In order to confirm that the JICD construct was retained intracellularly, conditioned medium was also immunoblotted with the anti-C-terminal-Jagged1 antibody (Fig. 2E) and, as expected, no JICD was detected; WT-JAG1 cell lysate was used as a positive control in this experiment.

Next, we examined the effect of the various Jagged1 constructs on the expression and proteolysis of the E-cadherin protein (Fig. 3). Full-length E-cadherin was detected as a 120 kDa protein in cell lysates (Fig. 3A) consistent with a previous report [33] and levels of the protein were reduced 30% in WT-JAG1-transfected cell lysates. Notably the soluble form of Jagged1 in the sJAG1-transfected cells also reduced levels of cell-associated E-cadherin by 38%. Although, relative to the mock transfectants, the level of E-cadherin remained unaltered in lysates from the JICD-transfected cells, a significant increase in the protein level was observed in the latter cells relative to sJAG1-transfected cells. The impact of Jagged1 on the shed form of E-cadherin was even more apparent with levels of the latter protein reduced by 57% and 80%, respectively, in the conditioned medium from WT-JAG1- and sJAG1-transfected cells (Fig. 3C). Whilst there was a general trend towards an increased level of soluble E-cadherin in the conditioned medium from the JICD-transfected cells (relative to the mock controls), no statistical significance could be attached to this observation. However, there was a statistically significant increase in shed E-cadherin in the medium of the JICD-transfected cells relative to that of the sJAG1-transfected cells.
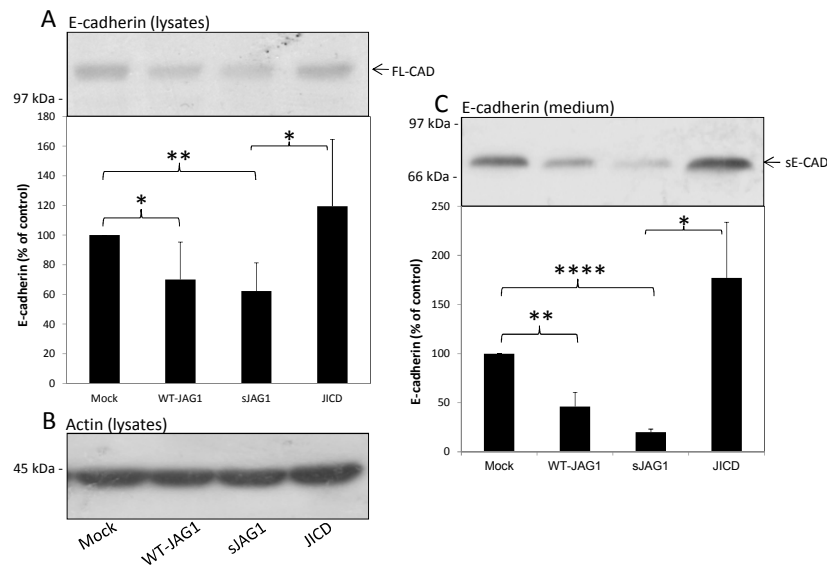

Fig. 3. The effect of Jagged1 constructs on E-cadherin protein expression and proteolysis

*HEK cells stably transfected with the various Jagged1 constructs were incubated for 5 h in reduced serum medium and lysates and conditioned medium were subsequently processed as described in the Materials and Methods section. **A** and **B**, lysates were immunoblotted with anti-E-cadherin and anti-actin antibodies, respectively. **C**, conditioned medium was immunoblotted with anti-E-cadherin antibody. Multiple E-cadherin immunoblots of lysates and medium were quantified by densitometric analysis.*
*Values are expressed as the mean ± S.D. (n=6). *,** and **** denote significant differences at P = 0.05, 0.005 and 0.000001, respectively. Unless otherwise indicated results were not significantly different*

In order to determine whether the levels of E-cadherin were being affected at the transcriptional level we next analysed the amount of E-cadherin RNA in the various Jagged1-transfected cell lines by RT-qPCR. The results (Fig. 4) demonstrated a 35% and 43% reduction in E-cadherin RNA levels, respectively, in the WT-JAG1- and sJAG1-transfected cell lines consistent with regulation of E-cadherin at the transcriptional level. In contrast, the level of E-cadherin RNA in the JICD-transfected cells was increased 2.2-fold.
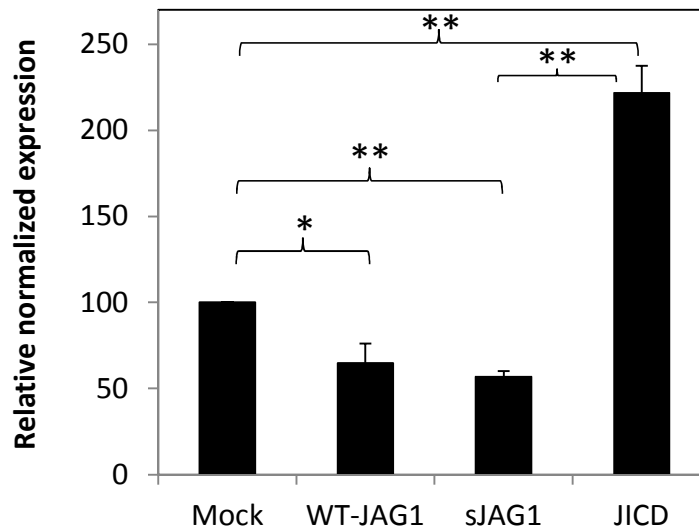

Fig. 4. RT-qPCR analysis of E-cadherin mRNA levels
mRNA was extracted from HEK cells stably transfected with the various Jagged1 constructs and subjected to RT-qPCR analysis as described in the Materials and Methods section.
*Values are expressed as the mean ± S.D. (n=3). * and ** denote significance at P = 0.05 and 0.005, respectively*

4. DISCUSSION

It has previously been reported that Jagged1-mediated Notch activation facilitates the repression of E-cadherin expression and concomitantly induces EMT in human breast epithelial cells [15]. However, Jagged1 is subject to RIP, generating soluble extracellular and intracellular domains the putative roles of which, in the regulation of E-cadherin expression and proteolysis, have not previously been investigated. In the current study, the over-expression of WT-JAG1 in HEK cells reduced the expression of the full-length E-cadherin protein by 30% (Fig. 3A); a level comparable to that previously observed [15].

Of particular interest in the current study was the fact that sJAG1 reduced E-cadherin protein expression by even more (38%) than the full-length Jagged1 protein. It should be noted, however, that the combined expression level of the soluble construct in lysate and medium was higher than that of the WT-JAG1 construct. Nonetheless, the soluble form of Jagged1 clearly exerted an effect on E-cadherin expression consistent with that of its full-length counterpart. One possible explanation for this observation might be that sJAG1 is capable of activating Notch signaling and that membrane anchorage of the ligand is not a prerequisite in this respect. This is in agreement with previous reports in which soluble forms of Jagged1 have been shown to induce keratinocyte differentiation consistent with Notch activation akin

to that by the full-length ligand [23,24]. Such observations contrast with the 'pulling force' model of Notch activation whereby the force produced through ligand endocytosis deforms bound Notch facilitating ADAM-mediated proteolysis of the receptor [34,35]. This latter model might predict that soluble Jagged1 would competitively reduce Notch activation by the full-length ligand. Certainly the repression of transmembrane Jagged1 signaling by the soluble form of the protein has been implicated in the induction of hematopoietic stem cell self-renewal [36] and the clonal expansion of neural crest stem cells [37]. However, in the current cell context, it would seem that sJAG1 is capable of repressing E-cadherin expression in much the same way as its membrane-anchored counterpart supporting the possibility of Notch activation on cells distal to the site of soluble ligand generation.

In the current study, the decreases in cell-associated E-cadherin following WT-JAG1 or sJAG1 over-expression clearly originated at the transcriptional level. However, it is notable that the impact of Jagged1 expression on soluble E-cadherin in conditioned medium (Fig. 3B) was significantly greater than on levels of the cell-associated protein. These data suggest that proteolytic shedding of E-cadherin from the cell surface might also be impaired following Notch activation. Several different metalloproteinases have been implicated in the shedding of E-cadherin including ADAM10 [20] which also acts as a Notch sheddase [38]. It is, therefore, possible that ADAM10 expression might itself be suppressed following Notch activation by way of a feedback mechanism to reduce further activation of the receptor.

In contrast to the effect of sJAG1, the JICD construct employed in the current study did not decrease E-cadherin expression. In fact, this fragment actually enhanced E-cadherin RNA levels 2.2-fold compared to mock-transfected cells (Fig. 4). Although there was no corresponding change in the level of E-cadherin protein in the JICD-transfected cells, there did appear to be a trend (albeit not statistically significant) for an increased level of soluble E-cadherin in the conditioned medium from these transfectants (Fig. 3C). This latter observation might suggest that JICD does in fact enhance E-cadherin protein synthesis but that levels of the cell-associated protein are kept constant through its enhanced shedding into medium. Indeed, a construct similar to the JICD employed in the current study has previously been shown to stimulate Activator Protein-1 (AP-1)-driven gene expression in HEK cells thereby demonstrating transcriptional activity [11].

5. CONCLUSION

Whether soluble Jagged1 is a competitive inhibitor of Notch activation or whether it is capable of receptor activation in its own right seem somewhat context-dependent. However, sJAG1 is clearly capable of repressing E-cadherin expression in the same way as the full-length ligand in the current context. Furthermore, it would seem that both the full-length ligand and sJAG1, in addition to repressing E-cadherin transcription, also impair shedding of the protein from the cell surface. In contrast, the level of E-cadherin RNA in JICD-transfected cells was increased in the current study raising the possibility of 'bi-directional' regulation of E-cadherin transcription by Jagged1 in adjacent cells. The regulation of E-cadherin by soluble Jagged1 raises the interesting possibility that this proteolytic fragment might be able to induce EMT in cells distal to its site of generation. Future studies in cancer epithelial cells are required in order to test this hypothesis.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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