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Biological function of mutant forms of JAGGED1 proteins in Alagille syndrome: inhibitory effect on Notch signaling

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Heterozygous mutations in *JAGGED1*, encoding a single-pass transmembrane ligand for the Notch receptors, cause Alagille syndrome (AGS), a polymalformative disorder affecting the liver, heart, eyes and skeleton and characterized by a peculiar facies. Most of the *JAGGED1* mutations generate premature termination codons, and as a result, two pathogenic mechanisms causing AGS have been proposed: haploinsufficiency or a dominant-negative effect of putative truncated proteins. To determine whether missense or protein-truncating mutations in *JAGGED1* can lead to the synthesis and function of abnormal proteins, we performed cell culture experiments. We showed that human JAGGED1 undergoes a metalloprotease-dependent cleavage resulting in the shedding of its extracellular domain and that this domain seems able to fulfill a biological function *in vitro*, probably by antagonizing Notch signaling. Moreover, the soluble form of JAGGED1 was able to compete with the transmembrane ligand. Mutant proteins with missense or nonsense mutations were synthesized and gave rise to a chord-like phenotype and a migration defect when expressed by stably transfected cells. These chord-like structures were similar to the phenotype exhibited by fibroblasts isolated from a fetus with a protein-truncating mutation. Results obtained from Notch signaling inhibition and Notch reporter assays showed that this chord-like phenotype, exhibited by mutant JAGGED1 transfectants, may result from an inhibitory effect on Notch signaling. Altogether, our results favor a dominant-negative mechanism of some *JAGGED1* mutations in AGS.

INTRODUCTION

Heterozygous mutations in *JAGGED1* (*JAG1*) (1,2), a cell-surface ligand of the Notch receptors, cause Alagille syndrome (AGS) (MIM 118450), an autosomal dominant disorder characterized by the association of five major features: a chronic cholestasis caused by a paucity of interlobular bile ducts, a peripheral stenosis of the pulmonary artery, butterfly-like vertebral arch defects, a posterior embryotoxon and a peculiar facies (3,4). Since its original description, many vascular anomalies have been associated to AGS. Although peripheral pulmonary stenosis is the most common cardiovascular feature of the syndrome, other abnormalities of the circulatory system have been described including complex intracardiac defects and stenosis of other systemic arteries (5–7). Because some of these defects can remain asymptomatic until adulthood, the prevalence of cardiovascular abnormalities in AGS may have been underestimated.

Consistent with these observations, *JAG1* is mainly expressed in the cardiovascular system, in particular the systemic arteries, during human development (8,9). More generally, Notch signaling has been involved in multiple aspects of

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vascular development. *In vitro*, it controls the proliferation and migration of endothelial cells, the differentiation of smooth muscle cells and the formation of endothelial tubes. *In vivo*, Notch signaling promotes epithelial-to-mesenchymal transformation during heart development (10,11) and is involved in the formation of angiogenic processes as well as in arterial-venous cell fate determination (12).

Jag1 belongs to the Delta, Serrate, Lag-2 (DSL) family of single-pass transmembrane ligands that activate the Notch receptors (13,14). Interaction between the extracellular domains of the DSL ligands and of the Notch receptors triggers some proteolytic cleavages within the receptors and the subsequent activation of the pathway (15). Delta ligands are also sequentially cleaved by ADAM proteases and a γ-secretase, resulting in the release of extracellular and intracellular fragments (16–18). The physiological function of these fragments is still unclear and controversy persists about the agonist or inhibitory function of the soluble forms of Delta on Notch signaling (19–22). The proteolytic processing of Jag1 has been less studied, and the significance of the shedding of the extracellular domain of the ligand is still controversial (23,24).

All the *JAG1* mutations detected in AGS patients map to the extracellular and transmembrane domains of the protein and lead in 70% of cases to a premature termination codon (PTC) (25,26). The pathogenic mechanism causing AGS remains unclear. Haploinsufficiency has been proposed as the primary mechanism responsible for AGS (2) and is supported by three main observations. First, heterozygous deletions of the whole *JAG1* gene have been reported in 5% of AGS patients. Secondly, some missense mutations (R184H, L37S and G274D) generate proteins with abnormal glycosylation patterns, which fail to reach the cell surface (27,28). Finally, most of the transcripts with PTC may be degraded by the nonsense-mediated mRNA decay (NMD) pathway. We have recently shown that NMD is indeed able to act on *JAG1* mRNAs with nonsense mutations but that some of these mutant transcripts escape degradation (29) and can then lead to the synthesis of truncated proteins. In addition, truncated forms of the Serrate/Jag protein have been shown to have a dominant-negative effect on both transgenic *Drosophila in vivo* and hematopoietic stem cell self-renewal, as well as on NIH3T3 cells, which exhibit *in vitro* a chord-like phenotype (30–32). Thus, a dominant-negative effect of some *JAG1* mutations causing AGS cannot be ruled out.

Here we report the chord-like phenotype of fibroblasts isolated from an AGS fetus with a PTC mutation. To gain insight into the mechanism responsible for AGS, we have studied the synthesis and function of wild-type (WT) and abnormal JAG1 proteins (with missense or PTC mutations) in cell culture experiments. We report that cleavage of the human WT JAG1 protein leads to the shedding of its extracellular domain, which is then able to fulfill a biological function, probably by antagonizing Notch signaling. Moreover, we show that mutant JAG1 proteins can induce a chord-like phenotype and a decrease in cell migration in stably transfected NIH3T3 cells. Finally, stable expression of mutant JAG1 proteins in NIH3T3 cells results in the inhibition of endogenous Notch signaling. Taken together, these results favor a dominant-negative effect of some mutant JAG1 proteins in AGS.

RESULTS

Shedding of human JAG1 protein from the cell surface

In order to detect a possible cleavage of the extracellular domain of the JAG1 protein, COS cells were transiently transfected with an expression vector encoding the human WT *JAG1* cDNA (COS-WT JAG1). Cell lysates (L) and culture media (CM) were analyzed by western blot (Fig. 1A). A protein of lower molecular mass than full-length JAG1 ($JAG1^{FL}$) was detected in the culture medium, when using an anti-Jag1 antibody directed against the N-terminus (Fig. 1Aa). When using an anti-Jag1 antibody directed against the C-terminus, two C-terminal immunoreactive fragments (CTFs) were identified in the cell lysate (Fig. 1Ab). The relative molecular masses of these two fragments correspond to the membrane associated (CTF_1) and intracellular (CTF_2) fragments of JAG1, respectively. Similar results were obtained from the culture of lymphoblastoid cell lines (LCLs) established from control individuals, in which *JAG1* is expressed endogenously (Fig. 1Aa and b). Treatment with a γ-secretase inhibitor (DAPT) eliminated CTF_2 production and increased CTF_1 signal in COS-WT JAG1 (Fig. 1B). These observations suggest that JAG1 is initially cleaved at the cell surface to produce a soluble $JAG1^{EC}$ fragment and CTF_1 and that subsequent intramembrane proteolysis releases CTF_2 (Fig. 1E).

We next analyzed the kinetics of $JAG1^{EC}$ shedding in the culture medium which was observed as early as 1.5 h and reached maximal levels 12 h after transfer in serum-free conditions (Fig. 1C). Production of $JAG1^{EC}$ was markedly reduced when COS-WT JAG1 cells were treated either with EDTA, a chelator of divalent cations, or with 1,10-phenantrolin, a zinc chelator (Fig. 1D), suggesting that the cleavage is mediated by a Zn^{2+}-metalloprotease. These results were supported by incubation of COS-WT JAG1 with APMA, an effective activator of cystein-switch metalloproteases, which resulted in a sharp increase of $JAG1^{EC}$ in the culture medium (Fig. 1D).

Characterization of mutant JAG1 proteins

In order to analyze the behavior of mutant JAG1 proteins, we generated expression vectors containing three different mutations found in AGS patients (one missense: C284F, and two nonsense mutations: E1003X and Δ4; Fig. 2A). When transfected transiently in COS cells, C284F proteins underwent cleavages: $JAG1^{EC}$ was detected in the culture medium (Fig. 2Ba) and CTF_1 and CTF_2 in the cell lysate (Fig. 2Bb). Truncated proteins (E1003X and Δ4) were synthesized and detected in both the culture medium and the cell lysate (Fig. 2Bc and d). They appeared slightly larger in size when identified from the medium (160 and 65 kDa for E1003X and Δ4, respectively) compared with the cell lysate (150 and 62 kDa), suggesting that the secreted form of the proteins underwent additional post-translational modifications compared with the form identified in the cell lysate.

The glycosylations of cellular and soluble proteins were different. Indeed, utilization of peptide-N-glycosidase F (PNGaseF), an endoglycosidase that removes all N-linked carbohydrates led to the total digestion of WT and mutant proteins from the culture medium or the cell lysate, showing that

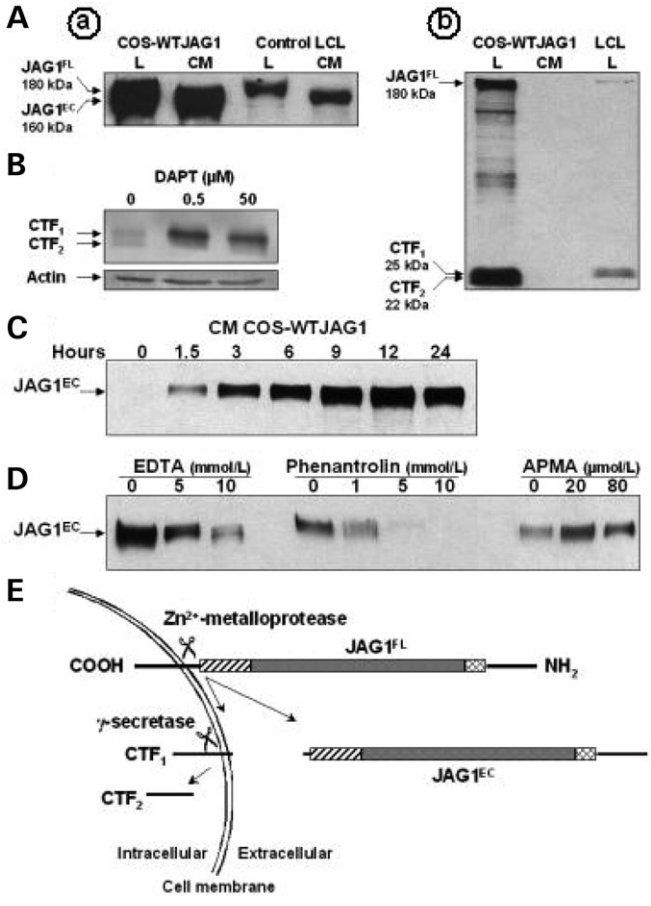

Figure 1. Human WT JAG1 protein undergoes proteolytic cleavages leading to the shedding of its extracellular domain. (**A**) Cell lysates (L) and CM of COS cells transiently transfected with human WT *JAG1* or of LCLs from control individuals were analyzed by western blot incubated with an anti-Jag1 antibody directed against (a) the N-terminus or (b) the C-terminus of the protein. Note the presence of shortened forms of JAG1 in the CM and in L. (**B**) COS cells expressing human WT JAG1 were treated for 4 h with various concentrations of the γ-secretase inhibitor DAPT. The C-ter Jag1 antibody revealed a decrease of CTF2 signal. Actin is shown as an internal control. (**C**) Transfected COS-WT JAG1 cells were first cultured for 24 h in complete medium and then in serum-free medium for 1.5–24 h. The level of $JAG1^{EC}$ in the medium was maximal at 12 h. (**D**) In cells treated for 90 min with EDTA (left) and 1,10-phenantroline (middle), $JAG1^{EC}$ level decreased. The protein level increased after APMA treatment (right). (**E**) Schematic representation of the proteolytic cleavages of the JAG1 protein.

all JAG1 proteins studied bear N-glycosylations (Fig. 2C). When both fractions were treated with endoglycosidase H (EndoH), which only cleaves simple carbohydrate structures such as 'high mannose' or 'hybrid' type structures, only proteins present in the cell lysate were processed, showing that proteins in the culture medium bear complex carbohydrate structures (Fig. 2C). Digestion by EndoH of cell lysates was complete for C284F and E1003X mutant proteins but only partial for WT JAG1. Complex glycosylations borne by proteins from the culture medium were compatible with a biological activity of soluble ligands.

The cellular localization of WT and mutant JAG1 in transiently transfected COS cells was subsequently studied by confocal microscopy. Immunofluorescence on non-permeabilized COS cells demonstrated that both WT and C284F JAG1 proteins are properly addressed to the plasma membrane but no Δ4 protein is present at the cell surface (Fig. 3Aa–c). After cell permeabilization, an intracellular staining was also observed, which could correspond to immature proteins in the endoplasmic reticulum (Fig. 3Ad and e). Although only very low levels of Δ4 JAG1 protein were detected by western blot in cell lysate (Fig. 2Bd), an intracellular staining that likely corresponds to truncated proteins was present in the cell cytoplasm before processing and secretion (Fig. 3Af). The cell surface localization of WT and C284F JAG1 proteins was confirmed by trypsin treatment (Fig. 3B). In these conditions, only proteins present at the cell surface should be digested. In both cases, the amount of $JAG1^{FL}$ decreased and products of lower apparent molecular mass appeared. Moreover, CTF_1 disappeared in favor of a protein of a smaller mass, confirming that CTF_1 is a membrane-bound fragment.

Thus, the C284F protein behaves like the WT protein: both proteins are present at the cell membrane and undergo cleavages leading to the shedding of the extracellular domain, and both soluble proteins, resulting from extracellular cleavage, bear complex glycosylation. The truncated proteins E1003X and Δ4 secreted into the culture medium also show glycosylation.

Soluble WT and mutant JAG1 proteins induce the formation of a chord-like phenotype

Fibroblasts isolated from an AGS fetus with a Δ4 *JAG1* deletion show the formation of a network of chord-like structures when compared with control fetal fibroblasts (Fig. 4A and B). In order to determine whether the truncated JAG1 protein could be responsible for this phenotype and to test whether other mutant JAG1 proteins could have a similar physiological function, we established stable NIH3T3 transfectants, carrying either an empty vector (mock), WT, C284F, E1003X or Δ4 *JAG1* cDNAs. JAG1 proteins were analyzed by western blot (data not shown): although all the stable transfectants expressed the appropriate JAG1 protein, the expression level was lower than that in transient transfectants. As shown in Figure 4C–G, all mutant JAG1 transfectants but not the mock control nor the WT-expressing fibroblasts exhibited a chord-like phenotype similar to the one observed with the fibroblasts of the AGS fetus, when plated at low density on plastic dishes.

In order to test the physiological function of the soluble JAG1 proteins that we identified in the culture medium, we used the medium conditioned by each of the stable transfectants and tested it on normal NIH3T3 cells. When grown in conditioned medium from mutant tranfectants, NIH3T3 cells adopted a chord-like morphology, suggesting that the effect observed was the result of the activity of the mutant proteins contained in the CM (Fig. 5). Surprisingly, 3T3 cells grown in conditioned medium from WT but not from mock transfectants also had a chord-like morphology. Moreover, similar results were observed when conditioned media from any transfectant (to the exception of mock) were added to WT stable transfectants (data not shown). From these observations, we propose that soluble WT JAG1 proteins contained in the culture medium have a biological activity similar to mutant

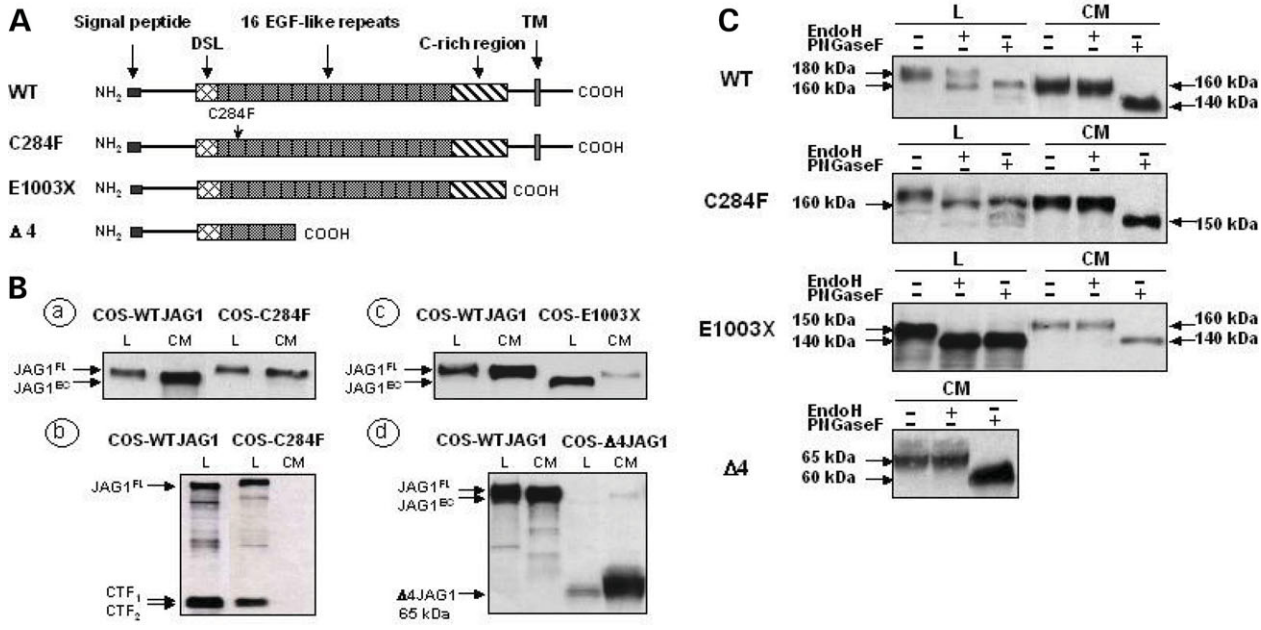

Figure 2. Mutant JAG1 proteins: cleavages, secretion and glycosylation. (**A**) Schematic representation of WT and mutant JAG1 proteins. (**B**) Cell lysates (L) and CM of COS cells transfected with WT, C284F, E1003X or Δ4 *JAG1* were analyzed by western blot incubated with the anti-N-ter Jag1 antibody (a, c and d) or the anti-C-ter Jag1 antibody (b). C284F protein underwent proteolytic cleavages. Truncated JAG1 was secreted into the CM. (**C**) Cell lysates and CM were denatured at 100°C for 10 min, digested either with EndoH or with PNGaseF for 2.5 h at 37°C and analyzed by western blot using the anti-N-terminal Jag1 antibody. In the culture medium, soluble JAG1 proteins bore complex glycosylations.

proteins and that all these soluble proteins are able to compete with the membrane-bound form of the ligand.

Soluble JAG1 competes with the immobilized protein

In order to confirm this competition, we used a synthetic soluble form of human JAG1 protein (syntJAG1$_{sol}$), either added to the culture medium or immobilized on the plastic surface (syntJAG1$^{imm}_{sol}$). Indeed, immobilization of soluble Notch ligands is known to mimic the effect of transmembrane ligands and to activate Notch receptors (20,32,33). WT transfectants cultured in conditioned medium from WT transfectants (containing soluble JAG1 resulting from the extracellular cleavage of the transmembrane ligand), as well as in presence of syntJAG1$_{sol}$, exhibited the chord-like morphology, confirming that this phenotype is the result of an excess of the soluble ligand (Fig. 6A and C). In both cases, the chord-like morphology of WT transfectants cultured in conditioned medium or in the presence of syntJAG1$_{sol}$ was abolished when cells were cultured on plates coated with syntJAG1$^{imm}_{sol}$, which provided an excess of transmembrane ligand (Fig. 6B and D). From these observations, we propose that soluble JAG1 has a biological activity when present in excess and is able to compete with transmembrane JAG1.

Mutant JAG1 proteins inhibit NIH3T3 migration

In order to understand the reason for such a chord-like phenotype and given the involvement of Notch signaling in cell migration (10,34,35), we next studied the mobility of transfectant cells by wound edge closure assay. All mutant JAG1 transfectants showed slower migration illustrated by delayed invasion of individual cells into the bared area, compared with mock and WT JAG1 transfectants (Fig. 7A). Since cell mobility is dependent on cell–matrix and cell–cell adhesions, we examined the expression of adhesion molecules in these transfectants by confocal microscopy. We observed normal abundant level of vinculin-positive focal adhesion sites in mock and WT transfectants, but the three mutant transfectants showed a significantly reduced number of focal adhesion sites (Fig. 7Ba–e), which may explain the slower mobility of the mutants. Furthermore, the mutant transfectants displayed a significant increase of cadherin expression at intercellular junctions (Fig. 7Bf–j). This abnormal expression of adhesion molecules in mutant transfectants strongly suggests the involvement of JAG1 in cell–matrix and cell–cell contacts.

Pharmacological inhibition of Notch signaling in NIH3T3 leads to characteristics similar to those obtained with mutant JAG1 transfectants

Upon ligand binding, Notch receptors undergo two proteolytic cleavages leading to their activation. DAPT inhibited the second and intracellular γ-secretase-dependent cleavage of the receptor, resulting in the inactivation of Notch pathway. NIH3T3 cells treated with this classical Notch signaling inhibitor exhibited characteristics similar to transfectant cells stably expressing mutant JAG1 proteins (Fig. 8). Indeed, NIH3T3 cells grown in the presence of DAPT adopted a chord-like morphology (Fig. 8A and B) and showed a significantly reduced mobility compared with control cells cultivated

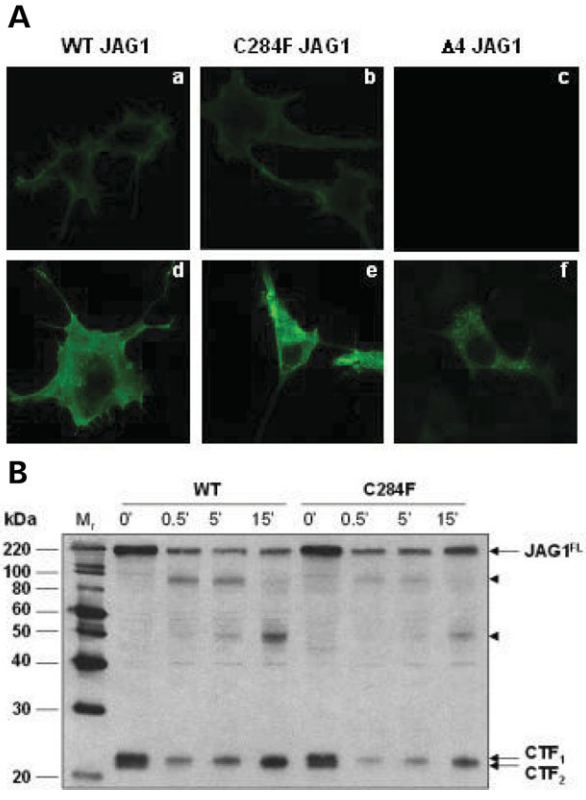

Figure 3. Cellular localization of WT and mutant JAG1 proteins. (**A**) Transfected cells were incubated without prior permeabilization, with the anti-N-ter Jag1 antibody which recognizes an extracellular epitope (a–c) or after permeabilization, with the anti-C-ter Jag1 antibody which recognizes an intracellular epitope (d–f). WT and C284F JAG1 were present at the cell surface but not Δ4 JAG1. (**B**) Twenty-four hours after transfection, COS cells transiently expressing WT or mutant C284F JAG1 were treated with 0.25% trypsin for 0–15 min, complete medium was added to inactivate trypsin. The cell lysates from each transfection were analyzed by western blot incubated with the anti-N-ter Jag1 antibody. Products generated by trypsin digestion are indicated by arrowheads. Note the decrease in the amount of $JAG1^{FL}$ and of CTF1.

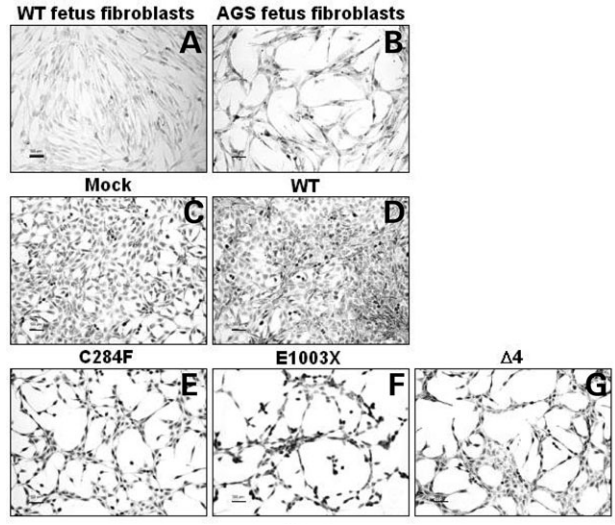

Figure 4. Fibroblasts from an AGS fetus and NIH3T3 transfectant cells expressing mutant JAG1 exhibit a chord-like phenotype. (**A**) Control fetal fibroblasts, (**B**) fibroblasts isolated from an AGS fetus, (**C**) NIH3T3 cells transfected with an empty vector, (**D**) with WT *JAG1*, (**E**) with C284F, (**F**) with E1003X and (**G**) with Δ4 constructs were grown on plastic. About 5×10^5 cells per 100 mM dish were plated. Two days after plating, cells were fixed and stained with hematoxylin–eosin.

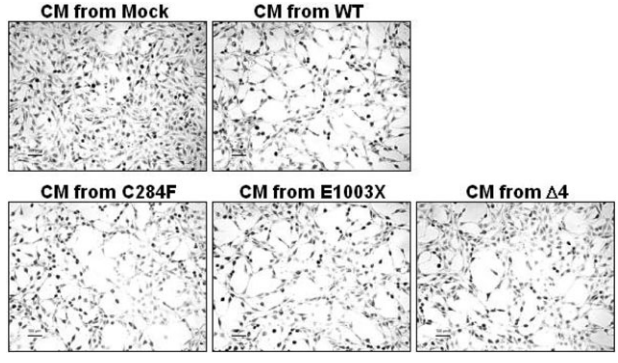

Figure 5. NIH3T3 cells grown with the conditioned media from WT or mutant transfectants form chord-like structures. About 5×10^5 cells per 100 mM plastic dish were plated. The following day, the culture medium was substituted for conditioned media from NIH3T3 cell transfectants. Conditioned media were changed every 12 h for 2 days. Three days after plating, cells were fixed and stained with hematoxylin–eosin.

in absence of the inhibitor (Fig. 8C and D), indicating that the phenotype of mutant JAG1 transfectants is mediated by the inactivation of the Notch signaling pathway.

Stable expression of mutant JAG1 proteins results in inhibition of Notch signaling

Activation of the Notch receptors by interaction with the ligands leads to the transcriptional activation of target genes after forming a complex between activated receptors and CSL (CBF1/RBP-Jκ in mammals, Su(H) in *Drosophila* and Lag-1 in nematodes) proteins (36,37). Therefore, to determine whether the chord-like phenotype is effectively associated with inhibition of the Notch pathway, we used a standard Notch reporter assay to measure the activation of the traditional RBP-Jκ-dependent Notch signaling pathway (38,39). NIH3T3 transfectants stably expressing WT JAG1 protein displayed a high level of RBP-Jκ-mediated transcription of the luciferase reporter gene when compared with mock and mutant transfectants which exhibited very low levels of Notch activation (Fig. 9A). The absence of the RBP-Jκ-mediated transcriptional activity in mutant NIH3T3 transfectants showed that mutant JAG1 proteins were unable to activate Notch signaling. Given the important standard deviation between mock and mutant transfectants, we cannot conclude on the inhibitory effect of mutant JAG1 ligands on the pathway. We then tested the capacity of mutant JAG1 proteins to inhibit Notch signaling in combination with the WT protein, using this Notch reporter assay, by transient transfection of each stable transfectant and NIH3T3 cells with the WT *JAG1* plasmid. RBP-Jκ-mediated transcription of the luciferase reporter gene mediated by WT JAG1 was inhibited when co-expressed with each mutant JAG1 protein (Fig. 9B). Thus, activation of

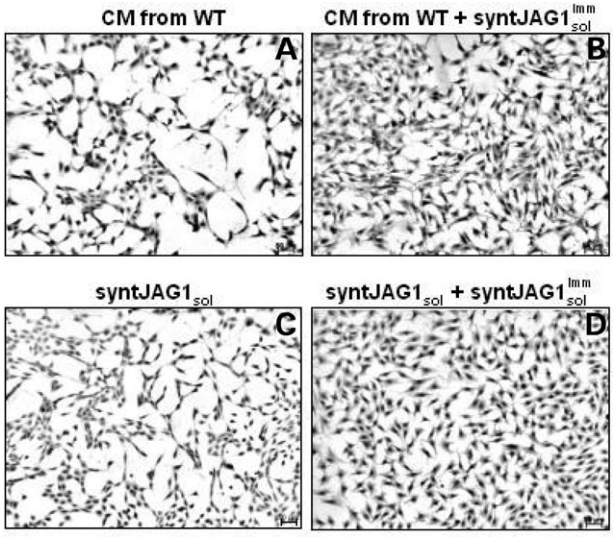

Figure 6. Competition between syntJAG1$_{sol}$ protein and syntJAG1$^{imm}_{sol}$. WT transfectants were cultivated with conditioned media from WT transfectants without (**A**) or with (**B**) syntJAG1$^{imm}_{sol}$. The chord-like morphology observed in the presence of conditioned media from WT transfectants was repressed in the presence of syntJAG1$^{imm}_{sol}$. WT transfectants were grown in the presence of syntJAG1$_{sol}$ (**C**) or syntJAG1$_{sol}$ + syntJAG1$^{imm}_{sol}$ (**D**). Note that only cells grown in the presence of syntJAG1$_{sol}$ exhibited a chord-like morphology.

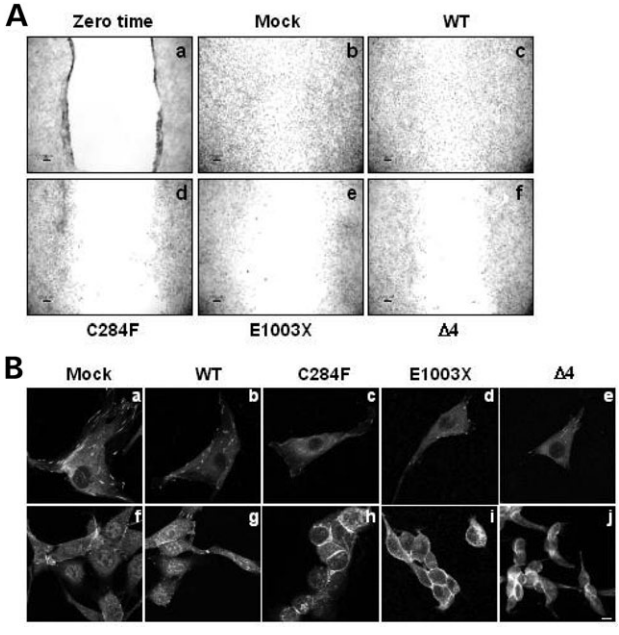

Figure 7. Cell migration is reduced in mutant transfectants. (**A**) Confluent monolayers of stably transfected 3T3 cells were scratched to create a clear area (a). The wound closure was monitored 22 h later for mock (b), WT (c), C284F (d), E1003X (e) and Δ4 *JAG1* (f) transfectants. Notice the low invasion of mutant transfectant cells compared with WT and mock transfectants. (**B**) Cell transfectants were stained and analyzed by confocal microscopy. Focal adhesion sites and intercellular contacts were visualized using an anti-vinculin antibody (a–e) and an anti-pan-cadherin antibody (f–j), respectively. Mutant transfectant cells showed less focal adhesion sites than mock and WT transfectants. There was a significant increase in pan-cadherin expression in mutant transfectant cells.

Notch signaling pathway by WT JAG1 was inhibited by co-expression with mutant proteins. These results demonstrate that the phenotype associated with the mutant transfectant cells

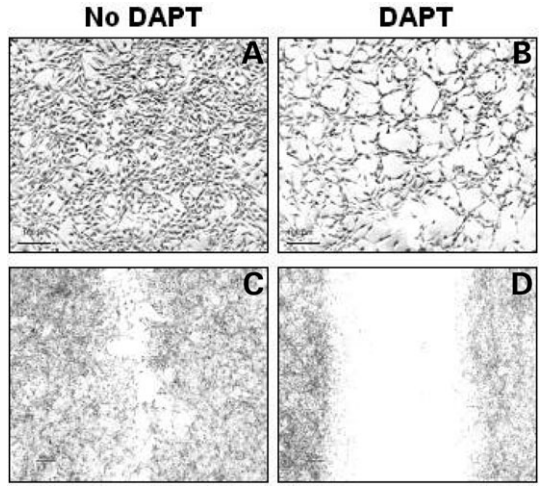

Figure 8. Inhibition of Notch signaling by DAPT mimics the effect of stable mutant JAG1 expression in NIH3T3. NIH3T3 cells were grown in standard culture medium (**A**) or in the presence of 50 μg of DAPT (**B**). Two days after plating, only cells grown in the presence of DAPT exhibited a chord-like morphology. Wound healing capacity of NIH3T3 cells grown in standard culture medium (**C**) or in the presence of DAPT (**D**), 22 h after the wound closure. Note the decreasing cell migration capacity when cells were grown with DAPT.

was due to the inhibition of the endogenous RBP-Jκ/Notch signaling. Moreover, the 2-fold increase of luciferase activity of WT stable transfectant transfected with WT *JAG1* plasmid compared with NIH3T3 transfected with WT *JAG1* plasmid indicates that JAG1 ligand acted in a dose-dependent manner.

DISCUSSION

AGS is caused by *JAG1* mutations that mainly lead to PTCs in the region encoding the extracellular domain of the protein. Our goal was to determine whether the mutations found in AGS could encode mutant proteins with a biological activity. For this purpose, we analyzed the function of WT and mutant JAG1 proteins in cell culture experiments. We showed that, similar to Delta1 (16–18), WT JAG1 is cleaved at the cell surface by a Zn^{2+}-metalloprotease activity in transfected cells as well as in LCLs (in which *JAG1* is expressed endogenously). Following this cleavage, we were able to recover an intact soluble protein corresponding to the extracellular domain from the culture medium. This contrasts with results obtained after transient transfection by full-length rat Jag1 in CHO cells, where a degraded form of the extracellular domain of Jag1 was observed in the medium (23).

The effect of soluble ligands on Notch receptors is debated. Previous data have suggested that the extracellular domain of Delta acts as an agonist of Notch activity (19,20), but other studies have presented the extracellular cleavage of Delta as an inactivation mechanism required for the distinction between the signaling and the receiving cells (21,22). In the case of JAG1, studies performed so far were conducted in transfected cells expressing the extracellular domain of JAG1 only, in the absence of its WT form. These cells exhibited a particular phenotype characterized by the formation of

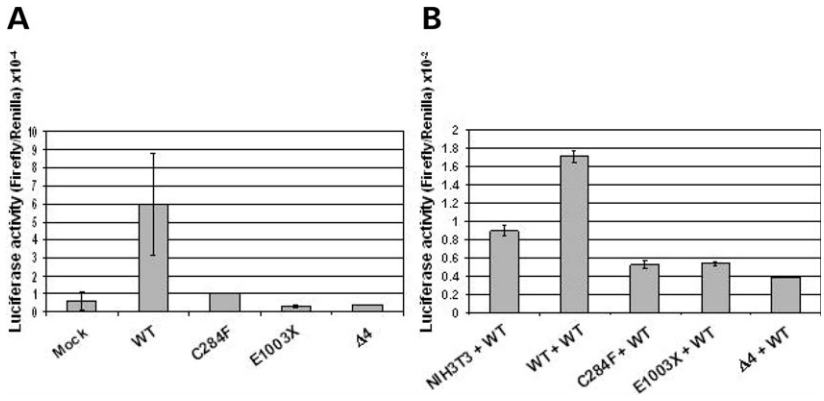

Figure 9. RBP-Jκ transcriptionnal activity is altered in mutant NIH3T3 transfectants. (**A**) NIH3T3 stably expressing mock, WT, C284F, E1003X or Δ4 JAG1 proteins were transiently cotransfected with firefly luciferase construct, driven by six tandem copies of the RBPJκ-response element, and the *Renilla* SV40 construct as an internal control for transfection efficiency. Cell lysates were assayed for both luciferase activities. Activity is shown as the ratio of firefly to Renilla luciferases. Note the decrease in the luciferase activity of mutant transfectants. (**B**) Normal NIH3T3 and NIH3T3 stably expressing WT, C284F, E1003X or Δ4 JAG1 proteins were transiently cotransfected with both luciferase and WT *JAG1* plasmids. Each mutant transfectant (transfected with WT *JAG1* plasmid) exhibited a decrease in the luciferase activity compared with cells expressing WT JAG1 protein alone.

chord-like structures and by the inhibition of cell migration through defective matrix and cell–cell interactions (23,31,34).

In this study, we have demonstrated that the soluble domain is able to compete with the membrane-bound protein. However, the physiological significance of this extracellular shedding remains unclear. It should be noted that the chord-like phenotype seems dependent on the relative amounts of soluble protein present in the medium and of membrane-tethered ligand. When NIH3T3 cells were transfected with WT cDNA, they did not show any phenotype; the phenotype appeared only after addition of a conditioned medium containing $JAG1^{EC}$ or a medium supplemented with syntJAG1. Two hypotheses are proposed to explain this competition. The soluble extracellular domain of JAG1 could interact with the extracellular domain of the transmembrane ligand, preventing interaction with the receptor. In a second model, soluble JAG1 interacts with the extracellular domain of Notch, thus occupying the interaction site of the receptor with the transmembrane ligand, leading to the sequestration of Notch receptors.

The function and the regulation of this extracellular cleavage are still unknown. An important question is whether this cleavage is constitutive or inducible. Results obtained on mouse Delta1 favored a constitutive cleavage of the ligand (17,18). However, several studies showed that Delta processing is up-regulated in co-culture with Notch-expressing cells, suggesting that interaction between ligand and receptor positively regulates the metalloprotease-dependent cleavage (23,40,41). NIH3T3 cells endogenously expressed Notch receptors (42,43), so we cannot exclude that JAG1–NOTCH interaction enhanced JAG1 processing. A recent study has shown that the extracellular matrix protein MAGP-2 interacts with DSL ligands and induces the shedding of JAG1 from the cell surface through an extracellular metalloprotease cleavage (24). MAGP-2 thus appears as a novel modulator of JAG1 shedding. It is then reasonable to hypothesize that protein interactions, such as the one between MAGP-2 and JAG1, regulate the activity of shed JAG1. Therefore, the generation of soluble Notch ligands by proteolytic cleavage appears as a regulatory mechanism of Notch signaling and our results suggest that the production of an excess of soluble JAG1 leads to a down-regulation of the pathway.

We have observed that fibroblasts from an AGS fetus spontaneously exhibited a chord-like morphology. We then analyzed the effect of three mutations identified in AGS patients (one missense and two PTC) in cellular models. The C284F missense mutation abolishes one disulfide bond in EGF2. We showed that the C284F mutant protein is expressed at the cell surface in spite of a disturbed conformation and is cleaved by a metalloprotease, resulting in the release of its extracellular domain. The C284F transfectants exhibited a chord-like phenotype. This is the first time that a JAG1 protein with a missense mutation exhibits a biological effect; proteins with other missense mutations (R184H, L37S and G274D) are not addressed to the cell surface and are thus unable to interact with the Notch receptors (27,28).

We then studied the effect of two nonsense mutations (E1003X and Δ4). The functional role of soluble JAG1 proteins corresponding to the entire extracellular domain of JAG1 (and thus very similar to E1003X JAG1) has already been studied (32,42,44), but the activity of a short protein, such as Δ4 JAG1 truncated in EGF5, was unknown. We showed that both truncated proteins have the capability to induce a chord-like phenotype and that this phenotype is also observed in the fibroblasts isolated from the AGS fetus with the Δ4 mutation (in which *JAG1* mutant transcripts are present, 29). Although we failed to detect the mutant protein in these AGS fibroblasts, the chord-like phenotype exhibited by the cells strongly suggests that the truncated protein is expressed at a level that is sufficient to induce the phenotype.

The three mutant transfectants also presented a slower cell migration illustrated by a decrease in focal adhesion sites and an increase in intercellular junctions, which could partly explain the chord-like morphology of the three mutant transfectants. Jagged/Notch signaling has previously been involved in the control of cell migration. Indeed, *Jag1* expression is considerably up-regulated in the regenerating endothelium of

injured arteries, where cells migrate and proliferate actively (34). Moreover, Notch1 mediates VEGF-induced endothelial cell proliferation and migration (35).

To determine whether the phenotype of mutant transfectants is related to an alteration of the Notch pathway, we used two different strategies. First, NIH3T3 cells treated with a pharmacological inhibitor of Notch signaling exhibit a phenotype similar to mutant JAG1 transfectant cells, which suggests that the phenotype of these cells is the result of the inhibition of the pathway. This hypothesis was confirmed using Notch reporter assays which showed an inhibitory effect of the three *JAG1* mutations on Notch signaling and suggested that these mutant JAG1 proteins act in a dominant-negative manner. These results are consistent with the chord-like phenotype and decreased migration rate displayed by NIH3T3 cells expressing dominant-negative forms of Notch1 and Notch2 (42). Although the molecular basis for this dominant-negative effect mediated by mutant JAG1 proteins is not known, we propose that the inhibition of Notch signaling by truncated ligand may result from the inability of the soluble JAG1-NOTCH complex to undergo endocytosis in the signal-sending cell, a mechanism essential for activation of the pathway (45,46). The physiopathological mechanism of C284F mutant JAG1 protein appears less evident than for soluble ligands and needs to be clarified by further experiments.

Overall, this work is the first evidence for a dominant-negative effect in AGS, whatever the mutation type studied. We propose that, in AGS, the effect of mutant JAG1 proteins results in an inhibition of Notch signaling, impairing either differentiation (47–49) or cell migration (35,50) during vascular development.

MATERIALS AND METHODS

Plasmids

Human WT, 1264G>T (C284F), 3420G>T (E1003X) and 1612–1615delGCCC (Δ4) *JAG1* cDNAs (positions 410–4140 on cDNA sequence GenBank accession no. HSU73936) were cloned into pcDNA3.1/Hygro(+) plasmid (Invitrogen) downstream of the CMV promoter (29).

Cell culture

COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 U/ml penicillin (Gibco/BRL), 100 μg/ml streptomycin (Gibco/BRL) and 2 mM glutamine (Gibco/BRL). NIH3T3 cells were grown in DMEM supplemented with 10% newborn calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine plus 500 μg/ml Hygromycin B (Invitrogen, Life Technologies) for stable transfectants. LCLs and fibroblasts from AGS fetus were grown in Roswell Park Memorial Institute medium (RPMI 1640, Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 U/ml penicillin (Gibco/BRL), 100 μg/ml streptomycin (Gibco/BRL) and 2 mM glutamine (Gibco/BRL).

Transfection

Transient transfections in COS7 or NIH3T3 cells (70% confluent) were performed using polyethylenimine (PEI) (Aldrich). Briefly, 13–160 ng of plasmid per cm^2 and 250 pg of PEI per cm^2 were mixed in serum-free medium for 15 min before being added to the cells. After a 4 h incubation, the transfection medium was removed, and complete medium was added for another 24 h. This medium was replaced by a serum-free medium a day later in order to study the shedding of JAG1 proteins.

Stable transfections in NIH3T3 cells were performed in essentially the same way. Two days after transfection, confluent cells were divided and supplemented a day later with 500 μg/ml Hygromycin B in DMEM (Invitrogen, Life Technologies). After the selection of resistant clones, cells were maintained in 50 μg/ml Hygromycin B.

Luciferase cotransfection assay

Each NIH3T3 transfectant cells, plated onto fibronectin-coated six-well tissue culture dishes, were transfected at 70% confluency by PEI with a mixture of 500 ng of the firefly luciferase construct driven by six tandem copies of the RBP-Jκ-response element (pGa981-6) (38) and 30 ng of the *Renilla* SV40 construct (Promega) as an internal control for transfection efficiency or a mixture of 1 μg of the firefly luciferase construct, 30 ng of the *Renilla* SV40 construct and 1 μg of the WT JAG1 construct. The medium was replaced with fresh complete medium 4 h after transfection. The cells were harvested 24 h after the medium change and firefly/*Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The efficiency of transfection was normalized to the activity of *Renilla* SV40 construct, and the activity was expressed as the ratio of firefly to *Renilla* luciferase activities. All assays were performed in triplicate for each experiment.

Competition between free soluble JAG1 and immobilized soluble JAG1

Synthetic soluble JAG1 protein (syntJAG1, R&D Systems) was immobilized (syntJAG1^{imm}) on 100 mM plastic dishes during 2 h of adsorption at 37°C, at a concentration of 5 μg/ml. Saturation was then performed with 1 mg/ml Bovine Serum Albumin (Roche Diagnostics) for 1 h at 37°C. Then, 3×10^5 NIH3T3 WT transfectant cells were seeded in 5 ml of medium containing 5 μg/ml free syntJAG1 in complete medium or in 5 ml of conditioned medium from WT transfectants. Conditioned medium was changed every 12 h for 4 days. Cells were then fixed and stained with hematoxylin–eosin.

Cell lysates and western blots

The serum-free CM of transfected cells were collected in the presence of protease inhibitors ('Complete', Roche Diagnostics) and concentrated ~20-fold using Amicon-Bioseparations Centrifugal Centriplus and Centricon spin filters (Millipore).

Cell lysates were prepared and protein concentrations estimated using the BIO-RAD protein assay (Bio-Rad Laboratories). Proteins were analyzed on SDS–PAGE gels (7.8–15%) and blotted to nitrocellulose membranes (Amersham Biosciences). Membranes were incubated with anti-rat N-terminal Jag1 (1:100, R&D Systems), anti-human C-terminal JAG1 C-20 (1:100, Santa Cruz Biotechnology) or anti-human actin antibodies (Santa Cruz Biotechnology), followed by incubation with the appropriate secondary antibody conjugated to horseradish peroxidase. Immunoblots were revealed by chemiluminescence (ECL™ Western Blotting Detection Reagents, Amersham Biosciences).

Cell migration assays

For each NIH3T3 transfectant, $\sim 5 \times 10^5$ cells were plated on 100 mm dishes and were grown to confluence under Hygromycin B selection. The cell monolayers were scratched using plastic tips, rinsed with PBS to remove detached cells and then fed with complete medium. Photomicrographs were taken immediately and 22 h after scratching.

Immunofluorescence

Cells were fixed in 3.7% paraformaldehyde and blocked in 1% BSA, PBS, $\pm 0.1\%$ Triton X-100 and incubated for 2 h with the appropriate primary antibody. We used polyclonal anti-JAG1 antibodies (1:200, Santa Cruz Biotechnology and R&D Systems), monoclonal anti-vinculin antibody (1:100, Sigma Chemicals Co.) and polyclonal anti-pan-cadherin antibodies (1:400, Sigma Chemicals Co.). The secondary antibodies used were Alexa Fluor® 488 donkey anti-goat IgG (Molecular Probes) and Rhodamine conjugated purified anti-mouse IgG. Stained cells were examined by confocal fluorescence microscopy (Zeiss LSM 410).

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Conflict of Interest statement. There are no conflicts of interest to declare.

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REFERENCES

1. Li, L., Krantz, I.D., Deng, Y., Genin, A., Banta, A.B., Collins, C.C., Qi, M., Trast, B.J., Kuo, W.L., Costa, T. *et al.* (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat. Genet.*, **16**, 243–251.
2. Oda, T., Elkahloun, A.G., Pike, B.L., Okajima, K., Krantz, I.D., Genin, A., Piccoli, D.A., Meltzer, P.S., Spinner, N.B., Collins, F.S. *et al.* (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat. Genet.*, **16**, 235–242.
3. Alagille, D., Odievre, M., Gautier, M. and Dommergues, J.P. (1975) Hepatic ductular hypoplasia with characteristic facies, vertebral malformations, retarded physical, mental and sexual development and cardiac murmur. *J. Pediatr.*, **86**, 63–71.
4. Alagille, D., Estrada, A., Hadchouel, M., Gautier, M., Odievre, M. and Dommergues, J.P. (1987) Syndromic paucity of interlobular bile ducts. *J. Pediatr.*, **110**, 195–200.
5. Berard, E., Sarles, J., Triolo, V., Gagnadoux, M.F., Wernert, F., Hadchouel, M. and Niaudet, P. (1999) Renovascular hypertension and vascular anomalies in Alagille syndrome. *Pediatr. Nephrol.*, **12**, 121–124.
6. Crosnier, C., Lykavieris, P., Meunier-Rotival, M. and Hadchouel, M. (2000) Alagille syndrome. The widening spectrum of arteriohepatic dysplasia. *Clin. Liver Dis.*, **4**, 765–778.
7. Kamath, B.M., Spinner, N.B., Emerick, K.M., Chudley, A.E., Booth, C., Piccoli, D.A. and Krantz, I.D. (2005) Vascular anomalies in Alagille syndrome: a significant cause of morbidity and mortality. *Circulation*, **109**, 1354–1358.
8. Crosnier, C., Attie-Bitach, T., Encha-Razavi, F., Audollent, S., Soudy, F., Hadchouel, M., Meunier-Rotival, M. and Vekemans, M. (2000) *JAGGED1* gene expression during human embryogenesis elucidates the wide phenotypic spectrum of Alagille syndrome. *Hepatology*, **32**, 574–581.
9. Jones, E.A., Clement-Jones, M. and Wilson, D.I. (2000) *JAGGED1* expression in human embryos: correlation with the Alagille syndrome phenotype. *J. Med. Genet.*, **37**, 658–662.
10. Noseda, M., Chang, L., McLean, G., Grim, J.E., Clurman, B.E., Smith, L.L. and Karsan, A. (2004) Notch activation induces endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. *Mol. Cell. Biol.*, **24**, 8813–8822.
11. Timmerman, L.A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J.M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J.C. *et al.* (2004) Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev.*, **18**, 99–115.
12. Iso, T., Hamamori, Y. and Kedes, L. (2003) Notch signaling in vascular development. *Arterioscler. Thromb. Vasc. Biol.*, **23**, 543–553.
13. Lindsell, C.E., Shawber, C.J., Boulter, J. and Weinmaster, G. (1995) Jagged: a mammalian ligand that activates Notch1. *Cell*, **80**, 909–917.
14. Fleming, R.J., Purcell, K. and Artavanis-Tsakonas, S. (1997) The Notch receptor and its ligands. *Trends Cell Biol.*, **7**, 437–441.
15. Bray, S.J. (2006) Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.*, **7**, 678–689.
16. Ikeuchi, T. and Sisodia, S.S. (2003) The Notch ligands, Delta1 and Jagged2, are substrates for presenilin-dependent 'γ-secretase' cleavage. *J. Biol. Chem.*, **278**, 7751–7754.
17. Six, E., Ndiaye, D., Laabi, Y., Brou, C., Gupta-Rossi, N., Israel, A. and Logeat, F. (2003) The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. *Proc. Natl Acad. Sci. USA*, **100**, 7638–7643.
18. Dyczynska, E., Sun, D., Yi, H., Sehara-Fujisawa, A., Blobel, C.P. and Zolkiewska, A. (2007) Proteolytic processing of delta-like 1 by ADAM proteases. *J. Biol. Chem.*, **5**, 282–444.
19. Qi, H., Rand, M.D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T. and Artavanis-Tsakonas, S. (1999) Processing of the notch ligand delta by the metalloprotease Kuzbanian. *Science*, **283**, 91–94.
20. Varnum-Finney, B., Wu, L., Yu, M., Brashem-Stein, C., Staats, S., Flowers, D., Griffin, J.D. and Bernstein, I.D. (2000) Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J. Cell Sci.*, **113**, 4313–4318.
21. Hicks, C., Ladi, E., Lindsell, C., Hsieh, J.J., Hayward, S.D., Collazo, A. and Weinmaster, G. (2002) A secreted Delta1-Fc fusion protein functions

both as an activator and inhibitor of Notch1 signaling. *J. Neurosci. Res.*, **68**, 655–667.
22. Mishra-Gorur, K., Rand, M.D., Perez-Villamil, B. and Artavanis-Tsakonas, S. (2002) Down-regulation of Delta by proteolytic processing. *J. Cell Biol.*, **159**, 313–324.
23. LaVoie, M.J. and Selkoe, D.J. (2003) The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. *J. Biol. Chem.*, **278**, 34427–34437.
24. Nehring, L.C., Miyamoto, A., Hein, P.W., Weinmaster, G. and Shipley, J.M. (2005) The extracellular matrix protein MAGP-2 interacts with Jagged1 and induces its shedding from the cell surface. *J. Biol. Chem.*, **280**, 20349–20355.
25. Crosnier, C., Driancourt, C., Raynaud, N., Dhorne-Pollet, S., Pollet, N., Bernard, O., Hadchouel, M. and Meunier-Rotival, M. (1999) Mutations in *JAGGED1* gene are predominantly sporadic in Alagille syndrome. *Gastroenterology*, **116**, 1141–1148.
26. Warthen, D.M., Moore, E.C., Kamath, B.M., Morrissette, J.J., Sanchez, P., Piccoli, D.A., Krantz, I.D. and Spinner, N.B. (2006) Jagged1 (JAG1) mutations in Alagille syndrome: increasing the mutation detection rate. *Hum. Mutat.*, **27**, 436–443.
27. Morrissette, J.D., Colliton, R.P. and Spinner, N.B. (2001) Defective intracellular transport and processing of JAG1 missense mutations in Alagille syndrome. *Hum. Mol. Genet.*, **10**, 405–413.
28. Lu, F., Morrissette, J.J. and Spinner, N.B. (2003) Conditional JAG1 mutation shows the developing heart is more sensitive than developing liver to JAG1 dosage. *Am. J. Hum. Genet.*, **72**, 1065–1070.
29. Boyer, J., Crosnier, C., Driancourt, C., Raynaud, N., Gonzales, M., Hadchouel, M. and Meunier-Rotival, M. (2005) Expression of mutant *JAGGED1* allele in patients with Alagille syndrome. *Hum. Genet.*, **116**, 445–453.
30. Sun, X. and Artavanis-Tsakonas, S. (1997) Secreted forms of DELTA and SERRATE defined antagonists of Notch signaling of *Drosophila*. *Development*, **124**, 3439–3448.
31. Wong, M.K., Prudovsky, I., Vary, C., Booth, C., Liaw, L., Mousa, S., Small, D. and Maciag, T. (2000) A non-transmembrane form of Jagged-1 regulates the formation of matrix-dependent chord-like structures. *Biochem. Biophys. Res. Commun.*, **268**, 853–859.
32. Vas, V., Szilagyi, L., Paloczi, K. and Uher, F. (2004) Soluble Jagged-1 is able to inhibit the function of its multivalent form to induce hematopoietic stem cell self-renewal in a surrogate *in vitro* assay. *J. Leukoc. Biol.*, **75**, 714–720.
33. Ohishi, K., Varnum-Finney, B., Flowers, D., Anasetti, C., Myerson, D. and Bernstein, I.D. (2000) Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. *Blood*, **95**, 2847–2854.
34. Lindner, V., Booth, C., Prudovsky, I., Small, D., Maciag, T. and Liaw, L. (2001) Members of the Jagged/Notch gene families are expressed in injured arteries and regulate cell phenotype via alterations in cell matrix and cell–cell interaction. *Am. J. Pathol.*, **159**, 875–883.
35. Takeshita, K., Satoh, M., Ii, M., Silver, M., Limbourg, F.P., Mukai, Y., Rikitake, Y., Radtke, F., Gridley, T., Losordo, D.W. and Liao, J.K. (2007) Critical role of endothelial Notch1 signaling in postnatal angiogenesis. *Circ. Res.*, **100**, 70–78.
36. Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R. and Israel, A. (1995) Signalling downstream of activated mammalian Notch. *Nature*, **377**, 355–358.
37. Schroeter, E.H., Kisslinger, J.A. and Kopan, R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature*, **393**, 382–386.
38. Minoguchi, S., Taniguchi, Y., Kato, H., Okazaki, T., Strobl, L.J., Zimber-Strobl, U., Bornkamm, G.W. and Honjo, T. (1997) RBP-L, a transcription factor related to RBP-Jkappa. *Mol. Cell Biol.*, **17**, 2679–2687.
39. Joutel, A., Monet, M., Domenga, V., Riant, F. and Tournier-Lasserve, E. (2004) Pathogenic mutations associated with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy differently affect Jagged1 binding and Notch3 activity via the RBP/JK signaling pathway. *Am. J. Hum. Genet.*, **74**, 338–347.
40. Bland, C.E., Kimberly, P. and Rand, M.D. (2003) Notch-induced proteolysis and nuclear localization of the Delta ligand. *J. Biol. Chem*, **278**, 13607–13610.
41. Delwig, A., Bland, C., Beem-Miller, M., Kimberly, P. and Rand, M.D. (2006) Endocytosis-independent mechanisms of Delta ligand proteolysis. *Exp. Cell Res.*, **312**, 1345–1360.
42. Small, D., Kovalenko, D., Kacer, D., Liaw, L., Landriscina, M., Di Serio, C., Prudovsky, I. and Maciag, T. (2001) Soluble Jagged1 represses the function of its transmembrane form to induce the formation of the Src-dependent chord-like phenotype. *J. Biol. Chem.*, **276**, 32022–32030.
43. Nie, L., Xu, M., Vladimirova, A. and Sun, X.H. (2003) Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *EMBO J.*, **22**, 5780–5792.
44. Trifonova, R., Small, D., Kacer, D., Kovalenko, D., Kolev, V., Mandinova, A., Soldi, R., Liaw, L., Prudovsky, I. and Maciag, T. (2004) The non-transmembrane form of Delta1, but not of Jagged1, induces normal migratory behavior accompanied by fibroblast growth factor receptor 1-dependent transformation. *J. Biol. Chem.*, **279**, 13285–13288.
45. Le Borgne, R., Bardin, A. and Schweisguth, F. (2005) The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development*, **132**, 1751–1762.
46. Nichols, J.T., Miyamoto, A., Olsen, S.L., D'Souza, B., Yao, C. and Weinmaster, G. (2007) DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J. Cell Biol.*, **176**, 445–458.
47. Doi, H., Iso, T., Yamazaki, M., Akiyama, H., Kanai, H., Sato, H., Kawai-Kowase, K., Tanaka, T., Maeno, T., Okamoto, E. *et al.* (2005) HERP1 inhibits myocardin-induced vascular smooth muscle cell differentiation by interfering with SRF binding to CArG box. *Arterioscler. Thromb. Vasc. Biol.*, **25**, 2328–2334.
48. Morrow, D., Scheller, A., Birney, Y.A., Sweeney, C., Guha, S., Cummins, P.M., Murphy, R., Walls, D., Redmond, E.M. and Cahill, P.A. (2005) Notch-mediated CBF-1/RBP-J{kappa}-dependent regulation of human vascular smooth muscle cell phenotype *in vitro*. *Am. J. Physiol. Cell Physiol.*, **289**, 1188–1196.
49. Proweller, A., Pear, W.S. and Parmacek, M.S. (2005) Notch signaling represses myocardin-induced smooth muscle cell differentiation. *J. Biol. Chem.*, **280**, 8994–9004.
50. Kodama, Y., Hijikata, M., Kageyama, R., Shimotohno, K. and Chiba, T. (2004) The role of Notch signaling in the development of intrahepatic bile ducts. *Gastroenterology*, **127**, 1775–1786.