Soluble Jagged1 Inhibits Pulmonary Hypertension by Attenuating Notch Signaling

Yongguang Xiao, Dan Gong, Wei Wang

Objective—Notch signaling has been implicated in the development of pulmonary arterial hypertension (PH) as reflected by increased expression of Notch member proteins that induce the proliferation of pulmonary arterial smooth muscle cells (PASMCs). Soluble Jagged1 (sJag1) has been shown to inhibit Notch signaling in vitro and in vivo; however, its capacity to suppress PH remains unknown.

Approach and Results—The mouse model of hypoxia-induced PH and the rat model of monocrotaline-induced PH induced high expressions of Notch1, Notch3, Jagged1, and Herp2. By attenuation and reversal of multiple pathological processes that were associated with PH, adenoviral sJag1 transfection significantly reduced the proliferation and enhanced the apoptosis of PASMCs in PH, whereas vehicle had no effect. The sJag1 inhibitory effect on Notch activation is likely related to its interference with ligand-induced signaling. Importantly, the administration with adenoviral sJag1 improved the survival rate of PH rats. Furthermore, sJag1 can restore the PH-PASMCs phenotype from the dedifferentiated to the differentiated state, by giving a positive effect on the physical binding of myocardin to the CArG-containing regions of vascular smooth muscle cells—specific promoters.

Conclusions—Our results demonstrated that the potential therapeutic use of the sJag1 may not only inhibit the proliferation of PASMCs but also restore the PH-PASMCs phenotype from the dedifferentiated to the differentiated state through interference with Notch–Herp2 signaling.

Key Words: hypertension, pulmonary ♦ phenotype

Pulmonary hypertension (PH) is a progressive disease of various origins that is associated with increased pulmonary vascular resistance and results in right ventricular hypertrophy and eventual right heart dysfunction. Structural remodeling of small pulmonary arteries and arterioles is progressive and is a diffuse that causes vessel wall thickening and obliteration of the distal pulmonary arterial tree mostly by excessive proliferation of pulmonary arterial smooth muscle cells (PASMCs). Furthermore, PH-PASMCs, similar to other adult vascular smooth muscle cells (vSMCs), are highly plastic and capable of modulating their phenotype in response to exogenous stimuli, cell-to-cell interaction, and cell-to-matrix signaling. And the transition from the contractile phenotype of normal PASMCs to proliferative and antiapoptotic phenotype of PH-PASMCs is a key pathologically responsible for all kinds of PH. Importantly, the unique phenotype of PASMCs remains duratively unchanged, even if isolated from a patient with PH or a PH animal model. However, the mechanism of how the genetic pathway regulates plasticity and proliferation of PASMCs remains unknown.

The Notch signaling is an evolutionarily conserved regulatory system that plays a prominent role in cell fate decisions including proliferation, differentiation, and survival. The effect of Notch signaling is based on the expression of certain genes in a cell-type—specific manner. Notch protein members are expressed on the surface of the cell that is activated by Notch receptors (Notch1, -2, -3, -4) interacting with ligands (Jagged1, -2, and delta-like [Dll]-1, -3, -4). Among the Notch receptors, only Notch1, 2, and 3 are expressed on vasculature and critical in regulation of vascular morphogenesis and function during development and disease. Ligand binding triggers a conformational change in proteolytic cleavage of the receptor by the presenilin–γ-secretase complex and release of the intracellular domain of Notch, which translocates into the nucleus and interacts with the DNA-binding protein, CBF-1 (also termed CSL or RBPJκ). This transcriptional activator complex induces transcription of target genes, most notably the Hey and Herp genes, which regulate cell fate.

Recently, the Notch signal pathway has been demonstrated to be involved in the development of pulmonary arterial hypertension associated with PASMC proliferation. Overexpression of Notch1 increased proliferation and inhibited apoptosis of vSMCs. Furthermore, members of the Notch family can have been fundamentally extended to regulate vSMC plasticity. Consistently, Jagged1 increased the expression of smooth muscle–myosin heavy chain, whereas the Notch downstream transcription factors HERP1 and HERP2 were able to stimulate vSMC proliferation and differentiation.
Jagged1 protein in lung tissue of rat-PH model was correlated in rat as time went on since the initiation of MCT treatment increased expression of Notch1, Notch3, and Jagged1 protein.

Western blot showed that there was progressively increased protein expression level on Notch1, Notch3, and Jagged1 correlated with the progression of PH, we studied PASMCs of rat model at serial time points during development of PH. Western blot data showed that the level of mPAP on day 42 in Ad-sJag1–treated rats was significantly lower than that in vehicle treatment (Figure 2B).

Next, we evaluated the therapeutic potential of sJag1 at a time point when PH pathology had already been established. The administration of Ad-Jag1 on day 21 after MCT treatment did not completely reverse but did halt the progression of PH, as judged on measurement of mPAP (Figure 2G), right ventricular hypertrophy (Figure 2E), and pulmonary arterial wall thickness (Figure 2I) on day 42 after MCT treatment. Taken together, those results demonstrated that sJag1 can inhibit the development of pulmonary arterial hypertension through interference Notch receptors interacting with Jagged1.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Notch/Jagged1 Signaling Was Highly Activated in PH
To investigate the expression pattern of Notch signaling pathway in normal and PH lungs, we studied 2 kinds of PH rodent models including hypoxia-induced mouse and MTC-induced rat. The Verhoeff–Van Gieson stain sections for lung tissue clearly showed that there was significantly increased vascular medial thickening in the PH models of rat (Figure 1A) and mouse. Consistent with this observation, our studies showed that the mean pulmonary arterial pressures (mPAP) by day 35 in hypoxia-mouse and by day 42 in MTC-rat were significantly higher than those in normal (Figure 2B).

Next, we isolated PASMCs from normotensive lung in mouse and rat and PH-PASMCs from hypoxia-induced mice and MTC-induced rat. Western blot data showed that these PH-PASMCs in rat and mouse exhibited a higher protein expression level on Notch1, Notch3, and Jagged1 than those PASMCs in normotensive lung (Figure 1C). However, we found no difference in Dll1 protein level between normal and PH in both rat and mouse. These results demonstrated that there was a direct involvement of Notch1, Notch3, and Jagged1 in the development of PH (Figure 1D and 1E).

To test whether the protein expression of Notch1, Notch3, and Jagged1 correlated with the progression of PH, we studied PASMCs of rat model at serial time points during development of PH. Western blot showed that there was progressively increased expression of Notch1, Notch3, and Jagged1 protein in rat as time went on since the initiation of MCT treatment (Figure 1F and 1G). Furthermore, high expression level of Jagged1 protein in lung tissue of rat-PH model was correlated with worsening disease severity (Figure 1H). In contrast to Jagged1, we found no difference in the expression of Dll1 protein in the normotensive lung compared with that of PH rat. Taken together, our results indicated that expression levels of Notch1, Notch3, and Jagged1 proteins correlated with the severity of PH in the rodent models.

sJag1 Ameliorated the Development of PH
To determine the effect of Jagged1 on the development of PH, we examined the therapeutic potential of sJag1 with adenoviral vector (Ad-sJag1) through interference Notch receptor (including Notch1 and Notch3) to interact with Jagged1.

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Because excessive proliferation of PH-PASMCs was the major underlying mechanism of vascular structure remodeling in the PH, we examined whether Ad-sJag1 can downregulate the proliferative properties of PH-PASMCs. Four sets of experiments were performed. First, using intracellular staining with proliferating cell nuclear antigen, we evaluated the effect of Ad-sJag1 on PH-PASMCs proliferation in vivo. Expression of proliferating cell nuclear antigen was significantly higher in PH rats that receive vehicle treatment compared with normal pulmonary arterial wall, whereas Ad-sJag1 drastically reduced proliferating cell nuclear antigen-positive PH-PASMCs nearly to a basal level (Figure 3A). This indirectly reflected
proliferative rates of PH-PASMCs in vivo. Second, we found that treatment with Ad-sJag1 increased the number of apoptotic cells in PH-PASMCs by accounting the number of terminal deoxynucleotidyl transferase dUTP nick end labeling–positive PASMCs (Figure 3B). Third, we isolated PH-PASMCs from MCT-induced rats with Ad-sJag1 and vehicle treatment. Western blot showed that Ad-sJag1–treated lung exhibited a high expression level of Jagged1 protein in PASMCs, whereas nil to minimal level of Jagged1 protein expression was found in lung tissue that received vehicle treatment (Figure 3C). Western blot data were reflected by the reverse transcription polymerase chain reaction data revealing that Ad-sJag1–treated lung expressed approximately twice more Jagged1 mRNA compared with vehicle-treated lung (Figure 3D). Finally, the direct evidence for the inhibitory activity of Ad-sJag1 on PH-PASMC proliferation was obtained from a cell subculture experiment. Our studies showed that subculture PH-PASMCs from Ad-sJag1–treated lung had roughly half the proliferative rate as that of PH-PASMCs from vehicle-treated lung (Figure 3E). Collectively, these experiments indicated that Ad-sJag1 not only attenuates the proproliferative properties of PH-PASMCs but also induces apoptosis.
Jagged1 Mediated Phenotype of PASMCs Dependent on Notch Signaling

Because the administration of Ad-sJag1 can inhibit PH, we examined how the effect of Ad-sJag1 on the proliferation of PASMCs was dependent on Notch signaling. As shown in Figure 4A, Western blotting revealed that Ad-sJag1 inhibited the expression of Herp2 protein. To gain a better understanding of molecular pathways involved in Notch–Herp2 signaling, we first compared phenotypic pattern of PASMCs from normal lung, PH-lung, and PH-lung treated with Ad-sJag1. In PH-PASMCs, expression of proteins associated with differentiated/contractile phenotype, including SM 22α and SM MHC, were decreased in comparison with the PASMCs of normal lung and PH-PASMCs treated with Ad-sJag1 (Figure 4B). In contrast, the PASMCs dedifferentiated, or the synthetic-specific marker proteins, including syndecan-1, were more abundant in PH-PASMCs compared with that in normal and Ad-sJag1–treated PH-PASMCs (Figure 4B). The studies below were natural to show how Ad-sJag1 signaling can restore PASMCs differentiation. We studied myocardin expression by Western blotting and demonstrated that Ad-sJag1 upregulated myocardin protein expression in PH-PASMCs (Figure 4C).

Figure 2. Rats were instilled with 100 μL 5.0×10¹⁷ VP/mL adenoviral soluble Jagged1 (Ad-sJag1) and vehicle, respectively. A, Representative hematoxylin- and eosin-stained sections of lung tissue from rat on day 42 showed pulmonary arterial hypertension (scale bars, 30 μm). Mean pulmonary arterial pressure (PAP; B) and systemic blood pressure (SBP; C) in rats measured at various time points after administration of monocrotaline (MCT). Percentages of medial thickness of resistance pulmonary artery (D) and right ventricular hypertrophy (E) were measured at indicated time point. Survival rates of the different experimental groups were showed in F. In separate studies, Ad-sJag1 was given to animal in the same way on day 21 after the administration of MCT and the mean PAP (G). Percentages of medial thickness of resistance pulmonary artery (H) and right ventricular hypertrophy (I) were measured at indicated time points. B and G, *P<0.05 vs vehicle on day 21, **P<0.01 vs vehicle on day 42, *P<0.05 vs vehicle on day 42. D–F, H, and I, *P<0.05 vs vehicle.
Given the effect of Ad-sJag1 on myocardin, we then tested whether physical binding of myocardin to the CArG-containing regions of PASMCs-specific promoters was indeed affected. Using quantitative ChIP assays, we found a lower level of myocardin binding to the CArG-containing regions of SM22 and SM MHC promoters in PH-PASMCs as compared with that of normal and Ad-sJag1–treated PH-PASMCs (Figure 4D and 4E). The observed effects of Ad-sJag1 on PASMC-specific transcription may explain Notch signaling–dependent reverse modulation of PH-PASMC phenotype from the dedifferentiated to the differentiated state.

Discussion

In this study, we demonstrated that sJag1 effectively attenuates mPAP and pulmonary vascular resistance through attenuation of Notch signaling and subsequent reduction of PASMC proliferation on PH. To our knowledge, this is the first demonstration that Jagged1 pathway is activated in the development of PH. Importantly, we provide intriguing evidence that the pathological antiapoptotic and proliferative PASMC phenotype is reversed toward the normal PASMCs phenotype by the administration of Ad-Jag1.

The most important character in PH is pulmonary vascular remodeling that consists of the thickening of the vascular intima and media and generally thought to result from cell hypertrophy, proliferation, migration, and extracellular matrix deposition.21,22 This process involves many cell types, including endothelial cells, smooth muscle cells, fibroblasts, inflammatory cells, and platelets.21,22 To assess whether Notch signaling may regulate proliferation of PASMCs, we examined the protein expression during the development of PH. Our study demonstrated that this process is associated with an increased expression of Notch1, Notch3, and Jagged1 in both the mouse model of hypoxia-induced PH and the rat model of MCT-induced PH. Since the start of MCT induction on the rat-PH model, there is an obvious and gradient upregulation of Notch1 and Jagged1 in the pulmonary arterial wall, indicating a quick induction of Notch signal pathway expression and activation in the formation of PH. In agreement with previously published data, the upregulation of Notch members, such as Notch1, can induce the proliferation of PASMCs.2,23 However, the pathway by which notch signal regulates the proliferation of PASMCs remains unknown. Especially, the notch ligands Dll1 and Jagged1 have totally opposing effects, and Jagged1 is a critical positive regulator because of its ability to modulate Notch/Dlll signaling.24 Our studies showed that it was Jagged1 but not Dll1 that highly expresses in PH-PASMCs. After binding with ligand Jagged1, notch1 triggers proteolytic cleavage and release of intracellular domain of Notch, which increase the downstream Herp2 and interacts with the DNA-binding protein.

To our knowledge, this is first demonstration that Notch1/Jagged1 signal pathway is critical for proliferation of PH-PASMCs. Importantly, we provide intriguing evidence that the formation and development of pulmonary arterial hypertension can be inhibited by blocking Notch1/Jagged1 signal pathway.

The ligand is cleaved on receptor interaction by the same proteases, giving rise to a soluble extracellular ligand domain that
can influence notch signal as well. Notch ligands induce signaling only when expressed as immobilized forms, whereas they show no activity or antagonistic activity when as soluble forms. The free as well as immobilized Jagged1 promotes proliferation of PASMCS to induce PH. In contrast, sJag1 influences Notch ligand–binding results toward inhibition of downstream signaling. For example, the free sJag1 has a dominant negative effect on stem cell and macrophage progenitors. In line with these previous studies, our results also showed that sJag1 inhibits proliferation of PA-PASMCs and attenuated pulmonary arterial pressure and pulmonary vascular resistance. Moreover, these results suggest potential clinical use of recombinant Notch ligand, sJag1, for inhibiting proliferation PASMCS in vivo and in vitro.

Our studies also revealed that sJag1 can inhibit Notch downstream signaling, the expression of Herp2 in the development of PH. This finding may be significant because of a recent study that Ad-sJag1 infection in vitro reduced Herp2 mRNA expression in vSMCs, suggesting an inhibitory effect on Notch/Jagged1 signaling. From in vitro and in vivo studies, it has been demonstrated that Herp2, as downstream of the Notch/Jagged1 signaling, regulates vSMCs differentiation and migration, and induces vascular remodeling in response to stimuli. Furthermore, the observation that the hampered proliferation of the Ad-sJag–treated vSMCs could be partially rescued by Herp2 overexpression suggests that Notch–Herp2 signaling is required for vSMCs function, and that sJag1 is an inhibitor for notch signaling both in vivo and in vitro.

The unique PH-PASMC phenotype is important for the development of PH. Notch signaling has been reported to promote vSMCs differentiation by enhancing myocardin/SLF binding to CARG box. And sJag1 can reduce Notch signal member expression concomitant with vSMC marker αSMA expression in neointima formation of a rat carotid artery injury model. In line with these studies, our study provides a clear
demonstration that sJag1 gives a positive effect on the physical binding of myocardin to the CArG-containing regions of vSMCs-specific promoters, such as SM22 and SM MHC.

In summary, our work provides an intriguing therapeutic strategy for attenuation and reversal of multiple pathological processes that are associated with PH in the hypoxia-induced mouse model and the MCT-induced rat model by interfering with Notch–Herp2 signaling. The sJag1 inhibitory effect on Notch activation is likely related to its interference with ligand-induced signaling. Additionally, the potential therapeutic use of the sJag1 may not only inhibit the proliferation of PASMCs but also restore the PH-PASMC phenotype from the dedifferentiated to the differentiated state.

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Disclosures
None.

References


Significance

The notch signal pathway has been demonstrated to be involved in the development of pulmonary arterial hypertension associated with pulmonary arterial smooth muscle cell proliferation. Obviously, Notch proteins can be activated by 5 Notch ligands, including Jagged1 and -2, and delta-1, -2, and -3. However, notch signaling in pulmonary hypertension remains unknown. In this study, we used soluble Jagged1 to inhibit Notch signaling in pulmonary hypertension. Our study showed the potential therapeutic use of soluble Jagged1 may not only inhibit the proliferation of pulmonary arterial smooth muscle cells but also restore the pulmonary hypertension–pulmonary arterial smooth muscle cells phenotype from the dedifferentiated to the differentiated state through interference with Notch–Herp signaling.
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Materials and Methods

Animal studies
All animal experiments were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. For inducing PH in mice, 8-10 weeks-old male C57BL/6J (B6) was placed in an airtight plexiglass chamber that maintained with hypoxic air (10% O2 and 90% N2) continuously for up to 5 weeks and was opened twice a week for cleaning and replenishment of food and water. For induction of PH in rats, monocrotaline (MCT form Sigma-Aldrich) was given as a single 60mg/kg subcutaneous injection to male Sprague-Dawley (SD) rats weighing between 220 and 250g in body weight.

We performed rodent hemodynamic measurements and pulmonary angiography as followed. At the end of each experiment, animals were intubated orotracheally with a 14-(for rats) or 22-gauge (for mice) TeflonTM angiocatheter and mechanically ventilated with 2-3% isoflurane in air at a rate of 90 (for rats) or 110 (for mouse) breaths/min and a tidal volume of 10mg/kg, using the Harvard Rodent Ventilator (Harvard Apparatus, MA). The systolic BP (SBP) was recorded in the right common carotid artery using a Millar Micro-Tip® catheter (Millar Instruments, TX) with the PowerLab system (ADInstruments, CO). After SBP measurement, the thorax of animal was opened, and the same system was used for measurement of pulmonary artery blood pressure as we previously described. For the assessment of right ventricular hypertrophy, the right ventricle was dissected from the left ventricle and interventricular septum, and these were weighed separately. The index of right ventricular hypertrophy was then calculated as the ratio of weight of the right ventricle to that of the left ventricle plus the interventricular septum.

Histological and Immunofluorescence analyses
For pulmonary vascular morphometry, rodent lungs were fixed and embedded in paraffin. The block from each animal was cut into 4-um sections and stained with hematoxylin and eosin (H&E), then examined by a microscope digital camera system (Zeiss) at various magnifications to determine the severity of PH. Percent medial thickness was calculated by the followed formula: medial thickness (%) = (external area –internal area)/ external area×100, where external area and internal area are the areas bounded by external elastic lamina and lumen, respectively.

For pulmonary artery staining of PCNA, the 4-um section were permeabilized with 0.1% PBS for 30 min (4°C), and then incubated with anti-PCNA antibody (Abcam, 1:600) for 1 hour at room temperature, finally followed by incubation with an Alexa Flour labeled secondary antibody at 1:300 dilutions. The number of PCNA-positive cell was counted by a microscope digital camera system and was calculated by following formula: the percentage of PCNA-positive nuclei= (total PCNA-positive cells/total cell count) ×100%.

For pulmonary artery staining of TUNEL, pretreated sections were incubated with the TUNEL reaction mixture (Roche, Germany) for 1 hour at room temperature, next followed by incubation with DAB. Apoptotic cells that appeared as green color were visualized with a microscope.

Cell culture
The procedure for isolation of single smooth muscle cells from rat resistance (external diameter,
300 um) pulmonary arteries was described as followed. After Sprague-Dawley rats were killed, the lungs were harvested using sterile technique and washed with RPMI 1640 medium. Resistance pulmonary arteries were carefully dissected using a dissecting microscope, and then the endothelium removed by gentle rubbing with a cotton-tipped applicator stick. The vessels cut into small pieces (1×10 mm) and then were digested in DMEM containing collagenase subtype (1 mg/ml, Roche, USA) for 4 hrs at 37°C. The resulting solution was centrifuged, and the pellet was resuspended in DMEM containing 20% FBS and then seeded onto 100-mm culture dishes. The dishes was incubated upside down at 37°C in a humidified atmosphere of 95% air/5% CO2.

Quantitative real-time PCR, ChIP-PCR and Western blot
Quantitative real-time PCR was used for quantitative analysis of Jagged1 mRNA level in PASMCs infected with Jagged1 adenovirus or lacZ adenovirus. In brief, total cellular RNA was extracted using RNeasy Mini Kit (Qiangen Sciences) and reversed-transcribed into cDNA and amplified using TaqMan Reverse Transcription reagents (Applied Biosystems). Quantitative analysis of gene expression was performed using the ABI Prism 7700 Sequence Detection System with 18s rRNA used as an endogenous reference.

The chromatin immunoprecipitation (ChIP) assay was performed according to the Abcam X-ChIP protocol. In brief, cross-linked chromatin from subcultured vSMCs was sonicated generate 500-1000bp DNA fragments, followed by overnight incubation at 4°C with primary antibodies specific for myocardin (Abcam) or IgG (Santa Cruz, negative control). Immunoprecipitated DNA was purified and amplified by RT-PCR with primers flanking the CArG boxes of SM22 or SM MHC. Primer sequences were as follows: SM22: 5'-GGTCCTGCCCATAAAAGGTTT-3' and 5'-TGCCCATGGAAGTCTGCTTGG-3'; SM MHC: 5'-CTGCGCGGGACCATA TTTAGTCAGGGGGAGG-3' and 5'-CTGGGCGGGAGACAACCCAAAAAGGCCAGG-3'.

Protein levels of Notch1, Notch3, Jagged1, Dll1, Herp2, and SM22, SM MHC, syndcan-1, myocardin in cell lysate were evaluated by an ECL Western blotting analysis kit (Amersham Biosciences). The primary antibodies used in this study were against: β-actin (Santa Cruz, 1:2000), Notch1 (Abcam, 1:400), Notch3 (Abcam, 1:400), Dll11 (Abcam, 1:400), Jagged1 and Herp2 (Santa Cruz, 1:1000), SM22 α and SM MHC (Abcam, 1:800), Sydenan-1 (Santa Cruz, 1:600), myocardin (Abcam, 1:600).

Adenovirus vector production and transduction
CDA containing the entire full-length Jagged1 or LacZ was used to construct and produce recombinant AdNEP plasmid (AdEasy™ Adenoviral Vector System, Stratagene). Adenovirus vectors were amplified and purified by cesium chloride density gradient ultracentrifugation. The AdLacZ vector was used as a negative control. All rats received a single intravenous injection of 100µL 5.0x10¹¹VP/mL Adenoviral LacZ (vehicle), or Adenoviral Jagged1 (Ad-sJag1) by tail vein on day 0 after MCT injection.

Statistical analysis
Data are expressed as mean±SEM, and statistical analyses performed with the Prism statistical
program. Unpaired Student’s t test was used for comparisons between two means. One-way ANOVA with the Newman-Keul’s was used to evaluate differences between more than two means. In all case, P value less than 0.05 was considered significant.

References