Published in final edited form as:

Eur J Cell Biol. 2018 September ; 97(7): 512–522. doi:10.1016/j.ejcb.2018.09.001.

Notch signaling regulates cell density-dependent apoptosis of NIH 3T3 through an IL-6/STAT3 dependent mechanism

Yosuke Matsuno^{a,*}, Takumi Kiwamoto^a, Yuko Moroshima^a, Yukio Ishii^a, Nobuyuki Hizawa^a, and Cory M. Hogaboam^b

^aDepartment of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

^bDivision of Pulmonary and Critical Care Medicine, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048, United States

Abstract

Apoptosis is a physiological process that plays a critical maintenance role in cellular homeostasis. Previous reports have demonstrated that cells undergo apoptosis in a cell density-dependent manner, which is regulated, in part, by signal transducers and activators of transcription (STAT) 3. The molecular mechanisms regulating cell density-dependent apoptosis, however, has not been thoroughly investigated to date. Since Notch signaling is activated via direct cell-to-cell contact and plays a pivotal role in cell fate decisions, we examined the role of Notch signaling in cell density-dependent apoptosis of mouse embryonic fibroblasts NIH 3T3 cells. With the increase in cell density, IL-6 expression was induced, which was necessary for STAT 3 activation as well as apoptosis regulation. Notch signaling was also activated in a cell-density dependent manner. Blocking Notch signaling either through siRNA-mediated targeting of Jagged1 expression or γ -secretase inhibitor treatment demonstrated that Notch signaling activation was necessary for IL-6 induction. Constitutive activation of Notch signaling via the overexpression of Notch1 intracellular domain was sufficient for the induction of IL-6, which was mediated via direct transcriptional activation. Taken together, our study indicates that Notch signaling regulates cell density-dependent apoptosis through IL-6/STAT3-dependent mechanism. Consequently, Notch signaling might represent an novel therapeutic target in diseases characterized by dysregulated apoptosis.

Keywords

Cell density; Apoptosis; Notch signaling; STAT3; IL-6

1. Introduction

Apoptosis, or programmed cell death, plays a critical role in maintaining cellular homeostasis. Dysregulated apoptosis is implicated in the pathogenesis of diseases such as

*Corresponding author at: Department of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. yosukem@md.tsukuba.ac.jp (Y. Matsuno).

Competing interests

The authors declare no competing financial interests.

cancer, inflammation, and fibrosis. In fibrotic disorders characterized by fibroblast accumulation and excessive extracellular matrix deposition, resistance to apoptosis contributes to the persistence of fibroblasts and consequently to disease progression (Horowitz et al., 2004; Moodley et al., 2003; Wallach-Dayana et al., 2007). Understanding the mechanisms regulating apoptosis could provide a novel therapeutic strategy in a number of diseases.

STAT3 is a transcription factor that is involved in divergent cellular functions including cell proliferation, migration, angiogenesis, and survival (Kida et al., 2008; Shen et al., 2001; Yu et al., 2014, 2009). STAT3 is activated by various ligands such as IL-6 family cytokines and growth factors and environmental condition, via the phosphorylation of tyrosine 705. Upon activation, STAT3 translocates to the nucleus to bind to specific DNA regulatory sequence of target genes thereby activating transcription (Bromberg and Darnell, 2000). Previous reports have demonstrated that, in cells cultured at high density, STAT3 is phosphorylated and protects cells from undergoing apoptosis (Vultur et al., 2004). The molecular mechanism whereby STAT3 is activated in a cell density-dependent manner, however, remains elusive.

Notch signaling is a highly conserved pathway that plays a critical role in intercellular communication and regulates cell fate decisions. In mammals, four Notch receptors (Notch1–4) and five ligands (Delta-like1, 3 and 4, Jagged1 and 2) transduce signals. Receptor-ligand interaction induces a series of proteolytic processing of Notch receptors. At the final step of receptor cleavage, γ -secretase releases Notch intracellular domain (NICD) from the cell membrane into the nucleus, where NICD complexes with the transcriptional repressor CSL (RBP-J) and co-activators to induce the transcription of various target genes. Since Notch signaling is activated through physical cell-cell contact, Notch signaling might be involved in cellular responses induced at high cell density. Indeed, in certain cell types, Notch signaling is activated when cells are confluent and contributes to contact inhibition (Nosedá et al., 2004) and angiogenesis (Benedito et al., 2009). It remains still unclear, however, whether Notch signaling plays any role in apoptosis that is induced at high cell density.

In the current study, we investigated the role of Notch signaling in cell density-dependent apoptosis of NIH 3T3 cells. We demonstrate that Notch signaling regulates cell density-dependent apoptosis via IL-6/STAT-3 dependent mechanism.

2. Materials and methods

2.1. Cell culture

NIH 3T3 cells were purchased from American Type Culture Collection (Manassas, VA). Mouse lung fibroblasts were isolated from BALB/c mice as previously described (Hogaboam et al., 1999). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were placed in 12-well plates at a density of either 1.5×10^4 or 1.5×10^5 cells in 1 ml of medium/well and cultured for 72 h. For STAT3 inhibition, cells were cultured with 4 μ M STAT3 inhibitor (WP1066, EMD Millipore, Darmstadt, Germany) or DMSO for 72 h. In IL-6 receptor blocking experiment, cells were treated with either 100 μ g/ml of anti-IL-6

receptor antibody (LSBio, Seattle, W A) or rat IgG (R&D systems, Minneapolis, MN) for 72 h. In experiments with a γ -secretase inhibitor (GSI), cells at 10×10^4 cells/well were cultured with either 20 μ M GSI IX (Carbiochem, Darmstadt, Germany) or DMSO for 72 h. Recombinant mouse IL-6 (R&D systems) was added at 50 ng/ml to other wells (containing 10×10^4 cells/well) to examine the direct effects of this cytokine on these cells.

2.2. Detection of apoptosis

NIH 3T3 cells were stained with annexin V and propidium iodide (BD Biosciences, San Jose, CA) and analyzed by flow cytometry on a BD FACS Calibur flow cytometer (BD Biosciences). Cell Quest software (BD Biosciences) was used to analyze the proportions of apoptotic cells. Cells were defined as apoptotic based on the positive staining with annexin V. Apoptotic cells were imaged by photomicrographs taken at x40 magnifications with a phase contrast microscope.

2.3. Small interfering RNA (siRNA) treatment

The siRNA targeting Jagged1 (160949) and the negative control (12935115) were obtained from Invitrogen (Carlsbad, CA). Cells were transiently transfected with 20 nM siRNA by using Lipofectamine RNAiM AX reagent (Invitrogen) and cultured for 48 h.

2.4. Plasmid constructs and gene transduction

The pGL3-CSL construct was kindly provided by Dr. Hyunggee Kim (Korea University, Seoul, Republic of Korea) and contains a CSL binding site. The mouse Notch1 intracellular domain construct, p3xFLAG-CMV7-N1ICD, was kindly provided by Dr. Raphael Kopan (Washington University, St Louis, MO) and contains a N-terminal FLAG epitope tag and amino acids 1744–2531 of the full length Notch1. IL-6 promoter sequence located between –1243 to + 78 from the transcription start site was PCR-amplified from genomic DNA purified from NIH 3T3 cells using primers 5'-GGCTCGAGGTGATCCTGAGAGTGTGTTTTG-3' and 5'-GGAAGCTTAGCGGTTTCTGGAATTGACTATCGTTCTTGG-3', which includes a XhoI and a HindIII site on the 5' and the 3' end, respectively. Promoter fragments were ligated into the XhoI/HindIII site of the pGL4.10 luciferase vector (Promega, Madison, WI) to generate pGL4.10-IL6p. Site-directed mutagenesis was performed using a KOD-Plus-Mutagenesis Kit (TOYOBO) according to the manufacturer's protocol, to generate an IL-6 promoter reporter construct with mutations in putative CSL binding site located at –116 to –110 (in which 5'-TTTCCCA-3' was changed to 5'-TTTCCGG-3'). The resultant construct was verified through complete DNA sequencing of both DNA strands. Cells were transduced with empty vector or N1ICD expression vector using Lipofectamine LTX reagent (Invitrogen).

2.5. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) and 1 μ g of total RNA was converted into cDNA. qRT-PCR was performed with the GeneAmp 7500 Sequence Detection System

(Applied Biosystems, Foster City, CA). All primers for qRT-PCR were purchased from Applied Biosystems and results were expressed as 2^{-CT} using GAPDH as the reference.

2.6. Western blot analysis

NIH 3T3 cells were lysed in Cell Lysis Buffer (Cell Signaling Technology) containing PMSF. Proteins in the cell lysates were quantified using the Bradford assay. SDS sample buffer was added to cell lysates and boiled for 10 min. All protein samples were resolved using 12% SDS-PAGE in 25 mM Tris, 192 mM glycine, 0.1% SDS buffer. Proteins were then transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol buffer. Blots were incubated with primary antibody in 5% milk in TBS at 4°C overnight. After three washes, the membranes were incubated with secondary antibody diluted 1:2000 in TBS for 1 h at room temperature. After three washes, proteins were visualized with ECL Western blotting reagents (GE Healthcare, Little Chalfont, UK).

2.7. Enzyme-linked immunosorbent assay (ELISA)

IL-6 protein levels in culture supernatants were measured using an IL-6 ELISA kit (R&D systems) according to the manufacturer's protocol.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a kit from EMD Millipore according to the manufacturer's instructions. Briefly, NIH 3T3 cells were transfected with FLAG-tagged N1ICD expression vector. 48 h later, cells were crosslinked using 1% formaldehyde and then lysed in SDS lysis buffer. After sonication to shear crosslinked DNA to 200–1000 base pairs in length, the lysates were diluted, and equal amounts of chromatin were used for immunoprecipitation with 4 µg of rabbit anti-FLAG antibody, anti-histone H3 antibody as a positive control or rabbit IgG as a negative control. In another experiment, NIH 3T3 cells were cultured in 12-well plates at a density of 1.5×10^5 cells in 1 ml of medium/well for 72 h and chromatin immunoprecipitation was performed using anti-Notch1 antibody. PCR was performed using primer pairs 5'- GATTCT TTCGATGCTAAACGACG -3' and 5'- AGCTACAGACATCCCCAGTCTC -3' to detect enrichment of DNA flanking the putative CSL binding site of IL-6 promoter region (-153 to + 31). The PCR products were analyzed by gel electrophoresis in 2% agarose.

2.9. Luciferase assay

To determine cell-density dependent Notch transcriptional activity, cells were cultured at either 1.5×10^4 or 1.5×10^5 cells in 1 ml of medium/well. Cells were then incubated for 24 h and transfected with 1 µg of pGL3-CSL and 0.01 µg of Renilla expression vector pGL4.74 (Prom ega) in 1 ml of medium using Lipofectamine LTX reagent (Invitrogen). To determine Notch1 dependent IL-6 promoter activity, cells cultured at 8×10^4 cells/well were incubated for 24 h, transfected with 1 µg of either N1ICD or empty vector, IL-6 promoter reporter construct without or with point mutations in CSL binding site, and 0.01 µg of Renilla expression vector pGL4.74 (Prom ega) in 1 ml of medium using Lipofectamine LTX reagent (Invitrogen). After 48 h, the cells were harvested and the activity of firefly and Renilla luciferase was determined using the Dual Luciferase Assay System (Promega). The relative

luciferase activity was calculated by normalizing firefly luciferase activity to that of Renilla luciferase to correct for the transfection efficiency.

2.10. Antibodies

Primary monoclonal antibodies directed against STAT3, phospho-STAT3 (Tyr705), cleaved caspase-3, Notch1 and cleaved Notch1 (Val1744) were purchased from Cell Signaling Technology (Danvers, MA). Jagged1 and GAPDH specific antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FLAG monoclonal antibody was obtained from Sigma Aldrich (St. Louis, MO). The secondary antibodies against mouse and rabbit IgG labeled with horseradish peroxidase were purchased from Cell Signaling Technology.

2.11. Statistical analysis

Two-tailed *t* tests were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Values were considered statistically significant at $p < 0.05$.

3. Results

3.1. Cell density-dependent apoptosis is regulated by IL-6-mediated STAT3 activation in NIH 3T3 cells

Previous reports have shown that both proportion of cells undergoing apoptosis (Brezden and Rauth, 1996; Fiore and Degraffi, 1999; Long et al., 2003; Saeki et al., 1997) and the expression of phosphorylated STAT3 (Vultur et al., 2004) increase in a cell density-dependent manner. In our study, phosphorylated STAT3 expression increased in a time-dependent manner with its highest expression at 48 h when cells were confluent (Fig. 1A, left). In addition, the expression of phosphorylated STAT3 was higher in cells cultured at 1.5×10^5 cells/well compared with cells cultured at 1.5×10^4 cells/well at 72 h (Fig. 1A, right). Both the percentage of apoptotic cells as measured by annexin V staining (Fig. 1B, left) and the expression of cleaved caspase 3 (Fig. 1B, right) were higher in cells cultured at the higher cell density. To examine whether these findings were also observed in other type of fibroblasts, fibroblasts isolated from the lungs of BALB/c mice were cultured at different densities. In line with the results from NIH 3T3 cells, a cell density-dependent increase in phosphorylated STAT3 and cleaved caspase3 expression (Fig. 1C, right) as well as in the proportion of apoptotic cells (Fig. 1C, left) were observed in primary lung fibroblasts. WP1066, an inhibitor of STAT3 phosphorylation (Horiguchi et al., 2010), increased the fraction of apoptotic cells (Fig. 1D, left), which was associated with an increase in the number of cells with rounded morphology (Mills et al., 1999), in cells cultured at 1.5×10^5 cells/well, but not in cells cultured at 1.5×10^4 cells/well (Fig. 1D, right). These results suggest cell density-dependent activation of STAT3 confers resistance to apoptosis. Since IL-6 is a major contributor for STAT3 phosphorylation, we next analyzed the expression of this cytokine. IL-6 mRNA expression was significantly higher in cells cultured at 1.5×10^5 cells/well than in cells cultured at 1.5×10^4 cells/well (Fig. 1E, left). IL-6 protein was detected only in the supernatants of cells cultured at 1.5×10^5 cells/well (Fig. 1E, right). To examine whether IL-6 regulated apoptosis through the activation of STAT3, cells were treated with anti-IL-6 receptor antibody to suppress IL-6 signal transduction. Anti-IL-6 receptor antibody significantly decreased the expression of phosphorylated STAT3 (Fig. 1F,

left) and increased the fraction of apoptotic cells (Fig. 1F, middle) as well as the number of cells with round shape (Fig. 1F, right) in cells cultured at 1.5×10^5 cells/well, but not in cells cultured at 1.5×10^4 cells/well. Together, these results demonstrate that cell density-dependent apoptosis is regulated by IL-6-mediated STAT3 phosphorylation in NIH 3T3 cells.

3.2. Notch signaling is activated as cell density increases

Next, we analyzed the expression profile of Notch signaling molecules in cells grown at differing densities. Real-time RT-PCR indicated that the expression of Jagged1, Jagged2 and Delta-like4 mRNA was upregulated in cells cultured at 1.5×10^5 cells/well compared to those cultured at 1.5×10^4 cells/well (Fig. 2A). Delta-like1 mRNA expression was not detected in these cultures. As for Notch receptors, Notch1, Notch2 and Notch3 transcript expression was significantly higher in cells cultured at a higher density (Fig. 2A). The increased expression of Notch1 and Jagged1 was confirmed at the protein level as well by immunoblotting (Fig. 2B). Activation of Notch signaling at a higher cell density was demonstrated by an increase in N1ICD expression (Fig. 2B). However, transcript expression of Hey1 (one of the canonical Notch target genes) was not increased under dense culture condition (Fig. 1C). Since Notch signaling can exert its effect via CSL without affecting Hey genes (Stockhausen et al., 2010), we then analyzed the activity of a CSL luciferase reporter construct in cultures of differing cell densities. Reporter activity was significantly higher in cells cultured at higher density (Fig. 2D), providing additional evidence of cell density-dependent Notch signaling. Time-dependent increases in both Jagged1 and Notch1 transcript and protein expression along with N1ICD protein expression were also observed (Fig. 2E). Thus, these results demonstrate that Notch signaling is activated with increased NIH 3T3 cell density with the induction of Jagged1 and Notch1.

3.3. Notch signaling activation is necessary for IL-6-mediated apoptosis regulation

To address the role of Notch signaling in cell density-dependent apoptosis, the effect of Notch pathway inhibition was evaluated. First, expression of Jagged1, which was most significantly induced among Notch ligands at high cell density, was suppressed by transiently transfecting siRNA against this gene. Inhibition of Jagged1 expression, which was confirmed by immunoblotting (Fig. 3A), significantly reduced IL-6 (Fig. 3B) and phosphorylated STAT3 (Fig. 3A) expression and increased the number of apoptotic cells (Fig. 3C). Next, the effect of GSI was examined. GSI significantly decreased the expression of N1ICD, showing that Notch1 activation was actually suppressed by GSI (Fig. 4A). In cells treated with GSI, IL-6 mRNA expression was significantly lower than in vehicle-treated cells during the 72 h of incubation (Fig. 4B, left). IL-6 protein level in culture supernatant was also reduced in GSI-treated cells (Fig. 4B, right). To examine whether Notch signaling regulation of apoptosis is dependent on IL-6, the effect of IL-6 replacement was analyzed in cells cultured with or without GSI. As expected, recombinant IL-6 markedly increased STAT3 phosphorylation in vehicle-treated cells (Fig. 4C), and GSI significantly reduced phosphorylated STAT3 expression (Fig. 4C) and increased the number of cells undergoing apoptosis (Fig. 4D). Addition of recombinant IL-6 to GSI-treated cells notably increased STAT3 phosphorylation (Fig. 4C), which was accompanied by a significant reduction in the number of apoptotic cells (Fig. 4D). Collectively, these results

indicate that Jagged1-mediated Notch1 activation is necessary for IL-6/STAT3-mediated regulation of cell density-dependent apoptosis in NIH 3T3 cells.

3.4. Constitutive activation of Notch1 is sufficient for the induction of IL-6

As demonstrated above, Notch signaling activation was required for cell density-dependent induction of IL-6. To determine whether Notch signaling activation was sufficient for the induction of IL-6, N1ICD was transiently transfected into NIH 3T3 cells to mimic activation of Notch1. Overexpression of N1ICD (Fig. 5A) induced IL-6 expression at both the mRNA and protein levels, which was accompanied by an increase in STAT3 phosphorylation (Fig. 5A and B). Interestingly, transduction of N1ICD also induced Jagged1 mRNA and protein expression (Fig. 5A and C). These results demonstrate that constitutive activation of Notch1 is sufficient for the induction of IL-6 and Jagged1.

3.5. Notch signaling directly activates IL-6 transcription

Finally, to elucidate the molecular mechanism through which Notch signaling induced IL-6 expression, we focused on the promoter region of IL-6. Previous reports have shown a functional CSL binding sequence in the IL-6 promoter located at -67 to -60 from the transcription start site (Palmieri et al., 1999; Plaisance et al., 1997). To determine whether N1ICD actually bound to this sequence in NIH 3T3 cells, ChIP assay was performed using cells overexpressing FLAG-tagged N1ICD. PCR was performed using purified DNA as a template and primers targeting the CSL binding sequence in the IL-6 promoter (Fig. 6A). DNA fragments flanking the CSL binding sequence were enriched in the FLAG immunoprecipitates (Fig. 6B). Furthermore, in ChIP assay using cells cultured at high cell density and antibody against Notch1, DNA fragment containing the CSL binding site was enriched in the Notch1 immunoprecipitates, suggesting the recruitment of Notch1 to the IL-6 promoter. Next, to examine whether Notch signaling directly modulated IL-6 transcription, luciferase assay was performed using cells transfected with luciferase reporter construct and either N1ICD or empty vector. Reporter constructs containing IL-6 promoter with or without point mutations in CSL binding sequence were prepared (Fig. 7A). N1ICD significantly increased the activity of the IL-6 promoter without mutations in the CSL binding sequence when compared with the empty vector (Fig. 7B). In contrast, N1ICD did not enhance the activity of the IL-6 promoter harboring mutations in the CSL binding sequence (Fig. 7B). Thus, these results demonstrate that activated Notch1 induces IL-6 transcription via CSL-mediated direct promoter activation.

4. Discussion

Cells sense changes in their surrounding environment and adapt via the modulation of gene expression. Dense culture condition increases the proportion of cells undergoing apoptosis (Long et al., 2003; Saeki et al., 1997; Vultur et al., 2004), which, together with the regulation of cell cycle and proliferation, appears to be a physiologically critical mechanism to maintain appropriate cell density. Notch signaling is a highly conserved cell-to-cell communication mechanism that plays a critical role in cell survival under stress conditions (Croquelois et al., 2008; Mo et al., 2013). In the current study, we investigated the role of Notch signaling in cell density-dependent apoptosis of NIH 3T3 cells. At a high cell density,

activated Notch signaling induced IL-6 expression, which, with subsequent STAT3 activation, inhibited the induction of apoptosis. These results underscore a novel role for Notch signaling in cell survival during environmental stress induced by increased cell density.

Since impaired apoptosis of fibroblasts may promote tissue fibrosis (Desmoulière et al., 2003; Moodley et al., 2003), unraveling the molecular mechanisms leading to apoptosis resistance is of significant therapeutic importance. STAT3 is a transcription factor that plays a pivotal role in cell proliferation, differentiation, immune response, angiogenesis and apoptosis. Previous studies have shown that STAT3 regulates apoptosis via its modulation of Bcl-2 (Fukada et al., 1996) and p53 (Niu et al., 2005) transcription. In fibrotic lung disease, activated STAT3 was observed in fibroblasts in the areas of dense fibrosis (Pechkovsky et al., 2012). Furthermore, STAT3 activation conferred resistance to apoptosis in fibroblasts derived from patients with idiopathic pulmonary fibrosis in vitro (Pechkovsky et al., 2012), suggesting a link between cell density, STAT3 activation, and resistance to apoptosis as observed in our study. Cell density-dependent STAT3 activation might represent a mechanism by which fibroblasts develop a resistance to apoptosis and thus exert pathogenic properties in fibrotic diseases.

Cell density-dependent STAT3 phosphorylation was shown to be ligand-independent in cancer cells (Steinman et al., 2003), while, in NIH 3T3 cells, it was observed to be mediated by JAK kinases (a family of non-receptor tyrosine kinases that transduce cytokine signaling (Vultur et al., 2004)). These divergent findings suggest that pathways leading to STAT3 activation are cell type-specific. The JAK/STAT pathway is activated by a wide array of cytokines and growth factors, including IL-6. Previous studies have also shown crosstalk between Notch and JAK-STAT signaling (Kamakura et al., 2004). In the current study, we observed a significant contribution of IL-6 to STAT3 activation (Fig. 1F), suggesting the Notch-IL-6 axis as a predominant cause of cell-density dependent JAK/STAT3 activation in NIH 3T3 cells.

Dysregulated IL-6 expression plays a pivotal role in the pathogenesis of diseases such as chronic inflammation and cancer (Gao et al., 2007; Rossi et al., 2015; Sansone et al., 2007). Through the blockade of the Notch pathway either via genetic (Fig. 3B) or pharmacologic (Fig. 4B) methods, we demonstrated that Notch signaling activation was necessary for the cell density-dependent induction of IL-6 in NIH 3T3 cells. Furthermore, Notch activation through NICD overexpression was sufficient for the induction of IL-6 (Fig. 5B). Thus, these results clearly demonstrate the importance of Notch signaling in regulating IL-6 expression and suggest the Notch pathway might be a therapeutic target to modulate IL-6 expression.

CSL is a DNA binding protein acting as either a transcriptional repressor or an activator of target genes depending on the Notch signaling status. CSL regulates transcription when Notch is not activated, while, upon Notch signaling activation, it binds to NICD to induce transcription. In human dermal fibroblasts, both CSL silencing by siRNA and inducible expression of activated Notch1 induced IL-6 expression (Procopio et al., 2015), suggesting an importance of a canonical Notch pathway in IL-6 induction. A CSL binding motif has been discovered at -67 to -60 from the transcription start site of IL-6 and CSL binding to

this site represses IL-6 transcription (Kannabiran et al., 1997; Palmieri et al., 1999; Plaisance et al., 1997; Sethi et al., 2011). An induction of point mutations in this CSL binding site only slightly increased the IL-6 promoter activity in cells transfected with empty vector (Fig. 7B), suggesting that CSL plays a minor role in the repression of IL-6 transcription in NIH 3T3 cells. However, our study demonstrated that N1ICD bound to this CSL site (Fig. 6B and C) and activated IL-6 promoter activity via a CSL-dependent mechanism (Fig. 7B). Canonical (i.e. CSL-dependent) Notch activation of IL-6 transcription was also reported in RAW264.7 macrophages (Wongchana and Palaga, 2012), while in MCF-7 breast tumor cells, Notch upregulation of IL-6 was mediated by a non-canonical mechanism (Jin et al., 2013). Furthermore, in HeLa cervical cancer cells, Notch activation via NICD transfection failed to promote IL-6 transcription (Palmieri et al., 1999). These results suggest that the effect of Notch activation on IL-6 transcription as well as the manner in which Notch induces IL-6 expression are cell type dependent. A functional NF- κ B binding site is present at -73 to -64 in the IL-6 promoter, which plays a critical role in transcriptional activation by stimuli such as LPS and TNF- α (Libermann and Baltimore, 1990). This NF- κ B binding site partially overlaps with CSL binding site in the IL-6 promoter, implicating an interaction between these transcription factors. In fact, N1ICD induction of IL-6 expression was completely abrogated by NF- κ B inhibition (Wongchana and Palaga, 2012), suggesting a role for NF- κ B as a downstream effector of Notch pathway. Further studies are required to address the role of NF- κ B and its crosstalk with Notch pathway in cell density-dependent induction of IL-6.

Interestingly, constitutive activation of Notch signaling significantly increased Jagged1 expression (Fig. 5A and C), suggesting the induction of Jagged1 through a positive auto-regulatory mechanism. Since Notch signaling is activated via a direct cell-cell contact, Jagged1 induction through Notch activation itself might be effective in coordinately amplifying cellular responses between juxtaposed cells. Different Notch ligands and receptors might have different, even opposing, effect on downstream target genes (Amsen et al., 2004; Benedito et al., 2009; Cheng et al., 2007). Herein, we focused on Jagged1 and Notch1 because the expression of both was most significantly induced at higher density. While clarifying the role of other Notch ligands and receptors requires further investigation, the effect of Jagged1 knock down alone significantly reduced the expression of IL-6, suggesting that it was the major Notch ligand accounting cell density-dependent Notch signaling.

Previous studies are inconsistent regarding the role of Notch signaling in fibroblast apoptosis. Forced expression of N1ICD induced apoptosis in mouse embryonic fibroblasts (Ishikawa et al., 2008), whereas Notch1 activation had no effect on the incidence of apoptosis in human dermal fibroblasts (Liu et al., 2012). In NIH 3T3, cell-to-cell contact, which is a prerequisite for Notch activation, induced STAT3 phosphorylation (Vultur et al., 2004). To address the role of Notch activation independently of cell-to-cell contact in the induction of apoptosis, we examined the effect of N1ICD overexpression on the incidence of apoptosis in cells in which cell-cell contact was inhibited. Following transfection with either N1ICD or a control vector, cells were dissociated with EGTA/EDTA treatment and incubated with calcium-free DMEM to inhibit cell-to-cell adhesion (Vultur et al., 2004). Considerably high fraction of apoptotic cells (about 60%) was observed in both groups, making the analysis of the role of Notch signaling difficult (data not shown). While the

contribution of other mechanisms is not precluded, Notch signaling via STAT3 appears to represent a major pathway downstream of cell-to-cell contact in the regulation of cell density-dependent apoptosis.

In conclusion, we demonstrate that Notch induction of IL-6 confers resistance to cell density-dependent apoptosis in NIH 3T3 cells. Dysregulated apoptosis is implicated in the pathogenesis of diseases with aberrant cell proliferation. Notch-IL6 axis might represent a mechanism whereby cells acquire apoptosis resistance and therefore might be a novel therapeutic target to regulate uncontrolled cell growth. The putative mechanisms through which Notch signaling regulates cell density-dependent apoptosis are summarized in Fig. 8.

Acknowledgements

We thank Dr. Hyunggee Kim (Korea University, Seoul, Republic of Korea) for the generous gift of pGL3-CSL and Dr. Raphael Kopan (Washington University, St Louis, MO) for the generous gift of p3xFLAG-CMV7-N1ICD.

Funding source

This work was supported in part by the Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (16K09571) (to Y.M.).

References

- Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA, 2004 Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117, 515–526. [PubMed: 15137944]
- Benedito R, Roca C, Sörensen I, Adams S, Gossler A, Fruttiger M, Adams RH, 2009 The Notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* 137, 1124–1135. 10.1016/j.cell.2009.03.025. [PubMed: 19524514]
- Brezden CB, Rauth AM, 1996 Differential cell death in immortalized and non-immortalized cells at confluency. *Oncogene* 12, 201–206. [PubMed: 8552393]
- Bromberg J, Darnell JE, 2000 The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19, 2468–2473. 10.1038/sj.onc.1203476. [PubMed: 10851045]
- Cheng P, Nefedova Y, Corzo CA, Gabrilovich DI, 2007 Regulation of dendritic-cell differentiation by bone marrow stroma via different Notch ligands. *Blood* 109, 507–515. 10.1182/blood-2006-05-025601. [PubMed: 16973960]
- Croquelois A, Domenghetti AA, Nemir M, Lepore M, Rosenblatt-Velin N, Radtke F, Pedrazzini T, 2008 Control of the adaptive response of the heart to stress via the Notch1 receptor pathway. *J. Exp. Med* 205, 3173–3185. 10.1084/jem.20081427. [PubMed: 19064701]
- Desmoulière A, Darby IA, Gabbiani G, 2003 Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab. Invest* 83, 1689–1707. [PubMed: 14691287]
- Fiore M, Degraffi F, 1999 Dimethyl sulfoxide restores contact inhibition-induced growth arrest and inhibits cell density-dependent apoptosis in hamster cells. *Exp. Cell Res* 251, 102–110. 10.1006/excr.1999.4542. [PubMed: 10438575]
- Fukada T, Hibi M, Yamanaka Y, Takahashi-Tezuka M, Fujitani Y, Yamaguchi T, Nakajima K, Hirano T, 1996 Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity* 5, 449–460. [PubMed: 8934572]
- Gao SP, Mark KG, Leslie K, Pao W, Motoi N, Gerald WL, Travis WD, Bornmann W, Veach D, Clarkson B, Bromberg JF, 2007 Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. *J. Clin. Invest* 117, 384–3856. 10.1172/JCI31871.

- Hogaboam CM, Bone-Larson CL, Lipinski S, Lukacs NW, Chensue SW, Strieter RM, Kunkel SL, 1999 Differential monocyte chemoattractant protein-1 and chemokine receptor 2 expression by murine lung fibroblasts derived from Th1- and Th2- type pulmonary granuloma models. *J. Immunol* 163, 2193–2201. [PubMed: 10438961]
- Horiguchi A, Asano T, Kuroda K, Sato A, Asakuma J, Ito K, Hayakawa M, Sumitomo M, Asano T, 2010 STAT3 inhibitor WP1066 as a novel therapeutic agent for renal cell carcinoma. *Br. J. Cancer* 102, 1592–1599. 10.1038/sj.bjc.6605691. [PubMed: 20461084]
- Horowitz JC, Lee DY, Waghray M, Keshamouni VG, Thomas PE, Zhang H, Cui Z, Thannickal VJ, 2004 Activation of the pro-survival phosphatidylinositol 3-Kinase/AKT pathway by transforming growth factor- β 1 in mesenchymal cells is mediated by p38 MAPK-dependent induction of an autocrine growth factor. *J. Biol. Chem* 279, 1359–1367. 10.1074/jbc.M306248200. [PubMed: 14576166]
- Ishikawa Y, Onoyama I, Nakayama KI, Nakayama K, 2008 Notch-dependent cell cycle arrest and apoptosis in mouse embryonic fibroblasts lacking Fbxw7. *Oncogene* 27, 6164–6174. 10.1038/onc.2008.216. [PubMed: 18641686]
- Jin S, Mutvei AP, Chivukula IV, Andersson ER, Ramsköld D, Sandberg R, Lee KL, Kronqvist P, Mamaeva V, Östling P, Mpindi J-P, Kallioniemi O, Screpanti I, Poellinger L, Sahlgren C, Lendahl U, 2013 Non-canonical Notch signaling activates IL-6/JAK/STAT signaling in breast tumor cells and is controlled by p53 and IKK α /IKK β . *Oncogene* 32, 4892–4902. 10.1038/onc.2012.517. [PubMed: 23178494]
- Kamakura S, Oishi K, Yoshimatsu T, Nakafuku M, Masuyama N, Gotoh Y, 2004 Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nat. Cell Biol* 6, 547–554. 10.1038/ncb1138. [PubMed: 15156153]
- Kannabiran C, Zeng X, Vales LD, 1997 The mammalian transcriptional repressor RBP (CBF1) regulates interleukin-6 gene expression. *Mol. Cell. Biol* 17, 1–9. [PubMed: 8972179]
- Kida H, Mucenski ML, Thitoff AR, Le Cras TD, Park K-S, Ikegami M, Müller W, Whitsett JA, 2008 GP130-STAT3 regulates epithelial cell migration and is required for repair of the bronchiolar epithelium. *Am. J. Pathol* 172, 1542–1554. 10.2353/ajpath.2008.071052. [PubMed: 18467707]
- Liebermann TA, Baltimore D, 1990 Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol. Cell. Biol* 10, 2327–2334. [PubMed: 2183031]
- Liu Z-J, Li Y, Tan Y, Xiao M, Zhang J, Radtke F, Velazquez OC, 2012 Inhibition of fibroblast growth by Notch1 signaling is mediated by induction of Wnt11-dependent WISP-1. *PLoS One* 7, e38811 10.1371/journal.pone.0038811. [PubMed: 22715413]
- Long H, Han H, Yang B, Wang Z, 2003 Opposite cell density-dependence between spontaneous and oxidative stress-induced apoptosis in mouse fibroblast L-cells. *Cell. Physiol. Biochem* 13, 401–414. 10.1159/000075128. [PubMed: 14631147]
- Mills JC, Stone NL, Pittman RN, 1999 Extranuclear apoptosis. The role of the cytoplasm in the execution phase. *J. Cell Biol* 146, 703–708. 10.1083/JCB.146.4.703. [PubMed: 10459006]
- Mo J-S, Yoon J-H, Ann E-J, Ahn J-S, Baek H-J, Lee H-J, Kim S-H, Kim Y-D, Kim M-Y, Park H-S, 2013 Notch1 modulates oxidative stress induced cell death through suppression of apoptosis signal-regulating kinase 1. *Proc. Natl. Acad. Sci. U. S. A* 110, 6865–6870. 10.1073/pnas.1209078110. [PubMed: 23569274]
- Moodley YP, Misso NLA, Scaffidi AK, Fogel-Petrovic M, McAnulty RJ, Laurent GJ, Thompson PJ, Knight DA, 2003 Inverse effects of interleukin-6 on apoptosis of fibroblasts from pulmonary fibrosis and Normal lungs. *Am. J. Respir. Cell Mol. Biol* 29, 490–498. 10.1165/rcmb.2002-0262OC. [PubMed: 12714376]
- Niu G, Wright KL, Ma Y, Wright GM, Huang M, Irby R, Briggs J, Karras J, Cress WD, Pardoll D, Jove R, Chen J, Yu H, 2005 Role of Stat3 in regulating p53 expression and function. *Mol. Cell. Biol* 25, 7432–7440. 10.1128/MCB.25.17.7432-7440.2005. [PubMed: 16107692]
- Noseda M, Chang L, McLean G, Grim JE, Clurman BE, Smith LL, 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. 10.1128/MCB.24.20.8813-8822.2004. [PubMed: 15456857]

- Palmieri M, Sasso MP, Monese R, Merola M, Faggioli L, Tovey M, Furia A, 1999 Interaction of the nuclear protein CBF1 with the kappaB site of the IL-6 gene promoter. *Nucleic Acids Res* 27, 2785–2791. [PubMed: 10373597]
- Pechkovsky DV, Prele CM, Wong J, Hogaboam CM, McAnulty RJ, Laurent GJ, Zhang SS-M, Selman M, Mutsaers SE, Knight DA, 2012 STAT3-mediated signaling dysregulates lung fibroblast-myofibroblast activation and differentiation in UIP/IPF. *Am. J. Pathol* 180, 1398–1412. 10.1016/j.ajpath.2011.12.022. [PubMed: 22322297]
- Plaisance S, Vanden Berghe W, Boone E, Fiers W, Haegeman G, 1997 Recombination signal sequence binding protein κ is constitutively bound to the NF- κ B site of the interleukin-6 promoter and acts as a negative regulatory factor. *Mol. Cell. Biol* 17, 3733–3743. [PubMed: 9199307]
- Procopio M-G, Laszlo C, Al Labban D, Kim DE, Bordignon P, Jo S-H, Goruppi S, Menietti E, Ostano P, Ala U, Provero P, Hoetzenecker W, Neel V, Kilarski WW, Swartz MA, Brisken C, Lefort K, Dotto GP, 2015 Combined CSL and p53 downregulation promotes cancer-associated fibroblast activation. *Nat. Cell Biol* 17, 1193–1204. 10.1038/ncb3228. [PubMed: 26302407]
- Rossi J-F, Lu Z-Y, Jourdan M, Klein B, 2015 Interleukin-6 as a therapeutic target. *Clin. Cancer Res* 21, 1248–1257. 10.1158/1078-0432.CCR-14-2291. [PubMed: 25589616]
- Saeki K, Yuo A, Kato M, Miyazono K, Yazaki Y, Takaku F, 1997 Cell density-dependent apoptosis in HL-60 cells, which is mediated by an unknown soluble factor, is inhibited by transforming growth factor beta1 and overexpression of Bcl-2. *J. Biol. Chem* 272, 20003–20010. [PubMed: 9242670]
- Sansone P, Storci G, Tavoroli S, Guarnieri T, Giovannini C, Taffurelli M, Ceccarelli C, Santini D, Paterini P, Marcu KB, Chieco P, Bonafè M, 2007 IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *J. Clin. Invest* 117, 3988–4002. 10.1172/JCI32533. [PubMed: 18060036]
- Sethi N, Dai X, Winter CG, Kang Y, 2011 Tumor-derived jagged1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. *Cancer Cell* 19, 192–205. 10.1016/j.ccr.2010.12.022. [PubMed: 21295524]
- Shen Y, Devgan G, Darnell JE, Bromberg JF, 2001 Constitutively activated Stat3 protects fibroblasts from serum withdrawal and UV-induced apoptosis and antagonizes the proapoptotic effects of activated Stat1. *Proc. Natl. Acad. Sci* 98, 1543–1548. 10.1073/pnas.041588198. [PubMed: 11171987]
- Steinman RA, Wentzel A, Lu Y, Stehle C, Grandis JR, 2003 Activation of stat3 by cell confluence reveals negative regulation of stat3 by cdk2. *Oncogene* 22, 3608–3615. 10.1038/sj.onc.1206523. [PubMed: 12789269]
- Stockhausen M-T, Kristoffersen K, Poulsen HS, 2010 The functional role of Notch signaling in human gliomas. *Neuro. Oncol* 12, 199–211. 10.1093/neuonc/nop022. [PubMed: 20150387]
- Vultur A, Cao J, Arulanandam R, Turkson J, Jove R, Greer P, Craig A, Elliott B, Raptis L, 2004 Cell-to-cell adhesion modulates stat3 activity in normal and breast carcinoma cells. *Oncogene* 23, 2600–2616. 10.1038/sj.onc.1207378. [PubMed: 15007380]
- Wallach-Dayana SB, Golan-Gerstl R, Breuer R, 2007 Evasion of myofibroblasts from immune surveillance: a mechanism for tissue fibrosis. *Proc. Natl. Acad. Sci* 104, 20460–20465. 10.1073/pnas.0705582104. [PubMed: 18077384]
- Wongchana W, Palaga T, 2012 Direct regulation of interleukin-6 expression by Notch signaling in macrophages. *Cell. Mol. Immunol* 9, 155–162. 10.1038/cmi.2011.36. [PubMed: 21983868]
- Yu H, Pardoll D, Jove R, 2009 STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat. Rev. Cancer* 9, 798–809. 10.1038/nrc2734. [PubMed: 19851315]
- Yu H, Lee H, Herrmann A, Buettner R, Jove R, 2014 Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat. Rev. Cancer* 14, 736–746. 10.1038/nrc3818. [PubMed: 25342631]

Fig. 1.

Cell density-dependent activation of IL-6/STAT3 regulates apoptosis in NIH 3T3 cells. Cells were cultured at a density of either 1.5×10^4 cells/well or 1.5×10^5 cells/well in 12-well plates for 72 h unless otherwise indicated. **(A)** Protein expression of STAT3 and phosphorylated STAT3 in NIH 3T3 cells cultured at a density of 1.5×10^5 cells/well at the indicated times (left) and in cells cultured at a density of either 1.5×10^4 cells/well or 1.5×10^5 cells/well at 72 h (right). **(B)** Fraction of annexin V-positive apoptotic cells (left) and protein expression of cleaved caspase 3 (right). **(C)** Fraction of annexin V-positive apoptotic

cells (left) and protein expression of STAT3, phosphorylated STAT3 and cleaved caspase 3 (right) in fibroblasts isolated from the lungs of BALB/c mice. **(D)** Fraction of apoptotic cells (left) and representative photomicrographs of cells (right) cultured in the absence or presence of WP1066 for 72 h (left). **(E)** IL-6 mRNA (left) and protein in the culture supernatant (right) as analyzed by real-time RT-PCR and ELISA, respectively. **(F)** Protein expression of STAT3 and phosphorylated STAT3 (left), fraction of apoptotic cells (middle) and representative photomicrographs of cells (right) cultured in the absence or presence of anti-IL-6 receptor antibody for 72 h. GAPDH was used as an internal or a loading control in **(A)**, **(B)**, **(C)** and **(F)**. Data are representative of three independent experiments and are shown as the mean $\pm$ SEM ($n = 3$). * $p < 0.05$.

Fig. 2.

Cell density-dependent Notch signaling activation in NIH 3T3 cells. Cells were cultured at a density of either 1.5×10^4 cells/well or 1.5×10^5 cells/well for 72 h. mRNA expression of Notch ligands (upper) and receptors (lower) (**A**), protein expression of Jagged1, Notch1 and Notch1 intracellular domain (**B**) and mRNA expression of Hey1 (**C**) were measured. (**D**) Luciferase assay of NIH 3T3 cells transfected with pGL3-CSL and pGL4.74. Results are expressed as the fold induction above cells cultured at a density of 1.5×10^4 cells/well. (**E**) mRNA expression of Jagged1 and Notch1 (left) and protein expression of Jagged1, Notch1

and Notch1 intracellular domain a (right) in NIH 3T3 cells cultured for the indicated times. GAPDH was used as an internal or a loading control. Data are representative of three independent experiments and are shown as the mean $\pm$ SEM (n = 3). * $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

Jaggedl expression is necessary for the induction of IL-6 in NIH 3T3 cells. NIH 3T3 cells were transiently transfected with small interfering RNA (siRNA) against Jaggedl or control siRNA for 48 h. Protein expression of Jaggedl, STAT3 and phosphorylated STAT3 (**A**), mRNA and protein expression of IL-6 (**B**) and fraction of apoptotic cells (**C**) were measured. GAPDH was used as an internal or a loading control. Data are representative of three independent experiments and are shown as the mean $\pm$ SEM ($n = 3$). * $p < 0.05$.

Fig. 4.

γ -secretase inhibitor regulates IL-6 expression in NIH 3T3 cells. NIH 3T3 cells were cultured in the presence or absence of γ -secretase inhibitor (GSI) (20 μ M) for 72 h. Protein expression of Notch1 intracellular domain (**A**), mRNA expression of IL-6 at indicated times and IL-6 protein in the culture supernatant (**B**) were measured. NIH 3T3 cells were cultured in the presence or absence of recombinant mouse IL-6 (50 ng/ml) with DMSO or GSI (20 μ M) for 72 h. Protein expression for STAT3 and phosphorylated STAT3 (**C**) and fraction of cells undergoing apoptosis (**D**) were measured. GAPDH was used as an internal or a loading control. Data are representative of three independent experiments and are shown as the mean $\pm$ SEM ($n = 3$). * $p < 0.05$. vs. corresponding DMSO. # $p < 0.05$ vs. DMSO, rIL-6-. § $p < 0.05$ vs. GSI, rIL-6-.

Fig. 5.

Constitutive activation of Notch signaling is sufficient for the induction of IL-6. NIH 3T3 cells were transiently transduced with either FLAG-tagged N1ICD or empty vector for 48 h. Protein expression of epitope tag FLAG, Jagged1, STAT3 and phosphorylated STAT3 (**A**), IL-6 mRNA and protein expression (**B**) and Jagged1 mRNA expression (**C**) were measured. GAPDH was used as an internal or a loading control. Data are representative of three independent experiments and are shown as the mean $\pm$ SEM ($n = 3$). * $p < 0.05$.

Fig. 6. Identification of a CSL binding site in mouse IL-6 promoter. **(A)** Schematic representation of the mouse IL-6 promoter covering 1000 bp upstream of transcription start site indicating the location of putative CSL binding site and DNA sequence amplified in ChIP assay. **(B)** ChIP analysis of NIH 3T3 cells transfected with FLAG-tagged N1ICD for 48 h. Rabbit anti-FLAG antibodies and control rabbit IgG were used to immunoprecipitate protein-DNA complexes. Enrichment of DNA was detected by PCR using primers spanning putative the CSL binding sequence.

Fig. 7.

Notch signaling directly activates IL-6 transcription. **(A)** Schematic representation of the IL-6 promoter constructs containing either intact (upper) or mutated (lower) CSL binding site. **(B)** Luciferase assay of NIH 3T3 cells co-transfected with p3xFLAG-CMV7 (empty) or p3xFLAG-CMV7-N1ICD (N1ICD), IL-6 promoter construct with intact (mut-) or mutated (mut+) CSL binding site, and pGL4.74 for 48 h. Results are expressed as the fold induction above cells transfected with p3xFLAG-CMV7 and IL-6 promoter construct with intact CSL

binding site. Data are representative of three independent experiments and are shown as the mean $\pm$ SEM of 3 determinations. * $p < 0.05$ vs. empty, mut-.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Under dense culture condition

Fig. 8.

Schematic representation of the regulation of cell density-dependent apoptosis by Notch signaling. Under dense culture conditions, activated Notch signaling, which is mediated by Jagged1 and Notch1, induces IL-6 expression through direct transcriptional activation. STAT3 is phosphorylated by secreted IL-6 and induces its target genes expression, resulting in the regulation of apoptosis induction.