

# Effect of soluble Jagged1-mediated inhibition of Notch signaling on proliferation and differentiation of an adipocyte progenitor cell model

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**Abbreviations:** C/EBP, CCAAT enhancer binding protein; EC, endothelial cell; EGF, epidermal growth factor; FABP, fatty acid binding protein; FGF, fibroblast growth factor; IL, Interleukin; NICD, Notch intracellular domain; PECAM, platelet endothelial cell adhesion molecule 1; PPAR, peroxisome proliferator-activated receptor; sDl1, soluble Delta-like 1; sJag1, soluble Jagged1; VEGF, vascular endothelial growth factor

Adipose tissue development is dependent on multiple signaling mechanisms and cell-cell interactions that regulate adipogenesis, angiogenesis and extracellular remodeling. The Notch signaling pathway is an important cell-fate determinant whose role in adipogenesis is not clearly defined. To address this issue, we examined the effect of inhibition of Notch signaling by soluble Jagged1 in the 3T3-L1 preadipocyte line. In vitro, soluble Jagged1 expression in 3T3-L1 cells altered cell morphology, increased the rate of cell proliferation and induced an early transcriptional response to differentiation stimuli. However, these cells did not form mature adipocytes due to their inability to exit the cell-cycle in response to serum-starvation and glucocorticoid-induced cell-cycle arrest. In contrast, subcutaneous allografts of soluble Jagged1 cells formed larger fat pads containing lipid-filled adipocytes with improved neovascularization compared with controls. Since adipogenesis is tightly associated with angiogenesis, we evaluated the influence of soluble Jagged1 on endothelial cells by culturing them in cell-free conditioned media from preadipocytes. Soluble Jagged1-mediated inhibition of Notch signaling increased levels of secreted cytokines, potentially contributing to the improved cell growth and proliferation observed in these cultures. Our findings demonstrate an initial requirement of Notch signaling inactivation for preadipocyte cell commitment and support the hypothesis that cell-to-cell crosstalk between the preadipocytes and endothelial cells is required for neovascularization and remodeling of the tissue to promote hyperplasia and hypertrophy of differentiating adipocytes.

## Introduction

Adipose tissue development is a complex process involving adipogenesis, angiogenesis and extracellular matrix remodeling.<sup>1–4</sup> It is therefore imperative to understand the dynamics of the responsible factors, the cell-cell signaling and cross talk.<sup>5</sup> While pro-angiogenic factors like plasminogen activator inhibitor-1 (PAI-1), angiotensin II, phospholipid transfer protein (PLTP), prostaglandin and cholesterol ester transfer protein (CETP)<sup>1,6</sup> secreted from adjacent adipocytes into the blood stream initiate endothelial cell recruitment to expanding adipocytes,<sup>7,8</sup> adipose tissue itself functions as an endocrine tissue secreting hormones with cytokine properties like the adipokines, leptin and adiponectin.<sup>9–13</sup> These hormones act in a paracrine fashion to support adipose tissue development, and also act on a variety of tissues to regulate the metabolism of the entire organism. Yet, the molecular

mechanisms regulating cross talk between adipogenic and endothelial cells within the tissue is not clearly understood.

An important modulator of vascular development and angiogenesis as well as adipogenesis, is the Notch signaling pathway.<sup>14–18</sup> Notch proteins are a family of transmembrane receptors whose intercellular signaling determines cell fate in almost all cell types and tissues. Four mammalian Notch receptors (Notch1–Notch4) and 6 Notch ligands (Delta-like 1, 2, 4, Jagged1, 2, F3/contactin) have been identified.<sup>19,20</sup> Notch ligand and receptor interaction leads to proteolytic cleavage and release of the Notch intracellular domain (Notch ICD) from the plasma membrane which translocates to the nucleus and regulates transcription of downstream targets.<sup>21</sup>

Notch signaling is complex and marked by diverse outcomes that reflect different combinations of specific receptor(s)/ligand(s) interaction, the cellular environmental context and developmental

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outcomes specific to the NotchICD (e.g., stem cell maintenance, progenitor selection, growth organizing boundaries, cell growth or inhibition, differentiation).<sup>19,22,23</sup> In the context of adipocytes, studies on Notch signaling functionality and target gene expression indicate inhibition of Notch signaling to promote adipocyte differentiation in mesenchymal stem cells (MSCs)<sup>24</sup> and preadipocyte cell line 3T3-L1 cells<sup>25</sup> in the early stages. The clonally expanded unipotent murine cell line 3T3-L1 is one of the classical and most extensively used cell models for studying adipocyte differentiation for in vitro evaluation in identifying molecular markers, transcription factors and various interactions that are required for preadipocyte differentiation.<sup>26</sup> Studies have shown that activation of Notch signaling inhibits adipocyte differentiation from MSCs and 3T3-L1.<sup>27-29</sup> While antisense Notch1 prevented 3T3-L1 differentiation, indicating a requirement of Notch signaling,<sup>30</sup> Notch was also shown to be dispensable for adipocyte specification and differentiation from either mesenchymal or epithelial progenitors.<sup>31</sup> In addition, the non-canonical Notch signaling pathway is also shown to regulate adipogenesis mediated by the ligand Delta-like 1 (Dlk-1/Pref-1).<sup>32-34</sup> Given these contrasting reports, the requirement and role of Notch signaling in adipose tissue development and adipocyte differentiation still needs clarification.

In this study we address the effects of inhibition of Notch signaling in adipocytes and the influence on adipocyte differentiation and interaction with endothelial cells (EC). We used the previously described<sup>35,36</sup> soluble Jagged1 (sJag1)-mediated suppression of Notch signaling in 3T3-L1 as the model system. In vitro suppression of Notch signaling by sJag1 increased proliferation in 3T3-L1 and EC and triggered the differentiation process in the preadipocytes. In vivo, subcutaneous fat pads formed by 3T3-L1 cells expressing sJag1 in nude mice displayed an increase in vessel formation and production of fat filled adipocytes. Together, these results show that inhibition of Notch signaling by sJag1 promotes both angiogenesis and adipocyte differentiation.

## Results

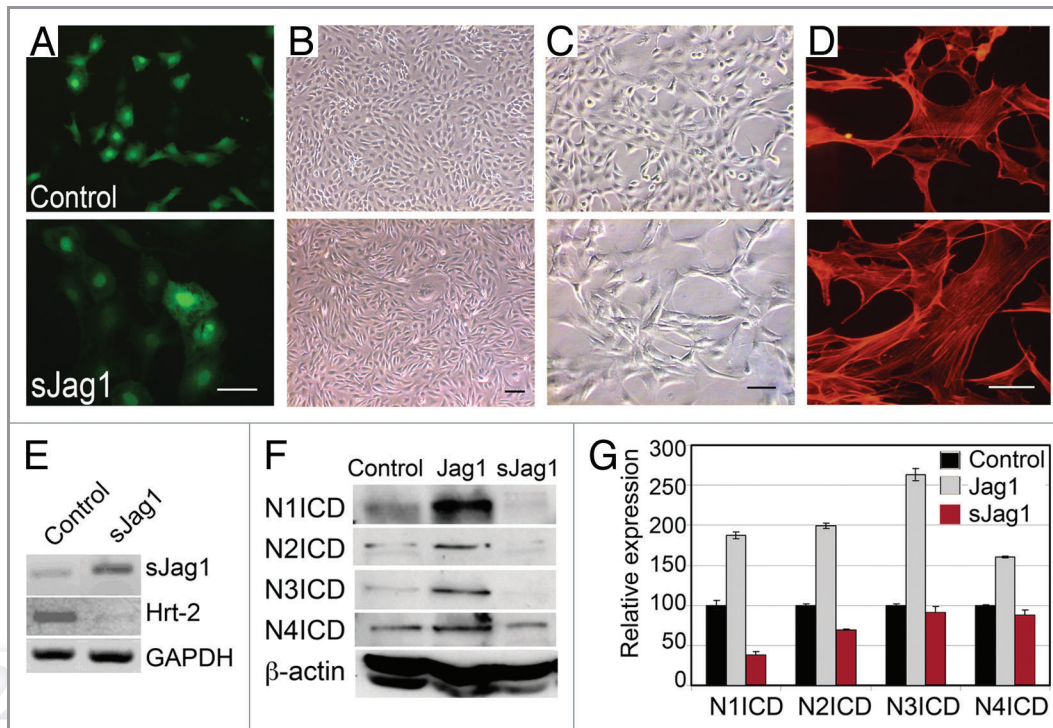
**sJag1 expression changes cell morphology.** Expression of sJag1 has previously been shown to inhibit endogenous Notch signaling.<sup>18,35</sup> Using the same principle, we utilized the 3T3-L1 cell model to define the mechanism of Notch inhibition in adipocyte differentiation. 3T3-L1 cells were transfected with the control vector or sJag1 ligand and a homogenous population of cells expressing sJag1 was selected by sorting for GFP expression (Fig. 1A). Cell passages were checked regularly to confirm GFP expression. sJag1 expressing cells cultured in regular growth media showed distinct phenotypic characteristics. Similar to the behavior of 3T3 fibroblasts expressing a sJag1 construct, stably transfected 3T3-L1 preadipocytes also exhibited a cord-like phenotype in growth media at confluence unlike the monolayer growth of empty vector expressing control cells (Fig. 1B and C). Additionally, similar to the previous report of sJag1 in 3T3 fibroblasts, sJag1 preadipocytes also lift off of the surface of the culture plate indicative of changes in the ability of the cells to adhere to

substrate. These cells showed loss of contact inhibition similar to the previous report of sJag1 in 3T3 cells<sup>35</sup> and typical fibroblast morphology with large elongated cells, and altered F-actin fiber organization illustrated by phalloidin stain (Fig. 1D).

**sJag1 expression reduces Notch activation in 3T3-L1 cells.** RT-PCR for regions specific to the ligands confirmed the expression of the sJag1 at the transcript level and corresponding decrease in Notch target gene *Hrt-2*, confirming functionality of sJag1 expression (Fig. 1E). Immunoblot for endogenous cleaved Notch intracellular domain (ICD) showed decreased levels in cells expressing sJag1 (Fig. 1F) indicating reduced Notch activation when compared with controls and Jag1 expressing 3T3-L1 cells used as a positive control. sJag1 expression repressed Notch1 and Notch2 activation, while Notch 3 and 4 were less affected as seen in the immunoblot quantifications based on integrated densities (Fig. 1F, graph).

**sJag1 expression increases cell proliferation.** sJag1 expression increased cell growth rate as measured by cell counts. Growth curve analysis showed sJag1-expressing cells had a 2-fold increase in cell numbers compared with control vector transformed cells as early as 2 d ( $p < 0.01$ ) (Fig. 2A). To determine if the increased growth rate is by virtue of secreted sJag1 acting as a Notch signaling inhibitor, non-transduced 3T3-L1 cells were cultured in cell-free conditioned medium from sJag1 overexpressing cells or controls (Fig. 2B). Growth curve analysis showed a 2-fold increase in cell numbers by 6 d in the sJag1 treated cells over the corresponding control. Although cell proliferation effects due to the conditioned media were not as dramatic as in sJag1 overexpressing cells, it must be noted that the conditioned media is expected to have lower amounts of available (not bound) ligand, and medium was collected after 24–48 h. This result indicates antagonism of the Notch receptor by sJag1 increases proliferation of the 3T3-L1 cells and confirms the earlier findings that the secreted sJag1 form functions as a dominant negative regulator inhibiting Notch signaling that can act in an autocrine and paracrine manner.<sup>37</sup>

To further explore cell growth dynamics, we performed cell cycle analysis using flow cytometry on proliferating cells stained with propidium iodide. Cells at ~60% confluence were serum starved (0.2% FBS) for 40 h to synchronize the cells prior to feeding complete media. Cells were collected at 4 h intervals for cell cycle analysis. More than 80% of the control cells were in G<sub>1</sub> phase at 0 h, while only ~40% of sJag1 cells were present in G<sub>1</sub> phase. This result indicates that serum starvation did not force the sJag1 cells into a resting or quiescence state as in the control line. Five to six percent of control cells were detected at G<sub>2</sub> phase, unlike the sJag1 expressing cells with over 20% in the G<sub>2</sub> phase (Fig. 2C). Higher numbers of cells were also present in the synthesis (S) phase in the sJag1 cells, indicating that sJag1 cells did not exit cell cycle even after 40 h of serum starvation. Corresponding to the increase in cell proliferation, immunoblot analysis showed increased levels of cyclin D3, a positive regulator of cell cycle progression (Fig. 2D). Quantification of the blots showed that at 0 h, cyclin D3 level was more than 2-fold elevated compared with controls (set at 100%) and subsequently showed 1.5-fold increase throughout the experiment up to 12 h (Fig. 2D,



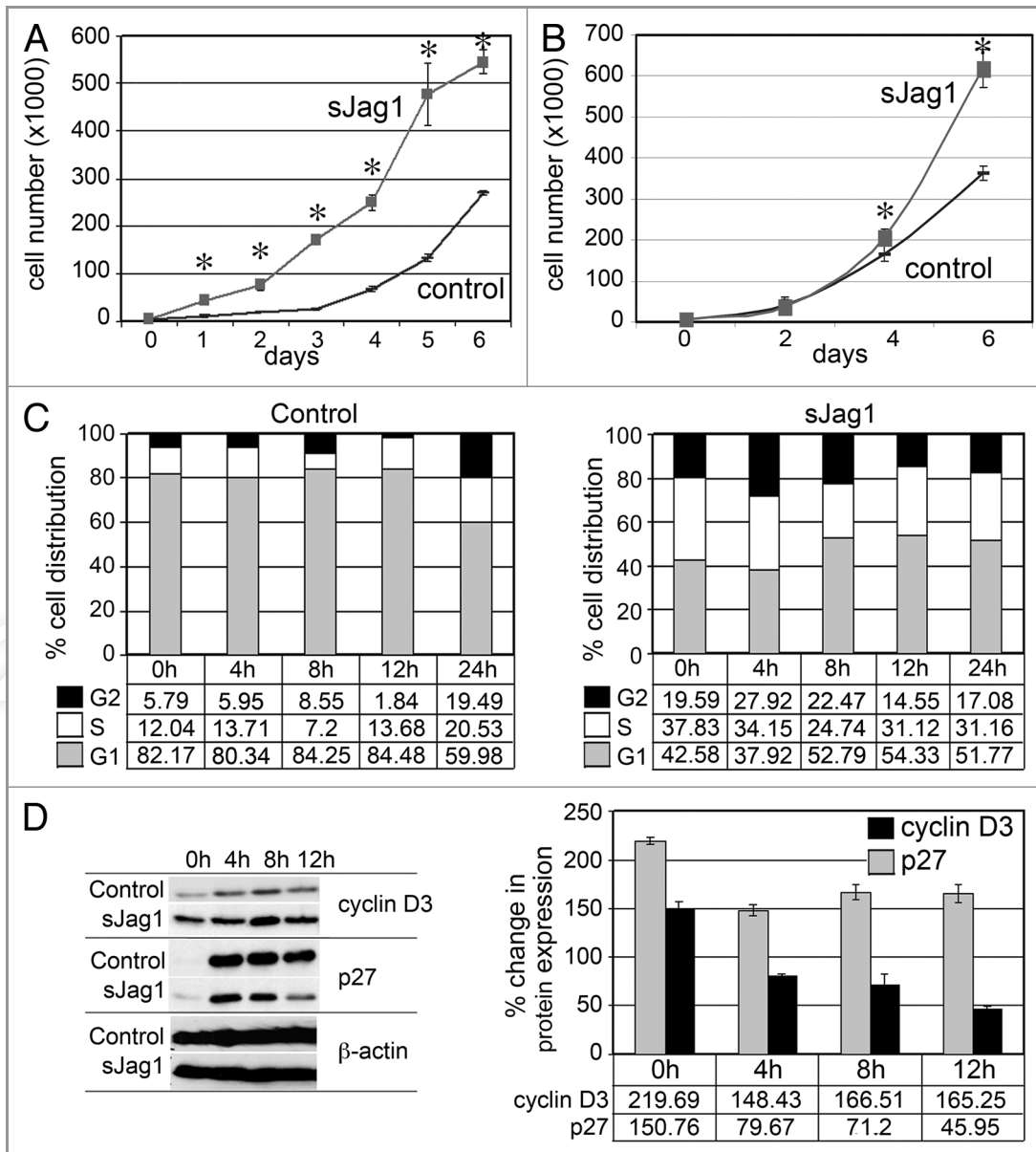
**Figure 1.** Expression of soluble form of Jagged1 (sJag1) in 3T3-L1 cells. (A) 3T3-L1 cells were transduced with vector control (control) or sJag1 and sorted for GFP expression. (B and C) Cells under low (B) and higher magnification (C) showing the fibroblastic phenotype in sJag1 cells. (D) Phalloidin staining to show altered F-actin organization in sJag1 expressing cells (bar indicates 50  $\mu$ m). (E) RT-PCR for sJag1 and Notch target gene Hrt-2 transcripts. (F) Immunoblot for endogenous levels of active Notch receptor activation (NICDs, Notch1, Notch2, Notch3 and Notch4) using whole cell lysates. Full-length Jagged1 overexpressing cells were used as positive control to indicate Notch activation. The graph shows quantification of the immunoblot based on integrated densities (graphed are means  $\pm$  SD).

graph). Unlike the controls, serum starvation did not bring down cyclin D3 levels in sJag1 expressing cells. Corresponding to the increased cyclin D3 levels, there was also a decrease in CDK inhibitor p27. Although the initial level at 0 h was 1.5-fold higher than control, serum stimulation dramatically lowered p27 levels in sJag1 cells (Fig. 2D). These results show that sJag1 cells proliferate at a higher frequency than control cells and do not exit cell cycle in response to serum starvation.

**sJag1 respond to differentiation signals but are unable to become mature fat cells.** To determine the influence of sJag1 expression on differentiating 3T3-L1 cells, sJag1 and control cells were stimulated with a cocktail containing IBMX, insulin and dexamethasone to induce differentiation. Control cells followed the expected preadipocyte differentiation pattern in which cells stopped proliferating, and changed morphology by becoming large, rounded acquiring lipid droplets (Fig. 3A). On the other hand, sJag1 expressing cells did not show changes in morphology characteristic of differentiation, but instead continued to multiply. After 4 d they became over confluent and began to lift off the surface. However, stimulation with differentiation media did cause the sJag1 cells to initiate the differentiation cascade, based on molecular markers. Two days post induction, quantitative PCR indicated increase in RNA transcript levels of adipogenic markers in sJag1 cells over control cells. Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and fatty acid binding protein

4 (FABP4) levels were upregulated 6-fold over the corresponding controls (Fig. 3B). However, 4 d post induction, the transcript levels were dramatically downregulated while C/EBP  $\alpha$  expression was upregulated in sJag1 expressing cells (Fig. 3B). Cell cycle analysis of cells in the differentiation conditions indicated that sJag1 cells did not exit cell cycle, similar to their behavior in normal growth conditions. On day 2 (48 h in differentiation medium), 56% of sJag1 cells were in G<sub>1</sub> phase, while 80% of the control cells were in G<sub>1</sub> phase, responding to glucocorticoid induced cell cycle arrest (Fig. 3C). By day 5, continued proliferation of sJag1 cells resulted in over confluence, loss of contact inhibition and cell detachment. These observations indicate that sJag1 cells respond partially to differentiation stimulus, but are unable to exit cell cycle and thus do not form mature adipocytes.

**sJag1 expression induces in vivo fat pad formation.** To determine the potential of sJag1 expressing cells to differentiate in vivo, we used the previously described<sup>8,38,39</sup> subcutaneous fat pad formation model in nude mice. Injection of 2.5 million proliferating control or sJag1 expressing cells showed fat pad formation within a week. Fat pad formation was visualized by MRI and fat pad volume was determined based on scanned images (Fig. 4A). At 3 weeks, fat pads in control mice measured a volume of 38.16 mm<sup>3</sup> while the sJag1 were 30.3 mm<sup>3</sup>. However, by 11 weeks, the control cells underwent transformation adopting

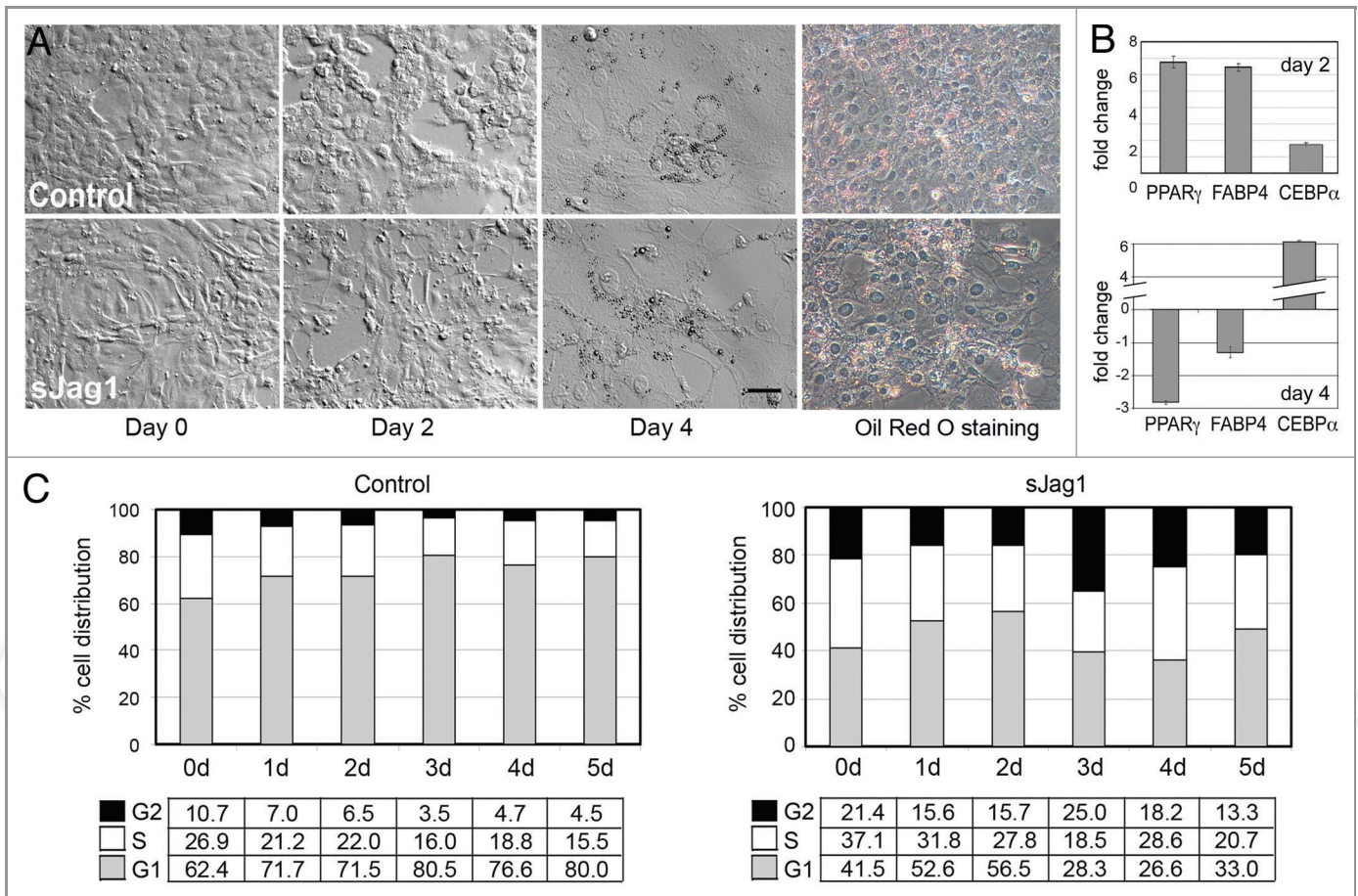


**Figure 2.** sjag1 expression increases cell proliferation and alters cell cycle. (A) Growth curves of control and sjag1 expressing 3T3-L1 cells cultured in regular growth media (bars indicate means  $\pm$  SD and \* indicates  $p < 0.05$ ). (B) Growth curve for 3T3-L1 cells cultured in cell-free conditioned media from control or sjag1 expressing 3T3-L1 cells (graphed are means  $\pm$  SD and \* indicates  $p < 0.05$ ). (C) Cell cycle analysis showing distribution of the cell population in the cell cycle stages G<sub>1</sub>, S and G<sub>2</sub> at different time intervals. (D) Immunoblot for cyclin D3 and p27 at different time intervals; the graph shows quantification of the immunoblots as percent change in expression relative to control at time 0 (graphed are means  $\pm$  SD).

a tumorigenic phenotype, grew larger and measured 218 mm<sup>3</sup> while the sjag1 allografts measured 55 mm<sup>3</sup> (Fig. 4A, graph). Localized spectroscopy was applied to detect lipid accumulation at 3 and 11 weeks. In the control cells, no peak was detected while in the sjag1 cells, a peak corresponding to the lipid was observed (Fig. 4B). This indicated that the sjag1 cells were accumulating lipid and differentiating into adipocytes. In the control allografts, cells became transformed, and caused excessive growth with histological features of rapidly growing tumors containing necrotic cores (Fig. 4D). Interestingly, although the sjag1 cells were highly proliferative in vitro, sjag1 allografts formed mature adipocytes.

The total volume based on MRI (Fig. 4A) therefore indicated the total region comprising the tumor and the fat pad. When the proportion of fat cells in each allograft was calculated, we determined sjag1 expressing allografts had almost half the area consisting of fat cells (48.47%) while in the control allografts, only ~10% of the total area was comprised of fat cells (Fig. 4C). Further, immunostaining of the allografts for GFP clearly shows positive staining of adipocytes and adjacent cells (Fig. 4E), but no staining in the host tissue.

Since angiogenesis often precedes or parallels the formation of adipose tissue<sup>8</sup> it is assumed that there is a direct link between



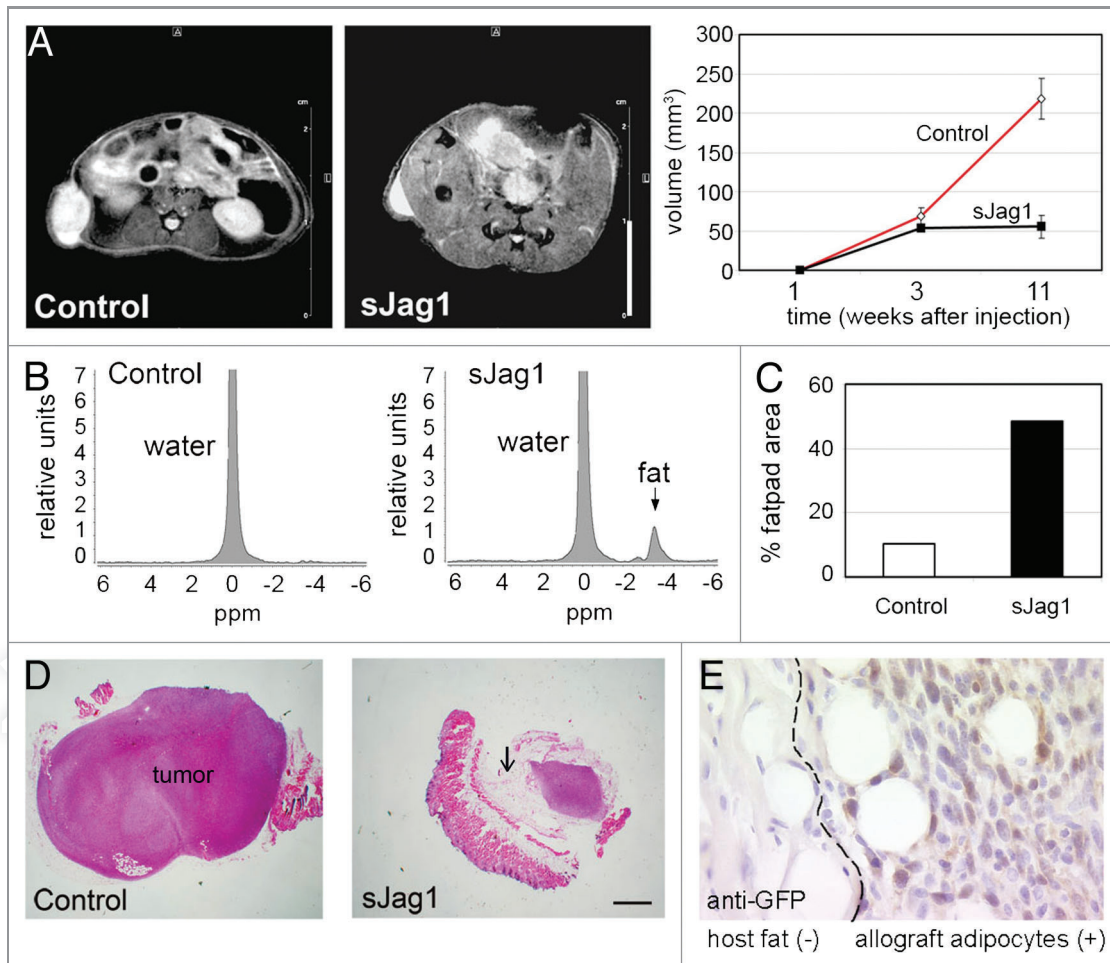
**Figure 3.** Effect of differentiation on sJag1 expressing cells. (A) Phase contract micrographs of control and sJag1 cells in differentiation conditions. Day 0 represents when cells were stimulated with differentiation media, day 2 represents, two days post stimulation when media was changed to maintenance media, and day 4 represents 4 d post stimulation (bar = 50  $\mu$ m) and Oil red O staining at 4 d. (B) qPCR for PPAR $\gamma$ , FABP4 and C/EBP  $\alpha$  gene transcripts represented as fold change after normalization to internal control and to corresponding control RNA (graphed are means  $\pm$  SD). (C) Cell cycle analysis showing distribution of cell populations at G<sub>1</sub>, S and G<sub>2</sub> stages for control and sJag1 cells under differentiation conditions.

adipogenesis and angiogenesis. We therefore used the endothelial specific PECAM staining to visualize vascularization in the fat pads that developed from the allografts (Fig. 5A). Quantification of vascular area normalized to the total fat cell area showed a 2-fold increase in vessel density in the fat pads in allografts from sJag1 cells (Fig. 5B). The vessel area normalized to the total fat pad area was 2.81 in sJag1 fat pads while the controls had 1.18, indicative of increased angiogenesis in the fat pads of sJag1 expressing allografts (Fig. 5B). This result indicates that sJag1 cells were capable of differentiating into adipocytes and form functional fat pads with neovascularization under in vivo conditions.

**sJag1 induces endothelial proliferation.** To determine the cause of the observed increase in vessel development within the sJag1 Vs control fat pads, we analyzed the effect of conditioned media from the control and sJag1 cells on endothelial cell proliferation. Endothelial cells treated with sJag1 conditioned media from 3T3-L1 cells showed increased proliferation in vitro. Cell counts on day 8 showed significant 2-fold increase with

sJag1 media over control cells (Fig. 5C). Cell cycle analysis of the endothelial cells grown in conditioned media showed differences similar to those found in the sJag1 cells. At 24 h, 68% of the sJag1 conditioned medium-treated endothelial cells were in G<sub>1</sub> while the control treated cells had 82% of the population in G<sub>1</sub> (Fig. 5D). This change in cell cycle progression indicates that the secreted proteins produced by the sJag1-expressing cells positively influenced endothelial cell growth and proliferation.

To further understand this paracrine signaling, the cytokines secreted by transfected preadipocyte cells were quantified using an ELISA based blot assay. Analysis revealed that there was a general increase in cytokines in conditioned media from sJag1 expressing cells. Quantification based on integrated intensities showed a ~2-fold increase in IL-1A, IL-1B, IL-4, IL-6, EGF, VEGF, FGF-A (FGF1) and FGF-B (FGF2) in sJag1 conditioned media over controls (Fig. 5E). Together, these results suggest that the proliferative effect of sJag1-mediated inhibition of Notch signaling on preadipocytes and endothelial cells may attribute to marked increase in the production of cytokines.

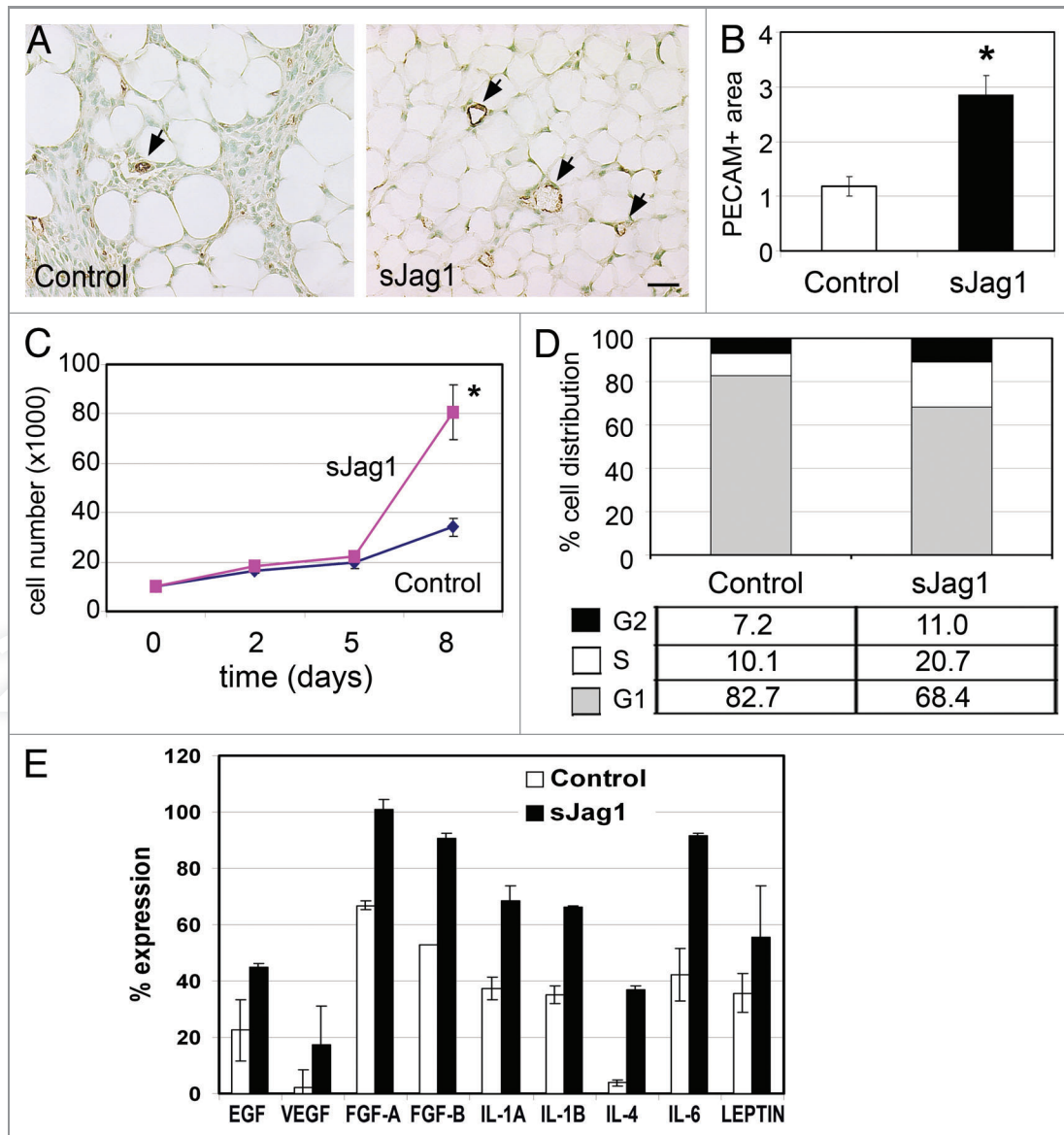


**Figure 4.** In vivo fat pad formation from 3T3-L1 allografts. (A) Axial images generated by magnetic resonance imaging (MRI) to determine fat pad volume in nude mice injected subcutaneously with control or sJag1 cells in the flanks (bar = 1 cm). The graph shows the fat pad volumes 3 or 11 weeks after injections ( $n = 6$ , graphed are means  $\pm$  SD and \* indicates  $p < 0.05$ ). (B) Localized spectroscopy acquired by placing a 1 mm<sup>3</sup> voxel at the center of the fat pad 3 weeks after injection to quantify water and fat content. (C) Graph indicates quantification of fat pad region in each group of mice. (D) Representative micrograph of hematoxylin and eosin stained sections from allografts showing transformation in control and significant fat differentiation in sJag1 allografts (bar = 5  $\mu$ m). Arrow indicates adipocytes. (E) Immunostaining indicating positive GFP staining in the allograft adipocytes and absence of GFP in the host tissue.

## Discussion

The primary goal of this study was to determine the functional role of Notch signaling in adipocyte differentiation. We used the well-established mouse embryonic fibroblast-derived preadipocyte cell line 3T3-L1 to test our hypothesis that suppression of Notch signaling would alter adipogenic differentiation in vitro and in vivo. The role of Notch signaling during adipogenesis is controversial, with data suggesting activities in either promoting or suppressing adipogenesis.<sup>28-31,40-42</sup> These in vitro studies support the hypothesis that Notch signaling has a dual role in adipogenesis and that its activity must be tightly controlled. Furthermore, non-canonical Notch signaling through Delta-like 1 (DLK-1) is implicated in regulating adipocyte differentiation.<sup>24,34,43,44</sup> While regulation of the Notch signal and its influence on the adipogenic program are still not completely understood, Notch signaling dynamics further increase the complexity of Notch involvement

by the multiple ligand-receptor mediated activation and cell type specificity. In this study, we used the previously described<sup>35</sup> preferential inhibition of Notch signaling via expression of dominant negative soluble form of the Jagged1 ligand (sJag1) to demonstrate changes in growth and differentiation characteristics in 3T3-L1 cells. The results from this study are not entirely unexpected and are similar to the previous report on sJag1 expression in 3T3 fibroblasts where it was shown that expression of soluble form of Notch ligands Jagged1 (sJag1) and Delta-like1 (sDL1) in NIH3T3 fibroblasts causes cell transformation, increased growth rate and tumors in vivo.<sup>18</sup> In smooth muscle cells<sup>24</sup> and chondrocytes,<sup>25</sup> sJag1 expression has an inhibitory effect on cell proliferation and migration, indicating cell specific effects of sJag1. As in NIH3T3 fibroblast, sJag1 has a similar proliferative effect on 3T3-L1 cells, also a fibroblast derivative. The increased proliferation rate correlated with changes occurring in cell cycle regulation and progression with upregulation of



**Figure 5.** sJag1 cells promote angiogenesis and endothelial cell proliferation. (A) Representative fat pad sections from subcutaneous injections stained with PECAM to determine vessel density (bar = 50  $\mu$ m). (B) The graph shows quantification of the vessel area (n = 6 tumors; 20 pictures each; graphed are means  $\pm$  SD and \* indicates  $p < 0.05$ ). In vitro effect of secreted sJag1 on endothelial cell. (C) Growth curve analysis of HUVEC cells treated with control or sJag1 conditioned media (graphed are means  $\pm$  SD and \* indicates  $p < 0.05$ ). (D) Cell cycle analysis showing distribution of endothelial cells at G<sub>1</sub>, S and G<sub>2</sub> phase cultured in cell-free conditioned media from control or sJag1 3T3-L1 cells. (E) ELISA based immuno-analysis of pro-angiogenic cytokines secreted from control and sJag1 cells. The graph shows quantification of the immunoblot based on integrated densities (graphed are means  $\pm$  SD).

cyclin D3. sJag1 expressing cells do not exit cell cycle even upon serum starvation, and instead are thrust into S-phase, resulting in excessive proliferation. Correspondingly, these cells do not respond to glucocorticoid-induced cell cycle arrest during differentiation, but, respond to insulin by initiating the transcription of the adipogenic regulators PPAR gamma and FABP4, albeit for a short period of time, as the cells continue to proliferate. Interestingly, cyclin D3 is also implicated in promoting adipocyte differentiation,<sup>45</sup> which could be a contributing factor stimulating the sJag1 cells to initiate differentiation. Earlier reports have implicated Notch1 in the commitment of 3T3-L1 cells to undergo adipogenesis by controlling the expression of the principal regulators of

the adipogenic program. Since impaired Notch1 expression blocks adipocyte commitment and differentiation in 3T3-L1 cells,<sup>30</sup> it is possible that sJag1 may not completely inactivate Notch1 signaling; however, it could inhibit either partial or basal level of Notch1 activation or Notch signaling via other Notch receptors. Since Jagged1 is not the only ligand binding to the Notch receptors, we cannot rule out activation of Notch signaling by other ligands through the other Notch receptors (Notch2–Notch4). On the other hand, it possible that sJag1 is not impairing Notch1 activation, but affecting other Notch receptors (Notch2–Notch4), since gamma secretase inhibitor pretreatment promotes adipogenesis and early differentiation in adipose tissue stromal cells.<sup>24</sup>

sJag1-mediated Notch inactivation elicited a similar response as gamma secretase inhibitor by promoting cell growth, proliferation and adipogenesis and early differentiation in 3T3-L1 cells, both *in vivo* and *in vitro*. Together, our results demonstrate that inactivation of Notch signaling is required to initiate the adipocyte differentiation cascade. As continued inactivation inhibits maturation of adipocytes, it is presumable that Notch signaling is required at a later stage during maturation. Our results support the hypothesis that a coordinated and temporal Notch signaling dynamics of initial Jagged1 mediated inactivation and subsequent activation is required for complete adipocyte differentiation.

The subcutaneous injection of preadipocytes in immunocompromised mice has been used for studying adipogenesis and angiogenesis in adult organisms.<sup>38,46</sup> With sJag1 expressing preadipocytes, we have to consider the paracrine effect of secreted sJag1 on surrounding endothelial cells in the event of neovascularization as seen in developing adipose tissue. There is substantial evidence of cross-talk between adipocytes and endothelial cells with bidirectional signaling mediated both by extracellular matrix components and direct cell-cell interaction promoting preadipocyte differentiation.<sup>2,8,47,48</sup> The expression of angiopoietin (1–2) and Tie (1–2) receptors and release of critical angiogenic factors by adipose tissue during its development and differentiation<sup>7,8</sup> provides considerable proof for implicating its role in neovascularization. Increased vessel area as seen in PECAM staining of the fat pads suggests the positive dynamics of angiogenesis and adipogenesis, although it cannot be confirmed if angiogenesis precedes adipogenesis or vice versa. The pro-angiogenic behavior of the sJag1 ligand in our *in vitro* and *in vivo* studies corroborates previous reports of sJag1 promoting fibroblast growth factor receptor (FGFR) dependent signaling and inducing a cord-like phenotype in NIH3T3 fibroblasts, and producing large, dilated vessels in chick chorioallantoic assays.<sup>18,49,50</sup> Although adenoviral sJag1 expression in endothelial cells did not affect proliferation and migration,<sup>51</sup> conditioned media from sJag1 3T3-L1 cells had a proliferative effect on endothelial cells as demonstrated by the increase in secreted cytokines and endothelial cell growth rate. Adipose tissue-secreted adipokines like leptin and adiponectin signal to the vascular endothelium, stimulating angiogenic response and are therefore believed to be the functional link between adipocytes and the vasculature.<sup>52,53</sup> Additionally, subcutaneous 3T3-F442A fat pads express high levels of leptin not attainable in cell culture.<sup>54</sup> Two distinct features of the *in vivo* study are (1) the use of the analogous preadipocyte cell line 3T3-L1 while most of the previous studies have used 3T3-F442A cells to form *in vivo* subcutaneous fat pads<sup>8,38,55,56</sup> and (2) use of lower number of cells ( $2.5 \times 10^6$  instead of  $3 \times 10^7$  cells/mouse) to avoid cell overdose and limit secreted factors. Nevertheless, the two cell lines are comparable and show similar fat pad dynamics including presence of a necrotic core.<sup>8</sup> It is important to note that 3T3-L1 cells underwent transformation in the presence or absence of matrigel, the latter being less aggressive. This raises the question of whether the presence of sJag1 *in vivo* prevents 3T3-L1 cells from becoming tumorigenic, possibly by favoring adipocyte differentiation. Additionally, matrigel has been shown to influence vessel development, decrease adipocyte maturation

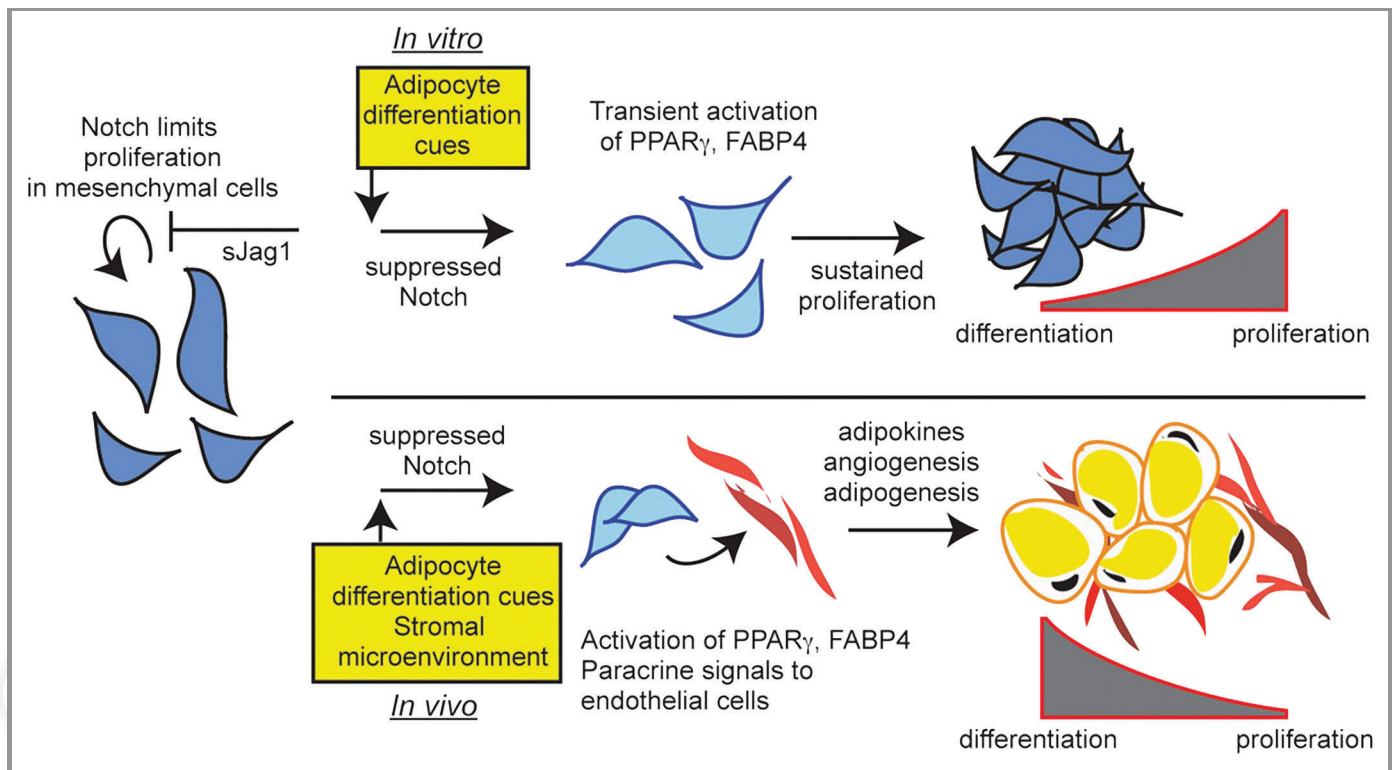
and microvessel maturation in 3T3-F442A cells, facilitating transformation to form tumors and dense connective tissue which suppress adipogenesis by physically impairing the development of capillaries.<sup>8</sup> In the tissue perspective, the cell-cell interactions and cell-extracellular matrix play a regulatory role in cell differentiation and formation of the mature adipocytes.<sup>38</sup> Studies have shown that influence of extracellular matrices on preadipocyte development is dependent on the source of substrate.<sup>57,58</sup> Remodeling of the adipose tissue matrix is an essential prerequisite for creating the space for triglyceride deposition. In particular, laminin has been implicated in matrix-enhanced preadipocyte attachment and spreading, indicating a pivotal role in preadipocyte differentiation.<sup>58</sup> Remodeling also involves a decrease in the key matrix protein fibronectin brought about by degradation by cathepsin-K, itself an adipokine. Cathepsin-K also degrades other matrix constituents such as collagen types I and II and osteonectin, which is a non-structural extracellular matrix protein modulating cell matrix interactions.<sup>59</sup> Additionally, enhanced proliferation leads to impaired adipocyte development since proliferation is inversely related to differentiation. Thus, it becomes necessary to consider these requirements for structural modifications to support adipocyte differentiation along with the cell cycle arrest. In the context of sJag1 overexpressing preadipocytes, there is continuous proliferation in the absence of any matrix changes as seen *in vitro*. However, *in vivo*, in the presence of a complex extracellular matrix and other cell-cell interactions (specifically, the endothelial cells), there is considerable remodeling of the matrix impacting the differentiation of preadipocytes, as evidenced by the formation of fat pads. Therefore, it is imperative to consider that *in vivo* preadipocyte differentiation is influenced by host response as triggered by matrix remodeling and neovascularization.

Given these complex interactions affecting *in vivo* differentiation of preadipocytes, we demonstrate that sJag1 cells secrete pro-angiogenic cytokines that can potentially support recruitment of endothelial cells and thereby neovascularization in the subcutaneous fat pads as represented in the schematic model (Fig. 6). In conclusion, our results confirm that (1) Notch signaling plays a role in preadipocyte cell response to proliferation and differentiation, (2) Inhibition of Notch signaling by soluble Jagged1 induces cell proliferation of preadipocytes and endothelial cells and (3) In the presence of sJag1, there is increased angiogenesis that correlates with the formation of a larger fat pad.

## Materials and Methods

**Cell lines and culture.** The stable cell lines were generated using the 3T3-L1 mouse preadipocyte cell line from ATCC (Catalog Number CL173) and transduced retrovirally to generate populations expressing the soluble and full length forms of the Notch ligands Jagged1 (Jag1). HUVEC cells (CC-2517) are from Lonza. The empty vector was used as the control. The retroviral constructs were a kind gift from Cathrin Brisken [Ecole Polytechnique Fédérale de Lausanne (EPFL); Swiss Institute for Experimental Cancer Research (ISREC), NCCR Molecular Oncology, Lausanne, Switzerland]. The cells were selected for





**Figure 6.** Schematic model describing sJag1 induced Notch signaling modulation of adipogenesis. sJag1 mediated inhibition of Notch signaling promotes cell growth and initiates the adipogenic cascade. Under in vivo conditions, the secreted sJag1 stimulates angiogenesis by inhibiting Notch signaling in the endothelial cells. This cross-talk potentially supports extracellular matrix remodeling and development of the composite adipose tissue.

GFP positive expression by FACS Aria (BD BioScience) sorting. The cells were maintained at low passage numbers to maintain high level of expression. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin. For differentiation, cells were stimulated at 70–80% confluence in the same media supplemented with 0.25  $\mu\text{M}$  dexamethasone, 0.5 mM isobutylmethylxanthine and 166 nM insulin for 2 d. This medium was replaced with DMEM regular medium with 10% FBS and 166 nM insulin thereafter.

**Characterization of the stable cell line.** *Morphology.* Semi-confluent cells were fixed with 4% paraformaldehyde, washed with PBS and stained for 30 min with phalloidin to visualize F-actin, washed and mounted for imaging.

*Cell cycle analysis.* Cells were synchronized by culturing in media containing minimum serum (0.5% FBS) for 24–30 h. At time 0, fresh media with 10% FBS was added and cells collected at time intervals of 0, 3, 6, 9, 12 and 15 h, fixed in ice cold 70% ethanol, stained with propidium iodide (PI) and analyzed by flow cytometry (FACS, BD Bioscience) to determine  $G_1$ ,  $G_2$  and S phases. Cell cycle analysis was also done for longer intervals of time for each cell line.

*Evaluation of bioactivity of conditioned media.* Conditioned cell-free media from transduced stable cell populations (control and sJag1) were collected from confluent plates. The conditioned media was concentrated using vivaspin cell concentrators by centrifugation or used as such at a 1:1 concentration.

*Effect on growth of endothelial cells.* Primary human umbilical vein endothelial cells (HUVEC) at passage 4 or 5 were seeded at a density of 5,000 cells per well in a 24-well plate and cultured in endothelial basal media (EBM) with 10% FBS. After 12 h, the EBM medium was replaced with 50% fresh EBM medium and 50% cell-free conditioned medium harvested previously from transduced stable lines. The growth of endothelial cell was monitored by cell count for 6 d, and the data expressed as a growth curve.

*Evaluation of angiogenic factors.* Angiogenic factors in conditioned media were detected using an ELISA based blot assay (Panomics Inc.). Briefly, concentrated cell free conditioned media (1 mg) from the stable cell lines was added to the blot for 2 h, and binding visualized by streptavidin conjugated antibody following the protocol provided with the kit. Expression level was based on the blot intensity as quantified using image quant.

*In vivo tumor allografts in immunocompromised mice.* All protocols involving mice were evaluated and approved by our Institutional Animal Care and Use Committee, and performed under veterinary supervision. The in vivo model for subcutaneous tumor development in nude mice was performed as described.<sup>50</sup> Briefly, 3T3-L1 stable lines were grown to confluence and 24 h prior to injection the medium was changed to DMEM containing 10% FBS. The transfectants were washed with PBS, harvested by trypsin digestion and resuspended in PBS. Male athymic nude mice (*nu/nu*) from Taconic between 6–8 weeks of age were injected subcutaneously in the right flank with  $2 \times 10^6$  transfected cells in a total volume of 200  $\mu\text{l}$ . Tissue growth was

monitored by palpation, and the onset noted when nodules were palpable. Tissue nodule size was measured with calipers, and volume calculated assuming the shape as ellipsoid. Representative data were obtained from 5 mice/experimental group, and the entire experiment was repeated in three independent trials. Prior to collection, mice were injected subcutaneously with 200  $\mu$ l of 25 mg/ml BrdU solution at 15 h and 1 h before collection. The mice were euthanized and the tissue allografts overlying skin collected 3–5 weeks after injection, depending on growth. Allografts were individually weighed, half was snap frozen for RNA and protein, and the rest fixed in 4% paraformaldehyde for histological studies.

**Quantification of vessel area in allograft sections.** Non-counterstained Platelet endothelial cell adhesion molecule-1 (PECAM) stained sections (five tumors per condition) were quantified for vessel area. Ten pictures of comparable regions of each tumor were taken and quantified in a blinded fashion. In Photoshop 7.0, the vessels were outlined in a transparent layer and filled in with black. The outlined vessel image was opened in Scion Image, converted to binary, threshold set to constant, and area of black pixels measured. Shown is average percentage of vessel area per allograft, and results were analyzed by Student's *t* test to determine statistical significance compared with the control group.

**Quantitative reverse transcriptase-PCR (qRT-PCR).** Total RNA was collected using TRI Reagent (Sigma) following the manufacturer's protocol. RNA was reverse transcribed using oligodT in the presence of AMV reverse transcriptase to make cDNA. Successful cDNA production was verified using primers against GAPDH or  $\beta$ -actin. Quantitative PCR was performed using Bio-Rad (Bio Rad) cybergreen supermix and amplification on the Bio-Rad Icyler and quantified based on Ct values as described earlier.<sup>60</sup> Primers used for PCR reactions are as follows:

sJag1 Forward primer 5'-TGCCTCTGTGAGACCAACTG-3'  
Reverse primer 5'-TGGGCAACACTCACACTCAA-3'  
Hrt2 Forward primer 5'-CCAGAAAAAGACGGAGAGGA-3'  
Reverse primer 5'-GCGCGTCAAAGTAACCTTTC-3'  
GAPDH Forward primer 5'-GCCTCAAGATCATCAGC-AAT-3'  
Reverse primer 5'-GGACTGTGGTCATGAGTCCT-3'  
PPAR $\gamma$ 1+2: Forward primer 5'-TCATCTCAGAGGGCC-AAGGA-3'  
Reverse primer 5'-CACCAAAGGGCTTCCGC-3'  
FABP4: Forward primer 5'-TGGAAGCTTGTCTCC-AGTGA-3'

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Reverse primer 5'-AATCCCCATTTACGCTGATG-3'  
Cyclophilin: Forward primer 5'-CTCGAATAAGTTTGACT-TGTGTTT-3'

Reverse primer 5'-CTAGGCATGGGAGGGAACA-3'

**Immunoblot analysis.** Total cell lysate was prepared using cell lysis buffer consisting of 150 mM NaCl, 50 mM Tris pH = 8, 1% Triton X-100, and proteinase inhibitors. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted as indicated. Immunoblots were visualized using chemiluminescence (Amersham). Cytokine levels in cell free conditioned medium was quantified using an ELISA based dot-blot assay (Panomics Inc.). Expression level was quantified using image quant software. The intensity of the predetermined positive controls was set at 100.

**MRI and <sup>1</sup>H spectroscopy.** Axial and coronal images through the allograft area were acquired from isoflurane (1%, 0.4 l/min) anesthetized mice in a 7.0T Bruker PharmaScan magnet. A rapid acquisition method with enhanced relaxation (RARE) was used (TR 2500 ms, TE 10.64 ms, FOV 3.5 cm, matrix size 256  $\times$  256, slice thickness 1 mm, total scan time 5 min 30 sec). The allograft dimensions were measured at the largest width, length, and height. For localized spectroscopy PRESS (TR 2000 ms, TE 21.4 ms) was used. A voxel of 1 mm<sup>3</sup> was placed into the center of the tumor. Placement of the voxel had no influence on the spectrum. One spectrum is the sum of 800 fields with a total scan time of 26 min 40 sec.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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