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Notch Signaling Involvement in Endothelial Responses to Inflammation: *Jagged1 and Notch1 Mediate IL-1β - Induced Up-regulation of Adhesion Molecules*

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INDEX

ABSTRACT

Italian version
English version

INTRODUCTION

I. Endothelial Cells and Vascular system
1.1 Vascular Development: Vasculogenesis, Angiogenesis and Neoangiogenesis
1.2 Endothelial
1.3 Progenitor Cells
1.2.1 Antigens expression
1.2.2 Culturing Methods

II. Vascular Responses to Inflammation
2.1 Role of Vascular Endothelium
2.2 Adhesion Molecules
2.3 The Inflammatory Cytokine IL-1β
2.4 NF-kB Signaling

III. Notch Signaling
3.1 Notch Receptors
3.2 Notch Ligands
3.3 Notch Signaling Transduction

IV. Notch Signaling in the Vascular System
4.1 Expression Mapping of Receptor and Ligands in the Vasculature
4.2 Notch and Vascular System Formation in Embryo
4.3 Notch Signaling and Vascular Diseases

BACKGROUND

AIM OF THE PROJECT

PROJECT STRATEGY

RESULTS

DISCUSSION

MATERIALS AND METHODS

BIBLIOGRAPHY

ABSTRACT

Italian Version

Obiettivo--Il signaling di Notch, attraverso l'attivazione dei recettori Notch1 e Notch4, gioca un ruolo chiave nella determinazione e nell'omeostasi dell'endotelio. Il TNFα determina la down-regolazione di Notch4 e del target gene Hes1, e questa modulazione causa la perdita della quiescenza e l'induzione della molecola di adesione vasale (VCAM-1) nelle cellule endoteliali (ECs). Questo studio mira ad investigare la regolazione dei componenti del signaling di Notch indotta dall'IL-1β ed il loro coinvolgimento nell'up-regolazione delle molecole di adesione.

Metodi e Risultati--Abbiamo dimostrato che l'IL-1β induce una forte up-regolazione di Jagged1, e mentre l'attivazione di Notch4 diminuisce, la forma attiva di Notch1 (Notch1ICD) rimane costante. La complessiva inibizione del signaling di Notch, attraverso un inibitore della γ–secretasi, determina una riduzione nell'up-regolazione di VCAM-1 indotta dall'IL-1β. Inoltre, il silenziamento di Jagged1 ha un effetto negativo sull'up-regolazione delle molecole di adesione indotta dall'IL-1β e il doppio silenziamento di Jagged1 e Notch1 determina un effetto ancora maggiore. In aggiunta, la forzata over-espressione di Notch1ICD induce l'espressione di VCAM-1, e questo effetto aumenta dopo il trattamento con l'IL-1β. Nelle cellule over-esprimenti Notch1ICD, inibendo chimicamente la traslocazione nucleare di NF-kB indotta dall'IL-1β, non si riduce significativamente l'up-regolazione di VCAM-1. In fine, l'over-espressione di Hes1, durante il trattamento con l'IL-1β, inibisce l'up-regolazione delle molecole di adesione e reprime la trascrizione di Jagged1, indicando un loop regolativo negativo tra i componenti del signaling di Notch.

Conclusioni—Complessivamente i risultati indicano che Jagged1 segnala attraverso Notch1, e Notch1 e' il recettore piu' importante nella risposta infiammatoria delle ECs. Inoltre, in presenza di Notch1 l'induzione delle molecole di adesione risulta solo parzialmente dipendente da NF-kB.

English Version

Objective--Notch signaling plays a key role in endothelial determination and homeostasis through Notch 1 and Notch 4 receptors. In endothelial cells (ECs) the TNFα determines the down-regulation of Notch4 and Hes1 and this modulation are associated with the loss of quiescence and the up-regulation of vascular cell adhesion molecules-1 (VCAM-1), This study investigated the regulation of Notch signaling components upon IL-1β induction and their implication in the up-regulation of adhesion molecules.

Methods and Results-- We showed that IL-1β induced a strong up-regulation of the Notch ligand Jagged1. Strikingly, while Notch4 activation decreased, Notch1 active form (Notch1ICD) remained constant. The global inhibition of Notch activation through a γ–secretase inhibitor resulted in reduction of IL-1β-induced VCAM-1 up-regulation. Moreover, the silencing of Jagged1 partially affected the up-regulation of adhesion molecules induced by IL-1β, and the double silencing of Jagged1 and Notch1 led to a higher reduction of the adhesion molecules expression after IL-1β treatment. Interestingly, Notch1ICD forced expression induced VCAM-1 expression in ECs and increased the up-regulation induced by IL-1β treatment. Chemical inhibition of the NF-kB nuclear translocation induced by IL-1β treatment did not significantly reduce VCAM-1 expression in Notch1ICD expressing cells. Finally, Hes1 over-expression during IL-1β treatment inhibited the up-regulation of adhesion molecules and, concomitantly, repressed Jagged1 trascription, suggesting a negative regulative loop between Notch signaling component.

Conclusions--All together our results indicate that, Jagged1 sustains Notch1 activation that specifies the effects of IL-1β. Thus, Notch1 is the most important receptor involved in inflammatory responses of ECs, and its function is, at least in part, NF-kB-dependent.

INTRODUCTION

I. Endothelial Cells and the Vascular System

1.1 Vascular Development: Vasculogenesis, Angiogenesis and Neoangiogenesis

The vascular system is formed during the prenatal life in embryo through two different mechanisms, termed vasculogenesis and angiogenesis. In the adult life vascular remodeling or new vessel formation occurs physiologically in hairy skin, in female reproductive system and in tissues subjected to tissue repair, otherwise, is linked to pathological condition such as tumor growth, diabetes, and inflammatory disorders (Folkman, 1995; Folkman et al., 1971; Tamanini and De Ambrogi, 2004).

Until few years ago, the term vasculogenesis described a process that occurred essentially in embryo: the differentiation of stem cells in endothelial precursors followed by their maturation in endothelial cells (ECs) and the assembling in the first vascular structures *i.e.* the extra-embryonic sac followed by the primitive heart and primary vascular plexus. This last in embryo is formed in the blood islands, which are constituted by clusters of cells called hemangioblasts. The hemangioblast is the precursor of hematopoietic/endothelial cells. In the blood islands the outer cells (angioblasts) differentiate in endothelial cells, while the inner cells develop in haematopoietic cells (Figure I) (Patan, 2004; Ribatti, 2007; Risau, 1997). The further expansion and networking of primary vessels occurs by angiogenenesis (Kalka et al., 2000). Endothelial cells migrate, proliferate and form tubular three-dimensional structures. Endothelial cells of nascent vessels release factors (*i.e.* PDGF-BB) that recruited perivascular cells such as pericytes (PCs) and smooth muscle cells (SMCs), which in turn, produce TGFβ to inhibit the further proliferation of endothelial cells. This process, called arteriogenesis, is based on cell-cell cross-interactions and provide to tissue stabilization and regulation of vessel perfusion (Benjamin et al., 1998).

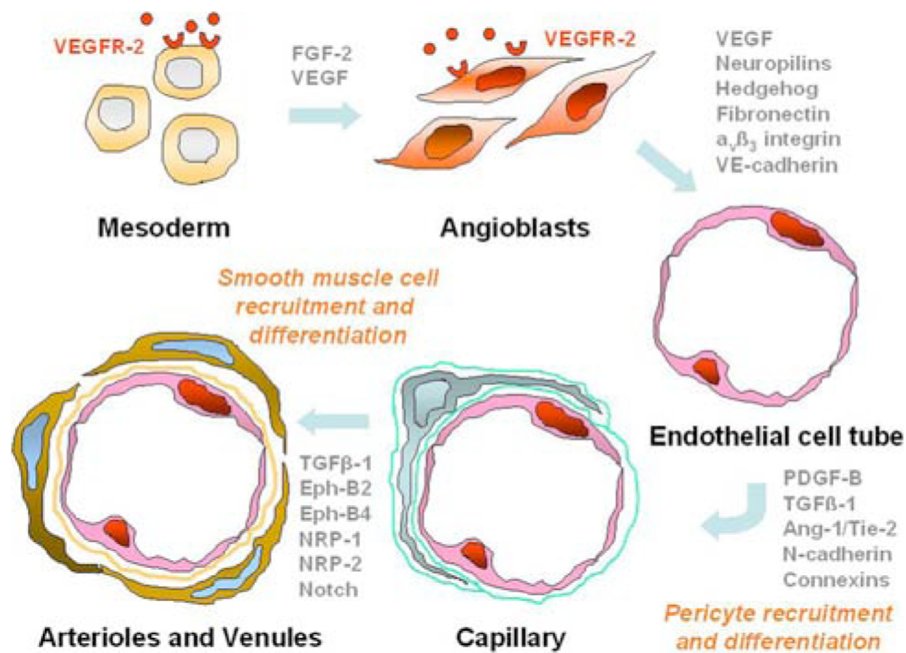


Figure I Vasculogenesis (Ribatti et al. 2009) The earliest blood vessels in the embryo originate from mesodermal cells that are specified into angioblasts most likely in response to Fibroblast Growth Factor-2 (FGF-2) and Vascular Endothelial Growth Factor (VEGF) signals. Angioblasts begin to differentiate into endothelial cells and assemble into tubes, principally as the result of VEGF signals from surrounding tissues and the expression of intercellular and cell-matrix adhesion molecules. Endothelial cell tubes are soon stabilized by pericytes recruited from the surrounding mesenchyme to form early capillaries. In microvessels, PDGF and TGFβ-1 signals are involved in the recruitment of pericytes. In larger vessels, arterioles and venules, the vascular wall is made up of endothelial cells and smooth muscle cells, which are recruited mainly through the Tie-2 and Angiopoietin-1 receptor-ligand pair,. Ephrin-B2 and Ephrin-B4 are implicated in arterialand venous endothelial cell specialization, respectively

1.2 Endothelial Progenitor Cells (EPCs)

For the first time, in 1994, the group of Asahara showed that clusters of endothelial cells were present in the center of an artificial graft *in vivo*. They hypothesized that besides the migration of mature endothelial cells from the edges, the re-endothelialization occurred through the differentiation of circulating endothelial-like (Shi et al., 1994)

The dogma that after birth new vessels in adult develop by angiogenesis was accepted until the late1990s, but more recent findings strongly suggested the new concept of postnatal development of blood vessels by vasculogenesis.

The same group few years later isolated from human peripheral blood mononuclear cells (CD34+), that injected in a mouse model of limb ischemia, they cooperate to the revascularization for a 25% (Asahara et al., 1997). These cells had the same antigenic profile of the cells that contributed to the fallout endothelialization observed in the previous work (Shi et al., 1994), and similar to embryonic angioblasts (Asahara and Kawamoto, 2004) (Shi et al., 1998). In transgenic mice it was shown that EPCs from bone marrow are involved in both physiological and pathological neovascularization (Asahara et al., 1997). Over the lasts years, several groups demonstrated the existence in postnatal life of progenitor cells, isolated from bone marrow, umbilical cord blood, peripheral blood and fetal liver, that were able to form *de novo* capillary structures by migrating and proliferating (Ceradini and Gurtner, 2005; Gehling et al., 2000; Grenier et al., 2007; Harraz et al., 2001; Jia et al., 2006; Kocher et al., 2001; Murga et al., 2004; Quirici et al., 2001; Takahashi et al., 1999).

Therefore now, it is accepted that endothelial progenitor cells exist in adult and cooperate in generation of blood vessels by a phenomenon called neo-angiogenesis (Asahara and Kawamoto, 2004; Eguchi et al., 2007; Luttun et al., 2002; Rafii and Lyden, 2003).

Ingram and colleagues, isolated from umbilical cord blood, progenitor cells with high proliferative potential and clonogenic capability, that were able to differentiate in mature endothelial cells (Case et al., 2007; Ingram et al., 2005; Ingram et al., 2004).

The term EPCs describes a group of cells in different stages of differentiation. It has been suggested that different subpopulations of EPCs co-exist in circulation, but the exact molecular signature of EPCs remains to be elucidated. Furthermore, several antigenic profiles have been proposed as

identifiers of EPCs, but a well characterized antigenic profile to define EPCs is still debate and controversial. Thus the *in vivo* contribute of progenitor cells to neovascularization is not really clear (Caplice and Doyle, 2005; Khakoo and Finkel, 2005; Larrivee and Karsan, 2007).

1.2.1 EPCs Identification

Originally, hematopoietic cells were regarded as source of EPCs, being both the hematopoietic progenitors and the endothelial progenitors derived from hemangioblast, and isolated from peripheral blood or bone marrow (Schatteman et al., 2004).

Common antigens such as CD34, and VEGFR2 are expressed by hematopoietic and endothelial progenitor cells. Mononuclear cells CD34+/VEGFR2+ have been reported to differentiate in mature endothelial cells and to be incorporated in new vessels (Asahara et al., 1999; Asahara et al., 1997). Nevertheless, subpopulations of CD34+/VEGFR2+ express additional antigens that defined the hematopoietic stem cells (HSCs) such as CD133, the angiopoietin receptor 2 (Tie-2) and CD117/c-kit in human and mouse and Sca-1 in mouse, (Gehling et al., 2000; Grant et al., 2002; Kritzenberger and Wrobel, 2004; Matsumoto et al., 2008; Peichev et al., 2000; Quirici et al., 2001; Shaw et al., 2004). Progenitor cells that express these characteristics are assumed to be multipotent hematopoietic stem cells, acting as progenitors for both hematopoietic and endothelial lineage. The commitment towards endothelial lineage is characterized by the expression of endothelial-specific cell surface antigens, such as von Willebrand Factor (vWF), platelet endothelial cell adhesion molecule (PECAM-1 or CD31), and Vascular Endothelial Cadherin (VE-Cad). Moreover, during their maturation, they acquired particular functions endothelium-specific, such as: the ability to up-take of Acetylated low-density lipoprotein (AcLDL), to bind certain lectins, and to form endothelial tubular structures (Kalka et al., 2000). To date, it is possible to distinguish HSCs with respect to EPCs, through the expression of CD133, but still lacks a specific epitope to discriminate between progenitor and mature endothelial cells. Indeed some of their antigens were found in embryonic progenitor endothelial cells, but also in mature or reactivated ECs (Choi, 1998; Krause et al., 1996; Ziegler et al., 1999). A definitive characterization of EPCs still has to be established.

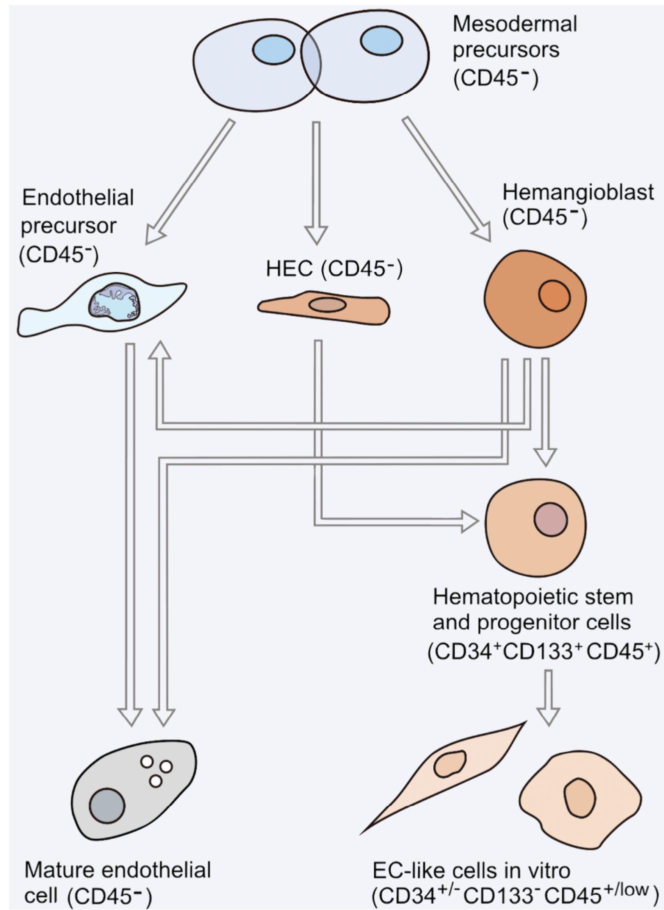


Figure II (Timmermans et al., 2009). Haemato-endothelial developmental pathways and their relation to the expression of CD45. In the embryo, CD45⁻ mesodermal precursors give rise to CD45⁻ endothelial precursors (EPCs), haemangioblasts and/or haemogenic endothelial cells (HECs). The CD45⁻ EPCs differentiate into functional and mature ECs. Embryonic haemangioblasts are CD45⁻ and differentiate to both CD45⁻ endothelial lineage cells and CD45⁻ HSCs/HPCs in vitro. Alternatively, or in addition to haemangioblasts, CD45⁻ HECs give rise to CD45⁻ haematopoietic stem/progenitor cells. HSCs/HPCs can give rise to EC-like cells in vitro and retain expression of CD45, whereas expression of the CD133 antigen is downregulated.

1.2.1 Culturing Methods

Several in vivo culture techniques have been developed to isolate and to culture EPCs (Figure III). Initially, EPCs were cultured from mononuclear cells, and in attempt to avoid possible contamination with monocytes, a preplating step of 24 or 48h was used to eliminate adherent cells. Non-adherent cells were re-plated, and after about 7 days in culture they formed colonies, that showed a typical central core composed by round cells and sprouting at the periphery spindle-shape cells. These cells, called early EPCs, expressed endothelial markers, but expressed also typical molecules of monocytic lineage (Figure II and IIIB). They disappeared after 14 days in culture and their clonal efficiency was very low, thus the non-adherent cells fraction could not be passaged in culture significantly ex-vivo (Fernandez Pujol et al., 2000; Fernandez Pujol et al., 2001; Gulati et al., 2003; Rehman et al., 2003; Schmeisser et al., 2001; Urbich et al., 2003).

Conversely, the adherent fraction gave rise to two different cell colonies. Cells with low proliferative potential appeared in culture within one week. Studies on patients that received sex-mismatch bone marrow transplant showed that these cells were probably circulating mature ECs (CECs) that sloughed from vessels wall (Lin et al., 2000).

Cells with high proliferative potential, with 1000 fold expansion for two months ex-vivo, and uniformly expressing endothelial but not hematopoietic marker, such as CD45 or CD14, appeared in culture in two or three weeks (Figure IIIC). These cells, defined late blood outgrowth endothelial cells (EOCs), were isolated from adult peripheral blood or cord blood, and were shown to originate from bone marrow (Figure IIIC) (Ingram et al., 2004; Lin et al., 2000).

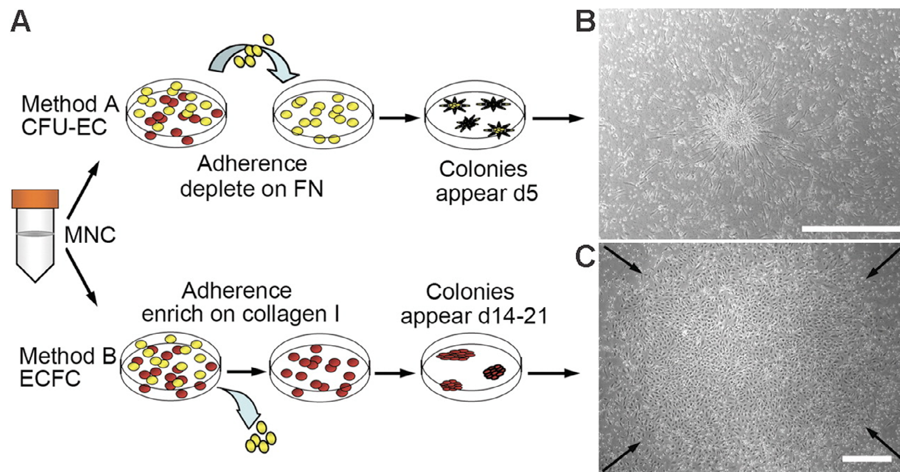


Figure III (Yoder et al., 2007) Culture of EPCs from human peripheral blood**.** (A) Two methods for isolating and culturing EPCs from human peripheral blood. Yellow cells represent non-adherent cells and red cells represent adherent cells. FN indicates fibronectin. (B) Representative phase-contrast photomicrograph of a CFU-EC colony (day 5) cultured from adult peripheral blood MNCs by method A. Similar colonies were observed from 29 other adult peripheral and 10 cord blood donors. Scale bar represents 500 μm. (C) Representative phase-contrast photo-micrograph of an ECFC colony (day 19) cultured from adult peripheral blood MNCs by method B. Similar colonies were observed from 29 other adult peripheral and 10 cord blood donors. Arrows indicate colony boundary and scale bar represents 500 μm.

EC-like cells	EOCs	CECs
(EPCs, ECs, CFU-ECs, CACs, ATs, early outgrowth CE-EPCs, CMMCs and early EPCs)	(EPCs, ECs, CFU-ECs, BOECs, ECFCs, EPDCs, EC-like, late EPCs, late endothelial outgrowth)	(Circulating endothelial cells)
1. Generated after 4–21 days in culture	1. Appear after > 7 days in culture	1. Low proliferative ECs, shed from the vascular wall into the circulation
2. Round (pancake) to spindle shaped appearance; no typical confluent monolayer	2. Typical polygonal cells in a confluent cobblestone monolayer	2. Have a similar phenotypical profile compared to EOCs
3. Express endothelial and haematopoietic markers (e.g. CD45, CD14)	3. Express CD31, CD34, CD105, CD146, VE-Cadherin, VEGFR-2, but not the haematopoietic surface markers CD133, CD14 or CD45	3. Do not express haematopoietic markers and have no apparent haematopoietic potential or function
4. Bind UEA-1 lectin and take up LDL	4. Bind UEA-1 lectin and take up LDL	
5. Maintain haematopoietic potential and/or functions	5. Have no apparent haematopoietic potential	
6. Have low proliferative potential	6. Bear high proliferative potential	
7. Do not generate vascular tubes *in vitro* in matrigel	7. Generate vascular tubes *in vitro*/*in vivo* in matrigel	
8. Improve neovascularization *in vivo*	8. Improve neovascularization *in vivo*	
9. Originate from CD45+ haematopoietic lineage cells ($CD34^{+}CD45^{+}$, $CD133^{+}CD45^{+}$, $CD34^{-}CD45^{+}$, $CD14^{+}CD45^{+}$)	9. Originate from $CD45^{-}CD133^{-}CD34^{+}$ cells, bone marrow (*) and probably the vascular wall	

Figure IV (Timmermans et al., 2009) Characteristic of human EC-like, EOCs, and CEC

II. Vascular Responses to Inflammation

2.1 Role of Vascular Endothelium

The quiescent vascular endothelium consists of a monolayer of endothelial cells (ECs) covering the luminal side of blood vessels (Carmeliet, 2003). ECs control the exchanges between the bloodstream and the neighboring tissues and form an anti-adhesive and non-thrombogenic surface for blood cells and platelets. Ablumenally, ECs are anchored to basement membrane, and mural cells, as pericytes and smooth muscle cells (SMCs), ensheath ECs penetrating the basement membrane and making direct contacts with them. ECs adhere to each other through junctional transmembrane proteins (Dejana, 2004) that regulate endothelial barrier functions. These proteins include vascular endothelial (VE)-cadherin and N-cadherin at adherent junctions, and occludins, claudins and junctional adhesion molecules (JAMs) at tight junctions (Lampugnani and Dejana, 1997; Luo and Radice, 2005; Vestweber, 2003). The organization of endothelial junctions varies in different vascular beds, which are susceptible of different exogenous stimuli.

In healthy adult, quiescent endothelium has turnover rates of months to years and proliferates only following angiogenic activation. Nevertheless, loss of quiescence is a common feature of inflammation (Carmeliet, 2005).

During inflammatory diseases leukocytes are stimulated by different mechanisms and secrete inflammatory cytokines such as TNFα and IL-1β. Under these stimuli, the endothelial layer can undergo a transition from the resting anti-adhesive state to the active adhesive state that results in change of ECs both adhesiveness and permeability. The activation of ECs involves rapidly acting, presynthesized and stored molecules, in addition to a subsequently slower transcriptionally regulated response program.

Pre-synthesized molecules, such as P-selectin and Angiopoietin-2, are stored in endothelial specific granules, known as Weibel-Palade bodies (WPBs), are released within seconds to minutes in response to inducers of inflammation and promote the subsequent activate state (Dikranian and Stoinov, 1991; Matsushita et al., 2004; Vischer et al., 1995)

Endotheliaum activation is associated with the loosening of inter-endothelial junctional complexes and the surface presentation of a specific set of adhesion molecules. These adhesion molecules mediate the interaction

between activated-ECs and leukocytes (*i.e.* monocytes, lymphocytes and macrophages) that express their counter-receptors, leading to their adhesion and the following transendothelial migration and extravasation into the underlying tissues (Dejana, 2004; Iso et al., 2001; Johnson-Leger and Imhof, 2003). Thus endothelial cells activation is the first step initiating the inflammation (Figure V).

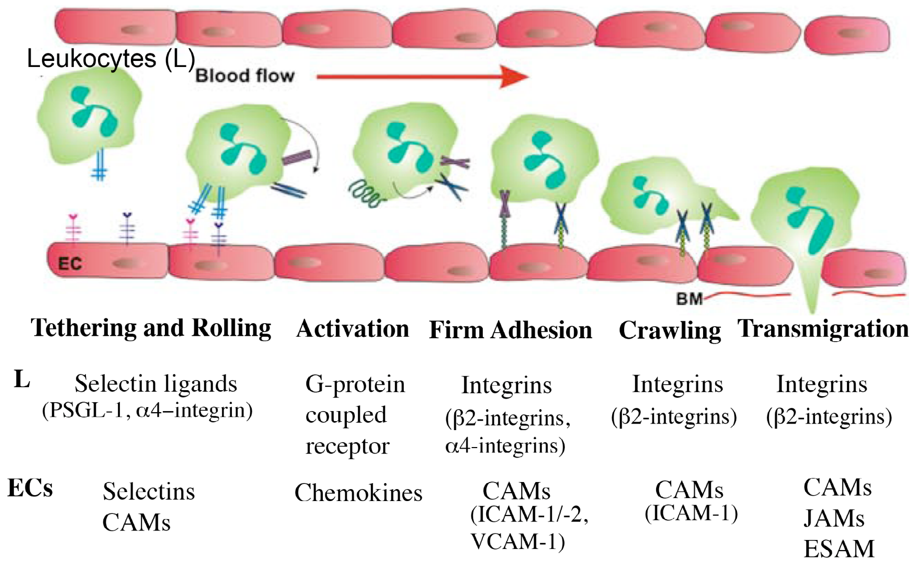


Figure V. Leukocytes transmigration (Petri et al., 2008) ECs, endothelial cells; L, leukocytes; BM, basal membrane; PSGL-1, P selectin glycoprotein ligand-1; CAMs, cellular adhesion molecules; JAM junctional adhesion molecules; ESAM, endothelial cell-selective adhesion molecules.

2.2 Adhesion Molecules

The adhesion molecules involved in the inflammatory endothelial response are: selectins, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM). They promote different phases of leukocytes transmigration: (i) the tethering and the rolling of leukocytes

along the endothelial surface is mediated by selectins, (ii) the firm adhesion is mediated by VCAM-1 and/or ICAM-1, and (iii) the definitive leukocytes transmigration through the opened junctions (Diapedesis) is mediated by ICAM-1 and other junctional molecules (Figure V) (Szmitko et al., 2003a; Szmitko et al., 2003b; Zhang, 2008).

Selectins are a family of calcium-dependent, type I transmembrane glycoproteins consisting of E-seletin and P-selectin expressed on venular endothelium, and L-selectin on leukocytes (Petri and Bixel, 2006). In human endothelium, TNFα and IL-1 induce primarily E-selectin *in vitro* (Pan et al., 1998). E-selectin expression overlaps with P-selectin enhancing the leukocytes recruitment and decreasing the rolling velocity (Kunkel and Ley, 1996). The binding of E- and P-selectin with their ligand, P-selectin glycoprotein ligand-1 (PSGL-1), expressed on the monocytes surface, signals to activate integrins (Ma et al., 2004; Weyrich et al., 1995). Integrins are heterodimeric cell surface receptors, constitutively expressed by lymphocytes and monocytes, and support both the rolling and the firm adhesion. Upon the activation integrins undergo a series of conformational changes that result in increased binding affinity for their respective ligands (Luo et al., 2007). The leukocytes integrins most relevant are very late antigen-4 (VLA-4) and β_2-integrins (LFA-1).

VLA-4, also known as $\alpha 4\beta 1$ integrin, mediates the rolling on its ligand, VCAM-1, expressed by ECs, when is in a lower affinity conformation, and the firm adhesion when is in a high-affinity state (Alon et al., 1995; Huo and Ley, 2001).

VCAM-1 is a member of the immunoglobulin-like superfamily of adhesion molecules and, it is not routinely expressed under physiologic conditions, but it is induced on cytokines-stimulated endothelium. The functional importance of VCAM-1 expression in atherosclerotic lesions is supported by studies in which antibodies blocking VLA-4 or VCAM-1 increase the velocities of the rolling, and monocytes adhesion was reduced by 75% (Huo and Ley, 2001; Ramos et al., 1999). Conversely, activation of VLA-4 in atherosclerotic lesions mediates the firm adhesion of monocytes to ECs (Huo and Ley, 2001).

The β_2-integrins are made up of a common β_2 subunit and one of four different α subunits (Luo et al., 2007). They mediate the firm adhesion in the high affinity state, binding ICAM-1 expressed on ECs surface. The exact role of ICAM-1 in monocytes recruitment has not been defined, but mice on high-fat diet lacking ICAM-1 show smaller atherosclerotic lesions than wild

type (Nageh et al., 1997), even if ICAM-1 expression is not detected in lesion sites (Cybulsky et al., 2001).

2.3 The Inflammatory Cytokine IL-1β

Cytokines are soluble and short acting proteins, glycoproteins and peptides, produced by various immune cells and vascular cells. Some cytokines may be membrane-bound or associated with extracellular matrix (ECM), and switching between soluble and membrane-bound forms is a regulatory event. Cytokines may be classified in pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines, such as TNFα, IL-1, IL-6, IFNγ, and TGFβ are involved in the up-regulation of inflammatory reactions and are produced predominantly by activated macrophages.. Anti-inflammatory cytokines are involved in the down-regulation of inflammatory reactions, and include IL-1, IL-10, IL-13, IFNγ and TGFβ. However, a clear-cut classification as pro- or anti-inflammatory may be difficult because the effects can be determined by local environment in which they are released, by the timing of the release, by the presence of synergistic or competing factors, by cytokines receptor density and tissue responsiveness.

IL-1α and IL-1β together with TNFα are defined as "alarm cytokines" secreted by macrophages and initiating the inflammatory reaction (Apte and Voronov, 2002; Dinarello, 1996, 2002). They stimulate their own and each other production, and induce the expression of pro-inflammatory genes. They increase the expression of high affinity adhesion molecules on ECs and stromal cells, promoting the infiltration of leukocytes from bloodstream into the tissues. Binding of cytokines with their receptors leads to the activation of transcription factors such as signal transducer and activators of transcription (STATs) (Ihle, 2001), and nuclear factor kB (NF-kB) (Tedgui and Mallat, 2006). NF-kB plays a central role in the development of inflammation activating the transcription of pro-inflammatory cytokines, adhesion molecules such as E-selectin, VCAM-1 and ICAM-1, chemokines, growth factors, inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide syntase (iNOS), and matrix metalloproteinases 1, 3 and 9 (MMP) (Bond et al., 2001).

Among cytokines, IL-1β plays a major role in the atherogenesis. It has been found in human plaques (Tedgui and Bernard, 1994) and *in vivo* studies had shown its involvement in the atherosclerotic process. It has been shown that

mice knockout for the transporter of LDL apoliprotein E (apoE), and additionally silenced for IL-1β (Kirii et al., 2003) or for IL-1β receptor (IL-1R), (Chi et al., 2004) or over-expressing the antagonist of IL-1β (IL-1Ra), showed significantly decreased tail lesions compared to mice $apoE^{-/-}$. Similar results were obtained in mice $apoE^{-/-}$ with subperitoneal injection of human recombinant IL-1Ra (Elhage et al., 1998).
Acting as autocrine and paracrine factor, IL-1β regulates: (i) macrophages infiltration by inducing the expression of adhesion molecules on ECs (Yamamoto et al., 1998); (ii) formation of fibrotic chapel, by provoking migration and proliferation of smooth muscle cells (SMCs) (Delbosc et al., 2008; Libby et al., 1988); (iii) plaque instability, by increasing apoptosis of SMCs and macrophages (Geng et al., 1996), (v) secretion MMPs (Delbosc et al., 2008; Gurjar et al., 2001), and (vi) amplification of inflammatory reaction by inducing the biosynthesis of prostaglandine E_2 in SMCs (Couturier et al., 1999; Jaulmes et al., 2006).

Besides to the direct effect on vessels wall dysfunctions, recent evidence highlights the involvement of IL-1β in the pathogenesis of several neurological disorders (Allan and Rothwell, 2001; Allan et al., 2005). IL-1β increases in brain of rodent following cerebral ischemia (Buttini et al., 1994; Davies et al., 1999; Minami et al., 1992; Pearson et al., 1999; Zhang et al., 1998), and several groups have shown that IL-1β induction contributes to neuronal death (for review see: (Loddick et al., 1998; Rothwell and Luheshi, 2000; Rothwell and Relton, 1993; Rothwell and Strijbos, 1995)). The exact pathways remain to be defined, but it has been shown that inhibition of IL-1β signaling resulted in decrease of adhesion molecules production, and consequently, reduced leukocytes infiltration leading to a significant reduction of infarct size in rat models of cerebral ischemia (Garcia et al., 1995; Yamasaki et al., 1995; Yang et al., 1999).

In cancer patients, increased local IL-1β levels have been shown correlated with tumor invasiveness and poor prognosis (Apte and Voronov, 2002). Little is known about the effects of IL-1 on carcinogenic process, but it may enhance the invasiveness of already existing tumor cells by switching on the angiogenesis and leading to tumor dissemination and metastasis. Indeed, infiltration of macrophages is a common feature of inflammation, angiogenesis and cancer.

2.4 Cytokines-Induced Inflammatory Signaling.

The main intracellular mediators of the inflammatory responses belong to the protein family of NF-kB. These proteins are transcriptional factors that, in a cell-specific manner, activate the downstream cascade of inflammatory genes, such as adhesion molecules in endothelial cells (Chen et al., 2009; Madge and Pober, 2001; Ronald et al., 2001; Senftleben and Karin, 2002; Yang et al., 1999).

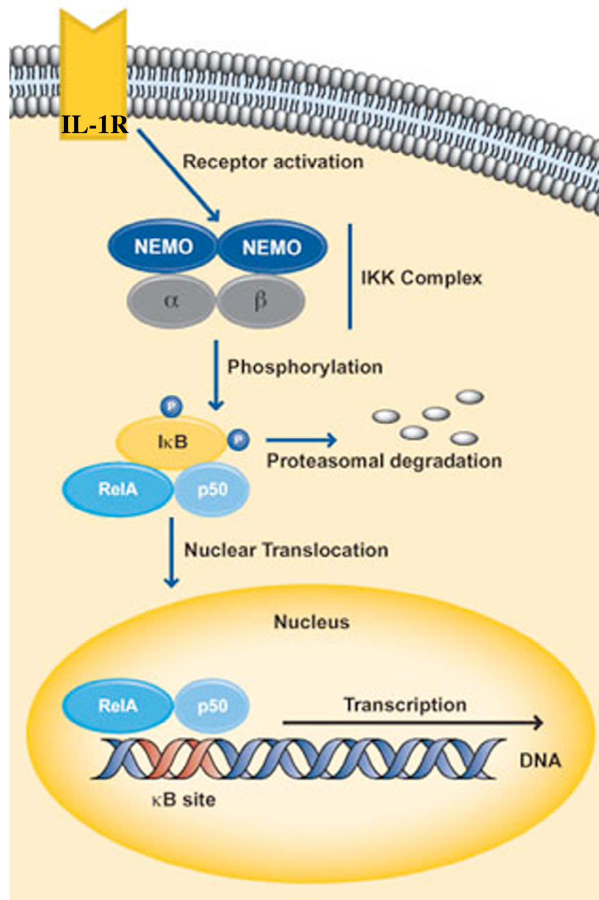


Figure VI. NF-kB Signaling

NF-kB family includes homo- and hetero-dimers formed by p50, p65 (RelA), c-Rel, p52 and RelB. The Rel domain (RHD), which contains a nuclear localization sequence (NLS) is present in all five proteins and is involved in the DNA binding. In non-stimulated condition, NF-kB dimers are sequestered in the cytoplasm through the binding with proteins belonging to the family of Inhibitor of kB (IκB). These include IκBα, IκBβ, IκBε, the two precursors of NF-kB p100 (p52 precursor) and p105 (p50 precursor), as well as Bcl3. These proteins physically interact with the RHD of NF-kB masking the NLS and thus, preventing the NF-kB nuclear import and its transcriptional activity (Figure VI) (Hayden and Ghosh, 2004).

NF-kB activation may occur by different pathways: the canonical IKK pathway, the non-canonical pathway and the atypical pathway.

IL-1β activates NF-kB through the canonical IKK pathway. The IKK signalosome, is a multiproteic complex made up of three subunits, IKKα, IKKβ, and IKKγ/NEMO. Activation of the canonical pathway is mediated primarily through the activation IKKβ, which in turn, phosphorylates IκBα, on the residues Ser 32 and Ser 36, and IκBβ and IκBε, on equivalent residues, triggering to their polyubiquitination and proteosomal degradation. This results in the NF-kB release and translocation into the nucleus, where it activates the transcription of target genes (Bonizzi and Karin, 2004; Pasparakis et al., 2006).

The non-canonical pathway is activated by ligands such as CD40L and lymphotoxin and leads to IKKα activation, which in turn phosphorylates p100 and p105 causing its partial degradation to generate p52 heterodimers (NF-kB2) (Bonizzi and Karin, 2004; Perkins, 2003).

A third pathway is the atypical or IKK-independent pathway, activated by different stimuli such as UV radiations, hypoxia or oxidizing radicals. In this case, the IκBα phosphorylation with consequent proteosomal degradation occurs on Tyr 42 and on Ser/Thr residues on C-terminal PEST domain (Perkins, 2006; Perkins and Gilmore, 2006).

III. Notch Signaling

3.1 Notch Receptors

Notch signaling is an evolutionarily conserved pathway that modulates cell fate decisions through local cell-cell interactions. Notch receptors and, more in general all components of Notch signaling, display a tissue-specific expression pattern. Subtle differences in Notch expression and signaling can single out individual cells from a group of equivalent neighboring cells and thereby impose differential behavior and differentiation fates (Lai, 2004; Schweisguth, 2004).

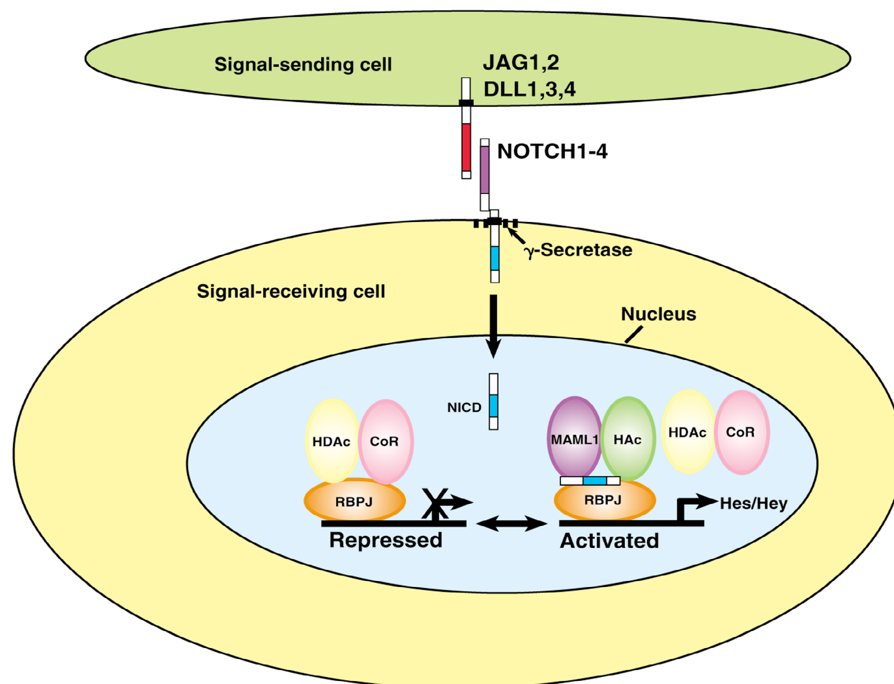


Figure VII. Core components of Notch signaling (Gridley, 2007). Signal-sending cell (green) expresses Notch ligands (Jagged1, 2 and Dll1, 3, 4); signal-receiving cell (yellow), expresses Notch receptor (1-4). After the binding with the ligand, the receptor is subjected to

two proteolitic cleavages. The second cleavage is mediated by γ–secretases complex, and releases the Notch intracellular domain (NICD), that translocates into the nucleus. NICD displaces the Histone Deacethylases (HDAc) and the co-repressor (CoR) from RBP-jK (also known as CSL for CBF/ Su(H)/ Lag-1) and allows to association with Histone Acethylases (HAc) and Mastermind-like 1 (MAML-1), and the following transcriptional activation of target genes (Hes and Hey)

Binding of Notch ligand to Notch receptor induces conformational change in the Notch extracellular domain resulting in the exposure of a cleavage site within the Notch extracellular domain. Following the cleavage Notch receptor undergoes to proteolysis, mediated by γ-secretases complex, at a conserved cleavage site in the intracellular domain. Hence, the Notch intracellular domain (NotchICD) is released into the cytoplasm and translocates into the nucleus where it activates the transcription of target genes (Hes/Hey) (Figure VII).

Genes of the Notch family encode a series of type I transmembrane receptors highly conserved among species (Fleming, 1998). In *Drosophila* a unique Notch receptor was identified (Wharton et al., 1985). In *C.Elegans* are known 2 homologues (LIN-12 and GLP-1) (Austin and Kimble, 1989) whereas mammals four Notch receptor homologues (Notch1 to 4) have been characterized (Artavanis-Tsakonas et al., 1999; Kopan and Weintraub, 1993; Lardelli et al., 1994; Weinmaster et al., 1991, 1992).

Notch receptors (Figure VIII) are synthesized as single-chain precursors, and after essential glycosylation in the endoplasmic reticulum, are cleaved by the protease furin-like covertase in the *trans*-Golgi network. The extracellular (NotchECD) and the transmembrane-intracellular subunits are bound, at the external membrane side, by Calcium-dependent non-covalent interactions (Bray, 2006; Hurlbut et al., 2007; Le Borgne et al., 2005; Weinmaster, 2000) .

The NotchECD domain contains: 29-36 epidermal growth factor (EGF)-like repeats, depending on the particular Notch receptor, of which the 11th and 12th are involved in the ligands binding (Haines and Irvine, 2003; Rebay et al., 1991); three cysteine-rich Notch-LIN12 repeats (LNR) are involved in the heterodimerization of the receptor and prevent the ligand-independent activation (Hurlbut et al., 2007; Le Borgne et al., 2005; Weinmaster, 2000).

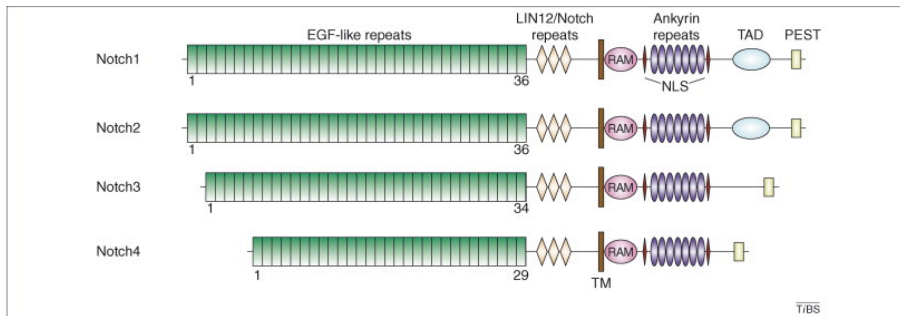


Figure VIII. Representation of mammal Notch receptors (1-4) (Wu and Bresnick, 2007) Notch extracellular domain contains between 29 to 36 tandem repeats of epidermal growth factor (EGF)-like repeats and three Lin12/Notch repeats. The Notch intracellular domain includes a RAM domain, seven cdc10/ankyrin repeats, two nuclear-localization sequences (NLS), a transactivation domain (TAD) (Notch1 and Notch2) and a PEST motif at the C-terminus. Abbreviation: TM, transmembrane domain.

Notch receptors are modulated by glycosylation within the EGF-like repeats by the glycosyltransferase Fringe in the Golgi. These modifications inhibit the ability of Notch to be activated by Jag ligands, whereas the activation by Dll proteins is potentiated (Bray, 2006; Haines and Irvine, 2003). The different number of EGF-like repeats in the receptor could permit a different post-transcriptional regulation leading to a particular specificity in the binding with ligand sub-types.
The NotchICD includes: a RBP-jK associated domain (RAM) which potentiates the interaction with a DNA binding protein, called RBP-jK in mammals; seven tandem ankyrin repeats (ANK); two nuclear localization signal (NLS); a glutamine-rich domain; a prolin, glutamate, serine, threonine-rich domain (PEST) domain involved in the receptor turnover; and the transmembrane domain (TM) (Lubman et al., 2004). Notch1 and Notch2 have in the NotchICD a transactivation domain (TAD) probably responsible for the higher transcriptional activity compared to Notch3 and Notch4 (Figure VIII) (Beatus et al., 2001)

3.2 Notch Ligands

In mammals are known five canonical Notch ligands, collectively referred to Delta/Serrate/Lag-2 (DSL), on the basis of structural homology to the two *Drosophila* ligands Delta and Serrate. They are named Delta-like (Dll1, Dll3, Dll4) and Serrate-like (Jagged1 and Jag2) (Fleming, 1998; Shutter et al., 2000). As well as for Notch receptors, the Notch ligands are type 1 transmembrane proteins, containing a variable number of EGF-like repeats in the extracellular domain (Figure IX) (Artavanis-Tsakonas et al., 1999). The DSL domain together with the N-terminal domain (NT) and the first and second EGF-like repeats are required for the binding to the receptor (Parks et al., 2006; Shimizu et al., 1999). Jagged1 and Jag2 have two EGF-like regions (Weinmaster, 1997) and an additional cysteine-rich region (CR) not found in Dll ligands (Vitt et al., 2001). The role of CR is unknown, but deletion of the region in *Xenopus* embryo resulted in inhibition of the ligand activity (Figure IX) (Kiyota and Kinoshita, 2002).

Figure IX. Schematic representation of DSL ligands (Bolos et al., 2007). The ligands cointain at the N-terminal one Delta /Serrate/Lag-2 domain (DSL); 1 or 2 epithelial growth factor(EGF)-like repeats; In Jagged1 and 2 is present a cysteine-rich region (CR).

DSL ligands can undergo proteolytic cleavage in the juxtamembrane and transmembrane region by ADAMs and γ−secretase, respectively (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Mishra-Gorur et al., 2002; Six et al., 2003). The ligand proteolysis affects Notch signaling by decreasing cell-surface expression, but it is less clear if the proteolytic product might have an intrinsic cellular activity, even if a nuclear localization was found for Delta in *Drosophila* S2 cells (Bland et al., 2003). On the other hand, the over-expression of the intracellular domain of Jagged1 increases the transcription of a reporter gene under a CBF-1 promoter, indicating that this ligand could act as a transcriptional activator. Furthermore, the co-transfection of the intracellular domain of both Jagged1 and Notch1 inhibits the CBF-1 promoter trans-activating, suggesting a possible crosstalk between Notch and DSL in same cells (LaVoie and Selkoe, 2003).

3.3 Notch Signaling Transduction Pathway

Notch signaling is an evolutionarily conserved pathway that modulates cell fate decisions through local cell-cell interactions.

The interaction of the ligands with the Notch extracellular domain results in a conformational change on the extracellular portion of the receptor exposing a motif recognized and cleaved (S2) by the Tumor Necrosis Factor-α (TNF-α) converting enzyme (TACE/ADAM17) (Baron, 2003; Brou et al., 2000). A further cleavage (S3) takes place within the transmembrane domain by the γ-secretases complex (Baron, 2003; Brou et al., 2000; Kimberly et al., 2003). Once the receptor is cleaved, the extracellular domain of the receptor is internalized in the signal sending-cell through the binding with the ligand, and the Notch intracellular domain (NotchICD) is released and migrates into the nucleus where it interacts with a DNA binding protein CSL, called RBP-jK in mammals (Schweisguth, 2004). CSL in the absence of NotchICD acts as a transcriptional repressor recruiting histone deacetylases (HDAc) and other components to form a co-repressor complex (N-CoR). The binding with NotchICD displaces the histone deacetylases and the co-repressor complex and recruits other proteins, such as mastemind-like1 (MAML1) protein and histone acetylases (HAc), leading to the transcriptional activation of Notch target genes (Figure X) (Bray and Furriols, 2001).

Notch target genes encode for basic helic-loop-helic (bHLH) proteins, belong to Hes (Hairy and enhancer of split) and HRT/Hey/HERP (Hairy-related transcription) gene families of transcriptional repressor and are critically involved in mammalian cell differentiation (Kopan and Ilagan, 2009).

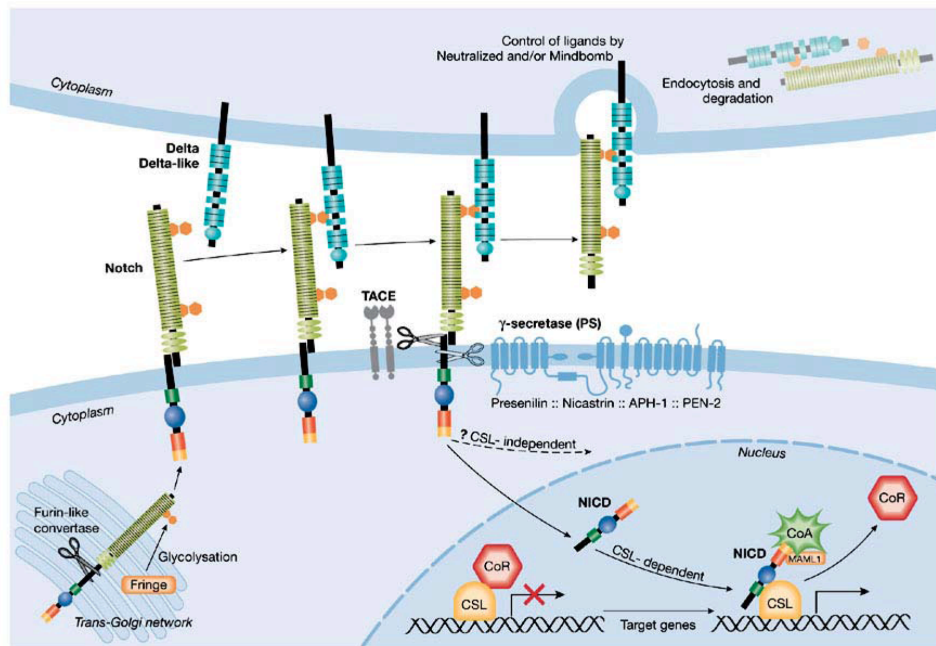


Figure X. Notch signaling transduction (Wu and Bresnick). Signal-sending cell expresses Notch ligands (Jagged1, 2 and Dll1, 3, 4); signal-receiving cell, expresses Notch receptor (1-4). After the binding with the ligand, the receptor is subjected to two proteolitic cleavages. The first cleavage is mediated by TACE, and the second by γ–secretases complex. The extracelluar domain of Notch receptor is internalized with the ligand in the signal-sending cell, whereas, the Notch intracellular domain (NICD), translocates into the nucleus. NICD displacing the Histone Deacethylases (HDAc) and the co-repressor (CoR) from RBP-jK (also known as CSL for CBF/ Su(H)/ Lag-1) allows the association with Histone Acethylases (HAc) and Mastermind-like 1 (MAML-1), and the following transcriptional activation of target genes (Hes and Hey).

The basic domain is necessary for the DNA binding, and present a conserved Proline residue (Massari and Murre, 2000). The HLH domain is involved in the dimerization and in the interactions with other proteins. The Orange domain,is formed by two α-helical stretches (helix3/4), that act as additional interface for proteins interactions, and seems to repress the transcription when fused to a DNA binding domain (Dawson et al., 1995).
Hes proteins, to form the transcriptional repressor complex, recruited the co-

repressor Groucho, called TLE in mammals, through the highly conserved domain in the carboxy-terminal, the WRPW (Figure XI) (Fisher and Caudy, 1998; Grbavec et al., 1998; Grbavec and Stifani, 1996). Hes proteins are supposed to repress by at least three mechanisms. The first mechanism is a DNA-binding-dependent transcriptional repression (Kageyama and Nakanishi, 1997; Kageyama et al., 2000). Hes proteins form a homodimers, bind class C (CACGNG) or N-box (CACNAG) consensus DNA sites and, by recruiting TLE, active repress the transcription of target genes (Fisher et al., 1996; Grbavec and Stifani, 1996; Oellers et al., 1994; Ohsako et al., 1994; Paroush et al., 1994; Sasai et al., 1992; Tietze et al., 1992; Van Doren et al., 1994). The second mechanism is passive repression involving protein sequestration (Hirata et al., 2000; Sasai et al., 1992). Hes1, for instance, can form a non-functional heterodimer with other bHLH factors, such as E47, a common heterodimers partner of tissue-specific bHLH fctors, such as MyoD and Mash1, thereby disrupting the formation of functional heterodimers (E47-Mash and E47-MyoD). The third mechanism is mediated by Orange domain, which is essential to repress its own (Hes1) promoter as well as the p21 promoter (Castella et al., 2000).

Among Hes proteins (Hes1-7) only Hes1, Hes5 and Hes7 can be induced by Notch pathway (Bessho et al., 2001; Hsieh et al., 1998; Jarriault et al., 1995; Nishimura et al., 1998; Ohtsuka et al., 1999).

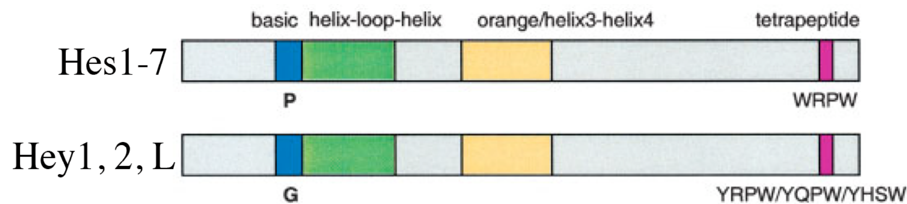


Figure XI. Schematic representation of amino acids sequence of Hes and Hey proteins (Iso et al., 2003b)

In contrast, all the three Hey genes (Hey1, 2 and L) can be induced by Notch. They are conserved during evolution and both the bHLH domain and the Orange domains are very similar to those of Hes proteins. However, they lack of the Proline residue in basic domain and do not bind N-boxes sequences on DNA (Iso et al., 2001). Moreover, they lack of the WRPW tetrapeptide and, thus, do not bind TLE. A related (YRPW) motif is found in

Hey1 and Hey2, or a degenerated (YXXW) sequence is present in HeyL (Figure XI) (Fischer et al., 2002; Iso et al., 2001). The bHLH domain of Hey proteins is necessary and sufficient for recruitment of a co-repressor complex including N-Cor (nuclear receptor corepressor), mSin3A and HDAC1 (histone deacethylses-1) (Iso et al., 2001). Hey, like Hes1, can bind the same DNA consensus sequences, albeit with different preferences than Hes1 (Nakagawa et al., 2000). A more intriguing possibility is that HES and Hey positively interact with each other to enhance DNA binding. Indeed, Hes and Hey associate with each other as a hetero-oligomer both in vitro and in intact cells in the absence of DNA (Iso et al., 2001; Leimeister et al., 2000).

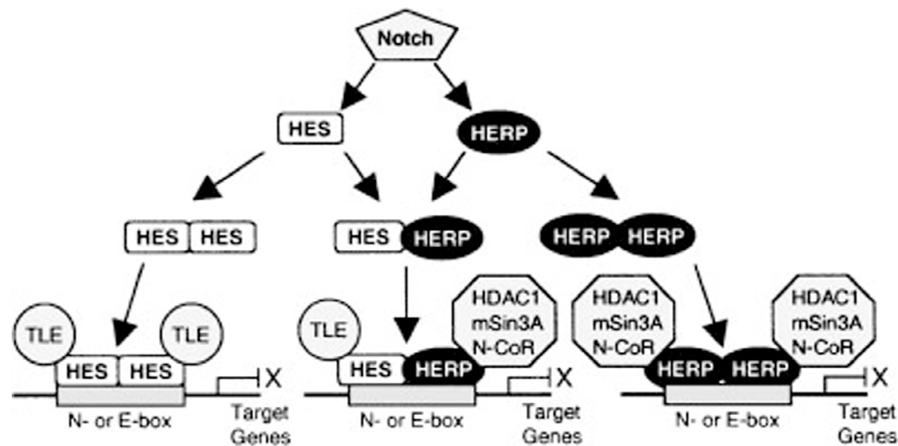


Figure XI. Model for HES and HERP/HEY cooperation in Notch signaling (Iso et al., 2003b). Upon notch stimulation, HES and HEY expression may both be induced. In tissues where only HES or HEY is expressed, the respective homodimer binds promoters of target genes. The HES homodimers recruit TLE via their C-terminal WRPW motif, whereas the HERP/Hey homodimers recruit a complex constituted by N-CoR (nuclear receptor corepressor), mSin3A and HDAC (Histone deacethylases) via their bHLH domain. In tissues where both HES and HERP/Hey are coexpressed, the HES-HEY heterodimers become the predominant complex that avidly binds a specific DNA site, which may be newly defined by the two heterologous basic domains of HES and HEY. Because of the higher DNA binding activity of the heterodimers, a lower concentration of HES and HEY may be sufficient to achieve repression. Repression by HES-HEY heterodimers may be reinforced by their ability to recruit a more diverse set of corepressors including both TLE and N-CoR/mSin3A/HDAC.

IV. Notch Signaling in the Vascular System

4.1 Expression Mapping of Receptor and Ligands in the Vasculature

Among Notch receptors Notch1 and Notch4 are expressed by ECs, and play the major role in endothelial physiology. Notch1 it has been also found in arterial SMCs during embryo development and in adult. Notch3 expression is restricted to VSMCs. The ligands Jagged1, Jag2, Dll1 and Dll4 are present in ECs and in SMCs, and their expression can temporally and spatially differ. Importantly, Notch4 and Dll4 appear to be specifically expressed in endothelium and maintain vascular homeostasis (Alva and Iruela-Arispe, 2004; Iso et al., 2003a; Karsan, 2005). During development Dll4 is the first ligand expressed in most embryonic capillary beds at mid-gestation and in the retina on the tip cells. Later in the development Dll4 segregates to the arterial vessel both in ECs and, to a less extent, in SMCs (Hofmann and Iruela-Arispe, 2007; Iso et al., 2003b; Shutter et al., 2000; Villa et al., 2001) Dll4 is re-expressed in neoangiogenesis in adult (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). Jagged1 distribution is complementary with that of Dll4, being present in resting (stalk) cells in retina, and excluded in tip cells (Beckers et al., 1999; Hofmann and Iruela-Arispe, 2007), and during vascular remodeling is present both in ECs and in SMCs. Differences in timing, location of expression, and loss of function phenotype for these two ligands suggest that they may have not overlapping functions but independent activities. Dll1 expression in ECs was found in embryo both in arteries and in veins. In adult, Dll1 is present in arteries (Beckers et al., 1999; Hofmann and Iruela-Arispe, 2007; Limbourg et al., 2007), and plays an important role in arteriogenesis associated with growth in post ischemic events (Limbourg et al., 2007). It seems possible that Dll1 in this process can activate Notch1, (Takeshita et al., 2007).

4.1 Notch and Vascular System Formation in Embryo

A large body of evidence indicates that Notch signaling plays a critical role in mammalian vascular development during embryogenesis. Gene inactivation strategies in mice have shown that Notch signaling is critical for the reorganization of vessels that derive from the primitive vascular plexus (Figure XII). It has been shown that Notch1$^{-/-}$ mice die at E9.5 with sever angiogenic defects, whereas the vasculogenesis was not affected (Krebs et al., 2000; Swiatek et al., 1994). Strikingly, Notch4$^{-/-}$ are viable and fertile and do not show defects in vasculature (Krebs et al., 2000). Interestingly, Notch1$^{-/-}$ and Notch4$^{-/-}$ mice show more sever vascular defects compared to the single Notch1$^{-/-}$ suggesting that Notch1 has non-redundant functions and can compensate for Notch4 loss (Krebs et al., 2000). Mice with endothelial-specific over-expression of the constitutively active form of Notch4 (Notch4ICD) displayed disorganized vascular network and dilated vessels, suggesting that a fine tuning of expression is necessary to properly drive morphogenesis (Uyttendaele et al., 2001). Notch2 expression is not reported in vasculature, and embryonic targeted deletion of Notch2 is lethal due to defects in cardiovascular (heart and hyaloid vasculature of eyes) and kidney (glomerular capillary) systems (Hamada et al., 1999; McCright et al., 2001). Finally, Notch3$^{-/-}$ mice are viable and fertile but show defects in vascular smooth muscle cells (VSMCs) maturation (Domenga et al., 2004).

In regard to the Notch ligand mutations, Dll4$^{+/-}$ mice present haploisufficiency and lethality at E9.5 (Gale et al., 2004) and defects in the remodeling of both the primitive vascular plexus and yolk sac, similar to Notch1$^{-/-}$, indicating that Dll4 could be the early critical ligand for Notch1 signaling in the vasculature (Krebs et al., 2004). Embryos lacking the Jagged1 gene die at E11.5., and although the lethality occurs two days later that Notch1$^{-/-}$ the phenotypic defects are reminiscent of Notch1$^{-/-}$ mice (Xue et al., 1999). This suggests that Dll4 and Jagged1 have functions neither overlapping nor redundant at least early in development. Dll1 expression is not predominant in the vasculature, and targeted deletion of Dll1 leads to lethality at E12.5 with generalized hemorrhagic events (Hrabe de Angelis et al., 1997).

The importance of Notch signaling is also confirmed by data on mice CSL$^{-/-}$. They displayed defects similar to the Notch1$^{-/-}$/Notch4$^{-/-}$ with severe growth retardation and lacking of remodeling of the primitive vascular plexus. Endothelial-specific CSL$^{-/-}$ resemble to Notch1$^{-/-}$, Notch1$^{-/-}$/Notch4$^{-/}$

and Dll4$^{-/-}$ mice, indicating the main role of Notch in vascular development (Krebs et al., 2004; Oka et al., 1995).

In mice, inactivation of Notch downstream target gene Hey2 revealed a role in heart development, and these mice were viable until one week after birth (Donovan et al., 2002; Sakata et al., 2002). In contrast, Hey1$^{-/-}$/Hey2$^{-/-}$mice showed embryonic lethality between E9.5 and E11.5 and display vascular defects, typical of disruption of Notch signaling, such as hemorrhage, defect in arterial/venous specification, enlarged pericardial sac, heart abnormalities, lack of vessel remodeling, enlarged vessel in embryo and yolk sac, suggesting a redundant role for these two genes (Fischer et al., 2004; Kokubo et al., 2005).

In summary the inactivation of Notch signaling pathway does not prevent vasculogenesis (*i.e.* differentiation of EPCs in mature ECs), but prevents the progression from the primitive vascular plexus to a structured network of arteries, capillaries and veins.

Adult neovascularization is also altered by deregulated Notch signaling. Notch signaling inhibition by genetic or pharmacologic approaches leads to increased endothelial proliferation with excessive vascular branching, with vessels with reduced or absent lumen. The outcome is the expansion of a non-functional vascular network that does not support tissue perfusion and tumor expansion (Hellstrom et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). In contrast, activation of Notch signaling in tumor, by over-expression of Jagged1, promotes angiogenesis and tumor growth (Zeng et al., 2005).

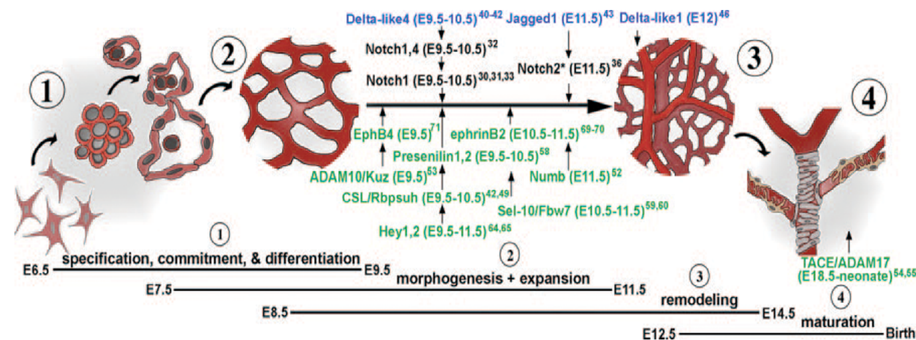


Figure XII. Vasculogenesis (Hofmann and Iruela-Arispe, 2007). Genetic inactivation of Notch ligands, receptors, downstream effectors, and modulators leads to embryonic lethality

as a result of vascular defects. The vascular system develops from mesenchymal progenitor cells that differentiate into hemangioblasts (1) and subsequently form the primitive vascular plexus. Later, this uniform network remodels into a hierarchical vascular system. (3) As indicated, inactivation of several Notch receptors, ligands, and genes associated with Notch signaling results in embryonic lethality at the developmental stages indicated in parenthesis. Loss-of-function experiments have not been done during vascular maturation. (4) The 4 stages in vascular morphogenesis are (1) specification, commitment, and differentiation of endothelial cells (E6.5 to E9.5); morphogenesis and expansion of the vasculature (E7.5 to 11.5); (3) remodeling (E8.5 to E14.5); and (4) maturation (E12.5 to birth).

4.3 Notch Signaling and Vascular Diseases

Due to the fundamental role of Notch signaling in tissue development and homeostasis, both gain- and loss- of- function mutations are linked to human pathologies (Gridley, 2007; Leong et al., 2007).

In human, Notch3 mutations in vascular smooth muscle cells (VSMCs) cause a vascular pathology termed Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). CADASIL is characterized by destruction and disorganization of VSMCs in cerebral arteries and arterioles with consequent arteriopathy that in turn leads to migraines and recurrent stroke (Louvi et al., 2006). All Notch3 mutations involved in CADASIL pathogenesis are present in the extracellular domain. To date, the functional mechanism of Notch3 mutations in CADASIL remains unclear.

Mutations in Jagged1 gene cause 60-70% of Alagille Syndrome (AGS). The AGS is a dominantly inherited multisystem disorder and the main clinical manifestations are abnormalities in liver, heart, eye and skeleton, and the cardiovascular defects. These lasts include: aortic coarctation, atrial and ventricular defects, and tetralogy of Fallot (Krantz et al., 1999). While AGS in human is caused by haploinsufficiency of Jagged1, mice heterozygous JAGGED1 do not recapitulate AGS, but the additional introduction of a single Notch2 hypomorphic allele leads to AGS-like abnormalities (McCright et al., 2002; McDaniell et al., 2006; Xue et al., 1999).

BACKGROUND

The first evidence for the involvement of Notch signaling in vascular inflammatory responses comes for the study of the Limon's group on de-differentiation induced by IL-1β in vascular smooth muscle cells (VSMCs) (Clement et al., 2007). They demonstrated that IL-1β down-regulating Notch3, by a NF-kB-dependent mechanism, induces the transition of VSMCs from a quiescent/contractile phenotype towards an inflammatory/de-differentiated state. Conversely, the over-expression of Notch3 active form (Notch3ICD) prevents this process. Notably, IL-1β induces the up-regulation of Notch1, by a NF-kB-independent mechanism, suggesting that a different signaling act on this receptor. Additionally, Notch1ICD over-expression did not show effects of VSMC phenotype. On the other hand, in VSMCs it was observed that Notch1 induces the preferential expression of the target gene Hey1, whereas Notch3 transcription mainly activated Hes1 (Clement et al., 2007). These data suggest different functions for Notch1 and Notch3 on the inflammatory phenotype of VSMCs and are consistent with opposite effects for Notch receptors as seen in different contexts (D'Souza et al., 2008; Fan et al., 2004; Graziani et al., 2008)

The involvement of Notch signaling in inflammatory context, it was reported also for Jagged1. It was shown that Jag1 gene transcription is activated by a mechanism NF-kB-dependent, and it is inhibited by over-expression of a dominant negative form of IkBα (Bash et al., 1999). Moreover, Sainson and co-workers demonstrated in ECs that TNFα up-regulates Jagged1 expression while down-regulates Notch4, Dll4 and Hes1, and the Jagged1 up-regulation has been related to the migratory phenotype of endothelial cells. , (Sainson et al., 2008).

A recent study showed that in ECs the TNFα treatment, besides the up-regulation of VCAM-1 and E-Selectin, induces down-regulation of Notch4 by a NF-kB dependent mechanism (Quillard et al., 2008). The Notch4 silencing or that of its target gene Hes1 was sufficient to up-regulate these adhesion molecules even outside of any inflammatory condition, suggesting that Notch4 may act as an anti-inflammatory endothelial molecule.

In several systems the NF-kB pathway has been correlated to the Notch1 activation or expression (Osipo et al., 2008). Indeed, Notch1 enhances the

NF-kB activity via transcriptional regulation of some NF-kB members in several tissues (Cheng et al., 2001; Oakley et al., 2003). Moreover, Notch1 intracellular domain has been shown to have IκB activity by associating with p50 subunit of p50/p65 heterodimers, and this effect resulted dose-dependent. Low amounts of Notch1ICD stimulate NF-kB transcriptional activity, while higher amounts inhibit it (Guan et al., 1996). Moreover, Notch1ICD can compete with the NF-kB inhibitor IkBα for binding to NF-kB, thus promoting the nuclear retention of NF-kB members (Shin et al., 2006). Accordingly, in SMCs Notch1 has been reported to facilitate NF-kB activation, decreasing IkBα expression (Clement et al., 2007).

Recent evidences clearly indicate that different Notch receptors can have divergent effects and that regulatory feed-back exists within the family of Notch receptors and their ligands in both physiologic and pathologic conditions (D'Souza et al., 2008).

In ECs during inflammatory stimuli down-regulation of Notch4 has been associated with loss of quiescence, but it can be hypothesized that other Notch receptors might act on ECs activation upon inflammatory cytokines stimulation, and data on Notch1 are still lacking.

AIM OF THE PROJECT

The main objective of this study focused on to dissect Notch signaling activation in inflammatory response of endothelial cells. To this end we evaluate the function of Jagged1 and the activation of Notch1 during IL-1β-induced up-regulation of adhesion molecules.

We treated different types of endothelial cells (ECs) with this cytokine. In fact, ECs show tissue-district specific characteristics. Different inflammatory vascular diseases target different macro-vascular or micro-vascular beds triggering diverse tissues response. The common feature of vessels-related pathologies is the resulting loss of quiescence of endothelial cells. Thus, we used: (i) Human Arterial Endothelial Cells (HAEC), which are target of atherosclerotic and degenerative vascular pathologies of large vessels; (ii) Human Umbilical Vein Endothelial Cells (HUVEC), as model for post-capillary venules and the most common model for ECs biology studies; (iii) Human MicroVascular Endothelial Cells (HMVEC), deriving from capillaries and involved in micro-angiopathies; and human cord blood-derived Endothelial Progenitor Cells (EPC), as model of ex-vivo transplant.

PROJECT STRATEGY

I) Characterization of modulation of Notch signaling components after IL-1β stimulus

II) Modulation of Notch signaling components (Jagged1, Notch1 and Hes1) in order to counteract or mime the IL-1β effect on endothelial up-regulation of adhesion molecules

- general inhibition of Notch signaling by using a γ-secretases inhibitor in ECs
- single or double knockdown for Jagged1 and Notch1 in ECs
- over-expression of Notch1 and Hes1 in ECs

III) Study of Notch signaling involvement in different models

- characterization of Jagged1 expression *in vivo* rat model of hepatic low-grade inflammation
- isolation and characterization of EPCs from human umbilical cord blood to study EPCs responses to IL-1β as model for ex-vivo transplants

RESULTS

IL-1β Modulates Components of the Notch Pathway in Different Types of ECs

To determine the role of Notch pathway during the inflammatory state induced by IL-1β, cultured quiescent HAEC were treated with IL-1β at 10ng/ml for 6h or 24h and the effects on Notch signaling components were analyzed both at RNA and protein levels.

RNA modulations were measured by quantitative Real Time PCR, and the values were given in fold changes versus the untreated control. Moreover, standardizing with an internal control we obtained the absolute mRNA copy number (see Material and Methods section for details).

The expression of all the Notch ligands was down-regulated, with exception of Jagged1. It showed a strong IL-1β-dependent mRNA up-regulation at both 6h and 24h (Figure 1A), that was confirmed at protein level (Figure 1E). Performing a time course experiment, an increase Jagged1 protein was evident as soon as 1h after IL-1β induction (Figure1F).

Notch1, Notch3, and Notch4 transcripts were at least 50% down-regulated at both time points, conversely, Notch2 mRNA was up-regulated. However, the more significant modulations regarded Notch1 and Notch4, since endothelial cells express a low copy number of Notch 2 and Notch 3 mRNA.

Western blot analysis showed that the protein level of the activated form of Notch4 (Notch4ICD) decreased as soon as 6h after IL-1β treatment and remained low up to 24h after treatment (Figure 1E), in agreement with recent data on TNFα effect on ECs (Quillard et al., 2008). The level of Notch4 full length form was undetectable in our conditions, probably since Notch4, maintaining the homeostasis of endothelial cells, needs to be constantly activated. Interestingly, while the Notch1 full-length protein was clearly decreased both at 6h and 24h of treatment, accordingly with the diminution of the Notch1 mRNA levels, the amount of Notch1ICD showed a slight diminution only 24h post-treatment (Figure 1E). This finding indicates that Notch1 signaling continues to be activated upon IL-1β treatment.

In order to assess the effects of IL-1β on canonical Notch target genes expressed by ECs, we analyzed Hes1, Hey1 and Hey2 mRNA expression. We observed that Hes1 and Hey2 mRNA were strongly decreased at 6h (Figure 1C), while Hey 1 resulted consistently increased, and its expression was further enhanced at 24h (Figure 1D). Of note, it has been found that TNFα induces induces down-regulation of Hey1 (Quillard et al., 2008; Sainson et al., 2008), and since IL-1β and TNFα are the most common

mediators of inflammation and act often in concert, additional studies should be addressed to clarify this aspect.

The same modulations for Notch signaling components were observed at both mRNA and protein level in HUVEC and HMVEC (data not shown).

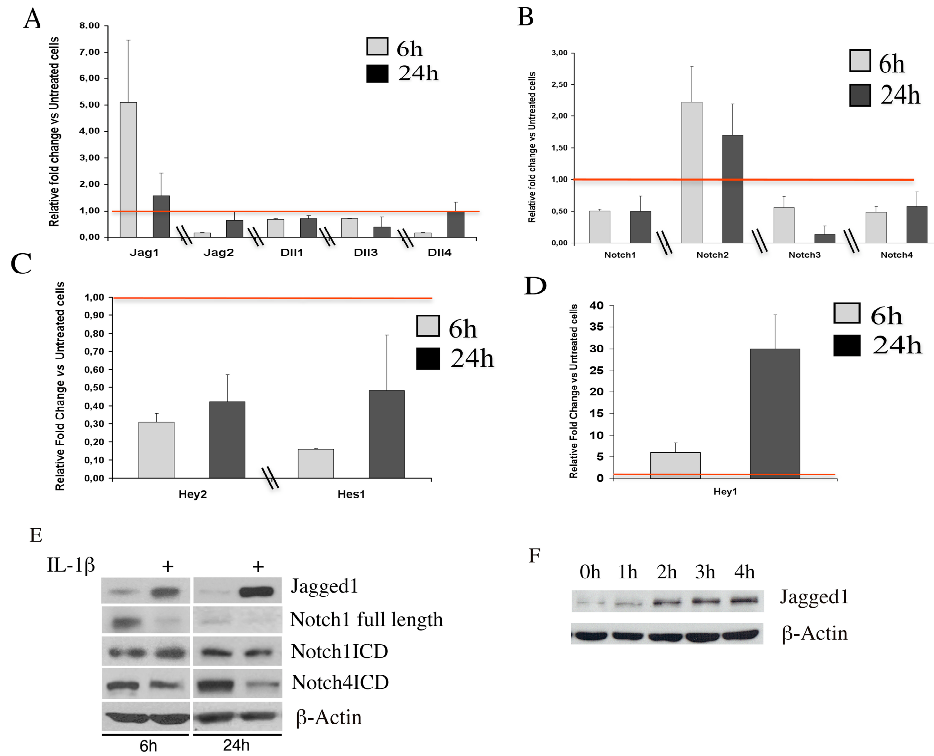


Figure1. *IL-1β induces modulation of Notch signaling components in HAEC*. Cells were grown to reach 100% confluence and treated with IL-1β 10ng/ml for 6h and 24h. Total RNA was extracted and analyzed by Sybr Green qRT-PCR. Relative expression of mRNA of ligands Jagged (Jag)1-2, and Delta-like (Dll)1, 3, 4 (A), Notch receptors 1-4 (B), and target genes Hes1 and Hey2 (C), Hey1 (D), was measured and standardized on β-Actin expression. The values represent the fold changes versus untreated control. The arbitrary value of 1 was assigned to untreated cells. A line at the level of the reference value of 1 is depicted in red. Results are a mean+/-SD of 3 independents experiments. E, Western blot analysis after 6h and 24h of IL-1β treatment shows protein expression of Jagged1, Notch1 full length and Notch1ICD, Notch4ICD. The amount of the active form of Notch1 and 4 was assed using

antibodies able to recognize the Intracellular domain of Notch. F, Immunoblot shows time course of Jagged1 expression in HAEC after IL-1β treatment.

DAPT Treatment Impaired IL-1β-Induced VCAM-1 Expression

The most important feature induced by inflammatory cytokines in the endothelial cells in the acquisition of the activated phenotype by endothelial cells is the up-regulation of the adhesion molecules, such as VCAM-1, ICAM-1 and E-selectin. These molecules are involved in the recruitment of leukocytes from bloodstream towards inflamed tissues (see section 2.1 and 2.2).

Recently, the down-regulation of Notch4 expression and activation, and consequently, that of its target gene Hes1 have been shown responsible for the transcriptional activation of the endothelial adhesion molecules after TNFα treatment (Quillard et al., 2008). However, it is not clear whether the activation of other Notch receptors can be involved in the up-regulation of adhesion molecules induced by IL-1β.

To verify whether activation of Notch signal participates in IL-1β–induced up-regulation of the adhesion molecules, we pharmacologically inhibited the activation of Notch receptors. To this end, we made use of an inhibitor of the γ–secretases complex, the DAPT, which inhibits the cleavage of Notch receptors and the formation of the Notch active form (NotchICD) (see section 3.3). The DAPT effect on inhibition of Notch activation was verified by assessing the down-regulation of target gene Hey1 by semi-quantitative RT-PCR on HAEC (Figure 2A). In IL-1β treated cells, the DAPT treatment partially reduced the transcriptional activation of Hey1 compared to vehicle (*i.e.* DMSO). In cells not treated with IL-1β (referred as untreated), Hey1 mRNA expression levels were undetectable, neither in vehicle, nor in DAPT treatment.

The expression of adhesion molecule was measured by cytofluorimetric analysis (FACS). In brief, we pre-treated the cells with either 5μM DAPT or the vehicle for 16h and, then, cells were co-treated 6h with IL-1β , detached and stained with an anti-VCAM-1 primary antibody (see Figure 2B for a representative FACS analysis). A fluorescent secondary antibody, conjugated with Cy5, was used to identify VCAM-1 expressing cells by FACS. Two parameters are measured by FACS analysis: the number of fluorescent cells (*i.e.* the percentage of cells that express the protein on their

surface) and the Mean of Fluorescence Intensity (MFI) that measures the fluorescence intensity level for each cell (*i.e*. the protein amount expressed by each cells)

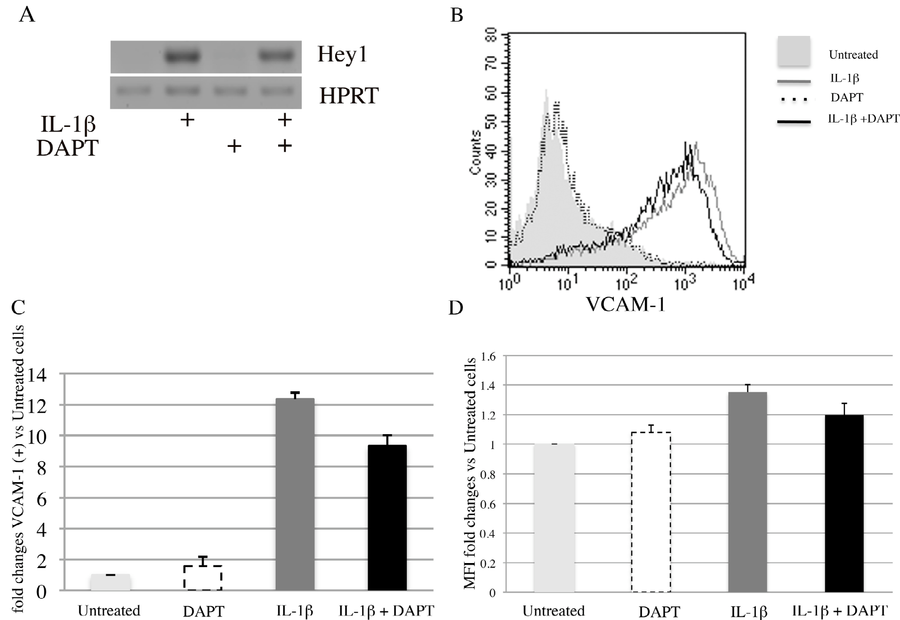


Figure 2. *DAPT treatment impaired IL-1β-induced VCAM-1 expression*. A, Semi-quantitative RT-PCR of Hey1 expression in HAEC treated with vehicle, with DAPT, with vehicle + IL-1β and DAPT+ IL-1β . B, HAEC were incubated with 5μM DAPT over night and treated 6h in presence or not of IL-1β. FACS analysis was performed and one representative experiment was shown. Vehicle treated cells (grey fill), or treated with IL-1β (grey line), DAPT treated cells (black dotted) or DAPT+ IL-1β treated (black line). C, Histogram shows the fold changes of VCAM-1 expressing cells versus control cells untreated. The arbitrary value of 1 was assigned to untreated control. Results are a mean+/-SD of 3 independents experiments. D, MFI for VCAM- 1 expression. The arbitrary value of 1 was assigned to untreated control. Results are a mean+/-SD of 3 independents experiment

The IL-1β induced 12-fold up-regulation of the percentage of VCAM-1 expressing cells and an ~ 30% increase of MFI, respectively (Figure 2C and 2D). DAPT treatment reduced to ~ 8.5 folds and to ~ 15% the IL-1β–induced up-regulations. In untreated cells the DAPT did not significantly modulate VCAM-1 expression. The reduction of IL-1β–induced VCAM-1 up-regulation on ECs, by globally inhibition of Notch activation, suggests

that an active Notch signal is partially responsible for the acquisition of the inflammatory phenotype.

Jagged1 Knock-Down Contrasts IL-1β-Dependent Up-regulation of the Adhesion Molecules Expression in ECs

The DAPT effect in restraining VCAM-1 up-regulation after IL-1β treatment (Figure 2B-D) was suggestive of a role for Notch receptors activation. Furthermore, we detected a strong IL-1β-induced up-regulation of Jagged1 (Figure1A and 1E), which acts activating Notch receptors. Therefore, we investigated the potential involvement of this ligand in the adhesion molecules up-regulation on ECs upon IL-1β treatment. To this end, we decided to down-regulate Jagged1 in ECs by using an RNA interference approach. We used three different small interference RNAs (siRNA) targeting Jagged1 mRNA and we choose the siRNA (Jag1RNAi) that caused the major reduction of mRNA levels (~ 70%; data not shown). A scrambled siRNA that does not match mammalian gene sequence was used as control (CtrRNAi).

After 48h of silencing, HAEC were treated with IL-1β for 6h and expression of VCAM-1, ICAM-1 and E-selectin was analyzed by FACS. In Jagged1 silenced cells treated with IL-1β, a ~ 20% reduction in MFI for all the three molecules examined was measured compared to CtrRNAi cells whereas the percentage of positive cells remained unaffected (Figure 3A, B and C and data not shown). No significant differences were observed in untreated cells after Jagged1siRNA treatment. Similar results were obtained in HUVECs (data not shown). Jagged1 protein level was strongly diminished in untreated Jagged1 silenced cells and remained under the level of detection also after the treatment with the cytokine (Figure 3D). Notch1ICD level resulted strongly diminished in Jag1RNAi cells, irrespective to IL-1β treatment, suggesting that Notch1 activation is, at least in part, controlled by Jagged1 in these cells. Notably, in Jag1RNAi cells the Notch4 activation was decreased to a lesser extent compared to that of Notch1, suggesting that Jagged1 may preferentially signal through the binding with Notch1 receptor.

Interestingly, Hes1 protein levels resulted up-regulated after Jag1siRNA transfection irrespective to cytokine treatment (Figure 3D).

Altogether, these results suggest that Jagged1 over-expression in ECs may regulate the Notch pathway to support the adhesion molecules expression upon IL-1β treatment.

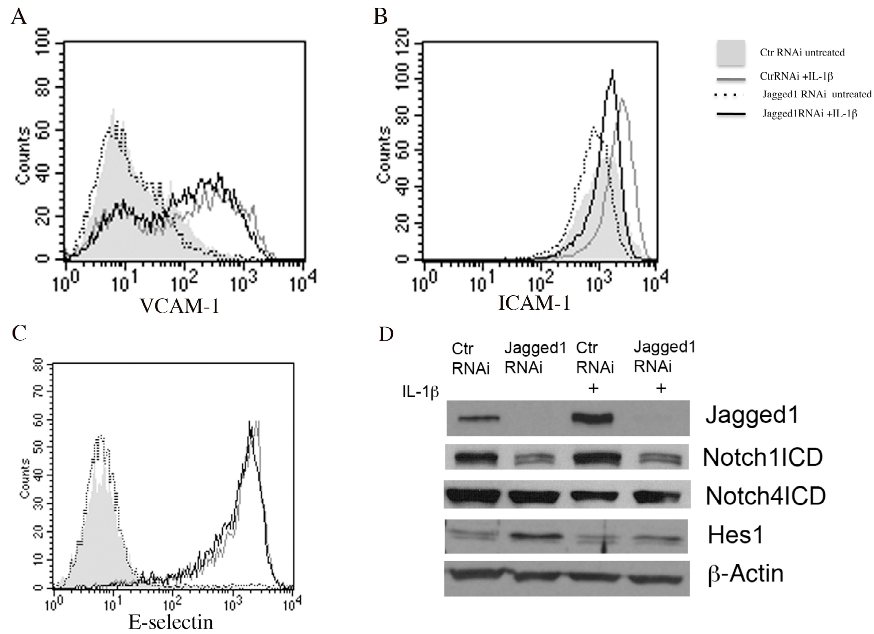


Figure 3. *Jagged1 Knockdown Contrasts IL-1β-dependent Up-regulation of the Adhesion Molecules Expression on ECs*. HAEC were transiently transfected with a specific siRNA targeting Jagged1 (Jag1RNAi) or with a control siRNA (CtrRNAi), and 48h later the cells were treated 6h with IL-1β. In histogram is shown one representative of three independent experiments for VCAM-1 (A), ICAM-1 (B) and E-Selectin (C). CtrRNAi untreated (grey fill) and IL-1β-treated (greyl ine), Jag1RNA untreated (black dotted) and IL-1β-treated (black line). D, Western blot analysis shows protein expression in silenced HAEC of Jagged1, Notch1ICD, Notch4ICD, Hes1 and β-Actin.

Jagged1 Mediates Inflammatory Responses to TNFα in ECs

In order to evaluate Jagged1 involvement in a more general inflammatory context, we evaluated its involvement in the up-regulation of adhesion molecules induced by TNFα. HAEC were silenced for Jag1RNAi and after 48h were treated with TNFα for 6h, and the expression of VCAM-1 was quantified by FACS (Figure 4A). After the TNFα treatment, a decrease (15%) in VCAM-1 expression was observed in Jag1RNAi cells compared to CtrRNAi cells. In western blot analysis (Figure 4B) the comparison of the two cytokines effects revealed a similar modulation of Jagged1, Notch1ICD and Notch4ICD expression. These findings confirm an involvement of the Jagged1 ligand in the leukocytes adhesion induced by both the cytokines.

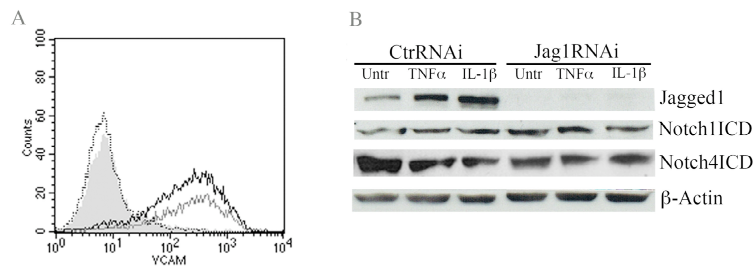


Figure 4. *Jagged1 mediates also inflammatory responses to TNFα in ECs*. HAEC were trasfected with control or Jagged1 siRNA, and 48h later were treated with TNFα for 6h. Three independent experiments were performed. A, FACS analysis of one representative experiment for VCAM-1 expression was shown: control siRNA untreated (grey fill) and treated (grey line), Jagged1siRNA untreated (black dotted) and treated (black line). B, Western blot analysis in HAEC transfected with CtrRNAi and Jag1RNAi shows Jagged1, Notch1ICD and Notch4ICD expression.

Double Knockdown for Jagged1 and Notch1 Impairs IL-1β-Induced VCAM-1 Up-regulation in ECs.

Jagged1 knockdown partially reduced VCAM-1 up-regulation after IL-1β stimulus with a concomitant reduction of Notch1 activation. This result strongly suggests a role for Notch1 signaling in this process. Therefore, to investigate the role of Notch1, we silenced this receptor to verify whether its silencing recapitulate the effect of Jagged1 silencing.
To this end, HAEC were silenced with a siRNA targeting Notch1 and, 48h later, were treated with IL-1β for 6h. VCAM-1 expression was measured by FACS analysis. VCAM-1 up-regulation, induced by IL-1β, was unaffected in Notch1-silenced cells (N1RNAi cells) compared to CtrRNAi cells (data not shown). In N1siRNA cells the amount of Notch1ICD was reduced compared to control siRNA, but when we treated with IL-1β, the N1RNAi cells showed only a slight reduction in the amount of Notch1ICD compared to CtrRNAi cells (data not shown). FACS analysis on silenced cell showed that the N1RNAi cells expressed VCAM-1 at the same extent of the CtrRNAi cells (data not shown). ,These findings indicate that IL-1β treatment induced a higher proteolysis of the receptor and sustains Notch1 activation suggesting that this receptor is probably involved in the activation of endothelial cells.

Among the Notch ligands, Jagged1 is induced by IL-1β (Figure 1A and 1E), this result suggests that it could be responsible for Notch1 activation.To elucidate this point, we silenced both Jagged1 and Notch1 in order to avoid that IL-1β treatment up-regulating Jagged1 activates the low amount of the Notch1 receptor that might be still present on the cell surface after Notch1 silencing. Thus, HAEC were silenced, for both genes (Jag1/N1RNAi cells) and, after 48h, were treated with IL-1β for 6h. VCAM-1 and ICAM-1 expression were evaluated by FACS analysis. After IL-1β treatment, VCAM-1 up-regulation was impaired in double silenced cells for a mean value of ~ 20% in MFI. In untreated conditions, no significant differences showed Jag1/N1RNAi cells compared to CtrRNAi cells (Figure 5A). The analysis of ICAM-1 showed a reduction (~ 30%) in MFI in Jag1/N1RNAi cells compared to CtrRNAi cells (Figure 5B). Protein levels showed that both Jagged1 and Notch1 were markedly reduced by siRNA in untreated cells and the treatment with IL-1β in absence of Jagged1 did not induced activation of Notch1 (Figure 5C), Interestingly, Hes1 expression was not diminished in Jagged1/N1RNAi cells, but even slightly enhanced, confirming results obtained for Jagged1 silencing.

All together these findings suggest that the up-regulation of Jagged1 induced by IL-1β in ECs is responsible for both the activation of Notch1 and for the following acquisition of the inflammatory phenotype .

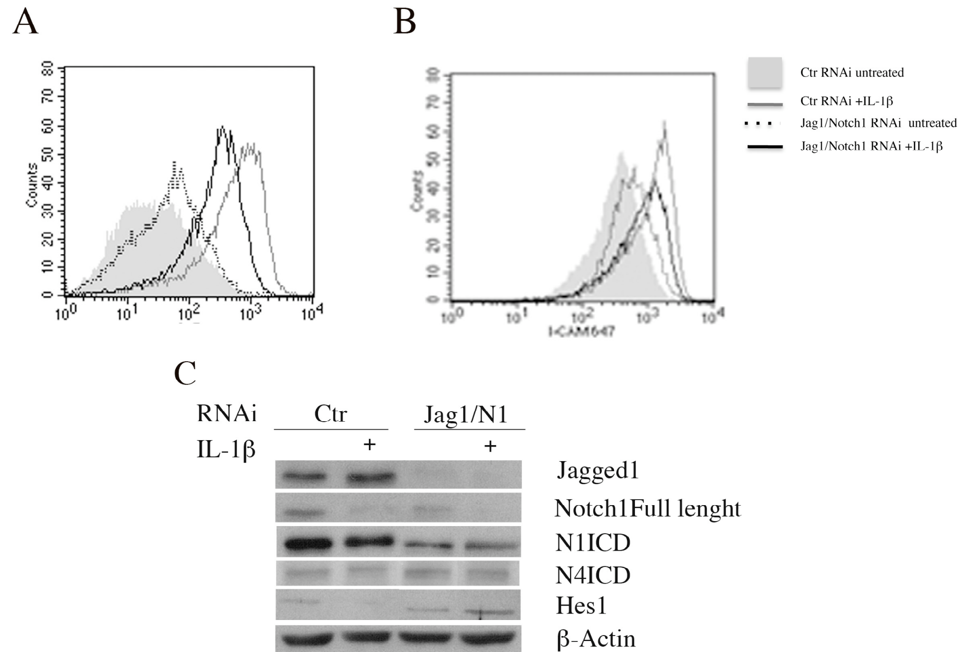


Figure 5. *Double Knockdown for Jagged1 and Notch1 Impairs IL-1β-induced VCAM-1 Up-regulation in ECs*. HAEC were transiently transfected with two specific siRNAs targeting Jagged1 (Jag1RNAi) and Notch1 siRNA (N1RNAi) or control siRNA (CtrRNAi), and 48h later the cells are treated 6h with IL-1β. In histograms is shown FACS analysis for one representative of three independent experiments for both A, VCAM-1 and ICAM-1. CtrRNA untreated (grey fill) and IL-1β-treated (grey line), Jag1/N1RNAi untreated (black dotted) and IL-1β-treated (black line). C, Western blot analysis in silenced HAEC shows Jagged1, Notch1ICD Notch4ICD and Hes1 and β-Actin.

Over-expression of Hes1 Counteracts IL-1β-Dependent Expression of Adhesion Molecules on ECs

It has been shown that TNFα decreased Hes1 expression and its silencing is sufficient to induce VCAM-1 up-regulation (Quillard et al., 2008). Here, we showed that also IL-1β induced a Hes1 down-regulation, while we observed an increase of Hes1 in both Jagged1RNAi and Jagged1/Notch1RNAi treated cells. To elucidate the Hes1 involvement in the IL-1β-induced phenotype, we investigated whether the forced over-expression of Hes1 could be able to interfere with the expression of endothelial adhesion molecules after IL-1β treatment. To this end, we infected HAEC with a GFP expressing adenoviral vector encoding human Hes1 (Ad-GFP-Hes1) or an empty GFP adenoviral vector (Ad-GFP) as control. The Adenoviruses used infected about 99% of cells (Figure 6A).

Twenty-four hours post-infection HAEC were treated with IL-1β for additional 6h and VCAM-1 and ICAM-1 expressions on cell surface were assessed by FACS analysis (Figure 6A-B). Interestingly, after IL-1β tretament, two cell sub-populations were identified in Ad-GFP-Hes1 infected cells. In one of these, the MFI overlapped with those of Ad-GFP IL-1β treated cells, whereas in the other, the MFI overlapped with those of Ad-GFP untreated cells. Analyzing the GFP fluorescence on these sub-populations we observed that the population with high expression of adhesion molecules presented lower GFP fluorescence, presumably corresponding to a lower expression of Hes1. Conversely, the population with both higher GFP and Hes1 expression, did not show any up-regulation of adhesion molecules. This result indicates that a high expression of Hes1 was able to inhibit the up-regulation of both VCAM-1 and ICAM-1 induced by IL-1β. mRNA analysis by semi-quantitative RT-PCR confirmed the up-regulation of Hes1 in both IL-1β treated and untreated cells. Notably, we observed a strong decrease of Jagged1 expression in Hes1 over-expressing cells, that is maintained also after IL-1β induction (Figure 6C). Western blot analysis showed the over-expression of Hes1 and the concomitant down-regulation of Jagged1 and Notch1ICD (Figure 6D).These results indicate a role for Hes1 in both the repression of both adhesion molecules and regulation of Jagged1 expression and suggest a regulative loop between these two proteins.

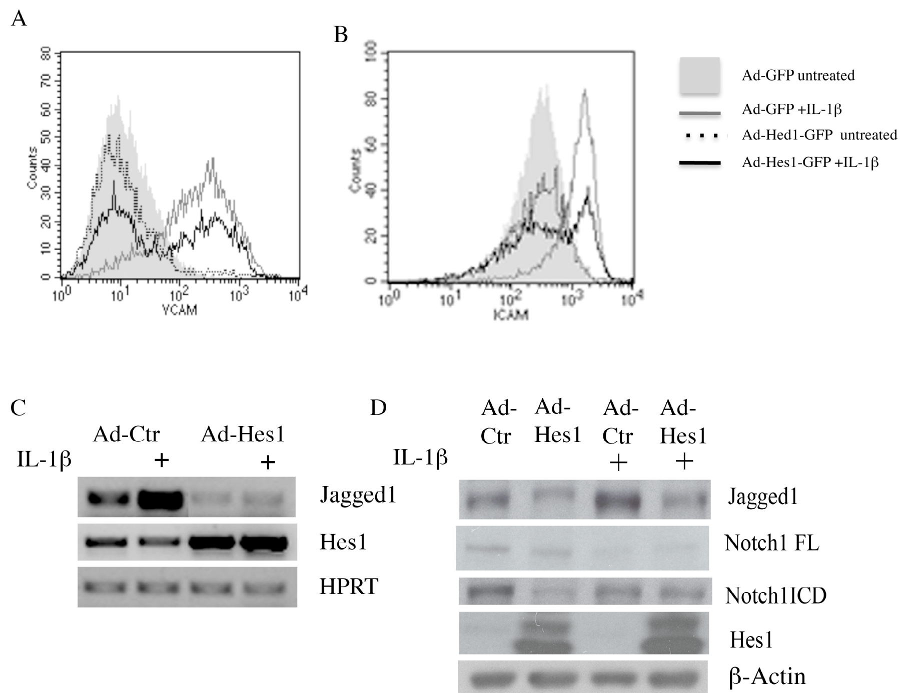


Figure 6. *Over-expression of Hes1 Counteracts IL-1β-Dependent Expression of Adhesion Molecules on ECs*. HAEC were infected with adenoviral vector coding Hes1, Ad-GFP-Hes1 , or Ad-GFP, and 24h post infection cells were treated for 6h with IL-1β 10ng/ml and analyzed by FACS to quantify the expression of VCAM (A) and ICAM (B) on cell surface. The histograms are representatives of 3 independent experiments. Ad-GFP untreated (grey fill), Ad-GPF treated with IL-1β (grey line), Ad-GFP-Hes1 untreated (black dotted) and Ad-GFP-Hes1 IL-1β (black line). C, Semi-quantitative RT-PCR shows mRNA expression of Hes1 and Jagged1, as housekeeping gene HPRT was used in HAEC Ad-GFP (ad-Ctr) and Ad-GFP-Hes1 (Ad-Hes1) 24h post-infection treated with IL-1β. E, Immunoblot shows the amount of Hes1 and Jagged1, Notch1 Full Length, Notch1ICD and β-Actin

Notch1ICD Over-Expression Increases VCAM-1 Expression in ECs.

To investigate the involvement of Notch1 activation in the up-regulation of adhesion molecules expression in ECs, we over-expressed the Notch1ICD to have a constitutively active Notch1 signal.
Efficiency of transfection (~80%) was assessed using a plasmid coding Green Fluorescent Protein (pCMV-GFP) (Figure 7A-B). HAEC were co-trasfected with the empty vector (pcDNA3) or Notch1 (pcDNA3/Notch1ICD) and a plasmid coding GFP (pCMV-GFP), in molar proportions of 3:1, to be confident that the cells GFP positive expressed also other plasmid. After 48h, HAEC were treated with IL-1β for 6h and VCAM-1 expression was evaluated by FACS analysis. A representative histogram is shown in Figure 7C. Analysis of GFP positive cells showed, in untreated cells that Notch1ICD increased the MFI of 3.3 fold compared to control cells (trasfected with empty vector) (Figure 7D). Indicating that Notch1 activation was able to up-regulate VCAM-1 in absence of any inflammatory stimulus. The IL-1β treatment increased the MFI of 10.6 fold in control cells and 21.8 fold in Notch1ICD expressing cells (Figure 7D). Moreover, Notch1ICD was able to induce the number of VCAM-1 expressing cells of about 28% (from 57.6% to 84.8%) with respect to untreated control cells, and after IL-1β treatment resulted a further slight increase (from 95% to 98%) (Figure 7E).

Western blot of Notch1ICD is shown in Figure 7F. Of note, Notch1ICD forced over-expression besides to up-regulation of VCAM-1 was able to up-regulate Jagged1.

Quantitative Real time PCR for Hes1 and Hey1 in Notch1ICD over-expressing cells showed a fold increase of Hey1 of 2.4 compared to increase of Hes1, suggesting that Hey1 could be the preferential target gene transcribed by Notch1 in these cells (Figure 7G). This result would be consistent with other findings indicating that Notch receptors do not equally activate target genes (Clement et al., 2007; Ong et al., 2006).

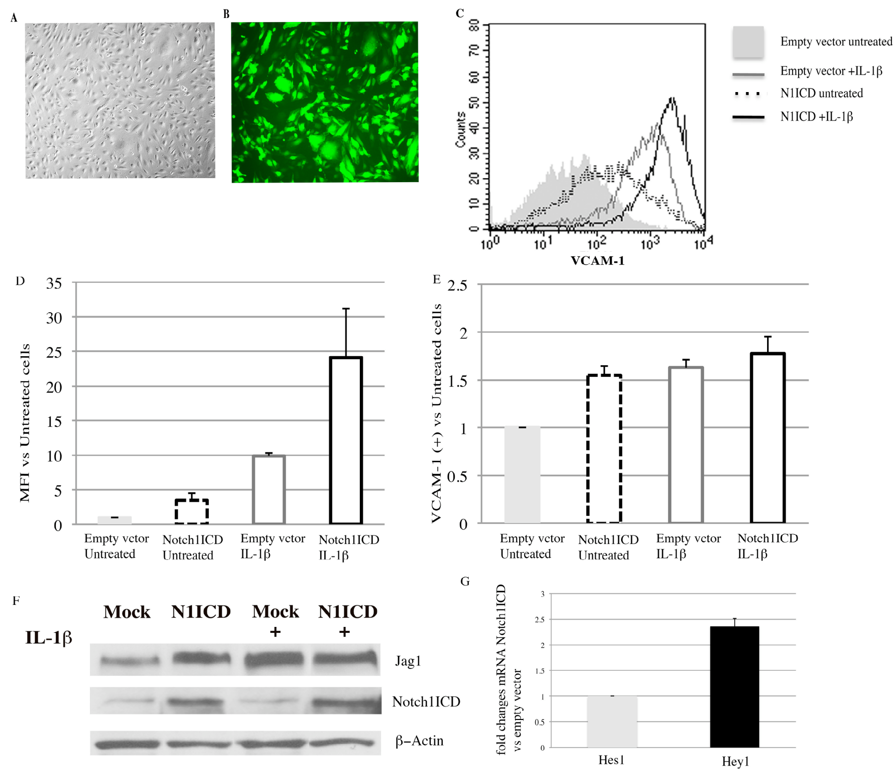


Figure7. *N1ICD over-expression increases VCAM-1 expression in ECs*. A and B, The efficiency of the nucleofection in HAEC using a plasmid coding GFP. HAEC were nucleofected with a plasmid coding Notch1ICD or the empty vector, in molar proportion of 3:1 with a plasmid coding GFP, and treated 48h later with IL-1β for 6h. C, VCAM-1 expression was analyzed by FACS and the histogram shows one representative of three independent experiments. In the population of GFP positive cells are shown: untreated (grey fill), and treated with IL-1β (grey line), Notch1ICD untreated (black dotted) and Notch1ICD treated with IL-1β.(black line). The values of MFI (E) and of percentage of positive cells for VCAM-1 expression (F) are given fold changes vs untreated control cells. Results are a mean+/-SD of 3 independents experiments. F, Western blot analysis for Jagged1 and Notch1ICD of HAEC trasfected with empty vector or Notch1ICD plasmid. G, HAEC over-expressing Notch1ICD and empty vector were analyzed by quantitative Real Time PCR for Hes1 and Hey1 expression. The mean values of three independent experiments are given in fold changes of Hey1 vs Hes1. Results are a mean+/-SD of 3 independents experiments

NF-kB Inhibition Does Not Hamper VCAM-1 Up-regulation Induced by Notch1ICD

The main intracellular mediator of the inflammatory responses is the protein family of transcriptional factors NF-kB. These factors, in a cell-specific manner, activate the downstream cascade of inflammatory genes, such as those encoding for adhesion molecules in endothelial cells (Chen et al., 2009; Madge and Pober, 2001; Ronald et al., 2001; Senftleben and Karin, 2002; Yang et al., 1999) Several groups reported in different systems a crosstalk between NF-kB and Notch1 (Guan et al., 1996; Nickoloff et al., 2002; Palaga et al., 2003; Rothwell and Luheshi, 2000; Shin et al., 2006; Song et al., 2008). To investigate whether Notch1 activity requires the NF-kB function for the up-regulation of adhesion molecules we decided to inhibit NF-kB signaling. We made use of an inhibitor, the BAY, which acts by blocking the phosphorylation of IkBα, resulting in the inhibition of IkBα degradation and the following retention of NF-kB in the cytoplasm (see section 2.4) (Zhu et al., 2008).

Preliminary experiments were performed to find the BAY concentration that inhibits the nuclear traslocation of NF-kB, with no toxic effect on ECs (7.5μg/ml). HAEC were treated for 24h with the BAY, and treated for 40 minutes with IL-1β to have the nuclear translocation of NF-kB. The immunofluorescence assay was performed, using an antibody recognizing the p65 subunit of NF-kB,,and the nuclear translocation of NF-kB induced by IL-1β was inhibited in BAY treated cells (data not shown).

To verify that the BAY treatment maintained NF-kB in the cytoplasm also in Notch1ICD over-expressing cells, we performed an Immunofluorescence on HAEC co-trasfected with the empty vector or Notch1ICD with pCMV-GFP (see the previous section). The cells, 16h post transfection, were treated for 24h with the BAY, and then activated with IL-1β for 40 minutes. The BAY inhibited the NF-kB nuclear translocation in both control and Notch1ICD cells (Figure 8A-F).
NotchICD

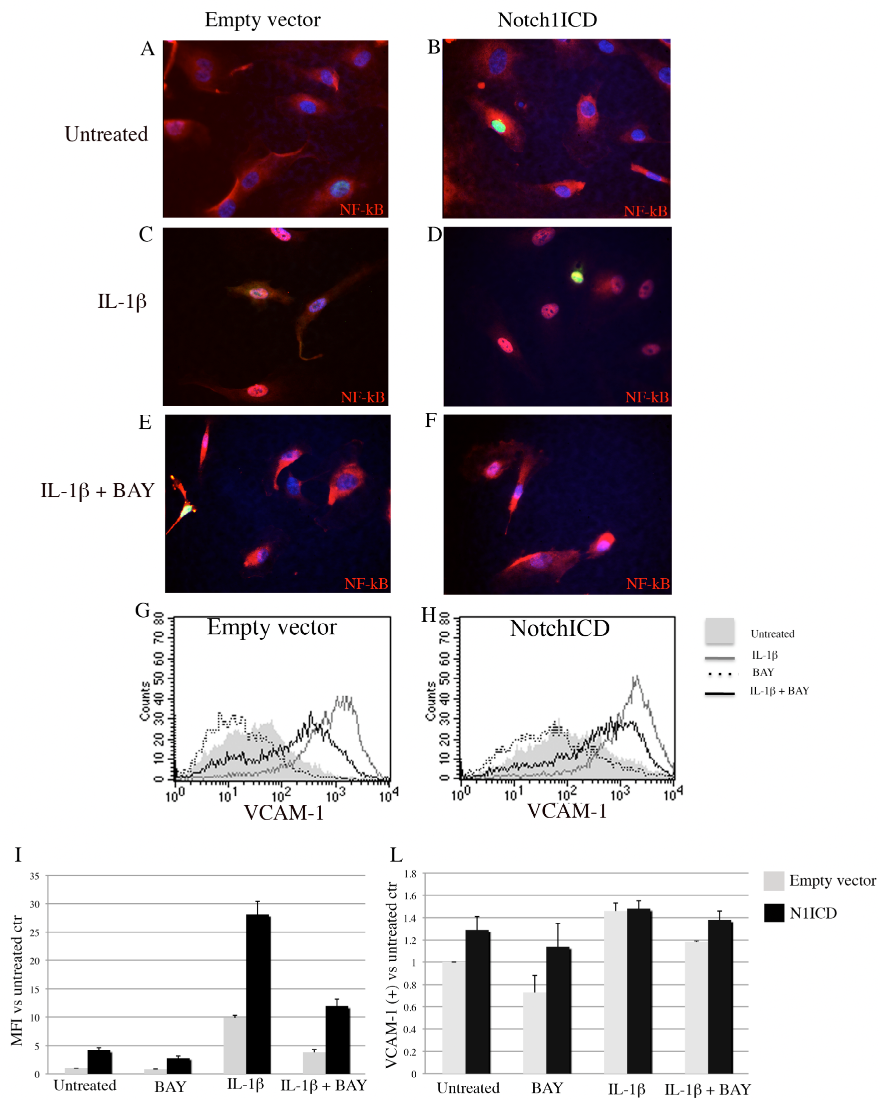


Figure 8. *NF-kB Inhibition Does Not Hamper VCAM-1 Up-regulation Induced by Notch1ICD***.** HAEC were nucleofected with a plasmid coding Notch1ICD or the empty vector, in molar proportion of 3:1 with a plasmid coding GFP, and treated 16h later with 7.5 μg/ml of the BAY inhibitor for 24h. For the Immunofluorescence the cells were treated for

further 40 minutes with or without IL-1β and p65 localization is shown in A, C and E, Empty vector trasfected cells untreated, treated with IL-1β and treated with IL-1β+BAY. B, D and F, Notch1ICD trasfected cells untreated, treated with IL-1β and treated with IL-1β+BAY. For FACS analysis of VCAM-1 expression, HAEC nucleofected with empty vector or Notch1ICD were treated for 24h with the BAY, and for further 6h with IL-1β. G and H, The histograms show one representative of three independent experiments. In the population of GFP positive cells are shown: untreated (grey fill), and treated with IL-1β (grey line), Notch1ICD untreated (black dotted) and Notch1ICD treated with IL-1β (black line). The values of MFI (I) and of percentage of positive cells for VCAM-1 expression (L) are given in fold changes vs untreated cells nucleofected with the empty vector. Results are a mean+/-SD of 3 independents experiments

To verify whether Notch1 was able to up-regulate adhesion molecules in absence of NF-kB, the cells were co-trasfected with the empty vector or Notch1ICD and the pCMV-GFP (see previous section) and after 16h were treated with the BAY for 24h and then activated with IL-1β for 6h. VCAM-1 expression was analyzed by FACS and representative histograms are shown in Figures 8G and 8H. In control cells, the BAY treatment decreased VCAM-1 up-regulation of 60% in MFI both in untreated and in IL-1β−treated cells. The same decrease in MFI was observed in Notch1ICD expressing cells treated with BAY both in untreated and in IL1β−treated cells (Figure 8I). The percentage of VCAM-1 expressing cells was reduced by the BAY treatment in control cells of 30% and 20% in untreated and IL1β−treated, respectively. Conversely, in Notch1ICD over-expressing cells the BAY treatment led to a not significant reduction both in untreated (12%) and in IL1β−treated cells (6%) (Figure 8L). In conclusion these findings show that VCAM-1 up-regulation induced by Notch1 is not influenced by NF-kB inhibition, neither after IL1β stimulus.

Jagged1 is Over-expressed in Vessels of Rats with Low-grade Chronic Inflammation

Animal models, when fed with a high-fat diet (HFD), develop metabolic features and histological patterns, which resemble a current common liver disease named non-alcoholic fatty liver disease (NAFLD) (Svegliati-Baroni, et al., 2006). NAFLD can be characterized by a simple liver steatosis or can progress to non-alcoholic steatohepatitis (NASH), a necro-inflammatory disease, which in turn may lead to cirrhosis (Larter and Yeh, 2008). Recently, it has been demonstrated that HFD rats express increased levels of serum TNF-α (Xu et al., 2009) and immunohistochemical staining of IL-1β (Fisher et al., 2009) compared to normal controls, representing a good model of low-grade chronic inflammation. We confirmed by ELISA assay that serum levels of TNF-α in HFD rats were higher than that of normal controls (Figure 9A). Additionally, we performed semi-quantitative RT-PCR on total RNA extracted from rat hepatic samples of both normal control and HFD to assess IL-1β production in liver, and we observed an increased level (70%) of IL-1β mRNA in HFD rats with respect to control ones (Figure 9B). Our results confirmed the inflammatory phenotype of this animal model at 12 weeks HFD rats. To assess whether this in vivo model could be used to study Notch modulation in low-grade inflammatory phenotype, immunohistochemical analysis was performed on liver of control and HFD rats to determine whether Jagged1 is up-regulated in ECs of the hepatic vessels of inflamed tissue. The endothelial specificity for the staining was assessed by immunohistochemistry with an antibody against the endothelial marker CD31 (9C, 9E) that stains ECs. The staining for Jagged1 was clearly present in ECs lining the liver vessels in HFD rats (Figure 9D, black arrow) whereas normal control animals did not showed any staining (Figure 9F). Further Jagged1 staining appeared in some cells of hepatic parenchyma. This result indicates HFD rats may represent a helpful in vivo model for investigating Notch signaling in low-grade inflamed tissues.

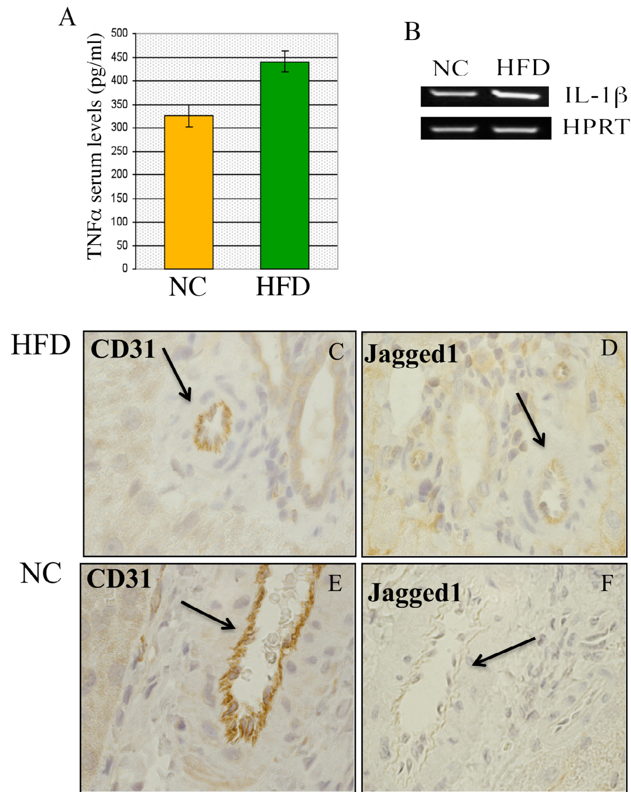


Figure 9. Jagged1 is over-expressed in animal model of low-grade inflammation. A, Quantification by ELISA of secretion of TNFα in serum of normal control (NC) and high fad diet (HFD) rats. B, Expression of IL-1β was verified by semi-quantitative RT-PCR on total RNA from NC and HFD rat livers. HPRT was used as housekeeping gene. Immunohistochemical staining shows expression of Jagged1 (C and E) and CD31 (D and F) in liver samples of NC and HFD rats.

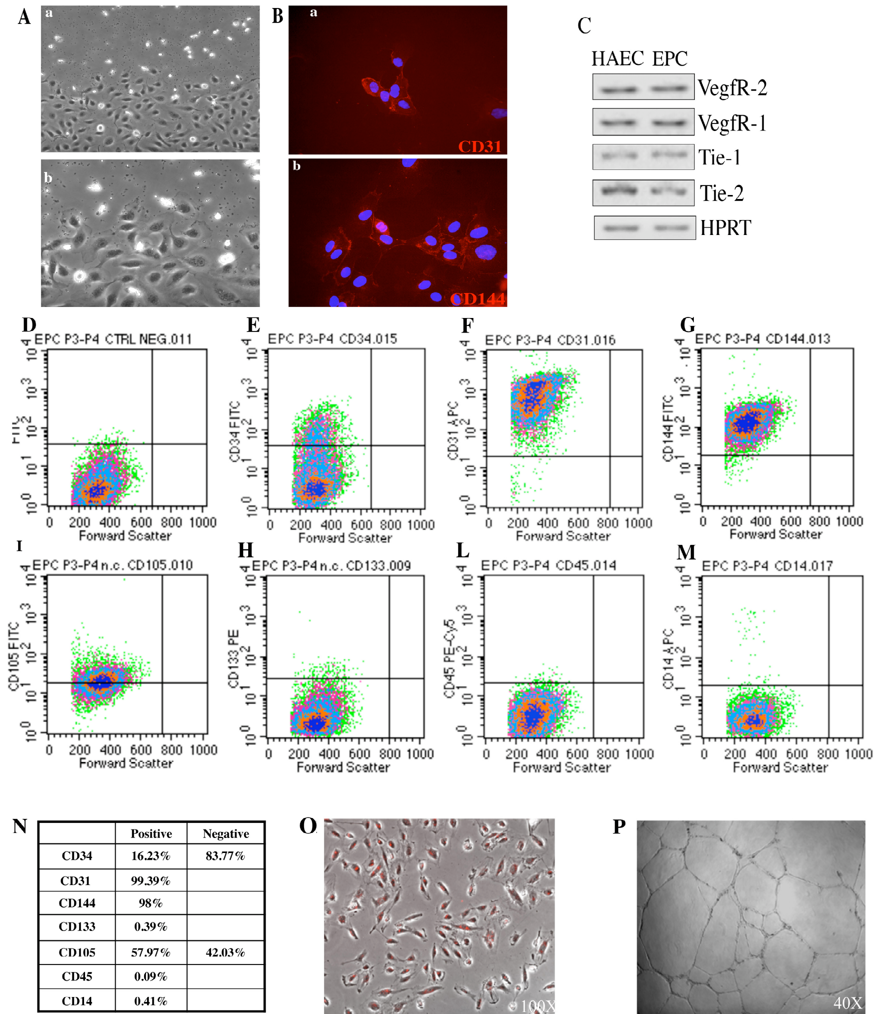
Endothelial Progenitor Cells (EPCs) isolation and characterization.

In the last years several groups isolated precursor cells able to differentiate in mature endothelial cells from different sources (peripheral blood, cord blood and bone marrow) (Timmermans et al., 2009). Endothelial progenitor cells (EPCs) originate in bone marrow and were mobilized upon a variety of stimuli to blood stream where they constitute a pool of cells that can repair endothelial damage (Kong et al., 2004). Alterations in EPCs number and functions have been correlated with a high number of pathologies, including cardiovascular disease and atherosclerosis (Ding et al., 2008; Jung et al., 2009; Krenning et al., 2009; Westerweel and Verhaar, 2009). Chronic treatment transplantation with ex-vivo BM-isolated EPCs in non-atherosclerotic $ApoE^{-/-}$ mice has been shown to prevent the development of atherosclerosis despite the persistent hypercholesterolemia (Rauscher et al., 2003). However, EPCs transplantation in the $ApoE^{-/-}$ mice, is not always beneficial. $ApoE^{-/-}$ mice following hindlimb ischemia, displayed increases the neovascularization in ischemic regions, but, also, accelerated atherosclerotic plaque formation in the new vessels. However, transfer of progenitor cells is not always beneficial. ApoE KO mice following hind limb ischemia, displayed increase neovascularization to those oxygen deficient regions, but also accelerated atherosclerotic plaque formation (Silvestre et al., 2003). Therefore, regenerative properties of EPCs could represent a promising therapeutic approach for the treatment of cardiovascular diseases, but little is still known, and understanding their biology would be essential to benefit for their potential. For this reason, to study the EPCs characteristics and the molecular specificity can help to better understand their patho-physiology behavior.

To this end, we isolated EPCs from mononuclear cells (MNCs) deriving from umbilical cord blood, by selective method of culture. MNCs were isolated by centrifugation on Ficoll gradient and were cultured on collagen substrate with endothelial specific medium that selectively sustain the growth of cells of the endothelial lineage. Adherent cells after 10-14 day gave rise to cell colonies. These cells were characterized through molecular and functional assays to establish their endothelial origin.

At phenotypic level, the EPCs formed colonies after 10-14 days, and showed the cobblestone morphology typical of endothelial cells (Figure 10 A). Immunofluorescence demonstrated the expression of Vascular Endothelial Cadherin (VE-Cad) and CD31 (PECAM), two specific endothelial junction molecules (Figure 10B: a, VE-Cad; b, CD31).

Molecular analysis by semi-quantitative RT-PCR showed the expression of the endothelial-specific receptors for VEGF, (VEGFR1 and VEGFR2), and for the angiopoietins (Tie1 and Tie2) (Figure 10C).



	Positive	Negative
CD34	16.23%	83.77%
CD31	99.39%	
CD144	98%	
CD133	0.39%	
CD105	57.97%	42.03%
CD45	0.09%	
CD14	0.41%	

Figure 10. *EPCs characterization*. A, morphology of colony at 20 days was shown a different magnifications (a, 100X; b, 200X). B, EPCs at the third passage in culture were fixed in PFA 4% and immunofluorescence was performed with antibodies for VE-Cad (a), and CD31 (b); C, semi-quantitative RT-PCR for Vegf Receptor 1, Vegf receptor 2, Tie 1 and Tie2. HPRT was used as housekeeping gene. A representative FACS analysis in EPCs for endothelial and hematopoietic lineage makers are shown in density plots: Negative Control (D), CD34 (E), CD31 (F), CD144 (G), CD105 (H), CD133 (I), CD45 (L), CD14 (M). N, Table showing the percentage of positive cells represented in density plots (D-M). O, EPCs, at third passage in culture were treated with DiI-Ac-LDL for 4h and the internalization is shown. P, EPCs were seeded on Matrigel in presence of complete medium and 8h later they differentiate.

FACS analysis showed the presence on EPCs plasma membrane of the endothelial markers CD34 and CD105, and confirmed the expression of VE-Cad and CD31 as well as the absence of mononuclear cells markers such as CD45 and CD14. The staining for CD133, supposed to be a marker of more immature cells, was negative. The reason could be the long time of growing in culture necessary to reach a consistent number of cells for FACS analysis, probably inducing an almost mature phenotype. Representative density plots of FACS analysis are shown in figure 9 D-M. The percentage of values of positive cells is shown in Figure 9N.

A typical function of endothelial cells is the capability of internalizing acetylated Low Density Lipoproteins (Ac-LDL). Thus, treating EPCs with a fluorescent Ac-LDL we observed up-take of this compound (Figure 10O) The most common function of endothelial cells is the ability to form capillary-like structures when seeded on Matrigel®, an *in vitro* reconstituted extracellular matrix. EPCs seeded on Matrigel differentiated on tubular structures as well as mature endothelial cells (Figure 10P). All together these functional and molecular assays confirmed the identity of EPCs and their capability to differentiate in mature ECs.

EPCs Show the Same Behavior of Mature Endothelial cells in Inflammatory Response to IL-1β.

After the characterization of EPCs we were interested to verify whether after IL-1β stimulus EPCs respond modulating the same pathways of mature endothelial cells. To this end, we treated EPCs for 6h with IL-1β and analyzed the modulation of Notch ligands, receptors and target genes by semi-quantitative RT-PCR. EPCs showed the same modulation of HAEC after induction with IL-1β (Figure11A). Indeed Jagged1 was the only ligand up-regulated at both mRNA and protein level (Figure 11A and 11B) by IL-1β. Among receptor Notch2 was up-regulated, and Notch1 and Notch4 were down-regulated at both mRNA and protein levels (figure 1A and 1B). In agreement with the data on HAEC, Hey1 was up-regulated, while Hes1 and Hey2 resulted down-regulated (Figure 11A). To verify whether Notch signaling is involved also in EPCs in the up-regulation of adhesion molecules induced by IL-1β, we silenced Jagged1 and analyzed VCAM-1, ICAM-1 and E-selectin expression by FACS (Figure 11B-D). The Jagged1 silencing contrasted the increase of adhesion molecules expression induced by IL-1β as observed for HAEC (Figure 3C-E). Western blot analysis (Figure 11D) of cells treated with control siRNA (CtrRNAi) showed that Jagged1 increased after 6h of IL-1β stimulus, and Notch1ICD did not show any modulation. In contrast, in Jagged1 silenced cells (Jagged1RNAi) Notch1 activation was decreased in untreated cells and this reduction was enhanced after IL-1β treatment.

Collectively, these results suggest the involvement of Jagged1 and of Notch1 modulation in the EPC responses to IL-1β, supporting a model of a similar behavior in EPCs and ECs during inflammatory stimuli.

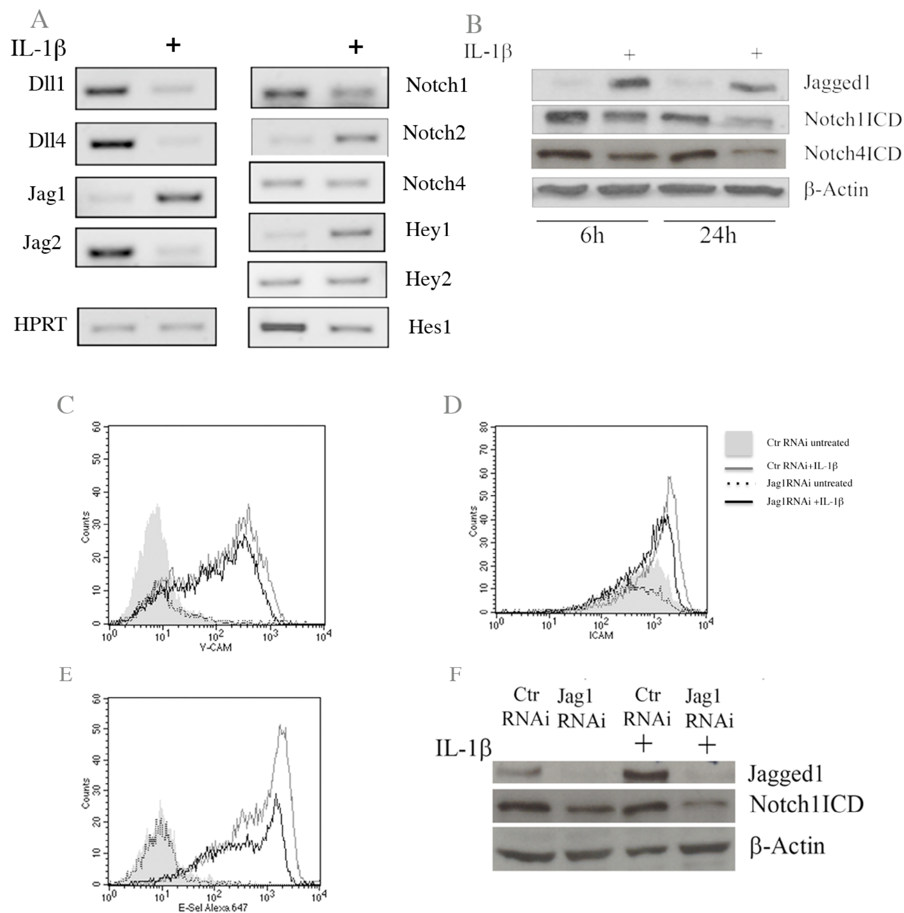


Figure 11. *EPCs show the same properties of mature endothelial cells in inflammatory response to IL-1β*. A, Notch receptors, ligands and target genes expression was analyzed by semiquantitative RT-PCR during exponential amplification cycles in EPCs treated 6h with IL-1β. HPRT was used as housekeeping gene. Results are representative of 3 independents experiments. B, Western blot analysis shows after 6h and 24h of IL-1β treatment Jagged1, Notch1ICD and Notch4ICD. EPCs were trasfected with control siRNA (CtrRNAi) or Jagged1 siRNA (Jagged1RNAi) and after 48h treated 6h with IL-1β and expression of VCAM-1, ICAM-1 and E-selectin was analyzed by FACS analysis and a representative histogram of three independent experiments is shown in C, D and E, respectively: Ctr RNAi untreated (grey fill), CtrRNAi + IL-1β (grey line), Jagged1RNAi (black dotted) and Jagged1RNAi + IL-1β (black line). Western blot analysis for Jagged1 and Notch1ICD of EPCs silenced for Jagged1 is shown in F.

DISCUSSION

The Notch signaling, through the interaction between ligand and receptor expressed on neighboring cells, controls vascular morphogenesis and remodeling, regulating processes such as migration, apoptosis and differentiation (Roca and Adams, 2007). During the endothelial quiescence, cell contact-mediated Notch activation up-regulate Dll4 in cultured endothelial cells (ECs), but Jagged1 transcript levels were unaffected. In other contexts has been reported that different ligands, even activating the same receptors, can lead to different responses (Benedito et al., 2009; Brooker et al., 2006; Urs et al., 2008).

A previous report demostrated recognition sequences for NF-kB in the Jagged1 promoter (Bash et al., 1999). More recent findings highlighted the up-regulation of Jagged1 during inflammation induced by different inflammatory stimuli, such as TGFβ (Zavadil et al., 2004), TNFα (Sainson et al., 2008) and LPS (Morga et al., 2009). In agreement, we found that IL-1β induces an immediate Jagged1 up-regulation.

Jagged1 expression has been correlated with the acquisition of migratory phenotype in TNFα– induced ECs (Sainson et al., 2008). We demonstrate that loss of Jagged1 affects both the IL-1β– and the TNFα–induced up-regulation of adhesion molecules, indicating Jagged1 as the ligand responsible for the inflammatory responses of ECs. Accordingly, the inflammatory function of Jagged1 is found in other systems. Loss of Jagged1, leads to decrease in the production of pro-inflammatory cytokines in activated glial cells (Morga et al., 2009), and inhibits the TGFβ-induced epithelial-to-mesenchymal transition in epithelial cells (Zavadil et al., 2004).

Notch activation in ECs is involved the maintaining of the quiescence. A recent study has shown that TNF-α induces down-regulation of Notch4 its target gene Hes1, in a NF-kB-dependent manner, and silencing of Notch4 induces up-regulation of VCAM-1 (Quillard et al., 2008).

Strikingly, we observed that the global inhibition of Notch signaling negatively affects the IL-1β-induced VCAM-1 and ICAM-1 up-regulation, indicating that a Notch receptor plays a predominant pro-inflammatory role in the activation of ECs. Loss of Jagged1 causes reduction of Notch1 activation, and the concomitant loss of Jagged1 and Notch1 shows a stronger inhibition of the IL-1β–induced adhesion molecules up-regulation, compared to that observed after Jagged1 silencing. Here, we show that Jagged1 specifically activates Notch1 to exerting the effects of IL-1β in ECs.

Ours results are in line with data on inflammatory signaling in vascular smooth muscle cells (VSMCs). Clement et al., demonstrated that IL-

1β treatment down-regulates Notch3 in VSMCs, in a NF-kB-dependent manner, and over-expression of Notch3ICD counteracts the inflammatory phenotype induced by IL-1β. Interestingly, Notch1ICD over-expression in these cells does not hampers the acquisition of the inflamed phenotype but conversely, it sustains NF-kB activation by decreasing the mRNA of IκBα (Clement et al., 2007). Opposite roles of different Notch receptors have been shown in other cellular contexts (D'Souza et al., 2008; Fan et al., 2004; Graziani et al., 2008).

For the first time we demonstrated that Notch1 activation is able to induce adhesive properties in inflamed ECs, since forced over-expression of Notch1ICD, in untreated cells, leads to an increase of VCAM-1 expression, and this effect is drastically increased after IL-1β treatment. The inhibition of NF-kB, the key mediator of inflammatory, does not hamper at all the up-regulation of VCAM-1 expression, induced by Notch1ICD over-expression, compared to control cells both in untreated cells and after IL-1β treatment. These findings indicate a prominent role for Notch1 that, at least in part, results to be independent from NF-kB signaling.

Of note, recently has been shown that VCAM-1 increases in ECs of vessel walls exposed to physiological shear stress (O'Keef et al., 2009), and interestingly, Notch1ICD translocates into the nucleus 30 minutes after the application of shear stress in ECs (Masumura et al., 2009). Thus, we can speculate that different stimuli, such as inflammatory mediators or mechanical stress, signal through Notch1 leading to ECs activation.

In other systems was reported a differential transcription of Notch target genes by diverse receptors (Clement et al., 2007; Tang et al., 2008). In particular, the evidence of Hey1 transcriptional activation downstream of Jagged1 has been shown in epithelial cell during epithelial-to-mesenchymal transition induced by TGFβ (Zavadil et al., 2004).

In agreement, Notch1ICD over-expression induces the preferential up-regulation of the target gene Hey1 compared to Hes1. Thus, different functions of Notch ligands and receptors might be obtained through different set of target genes.

Forced over-expression of Hes1 partially inhibits the IL-1β–induced up-regulation of both VCAM-1 and ICAM-1, and reduces Jagged1 expression at both mRNA and protein level. These findings suggest that a negative regulative loop exists between Notch signaling components, and a fine tuning is necessary to regulate cells responses.

In the *in vivo* model of hepatic low-grade inflammation ECs over-express Jagged1 and a broaden analysis of Notch signaling could be helpful for the diagnosis of diseases initiating with a low inflammatory phenotype.

Understanding the EPCs behavior in the inflammatory contexts, to make them for ex-vivo transplants,we found that endothelial progenitor cells (EPCs) up-regulates of adhesion molecules upon an inflammatory stimulus, and Jagged1 is involved in this phenomenon,

In conclusions, our results clearly indicate a pro-inflammatory function for Jagged1-Notch1 signaling in endothelial response to IL-1β, and strongly point out Notch1 as the most important receptor involved in this process.

MATERIALS AND METHODS

Cell culture

Primary human artery endothelial cells (HAEC), human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were purchased from Cambrex, cultured in EGM-2 complete medium (Cambrex), and used between passages 4 to 7. Endothelial progenitor cells (EPCs) were isolated from umbilical cord blood from healthy term newborn donors, as previously described (Ingram et al., 2004) with minor modifications. Confluent cell layers were treated with IL-1β 10ng/ml or TNFα 10U/ml in EBM-2 (Cambrex) supplemented with 0.5% FCS (Hyclone), for the indicated times. Umbilical cord blood was collected in citrate phosphate dextrose (CPD) solution, diluted 1:1 with HBSS (Invitrogen) and overlaid onto Histopaque-1077 (Sigma). Cells were centrifuged at 740g for 30 minutes. Buffy coat MNCs were collected and washed three times in EBM-2 medium (Cambrex) supplemented with 10% FCS (Hyclone). MNCs 5 $x10^7$ were plated, on 6-well coated with rat tail collagen (50mg/ml) (Roche), in EGM-2 complete medium (Cambrex) and after 24h of culture, non adherent cells and debris were aspirated. Adherent cells were washed in EGM-2 medium and complete medium was added to each well. Medium was changed daily for 7 days and thereafter on alternate days. Endothelial colony forming cells (ECFC) also referred to as blood outgrowth endothelial cells (EOC) gave rise to colonies between 5 and 22 day in culture, and are identified as well-circumscribed monolayer of cobblestone-appearing cells.

Gene Transfer and RNAinterference

ECs were infected with an adenoviral vector carrying both human Hes1 and the reporter gene for the green fluorescent protein (Ad-Hes1-GFP) (gift of PP Dotto) at the concentration of 2000 plaque forming units/cell for 6h in EBM2 0.5% BSA. An adenovirus expressing the GFP, Ad-GFP, was used as control vector. pcDNA3 coding Notch1ICD or empty vector (gift of M. Bocchetta) was used for nucleofection with Nucleofector (Amaxa) using Amaxa basic nucleofector kit for primary endothelial cells (Amaxa) according to manufacturer instructions. siRNA duplexes for Jagged1 and scramble-FITC purchased from (SIGMA), were trasfected at 100nM, using Oligofectamine reagent (Invitrogen) in Opti-MEM according to manufacturer's instructions. Assays were performed 48h post transfection.

Antibodies and Reagents

CD133-PE conjugated, CD31- and CD14-APC conjugated and CD45-PE-Cy5 (Miltenyi), CD105- and CD34-FITC conjugated (Chemicon), Ve-Cadherin (Pharmingen), V-CAM (Pharmingen), ICAM-1 (Invitrogen,Carlsbag,Ca) and E-Selectin (Upstate), FITC-conjugated anti-mouse secondary IgG (H+L)(Chemicon) or Cy5 anti-mouse secondary IgG (Jackson Lab), Jagged1 1:1000, Notch1 1:1000, DHSB; Notch4 1:1000, R&D; β-Actin 1:10000, Santa Cruz; anti-rat and anti-rabbit 1:10000 (Santa Cruz). Jagged1 (28H8 from Cell Signaling Technology, Inc., Danvers, MA) or CD31 (E11111, from Spring Bioscience, CA). DAPT (Sigma) was used at 5μM, as control was used DMSO. IL-1β (Peprotech) was used 10ng/ml, or TNFα (Peprotech) was used 10ng/ml. Duplex RNAi targeting Jagged1 and Notch1 was purchased form SIGMA (sasi_Hs01_00100442 and sasi_Hs01_00052328, respectively), scramble RNAi was constructed mutating some nucleotides of the sequence of Jagged1 or Notch1 RNAi.

Fluorescence-Activated Cell Sorting (FACS) analysis

For antigen detection by FACS, cells were detached with versene (Invitrogen), washed and incubated with the primary antibodies opportunely diluted in PBS 5% FCS at 4°C 30 minutes. Isotype identical IgG antibodies served as negative controls. For primary unconjugated antibodies cells were incubated with FITC-conjugated anti-mouse secondary IgG (1:100), 30 minutes at 4°C. FACS acquisition was performed on FACS LSR1 (Becton Dickinson) interfaced with Macintosh Cell Quest Pro software.

Immunofluorescence

Cells were grown on chamber slides on collagen and fixed in 4%formaldehyde for 15 minutes at RT, washed twice with PBS, permeabilyzed with 0.1% Triton X-100 10 minutes and blocked with PBS/10% of normal goat serum. Primary mouse anti-human Ve-Cadherin (Pharmingen), mouse anti-human CD31 (Chemicon), and anti-human p65 (Santa Cruz) were used 1:100 for 1h at RT. Secondary anti-mouse or anti-

rabbit TRITC (Chemicon) antibody were used 1:500 for 40 minutes at RT. Nuclei were counterstained with DAPI 200ng/ml for 2 minutes at RT. Cover slips were mounted with non-fat mounting solution (Biomeda). Images acquired by an Eclipse E600 fluorescence microscope, using LUCIA software.

Up-take of acetylated-LDL

ECFCs were cultured on chamber slides coated with rat tail collagen, and incubated with DiI-Ac-LDL (Molecular Probes) for 4h according to manufacturer's instructions. The cells were fixed in 4% formaldehyde for 15 minutes at RT. Cells were visualised with an Eclipse TE2000 microscope (Nikon), and images acquired using Metamorph software (Crisel).

In vitro tube formation assay

24well culture plates were coated with Matrigel Growth Factor Reduced (BD) and allowed to polymerize for 1h at 37°C. ECFCs were detached with trypsin, and seeded at 10^5 cells for wells and incubated for 8-12h at 37°C in EGM-2 complete medium supplemented with FCS at a final concentration of 10%. After 8h images of differentiating cells were acquired at Eclipse TE2000 microscope (Nikon), using Metamorph software (Crisel).

Semiquantitative and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA extraction was performed using TRIzol reagent (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. For semi-quantitative RT-PCR, RNA DNase I-treated was converted in first-strand complementary DNA cDNA using Super Script II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. For amplification reactions were used 25ng of cDNA as template and PCR reaction conditions were set up for each gene, and the samples were analyzed in the linear phase of amplification. Hypoxantine phosphoribosyltransferase (HPRT) was used as housekeeping gene. Primers list is depicted in Table 1. PCR products

were visualized under UV illumination with gel star (Biotium) in 1.5% agarose gel.

Conditions for cDNA synthesis used in quantitative RT-PCR are the following: the annealing of oligodT (1μM) to template RNA (1μg) for 5 min at 70°C, primer extension was initiated by adding the 1 unit (U) RT-MMLV enzyme plus 0.5 mM dNTP, 1U RNAsin and 10 dithiothreitol (Invitrogen, Cergy Pontoise, France), and carried out for 45 min at 37°C. PCR was performed by using the LightCycler-DNA Master Plus SYBR Green (Roche Diagnostics). As PCR template were used 250ng cDNA or purified DNA standard. Primer sequences used to amplify the desired cDNA are detailed in Table 1. Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics). After the initial denaturation step at 95°C for 5 min, amplification was performed using 50 cycles of denaturation (95°C for 10 s), annealing (60°C for 15 s) and extension (72°C for 10 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies. To standardise messenger RNA (mRNA) levels, we amplified β-Actin as housekeeping gene for internal control. Gene expression values were normalized by calculating the ratio between cDNA copy number of the target gene and β-Actin.

Western Blot Analysis

Cells were lysed in RIPA buffer for 30 minutes at 4°C, followed by centrifugation (20000g, 20 min). The protein content of the sample was determined using BCA kit (Pierce) according manufacturer's instructions. Proteins (40µg) were loaded in SDS-polyacrylamide gels and blotted onto nitrocellulose membrane. After blocking with non-fat milk 10% at RT for 1h, the primary antibodies (Jagged1, Notch1, Notch2, Notch4, Hes1 and β-Actin) were incubated in TBS, 0.05% Tween 20, 5% milk at 4°C ON. After incubation with secondary antibody for 1h at RT, enhanced chemiluminescence (Amersham) was performed according to manufacturer's instructions

Animal Model of Low-grade chronic inflammation in Non-alcoholic Fat Liver Disease (NAFLD)

Male Sprague-Dawley rats weighing 120-140 g were obtained from (Harlan Italy, San Pietro al Natisone, UD, Italy). All animals received proper care in agreement with the guidelines of the local committee for care and treatment of laboratory animals. They were housed in plastic cages under standard conditions with free access to water and feed. After being fed with standard rat chow for 5 days, animals were divided randomly into normal control group (NC) and high-fat diet group (HFD). The NC group received the standard diet (from Harlan) whereas the HFD group received high-fat pellets (Laboratory of Dr Piccioni, Gessate, Milano, Italy). High-fat diet contained 58% of energy derived from fat, 18% from protein, and 24% from carbohydrates (5.6 kcal/g) with respect to the normal chow containing 5% of energy derived from fat, 18% from proteins, and 77% from carbohydrates (3.3 kcal/g). Rats were sacrificed after 3 months (3 for each group/time of treatment) of the experimental protocol. At the indicated time points body weights were measured and livers were taken, weighed and processed for analysis and blood serum was collected to measure the TNFα levels.

Immunohistochemistry and Enzymee-Linked Immunoadsorbent Assay Analysis

Liver tissues from NAFLD and control animals were fixed in 10% formalin at RT for 24h and then embedded in paraffin. Consecutive sections from paraffin-embedded specimens were cut at 3mM, deparaffinised in xylene and rehydrated through graded ethanol. Morphological analysis was performed after staining in haematoxylin (Gill's)/eosin (G eosin in 1% acetic acid) solutions (Bio-Optica, MI, Italy). Antigen retrieval was performed on sections in 10 mM citrate buffer, pH9 for Jagged1 or 10 mM citrate buffer, pH6 for CD31, for 10 min at 98°C, followed by cooling at RT for 20 min. After endogenous peroxydase blocking with 3% H2O2 in Tris buffered saline (TBS) for 30min at RT 3%, 5% bovine serum albumin (BSA) in TBS was applied for 1h at RT for aspecific sites blocking. Sections were then incubated with primary Abs for Jagged1 or CD31 diluted either 1:50 in 5% BSA in TBS or undiluted, and leaved at 4°C ON or 1h at RT, respectively. After washing, anti-rabbit secondary antibodies were used 30 min at RT (LSB kit from DAKO Carpinteria, CA). Positive reactions were visualized

by staining with diaminobenzidine (DAB chromogen kit, DAKO Carpinteria, CA) according to the manufacturer's instructions and then, sections were counter-stained with Gill's haematoxyline. Positive reactions were indicated by brown staining observed through an Eclipse E600 microscope (Nikon Instruments, FI, Italy) and images were acquired through LUCIA software, version 4.81 (Nikon Instruments, FI, Italy) with a Nikon Digital Camera DXM1200F. The TNFα serum levels of 12 weeks rats NC or HFD were analyzed by the TNFα ELISA (R&D systems) according to manufacturer's instructions.

TABLE1. Primers sequences.

Gene Bank No	Gene	Primer Sense Sequence 5'-3'	Primer Antisense Sequence 5'-3'	Amplicon size (bp)
NM_001101	Hβ-Actin	CATGGGTCAGAAGGATTCCTAT	ATGTCGTCCCAGTTGGT	108 bp
NM_000194	HHPRT	GAATAGGAAATAGTGATAGA	AGACATTCTTTCCAGTTA	120 pb
NM_017617	HNotch1	GATGACCTGGGCAAGTC	CCCTGTTGTTCTGCATATCT	109 bp
NM_024408	HNotch2	CGCACTCAGCTCCTCTATC	GATTGCTTGCATCTCGGC	130 bp
NM_000435	H Notch3	CTGGGTTTGAGGGTCAGA	AGGGCACTGGCAGTTAT	113 bp
NM_00455	HNotch4	CTGCCCCTCTGGTTTCACAG	CCCCCTAGCTCTGCCTCAG	220 bp
NM_000214	HJag	GCTGACTTAGAATCCCTGTGTTA	AGGGTACTGTTGACTAGCTTT	133bp
NM_145159	HJag2	ACAATGGAGTATTCTCGGATAG	CACAACCTCTGGTAACAAAC	129bp
NM_005618	HDll1	GTGATGAGCAGCATGGATT	CAGCCTGGATAGCGGATAC	101bp
NM_016941	HDll3	TCCTCGATTCTGTCCGTGA	GAGAAGATGGCAGGTAGC	

128 bp

NM_019074 HDll4 GCGAGAAGAAAGTGGACAGG ATTCTCCAGGTCATGGCAAG
184bp

NM_005524 HHes1 CACGACACCGGATAAACCA CTGGCTCAGACTTTCATTTATTC
104bp

NM_012258 HHey1 GCACGCCCTTGCTATGGA GATGCGAAACCAGTCGAAC
128bp

NM_012259 HHey2 GTTTGCCCATGCGGATTCA GGTGGCAGAGAGGGACA
100bp

NM_031512 Rat IL-1β CTCGTGGGATGATGACGACG GAATACCACTTGTTGGCTTA
150bp

NM_031144 Rat HPRT CTGCGTGTGGCCCCTGAGGA
GACCAGAGATCAGGGAC 150bp

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