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Review article

# The role of Notch pathway in cardiovascular diseases

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**ABSTRACT**

The recent increase in human lifespan, coupled with unhealthy diets and lifestyles have led to an unprecedented increase in cardiovascular diseases. Even in the presence of a wide range of therapeutic options with variable efficacy, mortality due to heart failure is still high and there is a need to identify new therapeutic targets. Genetic and *in vitro* studies have implicated the Notch signalling in the development and maintenance of the cardiovascular system through a direct effect on biological functions of vascular cells (endothelial and vascular smooth muscle cells) and cardiomyocytes. Notch signalling is also involved in the modulation of inflammation, which plays a major role in causing and exacerbating cardiovascular diseases. The Notch pathway could represent a new therapeutic target for the treatment of cardiovascular diseases.

*Keywords:* endothelial cells, cardiomyocytes, apoptosis, atherosclerosis, heart failure, cardiotoxicity

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## INTRODUCTION

Cardiovascular disorders are the most prevalent cause of death in the industrialized world.[1] Atherosclerosis, which results in the gradual accumulation of atheromatous plaques in the wall of coronary, cerebral artery and aorta, is the most frequent cardiovascular disorder. The accumulation of atheromatous plaques in the injured endothelium leads to coronary artery disease and to often-fatal cardiovascular accidents such as stroke, heart attacks or heart failure.[2] The identification of the complex molecular mechanisms underlying endothelial cell dysfunctions is necessary to develop new therapeutic approaches to block the onset and progression of atherosclerosis. Similarly a full understanding of the mechanisms involved in myocardium repair is needed to improve the existing unsatisfactory therapeutic approaches for the treatment of heart failure.

The Notch pathway transduces signals between cells in various tissues.[3] Evidences accumulated during the last 10 years implicate this signalling pathway in cardiovascular development and homeostasis,[4,5] and suggest a role for Notch in the aetiology of many cardiovascular diseases.[5,6] This short review focuses on the role of Notch pathway in the regulation of proliferation and survival of vascular cells (endothelial cells and vascular smooth muscle cells) and cardiomyocytes. The implications of the involvement of Notch signalling in cardiovascular system maintenance relative to the prevention and therapy of cardiovascular disease are discussed.

## THE BASICS OF NOTCH SIGNALLING

The Notch pathway is an ancient signalling system involved in cell fate decision.[7] Humans have four Notch receptors (Notch 1-4) and five ligands (Delta-like-1, 3, 4 and Jagged-1 and -2). Both receptors and ligands are located on the cell surface and regulate communication of adjacent cells. Notch receptors are synthesized as single-chain precursors and cleaved into an extracellular and a transmembrane subunit by furin in the Golgi apparatus. These two subunits are held together on cell membrane by non-covalent bonds. Binding of ligand present on adjacent signalling cell triggers the removal of the extracellular subunit by a disintegrin and metalloprotease (ADAM) followed by an intramembranous cleavage by γ-secretase, a multisubunit membrane protease. This proteolytic cleavage releases an intracellular domain, which is the active form of Notch (NIC). NIC translocates into the nucleus, where it modulates transcription in the receiving cells via RPB-Jκ (recombinant signal binding protein 1 for Jκ) transcription factor (Figure 1).[7] The most prominent Notch target genes are the Hes and Hey gene families, which are negative regulators of transcription. While Hes genes are crucial in neural and endocrine functions, Hey genes play a crucial role during the development of the cardiovascular system.[8] Other well-known Notch targets include p21Cip/Waf, cyclin D1, cyclin A and transcription factors of the NF-kB (Nuclear transcription Factor-kB) family. The set of directly and indirectly Notch-regulated genes and proteins is very large and still new targets are being discovered.[9] The result of Notch activation is cell context dependent and the output is strongly affected by timing, duration and dose of activation. This tight regulation is accomplished by post-translational modifications such as phosphorylation, glycosylation and by rapid ubiquitination-mediated degradation (9). Inflammatory cytokines also modulate Notch activity[7] as well as cross-talks with other key pathways such as NF-kB, estrogen receptor α and erbB-2[10] and VEGF (vascular endothelium growth factor) receptors.[11]

## NOTCH IN THE ENDOTHELIUM

The role of Notch pathway in the development of the vascular system is well established.[7] Mouse embryos carrying mutations inactivating Notch 1 or both Notch 1 and Notch 4 show severe vascular defects and are not viable. Molecular data indicate that during development, Notch acts mainly by determining arterial-venous specification. Notch receptors 1, 2 and 4 and Delta-like ligands (Dll) 1,4 and Jagged-1, -2 are expressed in the endothelium also during adult life and modulate postnatal angiogenesis.[7] According to widely accepted model, under ischemic conditions, the formations of new blood vessels is driven by VEGF-A which induces endothelial cells sprouting from the parent vessels, followed by migration, proliferation and tube formation. Notch activity, also induced by VEGF-A, modulates angiogenesis by limiting the number of sprouts through an inhibition of VEGF receptor and interfering with Notch signalling leads to dysregulated and unproductive angiogenesis. Thus, in the context of angiogenesis, VEGF is the driving force, whereas Notch can be considered the steering wheel.[7]

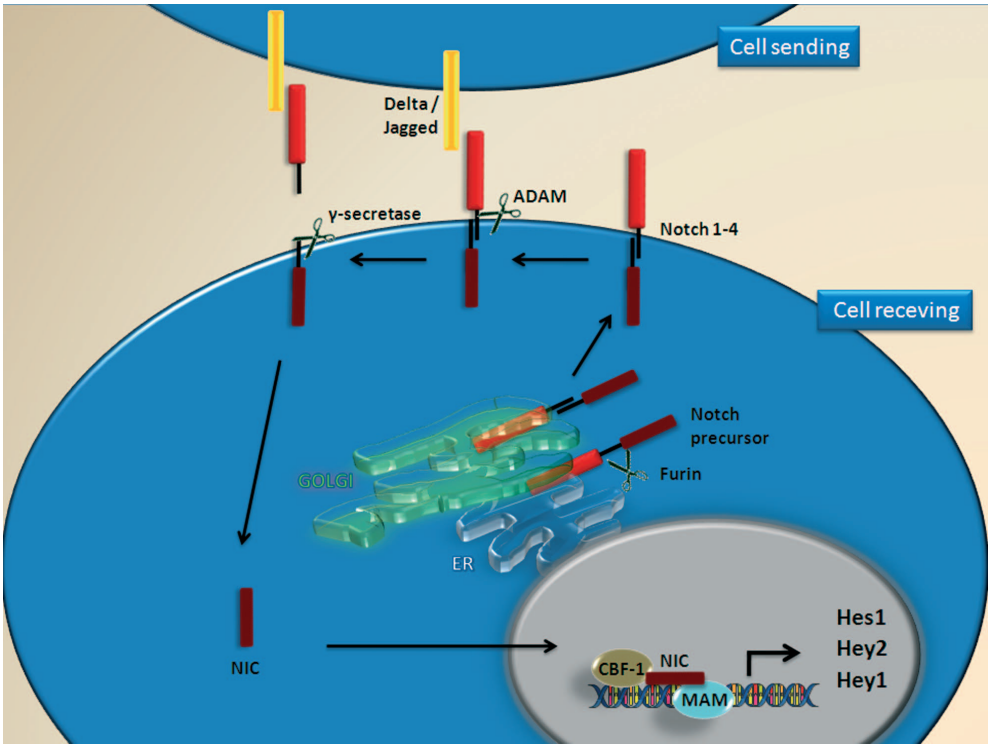


**Figure 1. Diagram showing activation of Notch signalling. Notch receptor precursor is processed in the Golgi apparatus before being translocated to the cell membrane. After binding to ligand present on adjacent cells, Notch undergoes two proteolytic cleavages which produce the active form of Notch (NIC). NIC moves into the nucleus where by interacting with transcriptional factor CBF1 induces the transcription of Notch target genes.**

Other than modulating angiogenesis, Notch plays an important role in preserving endothelium integrity by protecting endothelial cells from apoptosis induced by conditions such as inflammation, oscillatory blood flow and ischemia (Figure 2). *In vitro* treatment of endothelial cells with inflammatory cytokine TNF-α leads to dysregulation of Notch signalling and apoptosis.[7] A prominent role in particular for Notch 4 in protection of endothelial cell has been shown in cardiac allograft vessels in which impaired Notch4 expression, caused by pro-inflammatory cytokines, promotes endothelial cells dysfunction and transplant arteriosclerosis.[12] Notch dysregulation could be causing the observed increase in apoptosis of human umbilical vein endothelial cells (HUVEC) exposed to serum of heart failure patients.[13] It is well known that serum from these patients is characterized by an inflammatory unbalance with levels of TNF-α increasing with the worsening of the disease.

Disturbed blood flow conditions existing in regions of arteries bifurcation predispose the endothelium to atherosclerotic plaques formation by reducing expression of protective genes which leads to increased endothelial cell apoptosis.[14] Accordingly, inhibition of the survival pathway NF-kB has been observed in artery sites prone to plaques formation.[14] Exposure of microvascular endothelial cells to high laminar blood flow conditions (protective for the endothelium) results in upregulation of Notch 1 which increases cells survival by upregulating the antiapoptotic proteins Bcl2.[15]

Under ischemic conditions, VEGF-A promotes not only migration and proliferation but also protects endothelial cells from apoptosis. Experiments in cultures of HUVEC, grown in absence of serum to mimic an ischemic environment, have shown that VEGF-A treatment is unable to protect cells from serum deprivation-induced apoptosis in absence of a functional Notch 1 signalling.[7]

The Notch pathway modulates vascular endothelium integrity also by controlling endothelial cells proliferation[16] and by recruiting endothelial cells precursors from the bone marrow,[17] events which are both critical for the repair of injured endothelium.

## NOTCH AS A THERAPEUTIC TARGET IN ATHEROSCLEROSIS

As discussed in the previous paragraph, Notch signalling, by controlling the biology of endothelial cells, plays a major role in the first events leading to the formation of atherosclerotic plaques.

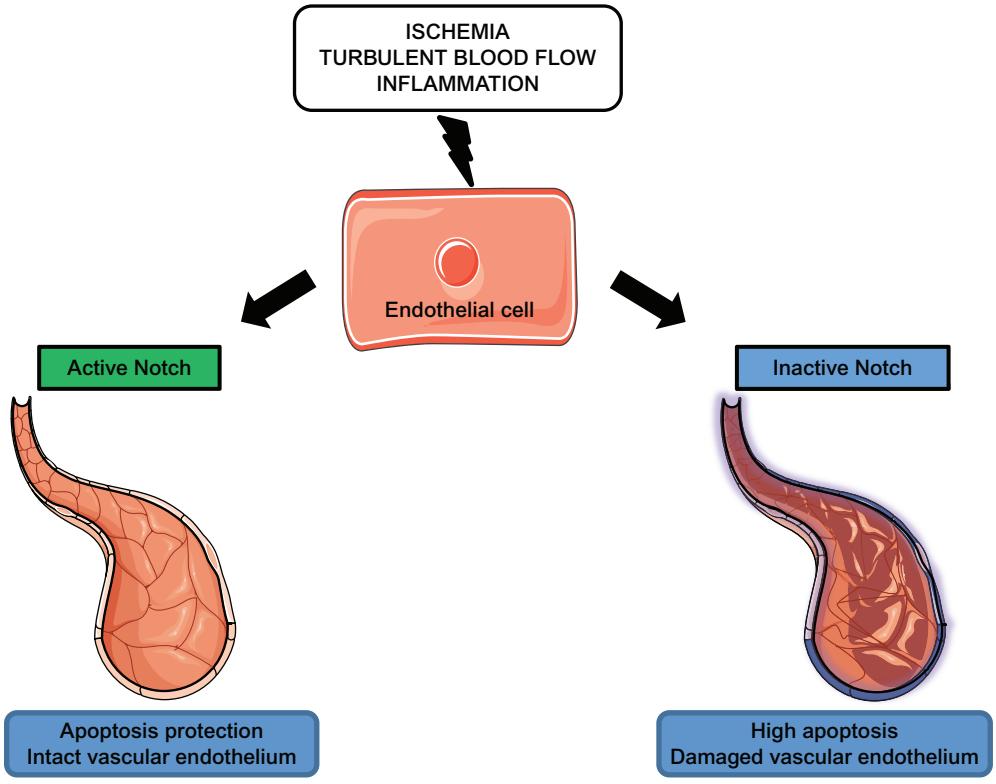


**Figure 2. Notch activation protects endothelial cells from apoptosis caused by different types of insult. Endothelial cells apoptosis and consequent vascular endothelium dysfunctions are the first steps of the formation of atherosclerotic plaques.**

In the context of atherosclerosis, activated macrophages contribute to lesion progression by enhancing inflammatory response in atheromatous plaques and promoting their instability. Macrophages in plaques express Notch 3 receptor and Dll4 ligand.[18] Inflammatory cytokines activate Notch signalling in macrophages and induce genes such as inducible nitric oxide synthase (iNOS), pentraxin 3 (PTX3), and inhibitor of differentiation (Id1), which are involved in the plaques burden, progression and trombogenicity.[18] Treatment of apolipoprotein E-deficient mice with DAPT, an inhibitor of Notch activation, reduced the number and size of plaques clearly indicating a role of Notch signalling in atherosclerosis progression.[19] Similarly, blockade of Dll4-Notch signalling using neutralizing anti-Dll4 antibody attenuated the development of atherosclerosis, diminished plaque calcification, improved insulin resistance, and decreased fat accumulation in LDL-receptor deficient mice fed a high-fat diet.[20]

Apoptosis, proliferation and migration of vascular smooth muscle cells (VSMC) also contribute to the pathogenesis of atherosclerosis and plaque rupture.[21] Additionally, VSMC proliferation and migration after vascular injury are major contributors to restenosis which often limits the success of revascularization procedures.[22] Notch receptors 1 and 3 play an important role in VSMC migration and proliferation and regulate secretion of matrix metalloproteinases (MMP) involved in the degradation of the extracellular matrix which is needed for VSMC migration.[23] These studies taken together indicate that Notch signalling could be targeted in different cell types to interfere with onset and progression of atherosclerosis.

## NOTCH AND CARDIOMYOCYTE SURVIVAL

Gene targeting studies conducted in mice have attributed a precise role to Notch target genes Hey 1 and Hes 1 in the formation of the atrioventricular canal and during cardiac neural crest development.[8] Additionally, Notch signalling is involved in valves, chambers, conduction system development[5] and in ventricular trabeculation.[24,25] Consistently with these observations, indicating a major role played by Notch during heart development, mutations in the Notch signalling pathway have been identified in human congenital defects such as Alagille syndrome, bicuspid aortic valve disease and calcification of

heart valve[26] and more recently in individuals affected by left ventricular noncompaction cardiomyopathy.[24] The role of Notch pathway in cardiomyocyte biology after birth is still not well characterized. Stem cells precursor of cardiomyocytes express high levels of Notch 1 and active Notch signalling is required for their proliferation.[27] Rat neonatal cardiomyocytes, isolated at birth, also express high levels of Notch 1 and are actively proliferating but after several passages in culture, Notch 1 expression becomes undetectable and these cells lose their proliferative ability.[28] These data indicate that Notch signalling is required for expansion of cardiac stem cells and immature cardiomyocytes but that needs to be downregulated to achieve terminal differentiation. Transient Notch 1 re-activation in cardiomyocytes induces transcription of genes conferring a contractile phenotype.[29] Additionally, Notch 1 re-activation in cardiomyocytes in ischemic heart reduces apoptosis by activating Akt, a pathway linked to cell survival.[30] In contrast, Campa et al. have shown that forced activation of Notch in mature cardiomyocytes is associated with cell cycle progression block and apoptosis, indicating that a prolonged and uncontrolled Notch activation can be lethal for these cells.[31] In conclusion, active Notch signalling is required for proliferation of cardiac stem cells and survival of cardiomyocytes (Figure 3), but timing and dosing of activation have to be tightly controlled to avoid negative effects on cell survival.

## NOTCH AS THERAPEUTIC TARGET IN CARDIAC REPAIR

### Heart failure

Expression of Notch signalling components has been observed in myocardium biopsies from heart failure patients.[32] Similarly, Notch signalling is absent under normal physiological conditions in adult rat myocardium, but it becomes transiently reactivated in cardiomyocytes, following a myocardial infarct.[30] These studies suggest a role for Notch signalling in the repair of damaged myocardium. In agreement with this hypothesis, scar formation following myocardial infarct is larger in a rat model of Notch 1 haploinsufficiency in comparison to wild type animals[33] and in transgenic mice overexpressing the Notch ligand Jagged1 on cardiomyocytes, reduced fibrosis and hypertrophic response induced by pressure overload has been observed compared to wild type animals.[34] Among the molecular mechanisms identified to explain Notch protective activity on the damaged myocardium there is

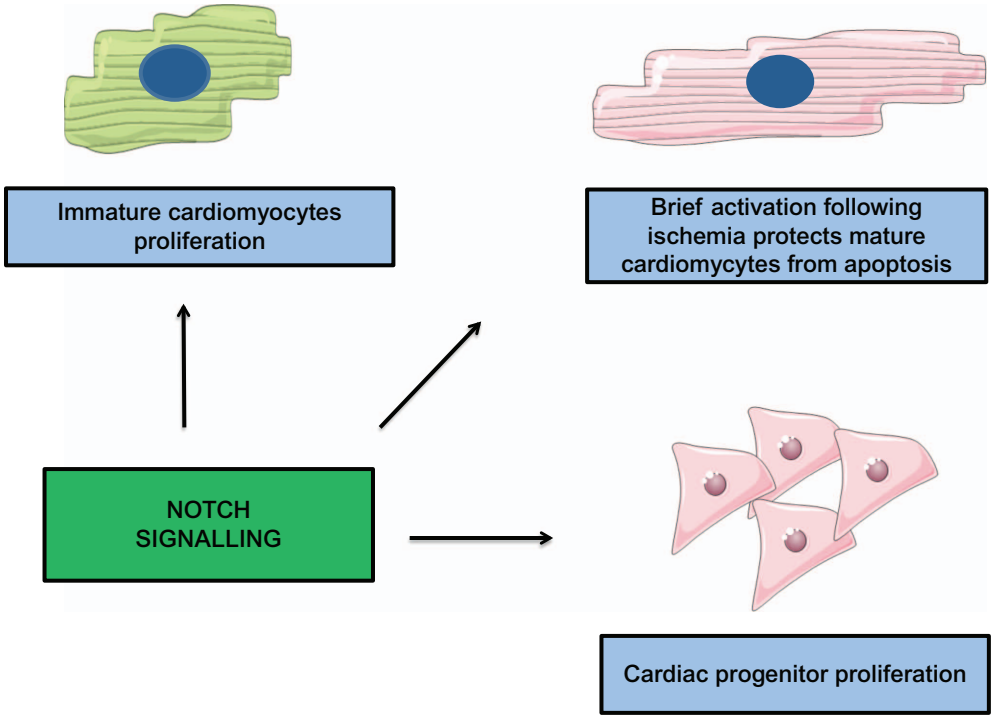


**Figure 3. Notch signalling controls proliferation of cardiac stem cells and of immature cardiomyocytes. In terminally differentiated cardiomyocytes Notch signalling is switched off and it becomes transiently activated following an ischemic insult, conferring apoptosis protection. Prolonged activation of Notch in mature cardiomyocytes lead instead to apoptosis.**

increased cardiomyocyte survival,[30] stimulation of angiogenesis[33] and augmented number of cardiac precursor cells.[34,35] These findings strongly suggest that the Notch pathway represents a unique therapeutic target that could be manipulated to improve the cardiac response to stress and to regenerate the damaged myocardium.

### Potential role of Notch in cancer drugs-induced cardiotoxicity

Cancer mortality has been steadily decreasing thanks to the development of treatments tailored for a particular type of cancer. Some of these treatments, including not only chemotherapeutics drugs but also targeted agent such as trastuzumab, are associated to cardiotoxicity that can be so extreme to cause death due to cardiovascular causes instead of cancer.

The anthracycline doxorubicin is used effectively in the cancer setting but its clinical use is limited by cardiotoxicity which has irreversible consequences after reaching the dose of 500 mg/m$^2$.[36] Although the reasons for cardiotoxicity induction by these drugs are not fully understood, several observations suggest that interactions of anthracyclines with iron are important. Anthracyclines form a complex with iron which catalyzes free radical production and leads to membrane disruption, widespread cellular dysfunction and, ultimately, cardiomyocyte death with consequent strong inflammatory response which enhances cardiac damage. Oxidative stress and inflammation are therefore hallmarks of anthracycline induced cardiotoxicity.[36] Since the Notch pathway is a major modulator of inflammation,[37] specific clinical studies should be able to determine whether cycles of a combined treatment of doxorubicin and Notch inhibitor would help to reduce cardiotoxicity associated to anthracycline treatment.

About 25 to 30% of all breast cancers overexpress HER2 (human epidermal growth factor receptor 2), a member of the epidermal growth factor receptor family involved in modulation of cell proliferation and survival.[38] Trastuzumab is a humanized monoclonal antibody that interferes with HER2 receptor. When administered with paclitaxel or anthracyclines in patients with metastatic HER2 overexpressing breast cancer, trastuzumab prolongs disease-free survival compared to chemotherapy alone. Trastuzumab treatment causes heart failure and asymptomatic decline in systolic function in 22 – 25 % of patient when administered sequentially or in combination with anthracyclines.[38] Cardiomyocytes express HER2 which activates survival pathways in response to stress or agents. According to some authors, trastuzumab induced cardiotoxicity would be a consequence and an exacerbation of anthracycline toxicity since inactivation of HER2 in cardiomyocytes would impair their ability to fix anthracyclines-induced damages.[36] On the other side, the use of trastuzumab alone or in combination with paclitaxel is also associated with cardiotoxicity.[39] Furthermore, differently from anthracyclines, trastuzumab-induced cardiotoxicity is reversible if treatment is interrupted. These observations indicate that the molecular mechanism of trastuzumab-induced cardiotoxicity is still unclear. Treatment of HER2-overexpressing breast cancer cells overexpressing with trastuzumab increases the levels of Notch activity.[40] Considering the role played by Notch in cardiomyocyte survival, it would be of interest to determine whether trastuzumab leads to uncontrolled activation of Notch in cardiomyocytes which would synergize with doxorubicin in inducing cardiomyocytes apoptosis and therefore cardiotoxicity.

Gamma secretase inhibitors (GSI) are small molecules that interfere with the activity of γ-secretase, the enzyme required for Notch activation. The Notch pathway has been found to be activated in the majority of solid tumors and leukemias where it inhibits cancer cells apoptosis induced by treatment. There are several clinical trials ongoing to evaluate the safety and efficacy of GSI administered in combination with standard care treatments of patients with solid tumors, central nervous system tumors, lymphoma or T-cell leukemia.[11] Intestinal toxicity has been observed in GSI treated patients since Notch regulates the balance between secretory and absorptive cell types in the intestine.[9] Considering the involvement of Notch in the maintenance of the cardiovascular system, the potential cardiotoxicity associated to GSI treatment in cancer patients should be addressed by specifically designed preclinical studies and by clinical trials with long follow up.

## CONCLUSIONS

The Notch pathway has important functions in embryonic development and maintenance of the cardiovascular system and its manipulation offers potential new therapeutic avenues to be pursued for the treatment of many cardiovascular diseases. Whereas the targeting of Notch pathway in cancer is already in clinical trials, the research in the cardiovascular field to establish if the targeting of components of the Notch pathways for cardiovascular disease will be successful in the clinic is still in

its infancy. The combined efforts of cardiovascular and cancer biologists and clinical investigators will be necessary to successfully and safely accomplish this task.

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