Targeting γ-secretases protect against angiotensin II-induced cardiac hypertrophy

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INTRODUCTION

Notch signalling plays key roles in vascular smooth muscle cells (VSMCs) during development and in vascular diseases such as pulmonary hypertension [1,2]. Five Notch-activating ligands and four receptors have been identified. Active Notch intracellular domains are generated by sequential proteolytic processing of the ligand-bound receptors in a process ultimately mediated by the γ-secretase complex.

Mice constitutively deficient for Notch3 receptor show a below-normal increase in blood pressure upon treatment with angiotensin II (Ang-II). However, these mice have a high mortality rate (65%) attributed to heart failure, thus casting doubt on the potential of antihypertension therapies based on Notch inhibition [3]. Here, we generated a mouse model for inducible genetic deletion of the γ-secretase complex.

Objective: The Notch pathway has been linked to pulmonary hypertension, but its role in systemic hypertension, and in particular in left ventricular hypertrophy (LVH), remains poorly understood. The main objective of this work was to analyse the effect of inhibiting the Notch pathway on the establishment and maintenance of angiotensin II (Ang-II)-induced arterial hypertension and LVH in adult mice with inducible genetic deletion of γ-secretase, and to test preclinically the therapeutic efficacy of γ-secretase inhibitors (GSIs).

Basic methods: We analysed Ang-II responses in primary cultures of vascular smooth muscle cells obtained from a novel mouse model with inducible genetic deletion of the γ-secretase complex, and the effects of GSI treatment on a mouse cardiac cell line. We also investigated Ang-II-induced hypertension and LVH in our novel mouse strain lacking the γ-secretase complex and in GSI-treated wild-type mice. Moreover, we analysed vascular tissue from hypertensive patients with and without LVH.

Main results: Vascular smooth muscle cells activate the Notch pathway in response to Ang-II both ‘in vitro’ and ‘in vivo’. Genetic deletion of γ-secretase in adult mice prevented Ang-II-induced hypertension and LVH without causing major adverse effects. Treatment with GSI reduced Ang-II-induced hypertrophy of a cardiac cell line ‘in vitro’ and LVH in wild-type mice challenged with Ang-II. We also report elevated expression of the Notch target HES5 in vascular tissue from hypertensive patients with LVH compared with those without LVH.

Conclusion: The Notch pathway is activated in the vasculature of mice with hypertension and LVH, and its inhibition via inducible genetic γ-secretase deletion protects against both conditions. Preliminary observations in hypertensive patients with LVH support the translational potential of these findings. Moreover, GSI treatment protects wild-type mice from Ang-II-induced LVH without affecting blood pressure. Our results unveil the potential use of GSIs in the treatment of hypertensive patients with LVH.

Keywords: angiotensin-II, cardiomyocyte, hypertension, left ventricular hypertrophy, notch, vascular smooth muscle cell, γ-secretase inhibitors

Abbreviations: 4-OHT, 4-hydroxytamoxifen; Ang-II, angiotensin II; GSI, gamma-secretase inhibitors; LVH, left ventricular hypertrophy; N3ICD, notch 3 intracellular domain; Physiological saline, sodium chloride 0.9%; VSMCs, vascular smooth muscle cells

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patients with left ventricular hypertrophy (LVH) supports the translational potential of these findings. Importantly, treatment with γ-secretase inhibitor (GSI) protects against LVH in wild-type mice infused with Ang-II and prevents the development of Ang-II-induced hypertrophy in mouse cardiomyocytes. Taking together, our findings unveil a protective role of targeting the γ-secretases in Ang-II promoted LVH.

MATERIALS AND METHODS

Mice
This study used CreERT2/ERT2 mice, in which transgenic tamoxifen-inducible Cre is systemically expressed under the RNA polymerase II promoter [4], and PsenF1/Psen2−/− mice, in which PS1 is flanked by foxP sites excizable by Cre recombination, and are defective for PS2 [5]. Both mouse lines were in the C57BL/6NCrl (obtained from Charles River) pure background and were crossed at the CNIO Animal Facility to generate PsenF1/Psen2−/−;CreERT2 mice, and male mice were used for experimental procedures at the CNIC. Age-matched littersmates were injected intraperitoneally (i.p) either with 1 mg 4-hydroxytamoxifen (4-OHT) (Sigma Aldrich, St Louis, Missouri, USA) in corn oil (Sigma Aldrich) or with corn oil alone (control group). The timing of injections is indicated in the figures. All animal procedures were approved by the CNIC-Instituto de Salud Carlos III (CNIC-ISCIII) Ethics Committee for Research and Animal Welfare.

Angiotensin II-induced hypertension and γ-secretase inhibitor treatment in mice
Mice were infused with angiotensin II (Ang-II) as described [6]. Briefly, 8-week-old male mice were anaesthetized with sevoflurane and a small incision was made in the interscapular area. Osmotic minipumps (Eugene, New Jersey, USA) at 37°C were inserted into the subcutaneous mid-scapular area. Osmotic minipumps (#2004; Durect Corporation, Cupertino, California, USA) loaded with Ang-II (Sigma Aldrich) were implanted subcutaneously into the B6.Ss (C57BL/6J) pure background and were crossed at the CNIC-Instituto de Salud Carlos III (CNIC-ISCIII) Ethics Committee for Research and Animal Welfare. Mice were infused with angiotensin II (Ang-II) as described [6]. Briefly, 8-week-old male mice were anaesthetized with sevoflurane and a small incision was made in the interscapular area. Osmotic minipumps (Eugene, New Jersey, USA) at 37°C were inserted into the subcutaneous mid-scapular area. Osmotic minipumps (#2004; Durect Corporation, Cupertino, California, USA) loaded with Ang-II (Sigma Aldrich) were implanted subcutaneously into the B6.Ss (C57BL/6J) pure background and were crossed at the CNIC-Instituto de Salud Carlos III (CNIC-ISCIII) Ethics Committee for Research and Animal Welfare.

Cell-culture assays
Primary cultures of mouse VSMCs were isolated and cultured as described [6]. Briefly, the thoracic aortas of 8-week-old mice were micro-dissected and digested with 2 mg/ml collagenase type IV (Worthington Biochemical Corp., Lakewood, New Jersey, USA) at 37°C for 15 min in a 5% CO2 incubator, to remove the adventitial and endothelial layers. VSMCs were released by a second collagenase digestion for 90 min at 37°C with constant agitation. Cells were then washed and suspended in minimal essential medium, supplemented with 1 mmol/l glutamine, 100 IU/ml penicillin, 100 μg streptomycin and 10% (v/v) foetal bovine serum and grown at 37°C in a humidified atmosphere at 5% CO2. Cells were grown to confluence and treated for 5 days with vehicle (0.1% DMSO; Sigma Aldrich) or 4-OHT (600 nmol/l; Sigma Aldrich) as indicated. For Ang-II stimulation, cells were first rendered quiescent by serum deprivation for 48 h and then stimulated with 1 μmol/l Ang-II for the indicated times. Experiments were performed with cells between passages 4 and 9 (1:3 splitting after trypsinization). Cultures of NkL-TAg cells, a mouse cardiac cell line, were maintained as described [9]. Briefly, cells were kept in DMEM/F12 supplemented with 10% FBS and plated into petri dishes coated with 12.5 μg/ml of fibronectin in 0.1% gelatin/PBS. To keep the cardiac phenotype only, two first passages after thawing cells were used. The GSI DBZ was added to the culture medium at 250 nmol/l and kept for 48 h before the addition of Ang-II. For Ang-II stimulation, cells were first rendered quiescent by serum deprivation for 48 h (with or without DBZ) and then stimulated with 1 μmol/l Ang-II for the indicated times. For the hypertrophic assay of the cardiomyocytes, we follow the protocol described before [10]. Briefly, cells were maintained in the described culture medium, supplemented with solvent carrier in control or with 1 μmol/l Ang-II and with or without DBZ. Twenty-four hours later, hypertrophy was assayed by measurement of the cell’s surface area using ImageJ software. Results represent the average of 85–100 cells from seven pictures from different areas in each group.

Protein analysis
Cell extracts were prepared by incubating cells on ice for 10 min in lysis buffer [50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, NP-40 0.5%, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l sodium fluoride, 10 mmol/l sodium orthovanadate, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 1 mg/ml pepstatin] followed by removal of cellular debris by centrifugation at 12 000 g for 10 min. Protein concentration was measured with the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, California, USA).

For immunoblots, 30 μg of protein was resolved on 4–20% SDS-PAGE gels, wet-transferred to nitrocellulose (Bio-Rad Laboratories) and immunoblotted. The following antibodies were used: anti-NOTCH3 (rabbit polyclonal sc-5593; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), antipresenilin1 (rabbit polyclonal 529592; Merck & Co., White House Station, New Jersey, USA), anti-γ-tubulin (mouse monoclonal GTU-88; Sigma Aldrich) and anti-GAPDH (mouse monoclonal MAB374; Merck & Co.). Horseradish peroxidase-linked secondary antibodies were from Agilent Technologies (Glostrup, Denmark). Immuno-complexes were visualized by chemiluminescence using the ECL detection system (GE Healthcare, Waukesha, Wisconsin, USA).

Quantitative real-time RT-PCR
Total RNA was purified from VSMCs and snap-frozen adventitia and endothelium-free mouse aorta with Trizol (Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was generated using Ready-to-Go (GE Healthcare). Quantitative real-time PCR was performed in an ABI PRISM 7700 thermal cycler (Life Technologies), using DNA Master SYBR Green I mix (Life Technologies).

The human primers used were as follows:
expression (2-ΔDD) of the corresponding 18S rRNA in Supplementary Figure 4, http://...

...heart was measured in M-mode with guided B-mode short-axis recordings at the mid-ventricular level. Left ventricular end-diastolic (LVIDd) diameter, left ventricular end-systolic diameter (LVIDs), end-diastolic interventricular septum (IVSd) and end-diastolic left ventricular posterior wall (LVPWd) thicknesses were measured. Left ventricular fractional shortening, left ventricular ejection fraction and left ventricular mass corrected were calculated following calculation definitions described in Visual Sonics Vevo 2100 Imaging System operator manual.

**Blood pressure measurements in mice**

Blood pressure (BP) was measured noninvasively with a tail-cuff device (BP-2000 Blood Pressure Analysis System; Visitech Systems Inc., Apex, North Carolina, USA) in trained conscious mice [12]. All measurements were taken at the same time in the morning. To increase accuracy, the first 10 measurements were discarded. Mean values for individual mice were used for analysis.

**Human arterial samples**

Carotid artery tissue was obtained from 10 men aged over 60 years who underwent revascularization via endarterectomy due to internal carotid stenosis more than 75%. All patients had a previous diagnosis of essential hypertension, and despite being under chronic treatment with antihypertensive medications (including angiotensin-converting enzyme inhibitors or angiotensin receptor blockers), SBP and DBP were maintained above 140 and 90 mmHg, respectively. Left ventricular mass (LVM) was estimated from measurements obtained by 2D-echocardiography. Patients were grouped into those with LVH (LVH; indexed LVM >131 g/m²; N=5) and those without (indexed LVM <131 g/m²; N=5) in accordance with the criteria reported by Lang et al. [13]. When atherosclerotic plaque was observed, it was immediately removed and the remaining tissue samples frozen in liquid nitrogen. The study protocol was approved by the Research Ethics Committee of the University Clinic of Navarra and written informed consent was obtained from all participants.

**Statistical analysis**

Values for each parameter within a group are expressed as mean±SEM. For comparisons between groups, statistical significance was assessed by unpaired two-tailed Student’s t-test. For within-group comparisons, a paired two-tailed Student’s t-test was used. A repeated measures two-way analysis of variance (ANOVA; mixed model) followed by Bonferroni posthoc test was performed (see Figs 2b and 3a). Differences were considered statistically significant at a P value less than 0.05.

**RESULTS**

Ang-II activates Notch signalling in vascular smooth muscle cells

Notch signalling is essential for proper vascular development in mammals [2]. To mimic pharmacological intervention in adulthood while avoiding possible effects of Notch inactivation during embryonic development, we generated the inducible mouse line Psen1f/f;Psen2+/i,CreERT2/ERT2, in which the γ-secretase complex is ubiquitously targeted...
after treatment with 4-hydroxytamoxifen (4-OHT). Using VSMCs isolated from Psen1f/f;Psen2−/−;CreERT2ERT2 mice, we investigated the ability of Ang-II to activate the Notch pathway by monitoring the activation of NOTCH3, which is highly expressed by VSMCs [1,14]. As expected, treatment of VSMCs with 4-OHT sharply reduced protein expression of PSEN1 (Fig. 1a) and prevented the strong Ang-II-induced activation of NOTCH3 (measured as the NOTCH3 intracellular domain; N3ICD) that rapidly occurred in vehicle-treated cells (Fig. 1a). Analysis of Notch downstream effectors by qPCR showed that while Ang-II had no effect on Hes1, the best-known Notch pathway effector, it upregulated Hey1 and Hes5, two essential NOTCH3 effectors in VSMCs [1,14] (Fig. 1b). 4-OHT significantly reduced Hes1 expression in control and Ang-II-treated VSMCs and blunted Ang-II-dependent Hey1 and Hes5 upregulation (Fig. 1b).

Next, we infused wild-type mice with Ang-II or physiological saline buffer using mini-pumps [6]. Ang-II infusion increased NOTCH3 activity, measured as nuclear NOTCH3 in VSMCs from the media of renal arteries (Fig. 1c). Accordingly, we also found higher expression of Hes5 in aortic tunica media cells from the same animals (Fig. 1d). However, aortic tunica media expression of Hes1 (data not shown) and Hey1 (Fig. 1d) was similar in saline and Ang-II infused mice.

**Targeting γ-secretases inhibit Ang-II-induced hypertension and left ventricular hypertrophy**

We next examined the effect of γ-secretase genetic deletion on the development of hypertension. As expected, VSMCs from 4-OHT-treated Psen1f/f;Psen2−/−;CreERT2ERT2 mice showed a markedly reduced Hey1 and Hes5 mRNA expression (Fig. 2a) indicating that the Notch pathway was affected. Neither 4-OHT nor vehicle treatments affected SBP over a period of 6 days (Fig. 2b), indicating that Notch signalling is not involved in the regulation of BP at homeostasis. In contrast, Ang-II promoted hypertensive SBP was significantly blunted in 4-OHT-treated mice compared with controls (Fig. 2b).

To analyse whether γ-secretase deletion protects against Ang-II-induced LVH, we performed echocardiographic longitudinal studies. Neither 4-OHT nor Ang-II affected ejection fraction or fractional shortening (Supplementary Figure 1, http://links.lww.com/HJH/A448). After Ang-II infusion in vehicle-treated mice, corrected LVM (LVMc) showed the expected progressive increase during follow-up (35% at 15 days after Ang-II infusion, \( P < 0.01 \) versus baseline). Importantly, this pathological response was blunted in 4-OHT-treated mice (no significant differences versus baseline and \( P < 0.01 \) when compared with vehicle-treated mice) (Fig. 2c, Supplementary Figure 2, http://links.lww.com/HJH/A448 and Supplementary Videos, http://links.lww.com/HJH/A444, http://links.lww.com/HJH/A445, http://links.lww.com/HJH/A446, http://links.lww.com/HJH/A447). The γ-secretase complex is needed to maintain Ang-II-induced hypertension and left ventricular hypertrophy

We next analysed whether targeting γ-secretases could be also beneficial in mice with established hypertension. Psen1f/f;Psen2−/−;CreERT2ERT2 mice were infused with

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**FIGURE 1** Ang-II activates Notch signalling in vascular smooth muscle cells. (a, b) Primary VSMCs from Psen1f/f;Psen2−/−;CreERT2ERT2 mice were treated with vehicle or 4-OHT and activated with Ang-II for the indicated times. Cells were analysed by western blot (a) and qPCR (b). (c) Representative images of immunostaining for NOTCH3 and α-smooth muscle actin (SMA) in cross-sections of paraffin-embedded kidney from saline or Ang-II-infused wild-type mice (n = 5 mice per group). Bar, 50 μm. The chart shows quantification of nuclear NOTCH3. (d) qPCR analysis of the indicated genes in adventitia and endothelium-free aorta from saline or Ang-II-infused Psen1f/f;Psen2−/−;CreERT2ERT2 mice (n = 5). ***P < 0.001; **P < 0.01; *P < 0.05.
Ang-II and, once SBP reached more than 150 mmHg, were randomized for treatment with vehicle or 4-OHT. In vehicle-treated mice, SBP continued to increase more than 170 mmHg, whereas 4-OHT-treated mice maintained the baseline systolic value of nearly 150 mmHg (Fig. 3a). Similarly, whereas vehicle-treated mice significantly increased LVMc during follow-up ($P < 0.05$ versus baseline at 24 days), LVMc in 4-OHT-treated mice showed no statistical differences between baseline and follow-up (Fig. 3b).

**Hypertensive patients with left ventricular hypertrophy have elevated HES5 expression**

In order to test the relevance of these findings in human patients, we analysed the activation of Notch pathway in a small cohort of 10 hypertensive patients. Despite being treated with the same pharmacological regime (Supplementary Table 1, http://links.lww.com/HJH/A448), half of patients display LVH, whereas the other half do not. Thus, we sought to investigate whether worst hypertensive condition correlates with an increase in the Notch pathway activity. Expression analysis of Notch effectors by qPCR demonstrated a markedly higher HES5 expression in patients with LVH ($P < 0.05$) than in those without LVH, whereas both groups showed similar expression in HES1 and HEY1 (Fig. 3c).

The γ-secretase inhibitor dibenzazepine protects against left ventricular hypertrophy in vivo and cardiomyocyte hypertrophy in vitro induced by Ang-II

To reinforce the translational capability of the results obtained in mice with genetic ablation of γ-secretase complex, we performed a preclinical analysis in Ang-II infused mice in which the Notch pathway was inhibited during 5 weeks of treatment with the GSI DBZ starting 7 days after pumps implantation. Interestingly, DBZ did not prevent BP elevation (Supplementary Figure 3, http://links.lww.com/HJH/A448), suggesting that secretase complex inhibition was less efficient after DBZ administration compared with genetic ablation likely due to inability for DBZ to reach small arteries. As Ang-II promotes cardiac hypertrophy and remodelling in the absence of increased BP [15], we tested the effect of DBZ administration on LVH by transthoracic echocardiography. Measurements at 18 and 38 days after Ang-II infusion (for a scheme of the experiment see Supplementary Figure 3, http://links.lww.com/HJH/A448) revealed a clear reduction in the LVH in DBZ-treated animals compared with vehicle-treated mice (Fig. 4a).

Prompted by this result, we analysed the effect of DBZ in the mouse cardiac cell line NkL-TAg [9]. Treatment of NkL-TAg cells with Ang-II upregulated NOTCH3 activity in a time-dependent manner, and DBZ abrogated this response.
termed with hypertension. Moreover, targeting genetically
limited the development of Ang-II-induced systolic hyper-
tension and LVH, an important pathological feature associ-
ating the clinical situation than germline knockout appro-
aches. We show that deletion of the
pharmacological intervention and thus more closely resem-
to the relative change between day 1 (dotted line) and day 24 after pump implantation. (c) qPCR analysis of NOTCH effectors in carotid endarterectomy
samples from patients with hypertension with or without LVH. Values correspond to the relative change between day 1 (dotted line) and day 24 after pump implantation. (c) qPCR analysis of NOTCH effectors in carotid endarterectomy specimens obtained from patients with hypertension with or without LVH (n = 5 each group). *P < 0.05.

FIGURE 3 The γ-secretase complex is needed to maintain Ang-II-induced hypertension and left ventricular hypertrophy. Psen1f/f;Psen2−/−;CreERT2/ERT2 mice were treated with vehicle or 4-OHT as indicated. (a) SBP in mice exposed to Ang-II (dotted black arrow) and subsequently treated (dotted gray arrows) with vehicle (n = 8) or 4-OHT (n = 9). (b) Quantification of LVMc measured by echocardiography 24 days after Ang-II pump implantation and treatment with vehicle (n = 8) or 4-OHT (n = 9) as in (a).

DISCUSSION
Herein, we report that Ang-II treatment activates NOTCH3 in vitro in murine VSMCs and NkL-TAg cardiac cells and in vivo in VSMCs. These findings are consistent with a previous report showing that Ang-II activates the Notch pathway through γ-secretase dependent cleavage in HEK293 cells ectopically expressing the Ang-II type 1 receptor [17]. We tested the relevance of these findings in vivo by generating a mouse model in which the γ-secretase complex is genetically targeted through the inducible ubiquitous elimination of Psen1/Psen2 genes in adults. This approach mimics a pharmacological intervention and thus more closely resembles the clinical situation than germline knockout approaches. We show that deletion of the γ-secretase complex limits the development of Ang-II-induced systolic hypertension and LVH, an important pathological feature associated with hypertension. Moreover, targeting genetically

γ-secretases in mice after hypertension is established prevents any further increase in SBP and protects against LVH. Notably, the Notch effector HES5 was strongly upregulated in carotid endarterectomy specimens obtained from patients with hypertension and LVH despite receiving antihypertensive treatment, supporting a translational potential of the results obtained in mice. To challenge this possibility, we have treated hypertensive mice with DBZ. This potent cell-
permeable GSI prevented the development of Ang-II-induced LVH without affecting BP. This, together with our observation that Ang-II-induced hypertrophy in NkL-TAg cells is inhibited by DBZ, suggest that the Notch pathway is important in the Ang-II response of both VSMCs and cardiomyocytes. Further studies are required to assess whether the reduction in LVH observed in 4-OHT-treated Psen1f/f;Psen2−/−;CreERT2/ERT2 was due, at least partly, to a synergistic effect between decreased BP and a direct inhibition in the hypertrophy by lack of Notch activity.

Constitutive Notch3 deletion in the mouse has been reported to protect against Ang-II-induced hypertension, but was associated with a high mortality rate soon after Ang-II infusion, which was attributed to heart failure [3]. We reasoned that the mortality associated with constitutive Notch3 ablation might be the result of developmental defects that manifest only upon challenge of the cardiovascular system with Ang-II. This is supported by our finding here that Notch inhibition in adult mice protects against Ang-II-induced LVH, with no increase in mortality or cardiac dysfunction. This protective outcome suggests that GSIs are a possible treatment for hypertension. Previously, Ozasa et al. [17] demonstrated that systemic GSI
administration reduces Ang-II-induced vascular remodeling in mouse aorta without reporting adverse effects, but information regarding LVH was not provided. In apparent conflict with our results, NOTCH1 was reported to have a cardioprotective role in LVH [18], but in this study, the Notch1 gene was eliminated specifically in the ventricles [9]. Our results suggest that GSI, which are under evaluation in clinical trials for several diseases, might be an effective therapy for patients with hypertension, especially those whose LVH is refractory to antihypertensive therapy, and who are therefore at an increased risk of heart failure [21].

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Li X, Zhang X, Leathers R, Makino A, Huang C, Parsa P, et al. Notch3 signaling promotes the development of pulmonary arterial hypertension. Nat Med 2009; 15:1289–1297.

2. Fouillade C, Monet-Leprêtre M, Baron-Mengu C, Joutel A. Notch signaling in smooth muscle cells during development and disease. Cardiovasc Res 2012; 95:138–146.

3. Boulou N, Helle F, Dussaule JC, Placier S, Milliez P, Djadja J, et al. Notch3 is essential for regulation of the renal vascular tone. Hypertension 2011; 57:1176–1182.

4. Guerra C, Mixomolle N, Dhawahir A, Dubus P, Barradas M, Serrano M, et al. Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. Cancer Cell 2005; 4:111–120.

5. Saura CA, Choi SY, Beglopoulos V, Malkani S, Zhang D, Shankaramanayana Rao BS, et al. Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. Neuron 2004; 42:25–36.

6. Esteban V, Mendez-Barbero N, Jimenez-Borreguero LJ, Roque M, Novella L, Garcia-Redondo AB, et al. Regulator of calcineurin 1 mediates pathological vascular wall remodeling. J Exp Med 2011; 208:2125–2139.

7. Cuilloton K, Draheim KM, Hermance N, Tammam J, Sharma VM, Ware C, et al. Targeting the Notch1 and mTOR pathways in a mouse T-ALL model. Blood 2009; 113:6172–6183.

8. Villa-Bellosta R, Rivera-Torres J, Osorio FG, Acin-Perez R, Enriquez JA, Djavadi-Olah G, et al. Defective extracellular pyrophosphate metabolism promotes vascular calcification in a mouse model of Hutchinson-Gilford progeria syndrome that is ameliorated on pyrophosphate treatment. Circulation 2013; 127:2442–2451.

9. Rybkin II, Markham DW, Yan Z, Basell-Duby R, Williams RS, Olson EN. Conditional expression of SV-40 T-antigen in mouse cardiomyocytes facilitates an inducible switch from proliferation to differentiation. J Biol Chem 2003; 275:19927–19934.

10. Alvarez BV, Johnson DE, Sowah D, Soliman D, Light PE, Xia Y, et al. Carbonic anhydrase inhibition prevents and reverts cardiomyocyte hypertrophy. J Physiol 2007; 579 (Pt 1):127–145.

11. Osorio FG, Navarro CL, Cadamnos J, Lopez-Mejia IC, Quiros PM, Bartoli C, et al. Splicing-directed therapy in a new mouse model of human accelerated aging. Sci Transl Med 2011; 3:106 ra7.

12. Krege JH, Hodgin JB, Haganman JR, Smithies O. A noninvasive computerized tail-cuff system for measuring blood pressure in mice. Hyper tension 1995; 25:1111–1115.

13. Lang RM, Bierig M, Devereux RB, Flachkampf FA, Foster E, Pollikka PA, et al. Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. J Am Soc Echocardiogr 2005; 18:1440–1463.

14. Campos AH, Wang W, Pollman MJ, Gibbons GH. Determinants of Notch-3 receptor expression and signaling in vascular smooth muscle cells: implications in cell-cycle regulation. Circ Res 2002; 91:999–1006.
15. Paradis P, Dali-Youcef N, Paradis FW, Thibault G, Nemer M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. Proc Natl Acad Sci U S A 2000; 97:931–936.

16. Dorn GW2nd, Robbins J, Sugden PH. Phenotyping hypertrophy: eschew obfuscation. Circ Res 2003; 92:1171–1175.

17. Ozasa Y, Akazawa H, Qin Y, Tateno K, Ito K, Kudo-Sakamoto Y, et al. Notch activation mediates angiotensin II-induced vascular remodeling by promoting the proliferation and migration of vascular smooth muscle cells. Hypertens Res 2013; 36:859–865.

18. Croquelois A, Domenighetti AA, Nemir M, Lepore M, Rosenblatt-Velin N, Radlke F, et al. Control of the adaptive response of the heart to stress via the Notch1 receptor pathway. J Exp Med 2008; 205:3173–3185.

19. Louvi A, Artavanis-Tsakonas S. Notch and disease: a growing field. Semin Cell Dev Biol 2012; 23:473–480.

20. Maraver A, Fernandez-Marcos PJ, Herranz D, Canadero M, Munoz-Martin M, Gomez-Lopez G, et al. Therapeutic effect of gamma-secretase inhibition in Kras(G12V)-driven non-small cell lung carcinoma by derepression of DUSP1 and inhibition of ERK. Cancer Cell 2012; 22:222–234.

21. Frohlich ED, Gonzalez A, Diez J. Hypertensive left ventricular hypertrophy risk, beyond adaptive cardiomyocytic hypertrophy. J Hypertens 2011; 29:17–26.

Referees’ Summary Evaluations

Referee 1
Strength: Providing evidence that genetic intervention on the Notch signaling pathway by deleting γ-secretase can prevent a further rise in blood pressure and development of left ventricular hypertrophy induced by angiotensin II.

Weaknesses: In vivo studies are restricted to adult mice. Genetic deletion of γ-secretase could prevent a further rise in angiotensin II-induced rise in blood pressure, but for unknown reasons this could not be accomplished by pharmacological inhibition of γ-secretase with diamino-benzidine, although this compound could prevent angiotensin II-induced left ventricular hypertrophy. Hence the suggested potential role of γ-secretase inhibition as a pharmacological tool to treat hypertension complicated by hypertension remains very speculative.

Referee 2
There is increasing evidence that Notch signaling regulates the cardiovascular system not just during development, but in adults as well. In this manuscript, the authors inactivate Notch signaling in adult animals by inhibiting γ-secretase both by genetic and pharmacologic means. Both interventions prevented the effects of angiotensin II on blood pressure and left ventricular hypertrophy. However, since others have shown that the Notch1 pathway may play cardioprotective roles and even mediate the beneficial action of angiotensin II inhibitors, additional studies are needed to determine the full extent of the effects of γ-secretase inhibition, and/or test whether Notch receptors activate secretase-independent beneficial noncanonical pathways.