

# The Notch pathway controls fibrotic and regenerative repair in the adult heart

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## Aims

In the adult heart, Notch signalling regulates the response to injury. Notch inhibition leads to increased cardiomyocyte apoptosis, and exacerbates the development of cardiac hypertrophy and fibrosis. The role of Notch in the mesenchymal stromal cell fraction, which contains cardiac fibroblasts and cardiac precursor cells, is, however, largely unknown. In the present study, we evaluate, therefore, whether forced activation of the Notch pathway in mesenchymal stromal cells regulates pathological cardiac remodelling.

## Methods and results

We generated transgenic mice overexpressing the Notch ligand Jagged1 on the surface of cardiomyocytes to activate Notch signalling in adjacent myocyte and non-myocyte cells. In neonatal transgenic mice, activated Notch sustained cardiac precursor and myocyte proliferation after birth, and led to increased numbers of cardiac myocytes in adult mice. In the adult heart under pressure overload, Notch inhibited the development of cardiomyocyte hypertrophy and transforming growth factor- $\beta$ /connective tissue growth factor-mediated cardiac fibrosis. Most importantly, Notch activation in the stressed adult heart reduced the proliferation of myofibroblasts and stimulated the expansion of stem cell antigen-1-positive cells, and in particular of Nkx2.5-positive cardiac precursor cells.

## Conclusions

We conclude that Notch is pivotal in the healing process of the injured heart. Specifically, Notch regulates key cellular mechanisms in the mesenchymal stromal cell population, and thereby controls the balance between fibrotic and regenerative repair in the adult heart. Altogether, these findings indicate that Notch represents a unique therapeutic target for inducing regeneration in the adult heart via mobilization of cardiac precursor cells.

## Keywords

Hypertrophy • Regeneration • Cardiac precursor cells • Notch signalling

## Introduction

In the mammalian heart, a rapid shift from myocyte hyperplasia to myocyte hypertrophy occurs shortly after birth.<sup>1</sup> Therefore, the heart has been considered a post-mitotic organ with a response to injury restricted to fibrosis and cardiomyocyte hypertrophy. However, new myocyte formation takes place in the adult heart due to the recruitment of pre-existing immature myocytes and cardiac precursor cells (CPCs).<sup>2–4</sup> Resident CPCs have been identified by the expression of surface markers such as stem cell antigen-1 (Sca-1) or c-Kit, and co-expression of early cardiac

markers such as Nkx2.5, GATA4, and Mef2c.<sup>5</sup> However, the adult heart has limited regenerative potential. Upon tissue damage, fibroblast proliferation far exceeds that of CPCs. Consequently, a fibrotic scar is generated instead of a functional myocardium derived from newly formed cardiomyocytes.

The reason why the mammalian heart adopts fibrosis as its primary repair process remains unknown. One of the key regulators of fibrosis is transforming growth factor (TGF)- $\beta$ , which induces myofibroblast differentiation.<sup>6</sup> Its downstream target, connective tissue growth factor (CTGF) promotes cardiomyocyte hypertrophy, enhances fibrosis, and stabilizes the fibrotic scar.<sup>7</sup>

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Periostin (Postn) is also a TGF- $\beta$ -inducible matricellular protein that regulates matrix production and stabilization.<sup>8</sup> Together, these factors orchestrate fibrotic repair after injury. Unfortunately, the fibrous scar represents a hostile environment, preventing regenerative processes to take place.<sup>9</sup> Experimental evidence indicates that limiting cardiac fibrosis improves function of the injured heart<sup>10–13</sup> and could tip the balance towards regeneration.<sup>9,14</sup> However, additional cues to induce cardiac precursor mobilization and myocyte proliferation need to be imposed. Signalling pathways that are important for cardiac morphogenesis during the foetal life and that are reactivated in the damaged adult heart, such as the Notch signalling pathway, could be used to promote CPC expansion.

The Notch pathway mediates signalling between adjacent cells expressing transmembrane ligands (Jagged1 and 2; Delta-like1, 3, and 4) and receptors (Notch1–4).<sup>15</sup> In the developing heart, Notch regulates trabeculation, myocyte proliferation, and valve formation.<sup>16</sup> Mutations in components of the Notch pathway lead to cardiac abnormalities such as ventricular septal defects and valve malformation.<sup>17,18</sup> In the postnatal heart, Notch regulates cardiac precursor expansion and differentiation.<sup>19</sup> Notch is essential for the maintenance of structural and functional integrity during the response to increased workload.<sup>20–23</sup> This pathway is activated in cardiac myocytes and mesenchymal precursors secondary to induced Jagged1 expression,<sup>20</sup> and sustains the proliferation of committed precursors and immature cardiomyocytes.<sup>20,24,25</sup> Pharmacological or genetic blockade of the Notch pathway exacerbates hypertrophy, and leads to the development of massive fibrosis.<sup>20</sup>

Altogether, these findings suggest that activation of the Notch pathway enhances myocyte production after damage. We have generated, therefore, transgenic mice overexpressing Jagged1 on the surface of cardiomyocytes to chronically activate Notch in adjacent myocytes and mesenchymal stromal cells such as fibroblasts and cardiac precursors, and evaluated the cardiac response to pressure overload. We show that Notch activation restrains cardiac hypertrophy and fibrosis, and promotes cardiac precursor expansion. Therefore, Notch could be used to shift the response to injury in the adult mammalian heart towards regeneration.

## Methods

All methods are available in Supplementary material online.

## Results

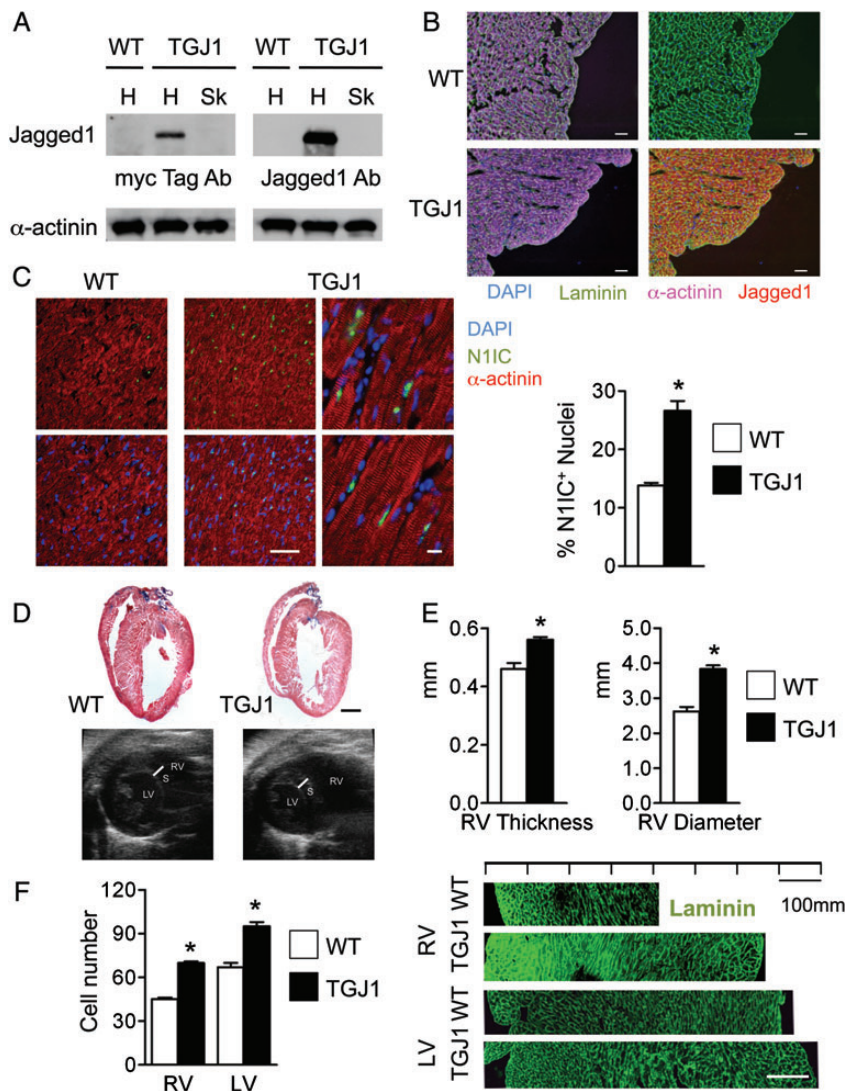
### Enhanced Notch activation in transgenic mice with cardiac-specific Jagged1 overexpression

Since adult cardiac precursor-specific markers are currently lacking, Notch signalling restricted to this lineage cannot be easily achieved in transgenic animals. To activate Notch in the adult myocyte and non-myocyte cells, we generated NMRI-Tg(*Myh6*:*Jagged1*)89<sup>Ped</sup> mice expressing Jagged1 under control of the  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter

(hereafter referred to as TGJ1 mice). Litters derived from heterozygote breeding contain ~40–45% transgenic mice. This is somewhat lower than the expected Mendelian ratio (i.e. 50%), suggesting that transgene expression produces a slight detrimental effect during development. RT-PCR analysis showed that adult transgenic mice expressed more Jagged1 than wild-type (WT) littermates (Supplementary material online, *Figure S1A*). Overexpression was already observed 1 week after birth, and peaks in adult hearts (Supplementary material online, *Figure S1B*). The myc-tagged Jagged1 protein was readily detected in the heart, but not in the skeletal muscle (*Figure 1A*). In the adult myocardium, Jagged1 was uniformly expressed on the surface of cardiomyocytes (*Figure 1B*), but not in interstitial non-myocyte cells (Supplementary material online, *Figure S1C*). Immunostaining using an antibody against the activated form of Notch1 (Notch1 intracellular domain; N1IC) showed that the Notch1 pathway was activated in  $\alpha$ -actinin-positive cardiomyocytes and in small interstitial  $\alpha$ -actinin-negative non-myocyte cells (*Figure 1C*). In transgenics, the percentage of N1IC-positive cells increased two-fold relative to WT, indicating that Jagged1 resulted in a sustained activation of the Notch1 pathway. Echocardiography and histological analysis revealed an enlargement of the right ventricle (RV) in adult TGJ1 mice (*Figure 1D*). The RV weight was also heavier in the TGJ1 mice than in control littermates (Supplementary material online, *Figure S2*). Interestingly, both the left ventricle (LV) and the RV had an increased number of cardiomyocytes across the full wall thickness in transgenic mice (*Figure 1F*). Cardiac function as assessed by echocardiography was normal (Supplementary material online, *Table S1*).

### Sustained myocyte proliferation in the neonatal heart of the TGJ1 mice

To determine whether the increased number of myocytes observed in transgenic hearts resulted from sustained postnatal proliferation, we determined the number of mitotic phosphohistone H3 (PH3)-positive cardiac cells in 1 and 2 week-old mice (*Figure 2A*). The hearts of 1 week-old TGJ1 mice contained twice as many PH3-positive cells than WT. This finding was confirmed by Ki67 and Cyclin D1 staining (Supplementary material online, *Figure S3A* and *B*). Quantitative RT-PCR also showed that the G2/M cyclin B1 was more expressed in the TGJ1 mice (2.5-fold increase;  $P < 0.05$ ; not shown). Importantly, these differences disappeared in 2 week-old animals (*Figure 2A*). To determine whether proliferating cardiac cells contributed significantly to cardiomyocyte production, mice were injected with BrdU at 1 week of age, followed by a 3-week chase period. Under these conditions, rapidly dividing cells quickly lose BrdU through successive cell divisions. Only cardiomyocytes reaching terminal differentiation shortly after BrdU administration and slowly dividing cells such as cardiac stem cells retain BrdU (label retaining cells). In WT and transgenic mice, BrdU incorporation was detected in cardiac myocytes ( $\alpha$ -actinin-positive cells) and non-myocyte cells ( $\alpha$ -actinin-negative cells; *Figure 2B*). Quantitative analysis showed that the number of BrdU-positive myocytes and non-myocytes was significantly increased in the TGJ1 mice (*Figure 2B*). Furthermore, the number of BrdU-positive Nkx2.5-positive cells was

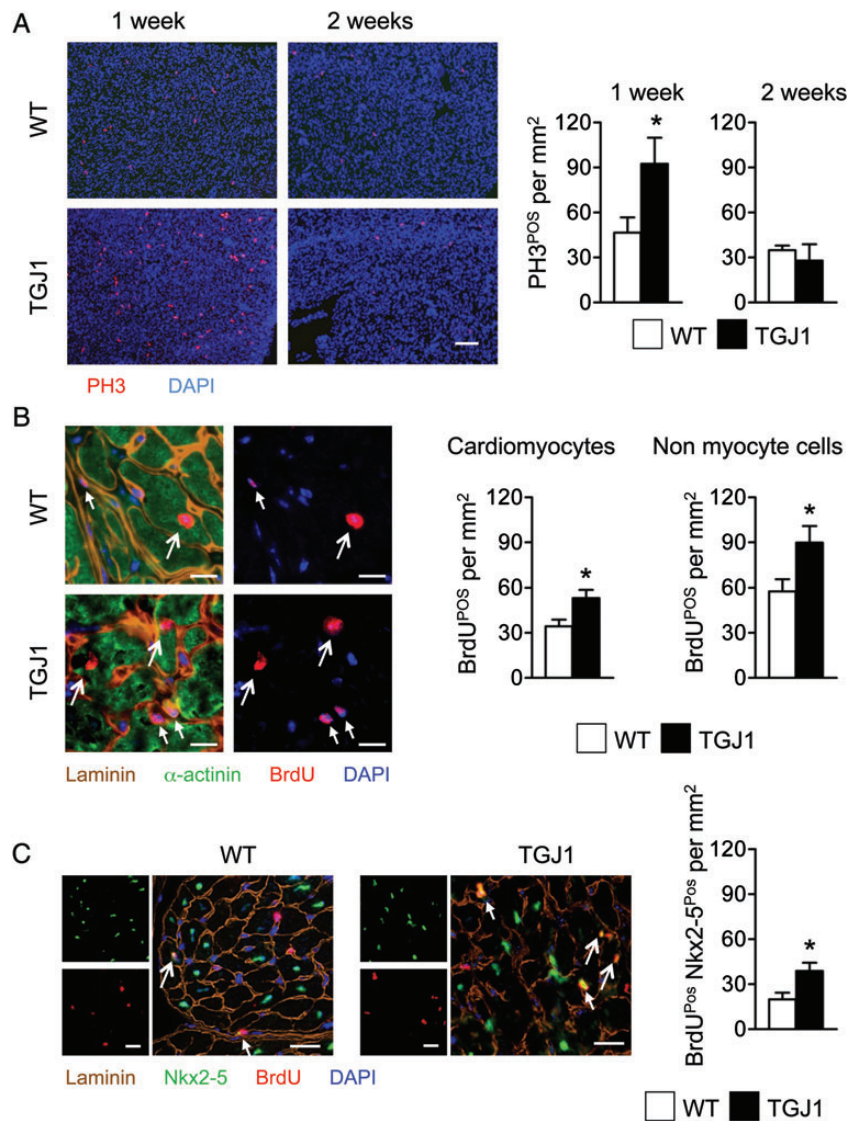


**Figure 1** Characterization of the TGJ1 mice. (A) Western blot analysis of Jagged1 expression in the heart (H), and skeletal muscle (Sk) of TGJ1 and wild-type mice using antibodies against myc-tag and Jagged1. (B) Jagged1 (red) expression in cardiomyocytes identified by  $\alpha$ -actinin immunostaining (purple) and laminin (green). DAPI (blue). (C) Notch1 activation detected with antibodies against activated Notch1 (N1IC; green);  $\alpha$ -actinin (red). Bar graph shows the percentage of N1IC-positive nuclei (mean  $\pm$  SEM; \* $P$  < 0.05, five mice per group). (D) H/E staining of heart sections and short-axis echocardiography images showing enlarged right ventricles in TGJ1 mice (LV, left ventricle; RV, right ventricle; S, septum). (E) Right ventricle wall thickness and diameter in TGJ1 and wild-type mice (mean  $\pm$  SEM; \* $P$  < 0.05, six mice per group). (F) Laminin immunostaining (green) across full thicknesses of left ventricular and right ventricular of wild-type and TGJ1 mice (right panel). Bar graph shows the number of cells in the left ventricular and right ventricular walls (mean  $\pm$  SEM; \* $P$  < 0.05, three mice per group). Scale bar in (B) and (C): 50  $\mu$ m; (D) 1 mm; (F) 100  $\mu$ m.

also increased in TGJ1 hearts (Figure 2C). Nkx2.5-positive cells represented  $26 \pm 4.0$  and  $45 \pm 6.6\%$  of total BrdU-positive cells in WT and in TGJ1 hearts, respectively. It is noteworthy that cardiac tissues in neonatal transgenic mice presented a less organized structure than WT (Figure 2B and C). Taken together, these data suggested that postnatal Notch activation promoted cardiomyocyte proliferation and expansion of a cardiac precursor pool that contributed to the higher cellularity seen in adult transgenic hearts.

### Attenuated cardiomyocyte hypertrophy in response to pressure overload in the TGJ1 mice

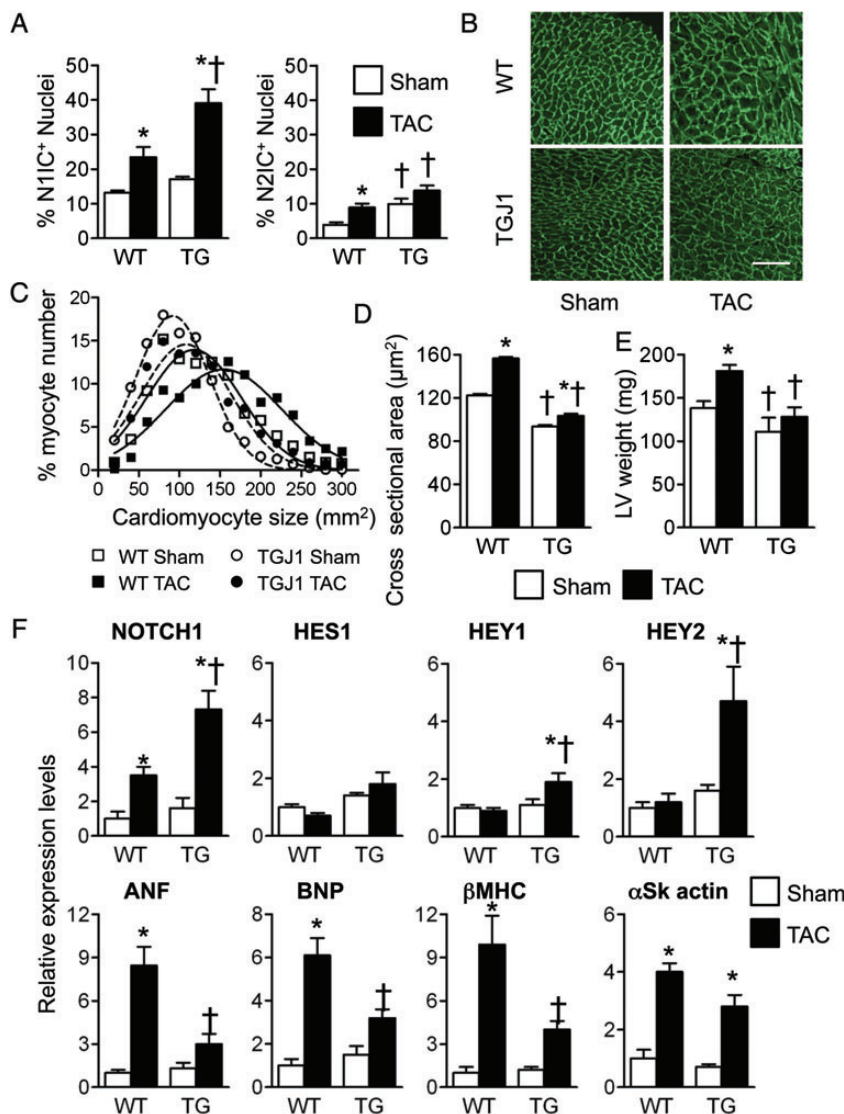
Mice were subjected to transaortic constriction (TAC) to produce pressure overload in the LV. Aortic velocity after TAC was identical in WT and in TGJ1 hearts (Supplementary material online, Figure S4A), indicating that the heart was subjected to a comparable load in both genotypes. Transaortic constriction activated Notch1



**Figure 2** Proliferation in the postnatal heart. (A) Number of proliferating cells in the heart of 1 and 2-week-old wild-type and TGJ1 mice. Heart sections stained with antibodies against phosphohistone-H3 (pink) and DAPI (blue). The quantification of PH3-positive cells (mean  $\pm$  SEM; \* $P < 0.05$ , six mice per group). (B) The identification of proliferating cells in postnatal wild-type and TGJ1 hearts. Mice received BrdU at p7 and were analysed for BrdU incorporation 3 weeks thereafter. Immunostaining: BrdU (red),  $\alpha$ -actinin (green); laminin (brown); DAPI (blue). Large arrows: cardiomyocytes; small arrows: non-myocyte cells. (C) The identification of Nkx2.5-proliferating cells in postnatal wild-type and TGJ1 hearts. Immunostaining: BrdU (red), Nkx2-5 (green); laminin (brown); DAPI (blue). BrdU was detected in Nkx2-5-positive cardiomyocytes (large arrows) and in non-myocytes (small arrows). The quantification of BrdU-positive cells per mm<sup>2</sup> (mean  $\pm$  SEM; \* $P < 0.05$ ; four to six mice per group). Scale bar in (A) 50  $\mu$ m; (B) 10  $\mu$ m; (C) 50  $\mu$ m in the single channel micrographs and 10  $\mu$ m in the merged images.

in WT and TGJ1 mice, but activation was more important in TGJ1, as judged by the increased number of N1IC-positive cells (Figure 3A and F). Notch2 was moderately stimulated (Figure 3A). The echocardiographic assessment of cardiac dimensions and function demonstrated the development of hypertrophy in WT controls after TAC (Supplementary material online, Table S1). The cross-sectional area of WT cardiomyocytes was increased in response to stress, and the size distribution shifted towards larger cells

(Figure 3B–D). As a consequence, LV weight was heavier in WT mice subjected to pressure overload than in sham-operated animals (Figure 3E). Markers of cardiac hypertrophy were up-regulated in the stressed WT hearts (Figure 3F). In sharp contrast to WT, the hypertrophic response in the TGJ1 mice was largely reduced (Figure 3 and Supplementary material online, Table S1, Figure S4B), suggesting that Jagged1-induced activation of the Notch pathway exerted anti-hypertrophic actions.



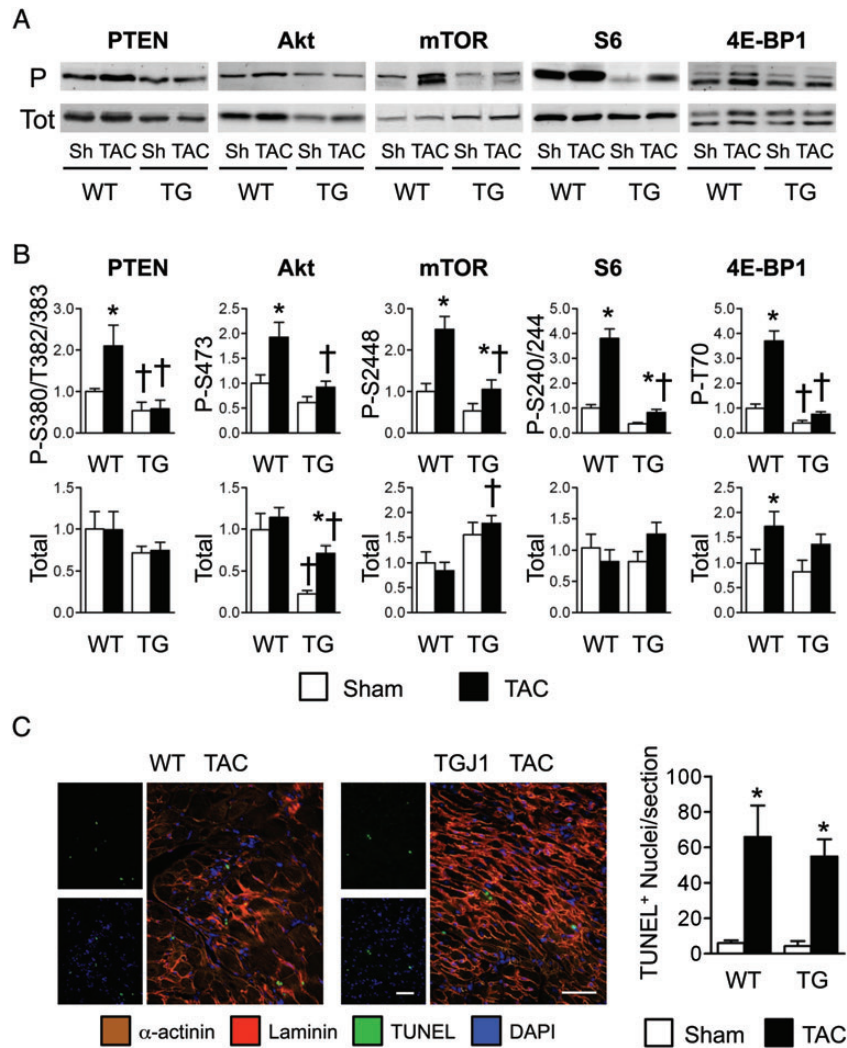
**Figure 3** The cardiac hypertrophic response to pressure overload. Wild-type and TGJ1 mice were subjected to transaortic constriction and analysed after 1 week. (A) The percentage of cardiac cells demonstrating Notch1 (left) or Notch2 (right) activation after transaortic constriction. (B) Heart sections stained with antibodies against laminin (green). (C) Cardiomyocyte size distribution. (D) Cardiomyocyte cross-sectional area (mean  $\pm$  SEM). (E) Post-mortem left ventricular weight in wild-type and TGJ1 mice. (F) Notch target gene expression using qRT-PCR (upper panel) and cardiac hypertrophy marker expression (lower panel) in wild-type and TGJ1 hearts. Data are expressed as fold change relative to wild-type Sham. Data in (A), (D), (E), and (F) are presented as mean  $\pm$  SEM (\* $P$  < 0.05 in transaortic constriction vs. Sham;  $^{\dagger}P$  < 0.05 in TGJ1 vs. wild-type; six mice per group). In (C) and (D), values are obtained from at least 700 cells per group. Scale bar in (B) 50  $\mu$ m.

The phosphoinositide 3-kinase (PI3K)/Akt pathway regulates cell growth and the development of cardiac hypertrophy through activation of the mammalian target of rapamycin (mTOR). Western blot analysis revealed that TAC induced phosphorylation of Akt, mTOR, and the effector proteins S6 and 4E-BP1 in the WT mice (Figure 4A and B). In contrast, the Akt pathway was only minimally activated after TAC in the TGJ1 mice (Figure 4A and B). The PI3K/Akt pathway is negatively regulated by the phosphatase and tensin homolog (PTEN), which is inactivated by phosphorylation. Phosphatase and tensin homolog was phosphorylated after TAC in WT but not in the TGJ1 mice, indicating that active PTEN likely

contributed to the down-regulation of the Akt pathway. The Akt pathway also promotes cell survival. Despite down-regulation of the Akt pathway, apoptotic cell death was identical to WT in TGJ1 mouse hearts, suggesting that transgenic hearts did not demonstrate higher susceptibility to stress (Figure 4C).

### Reduced cardiac fibrosis in response to pressure overload in the TGJ1 mice

The development of fibrosis is under control of soluble factors such as TGF- $\beta$ , CTGF, and Postn. We found that the expression

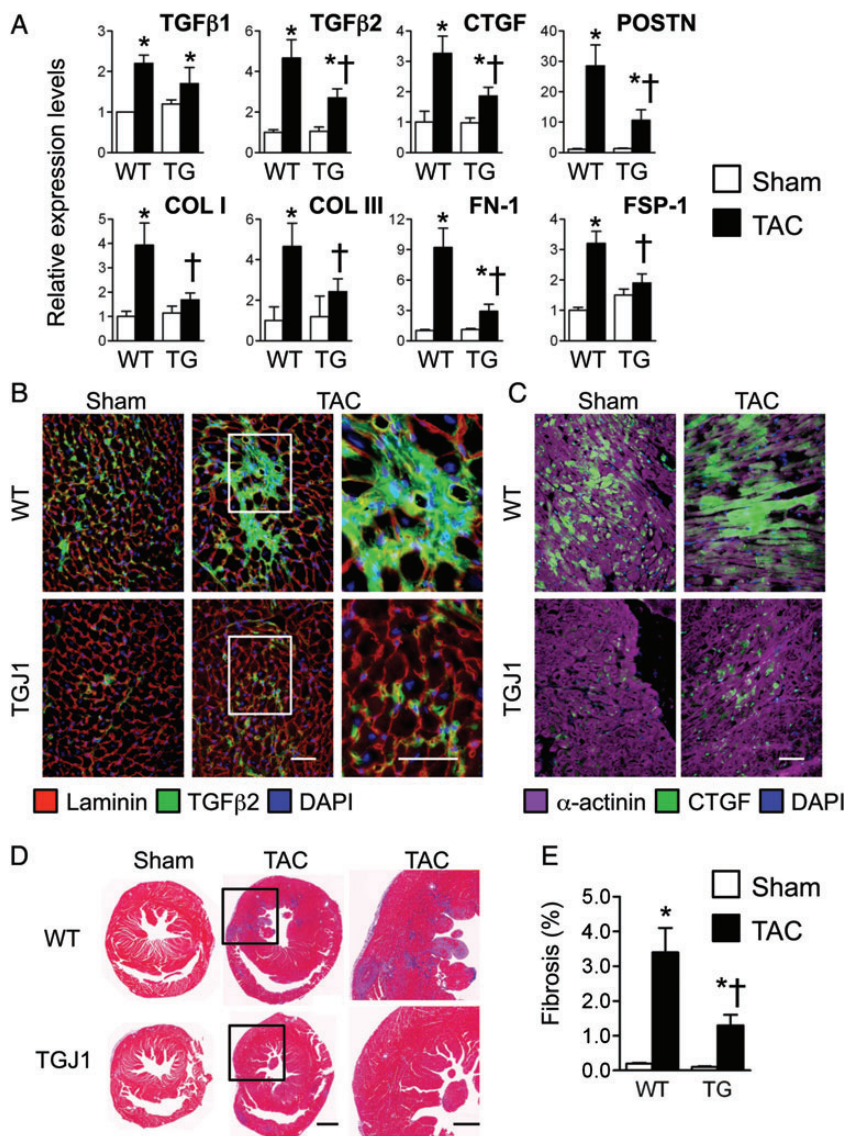


**Figure 4** Activation of the Akt pathway. (A) Western blot analysis of phosphorylated (P) and total (Tot) PTEN, Akt, mTOR, S6, and 4E-BP1 in wild-type and TGJ1 mice 1 week after transaortic constriction. (B) Quantitative data are presented as mean  $\pm$  SEM; six samples per group (\* $P < 0.05$  in transaortic constriction vs. Sham;  $^\dagger P < 0.05$  in TGJ1 vs. wild-type). (C) TUNEL staining (green) in wild-type and TGJ1 hearts after transaortic constriction; laminin (red),  $\alpha$ -actinin (brown). Bar graph: mean  $\pm$  SEM of TUNEL-positive nuclei per heart section (\* $P < 0.05$  in transaortic constriction vs. Sham;  $n = 6$  animals per group). Scale bars in (C) 50  $\mu$ m.

of TGF- $\beta$ 1, TGF- $\beta$ 2, CTGF, and Postn was induced by TAC in the WT mice (Figure 5A). In the TGJ1 mice, however, the induction of TGF- $\beta$ 2, CTGF and Postn was significantly lower. The expression of the extracellular matrix proteins collagen I, collagen III, and fibronectin-1 and of fibroblast-specific protein-1 (Fsp-1) was also significantly reduced. Transforming growth factor- $\beta$ 2 and CTGF were expressed by fibroblasts and cardiomyocytes, respectively, and these factors were up-regulated after TAC in WT, but not in the transgenic mice (Figure 5B and C). Finally, the quantification of collagen deposition showed that the TGJ1 mice accumulated significantly less interstitial fibrosis when compared with the WT mice (Figure 5D and E).

### Cardiac hypertrophy and fibrosis in mice lacking Jagged1 expression in cardiomyocytes

To further study the importance of Jagged1 on cardiac remodelling, we generated mice with cardiomyocyte-specific Jagged1 deletion (Jagged1<sup>Del/Del</sup>) by crossing mice carrying floxed Jagged1 alleles (B6; 129-Jagged1<sup>tmLoxPRad</sup>) with  $\alpha$ -MHC-MerCreMer mice [B6-Tg(Myh6-Cre/Esr1\*)1Jmk/J005657] expressing a Tamoxifen-inducible form of the Cre recombinase in cardiomyocytes. Tamoxifen-induced Jagged1 deletion was confirmed by PCR on cardiac genomic DNA and by immunostaining (not shown).



**Figure 5** Cardiac fibrosis. (A) Quantitative RT-PCR analysis of fibrosis marker genes in wild-type and TGJ1 mice 1 week after transaortic constriction. Data are expressed as fold change relative to wild-type Sham (mean  $\pm$  SEM;  $n = 6-9$  mice per group;  $*P < 0.05$  in transaortic constriction vs. Sham and  $\dagger P < 0.05$  in TGJ1 vs. wild-type). (B) Transforming growth factor- $\beta 2$  expression (green) in wild-type and TGJ1 heart sections; laminin (red), DAPI (blue). (C) connective tissue growth factor expression (green) in wild-type and TGJ1 heart sections;  $\alpha$ -actinin (magenta), DAPI (blue). (D) Masson Trichrome staining (blue) in wild-type and TGJ1 heart sections. (E) The per cent fibrotic area in wild-type and TGJ1 heart sections represented as mean  $\pm$  SEM ( $*P < 0.05$  in transaortic constriction vs. Sham;  $\dagger P < 0.05$  in TGJ1-transaortic constriction vs. wild-type-transaortic constriction; transaortic constriction,  $n = 6$ , Sham,  $n = 4$ ). Scale bars in (B) and (C) 50  $\mu$ m; in D = 1.0 mm and 0.5 mm in higher magnification insets.

Cardiomyocyte-specific Jagged1 knockouts were subjected to TAC, and analysed 1 and 4 weeks after surgery. No difference in the hypertrophic response to stress was observed between the two genotypes after 1 week (Supplementary material online, Figure S5). Of note, no compensatory expression of other Notch ligands was observed in the Jagged1<sup>Del/Del</sup> mice (Supplementary material online, Figure S6). After 4 weeks, mice lacking Jagged1 demonstrated a modest increase in susceptibility for hypertrophy after TAC, as judged by the increased expression of cardiac stress markers (Supplementary material online, Figure S5D). The

development of fibrosis was not affected by the deletion of Jagged1 in cardiomyocytes.

### Notch1 expression in cardiomyocytes is dispensable for Jagged1-induced anti-hypertrophic and anti-fibrotic responses

To evaluate the implication of Notch1 in the anti-hypertrophic and anti-fibrotic effects of Jagged1, we generated TGJ1 mice lacking

Notch1 in cardiomyocytes by crossing TGJ1 mice to floxed Notch1 mice (B6;129-*Notch1*<sup>tmLoxPRad</sup>) and  $\alpha$ -MHC-MerCreMer mice. Tamoxifen was injected to adult mice to delete Notch1 in cardiomyocytes (Notch1<sup>Del/Del</sup> mice). When subjected to TAC, WT; Notch1<sup>Lox/Lox</sup> mice (WT controls) developed a hypertrophic response, characterized by an increase in the LV mass (Supplementary material online, Figure S7 and Table S2). In the TGJ1 mice with or without Notch1 deletion, the hypertrophic response was absent or limited. The deletion of Notch1 was not compensated by up-regulation of other Notch receptors (Supplementary material online, Figure S7B). In addition, pressure overload induced Notch2 expression and activation but this induction was abrogated after the deletion of Notch1 (Supplementary material online, Figures S7B and C), indicating that activation and expression of Notch2 did not compensate for the lack of Notch1, and that Notch1 expression in cardiomyocytes is necessary for Notch2 activation and expression.

### Enhanced Notch activation favours the expansion of cardiogenic precursors and inhibits myofibroblast proliferation in the adult heart

The cell surface antigen Sca-1 has been used to identify CPCs in the heart. Sca-1-positive cells were found as individual cells or as cell clusters in interstitial spaces. Cells expressing Sca-1 were particularly present in the sub-epicardial region (Figure 6A). Under basal conditions, the number of Sca-1-positive cells was not different in WT and TGJ1 hearts (Figure 6B and C). Aortic constriction did not change the amount of Sca-1-positive cells in WT animals. In sharp contrast, TAC increased the number of Sca-1-positive cells, the number of Sca-1-positive cells per cluster, and the number of Sca-1-positive clusters in TGJ1 mice (Figure 6C). We next purified the mesenchymal fraction from adult heart, and demonstrated using cytofluorometry that these cells expressed Sca-1 (Figure 6D). After the induction of differentiation *in vitro*, cells up-regulated Nkx2.5 expression, and even differentiated into  $\alpha$ -actinin-positive cardiomyocytes. These data confirmed that these cells represent true cardiac progenitors. Interestingly, Sca-1-positive cells were also largely positive for N1IC (Figure 6E), suggesting that the Notch signalling pathway was activated in these subpopulation. We next determined whether Sca-1-positive cells expressed the early cardiac marker Nkx2.5 *in vivo*. Although Nkx2.5-positive cells can be found within clusters of Sca-1-positive cells, most of the Nkx2.5-positive cells were Sca-1-negative (Figure 6E). This finding is consistent with previous observation demonstrating a down-regulation of Sca-1 expression in cardiac committed precursors<sup>31</sup>.

To evaluate whether Jagged1 overexpression stimulated CPC production in the stressed heart, we quantified the number of proliferating  $\alpha$ -actinin-negative Nkx2.5-positive non-myocyte cells. WT and TGJ1 mice were subjected to TAC and given BrdU throughout the duration of the experiment. In sham-operated animals, the total number of BrdU-positive cells was higher in the TGJ1 mice compared with WT, and aortic constriction increased the number of BrdU-positive cells both in WT and TGJ1 hearts (Figure 7B). To identify proliferating cells, heart tissues were further analysed for

$\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression to detect myofibroblasts, and for Nkx2.5 expression to identify cardiogenic precursors and cardiomyocytes. In the WT mice, TAC induced massive proliferation of  $\alpha$ -SMA-positive myofibroblasts. In contrast, TAC induced no proliferation of  $\alpha$ -SMA-positive cells in the TGJ1 mice (Figure 7A and B). We next identified Nkx2.5 expressing cells (Figure 7C). Nkx2.5 expression was strong in the nuclei of cardiomyocytes (big arrows) but weaker in non-myocyte cells (small arrows). The BrdU-positive cells in the TGJ1 mice were essentially Nkx2.5-positive non-myocyte cells, indicating that transgene expression resulted in a preferential expansion of cardiac precursor cells (Figure 7C and D).

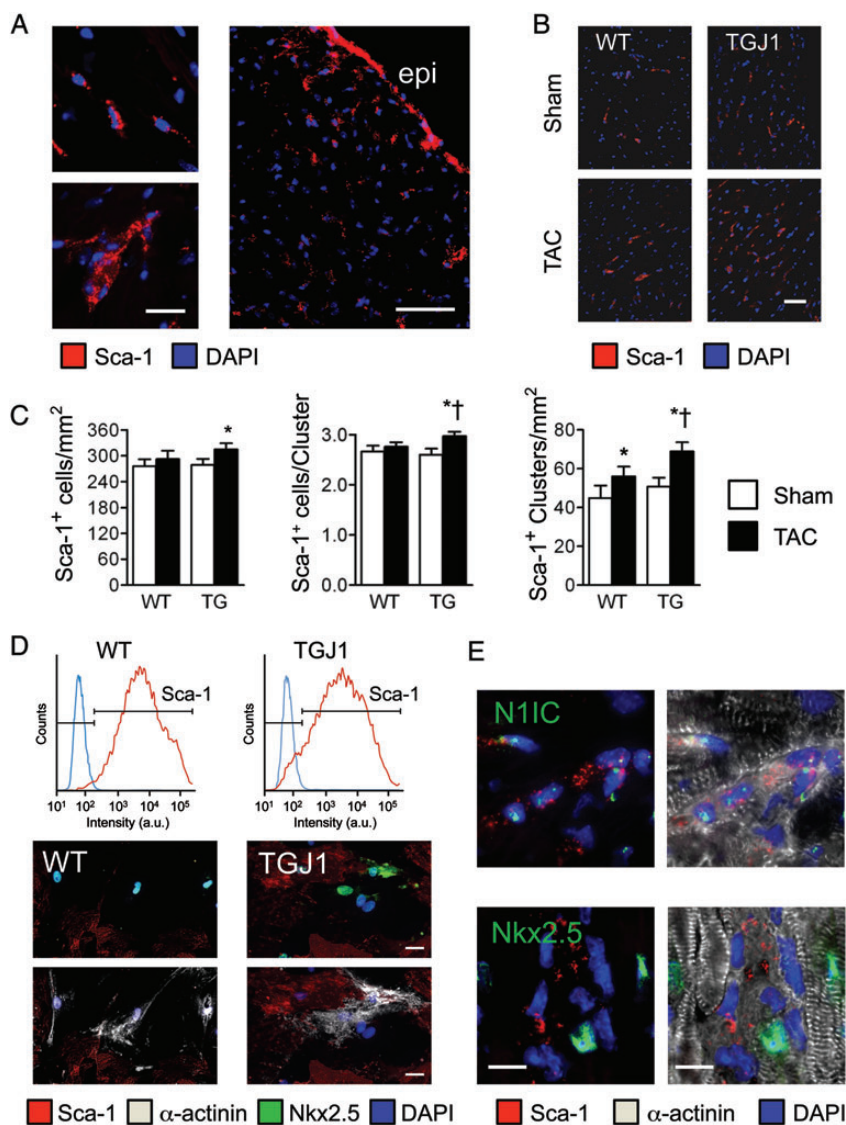
We next quantified Nkx2.5-positive myocytes and non-myocyte cells in the hearts of WT and TGJ1 mice (Figure 8). Nkx2.5-positive cardiac myocytes in transgenic hearts were characterized by a smaller size compared with WT, consistent with an immature phenotype (Figure 8A and B). Precursor cells were observed as clusters of Nkx2.5-positive non-myocyte cells in interstitial spaces. The total number of Nkx2.5-positive cells was increased in the TGJ1 mice, due to higher amounts of Nkx2.5-positive precursor cells (Figure 8Ca, c, and e). After TAC, the number of precursors in WT mice reached levels measured in TGJ1 animals (Figure 8Cc). The Nkx2.5-positive cardiomyocyte population was also larger in the transgenic mice after TAC than in WT (Figure 8Ce). We next identified cells with an activated Notch1 signalling pathway (N1IC-positive cells) (Figure 8Cb, d, and f). In the TGJ1 mice, the number of Nkx2.5-positive cardiomyocytes with an activated Notch1 pathway was increased under basal conditions and after TAC when compared with WT (Figure 8Cf). In contrast, Nkx2.5-positive CPCs in sham-operated TGJ1 were essentially N1IC-negative (Figure 8Cd).

## Discussion

New cardiomyocytes are produced in the adult heart after injury, and cardiac stem cells contribute to myocyte renewal.<sup>4</sup> However, the molecular mechanisms that could force the heart to adopt regeneration as a default pathway have not been identified. In the present study, we show that the Jagged1 expression on the surface of cardiomyocytes promotes the expansion of Nkx2.5-positive cardiac precursors and immature cardiomyocytes, which ultimately produce differentiated cardiomyocytes. In the adult heart, the amount of cells expressing Sca-1 (CPCs) is increased when Notch signalling is activated. A significant number of clusters containing Sca-1-positive cells contain also Nkx2.5-positive cells. However, Sca-1-positive cells are essentially Nkx2.5-negative. This is consistent with previous observation suggesting that cardiac committed CPCs down-regulate Sca-1 expression.<sup>31</sup> In addition, Notch activation, primarily via its actions on the fibroblast population, prevents pro-hypertrophic and pro-fibrotic factor production and limits cardiac hypertrophy and fibrosis in response to pressure overload. The effects of Notch on the cardiac microenvironment create favourable conditions for the production of cardiac precursors, and improve the capacity of the adult heart to respond to damage.

In WT animals, pressure overload induces cardiomyocyte hypertrophy. In contrast, TGJ1 mice, Notch activation limits myocyte

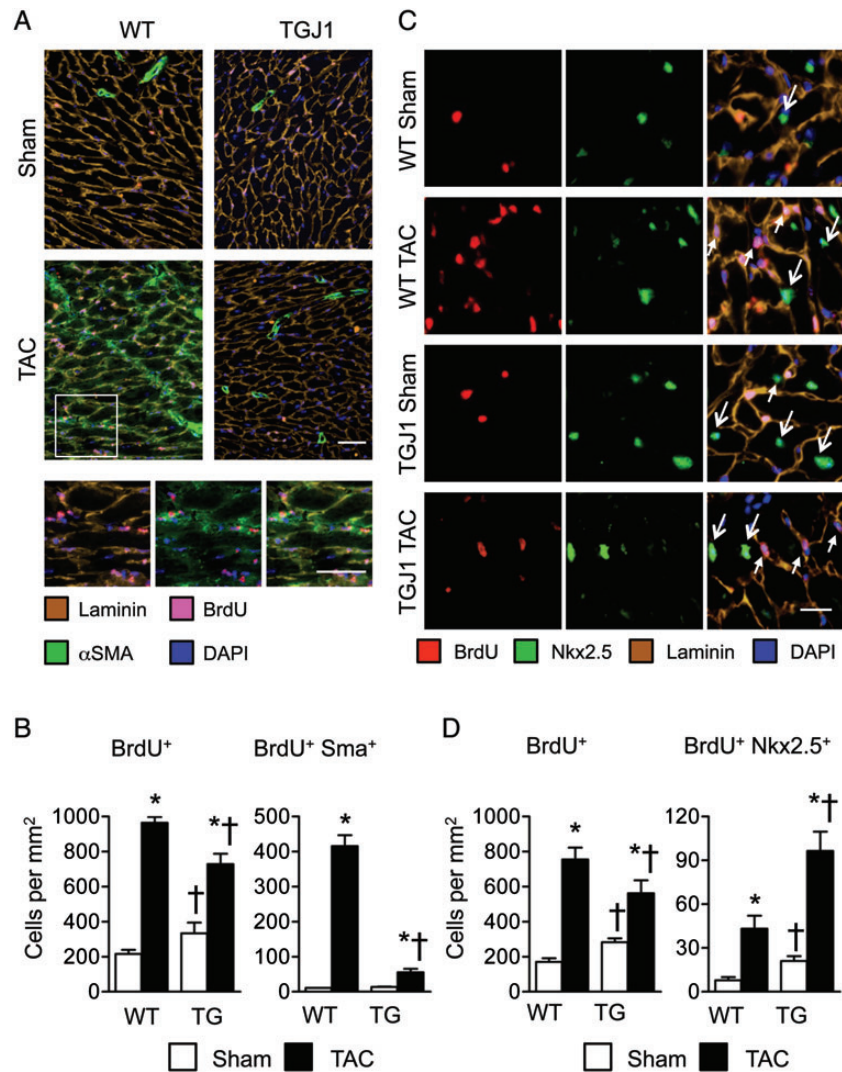




**Figure 6** Increased number of Sca-1-positive cells in the stressed TGJ1 heart. (A) Sca-1 expression (red) in TGJ1 heart sections identifying Sca-1-positive cells as single cells or multiple cell clusters; DAPI (blue). Epicardial region (epi). (B) Sca-1 expression (red) in wild-type and TGJ1 heart sections after transaortic constriction. (C) Quantification: total number of Sca-1-positive cells per mm<sup>2</sup> (left); number of Sca-1-positive cells per cluster (middle); number of Sca-1-positive clusters per mm<sup>2</sup> (right), in wild-type and TGJ1 mice after transaortic constriction. (mean  $\pm$  SEM; \* $P$  < 0.05 in transaortic constriction vs. Sham; † $P$  < 0.05 in TGJ1 vs. wild-type;  $n$  = 4–6 mice per group). (D) Upper panels: cytofluorometry analysis of Sca-1 expression in cardiac non-myocyte cells from adult wild-type and TGJ1 mouse hearts (blue: negative control antibody; red: anti-Sca-1 antibody); lower panels: cultured cardiac non-myocytes maintained in differentiation medium for 2 weeks. Cells were stained with antibodies against Sca-1 (red), Nkx2.5 (green), and  $\alpha$ -actinin (grey); DAPI (blue). (E) Upper panels: activated Notch1 in Sca-1-positive  $\alpha$ -actinin-negative cells (N1IC: green; Sca-1: red;  $\alpha$ -actinin: white; DAPI: blue); lower panels: Sca-1-positive  $\alpha$ -actinin-negative cells are Nkx2.5-negative (Nkx2.5: green; Sca-1: red;  $\alpha$ -actinin: white; DAPI: blue). Scale bar in (A) (left): 20  $\mu$ m; in (A) (right): 50  $\mu$ m; in (B): 50  $\mu$ m; in (D) 50  $\mu$ m, and in (E) 10  $\mu$ m.

hypertrophy in response to stress. This is consistent with previous data showing that the pharmacological or genetic blockade of Notch1 exacerbates the development of cardiac hypertrophy.<sup>20</sup> In the TGJ1 mice, Notch could suppress GATA-dependent gene transcription of sarcomeric proteins, possibly via Hes and Hey proteins.<sup>32</sup> Indeed, cardiac gene expression is lower in transgenic hearts when compared with WT. Nevertheless, this might reflect

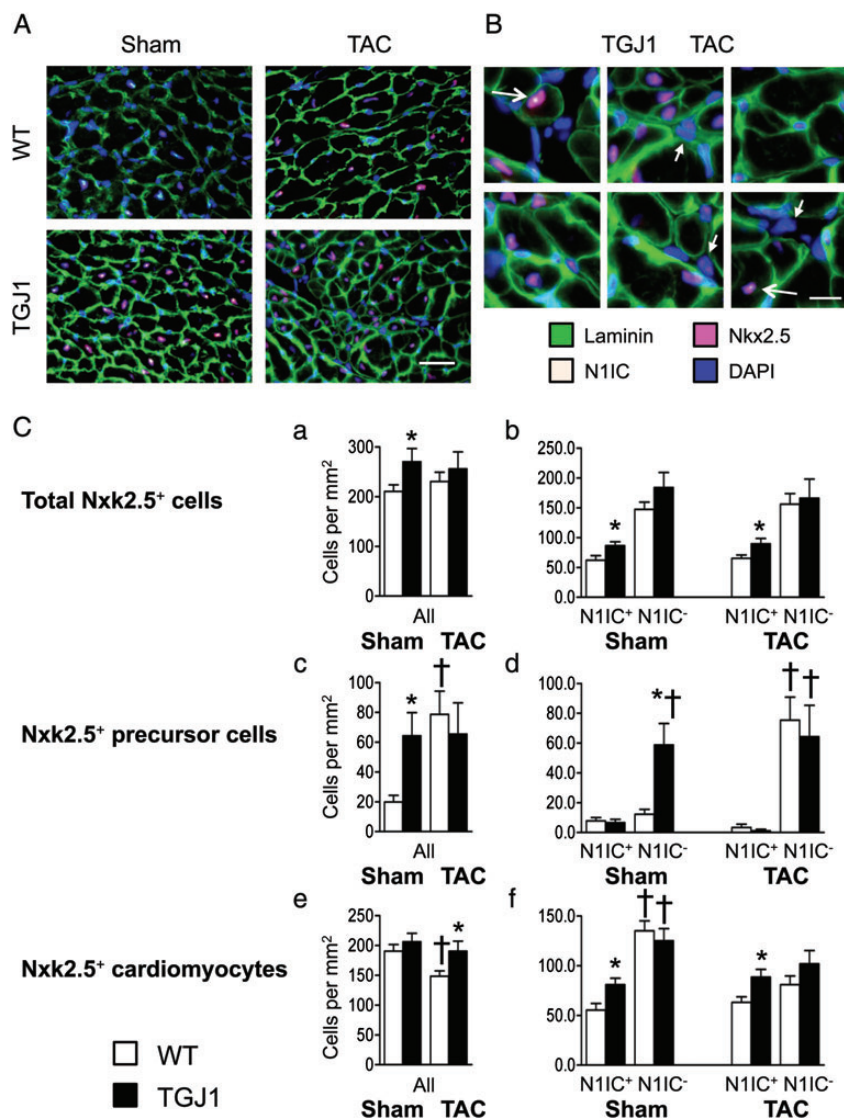
more an improved adaptation to stress than an actual cause for the absence of growth. The PI3K/Akt pathway is down-regulated in the heart of the TGJ1 mice. Increased activation of the Akt pathway can have detrimental and beneficial effects on heart function.<sup>33</sup> Sustained Akt signalling leads to hypertrophy and failure. On the other hand, activated Akt confers protection via the activation of survival pathways. Forced expression of N1IC or delivery of



**Figure 7** Jagged1-mediated cardiac precursor expansion. Mice were subjected to transaortic constriction and given BrdU for 2 weeks. (A)  $\alpha$ -smooth muscle actin expression (green) and BrdU incorporation (red) in wild-type and TGJ1 heart sections; DAPI (blue). (B) Quantification: numbers of BrdU-positive and BrdU- and  $\alpha$ -smooth muscle actin-positive cells in heart sections. (C) Nkx2.5 expression (green) and BrdU incorporation (red) in wild-type and TGJ1 heart sections; DAPI (blue). Large arrows indicate cardiomyocytes and small arrows indicate Nkx2-5-positive non-myocyte cells. (D) Quantification: numbers of BrdU-positive and BrdU- and Nkx2.5-positive cells in heart sections. Bar graphs represent mean  $\pm$  SEM (\* $P$  < 0.05 in transaortic constriction vs. Sham; † $P$  < 0.05 in TGJ1 vs. wild-type;  $n$  = 6 mice per group). Scale bars in (A) 50  $\mu$ m; in (C) 20  $\mu$ m.

Notch1 pseudoligand increase myocyte survival via Akt.<sup>21,22</sup> The fact that the number of apoptotic cells is similar in WT and TGJ1 mice indicates that the lack of Akt activation in TGJ1 hearts does not affect cardiomyocyte survival. Therefore, the transgenic heart must rely on different cellular mechanisms to improve its response to stress. Several reports have demonstrated a crucial role of Notch in stem cell expansion and survival.<sup>16,34</sup> Here, we show that transgenic hearts contain more cardiomyocytes and cardiac precursors, which, respectively, constitute additional functional reserve to cope with work overload, and a larger cardiac precursor pool with increased capacity to replenish lost cardiomyocytes.

Forced expression of N1IC in cardiomyocytes during development impairs cardiogenic differentiation and produces myocyte hyperplasia.<sup>22,35</sup> Notch activates also cell-cycle re-entry in immature cardiomyocytes.<sup>24,25</sup> Based on the myocyte size, persistent postnatal proliferation and a less organized cardiac tissue, neonatal TGJ1 hearts present a rather immature phenotype compared with WT. Hyperplasia occurs in both ventricles but is particularly pronounced on the right side, consistent with previous findings demonstrating a preferential role for Notch in cardiac structures derived from the secondary heart field.<sup>36</sup> In immature cardiomyocytes, the expression of components of the Notch pathway gradually declines after birth,<sup>25</sup> likely contributing to the progression of



**Figure 8** Jagged1-mediated Nkx2.5-positive cardiac myocyte and precursor expansion. (A) Nkx2.5 (pink) and laminin expression (green), and N1IC staining (white) in wild-type and TGJ1 heart sections; DAPI (blue). (B) High magnification photomicrographs of TGJ1 heart section after transaortic constriction. Large arrows indicate immature Nkx2.5- and N1IC-positive cardiomyocytes, and small arrows indicate Nkx2.5-positive non-myocyte cells. (C) Quantification: numbers of Nkx2.5-positive cells in heart sections. (a and b) Total numbers; (c and d), precursors, (e and f), cardiomyocytes. Bar graphs represent mean  $\pm$  SEM. mm<sup>2</sup> (\* $P < 0.05$  in TGJ1 vs. wild-type; † $P < 0.05$  in transaortic constriction vs. Sham;  $n = 4-6$  mice per group, five fields per heart). Scale bar in (A) 50  $\mu$ m; in (B) 20  $\mu$ m.

cardiomyocytes into a fully differentiated post-mitotic state. Likewise, embryonic and cardiac stem cells lacking RBP/Jk or Notch1 expression possess an increased capacity to differentiate into cardiomyocytes.<sup>20,37,38</sup> Therefore, by repressing cardiac gene expression and limiting the production of organized sarcomeres, Notch could maintain cardiomyocytes in an immature state that renders cytokinesis achievable.<sup>39</sup> In the TGJ1 mice, we observed increased PH3 and Ki67 staining and an up-regulation of cyclins B1 and D1 expression in the neonatal transgenic heart, indicating that cells advance into the G2/M phases. Cyclins and cyclin-dependent kinase inhibitors have been shown to be targets of Notch signaling.<sup>24</sup> Consistent with these findings, inhibition of Notch using

$\gamma$ -secretase inhibitor administration in neonatal mice results in a reduction of number of cardiomyocytes and dilated cardiomyopathy.<sup>19</sup> Therefore, the Notch pathway exerts combined actions in the myocyte lineage, which result in a sustained proliferation in the postnatal myocyte population.

The development of cardiac hypertrophy and fibrosis reflects cellular responses to autocrine and paracrine growth factors. Transforming growth factor- $\beta$  and CTGF, together with other factors such as Postn, regulate myocyte growth as well as myofibroblast proliferation and differentiation after injury.<sup>6,7,10</sup> A major finding in the current study is that TGF- $\beta$ 2, CTGF, and Postn are down-regulated by Jagged1-induced Notch signalling in the heart.

Cross-talk between Notch and TGF- $\beta$  pathways are crucial during the development of the heart, and has been described to regulate fibrosis in several organs in the adult.<sup>36,40</sup> In the TGJ1 mice, therefore, blunted factor expression prevents cardiomyocyte growth and fibroblast differentiation. Neutralization of TGF- $\beta$  appears to affect primarily interstitial non-myocyte cells, and suppresses fibrosis induced by pressure overload.<sup>10</sup> Interestingly, TGF- $\beta$ 2 enhances cardiogenic differentiation in embryonic stem cells.<sup>41</sup> Therefore, reduced TGF- $\beta$ 2 signalling might also contribute to prevent premature differentiation in cardiac precursors and favour expansion. Connective tissue growth factor has been shown to induce cardiomyocyte hypertrophy through the activation of the Akt pathway.<sup>42,43</sup> The down-regulation of CTGF expression and of the Akt pathway explains in part the absence of a hypertrophic response in the TGJ1 mice. Finally, cardiac Postn expression was also abolished in the TGJ1 mice, probably secondary to reduced TGF- $\beta$ 2 production.<sup>8</sup> Periostin does not affect the number of cardiac fibroblasts but enhances their profibrogenic capacity. Mice lacking Postn exhibit less cardiac fibrosis under pressure overload.<sup>12</sup> Periostin was claimed to induce myocyte cytokinesis but this finding has not been confirmed.<sup>44,45</sup> Since the TGJ1 mice demonstrate low Postn levels but still increased numbers of cardiac precursors and myocytes, a major role for Postn in stimulating proliferation in the cardiogenic lineage is not supported by our study.

Notch1 deletion specifically in cardiomyocytes does not alter the phenotype of the TGJ1 mice. Since increased Notch2 signalling is not observed in the stressed transgenic heart, it is unlikely that compensatory Notch2 signalling takes place in the myocyte population. Therefore, the cardiac mesenchymal fraction must represent the prime target of Notch1 signalling and a main integrator of stress during the response to injury. Resident cardiac fibroblasts originate from embryonic stromal cells. However, cardiac fibroblasts can also be generated from endothelial cells via endothelial-to-mesenchymal transition,<sup>46</sup> from the epicardium via epithelial-to-mesenchymal transition<sup>8</sup> or from the bone-marrow.<sup>47</sup> Likewise, cardiac stem cells share characteristics of mesenchymal stem cells (or mesenchymal stromal cells), and derive from similar cellular compartments in the heart.<sup>23,31,48–50</sup> Generation of either fibroblasts or cardiac precursors could, therefore, be regulated by common mechanisms. Depending on the microenvironment, reactivation of developmental pathways as well as autocrine and paracrine factors modulate the response towards resident cardiac precursor mobilization or fibroblast production.<sup>9,14</sup> Recently, a Notch-activated epicardium-derived cell population was shown to contribute to tissue repair in the heart.<sup>23</sup> These cells have characteristics of mesenchymal stem cells, and demonstrate fibrogenic and cardiogenic potential. Upon cardiac injury, fibrosis genes are up-regulated and muscle genes down-regulated, suggesting again that fibrogenesis represents a default pathway in the damaged myocardium. Along these lines, CTGF was recently shown to induce differentiation of multipotent mesenchymal stem cells into  $\alpha$ -SMA-positive myofibroblasts.<sup>51</sup> However, our results demonstrate that, in the absence of additional profibrotic cues such as TGF- $\beta$ , CTGF, and Postn signalling, Notch-activated cells preferentially contribute to the cardiogenic lineage. Interestingly, down-regulation of Notch signalling in

cardiac fibroblasts is necessary for their transformation into  $\alpha$ -SMA-positive myofibroblasts.<sup>52</sup> We conclude, therefore, that communication between Jagged1-expressing cardiomyocytes and Notch-expressing mesenchymal cells is crucial to shift the response towards cardiac precursor expansion. It remains that cross-talk between myocytes and non-myocyte cells is bidirectional. For example, Jagged1 on the surface of mesenchymal stem cells activates Notch in adjacent cardiomyocytes, and, together with secreted factors, promotes the proliferation of immature myocytes.<sup>53</sup> These additional signalling events could compensate for any disrupted communication in cardiomyocyte-specific Jagged1 knockouts.

In summary, we propose that Notch is pivotal in a complex network of interactions between cardiomyocytes and mesenchymal cells. Through regulating fibrogenesis and cardiogenesis, Notch is able to switch the cardiac repair mechanisms from a profibrotic default pathway to a pro-cardiogenic pathway. Altogether, our results suggest that the Notch pathway represents a unique therapeutic target that can be manipulated to improve the cardiac response to stress and regenerate the damaged myocardium via mobilization of cardiac precursor cells.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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