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Gap-134, a Connexin43 activator, prevents age-related development of ventricular fibrosis in *Scn5a*^{+/-} mice



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ABSTRACT

Down-regulation of Connexin43 (Cx43) has often been associated with the development of cardiac fibrosis. We showed previously that *Scn5a* heterozygous knockout mice (*Scn5a*^{+/-}), which mimic familial progressive cardiac conduction defect, exhibit an age-dependent decrease of Cx43 expression and phosphorylation concomitantly with activation of TGF- β pathway and fibrosis development in the myocardium between 45 and 60 weeks of age. The aim of this study was to investigate whether Gap-134 prevents Cx43 down-regulation with age and fibrosis development in *Scn5a*^{+/-} mice.

We observed in 60-week-old *Scn5a*^{+/-} mouse heart a Cx43 expression and localization remodeling correlated with fibrosis. Chronic administration of a potent and selective gap junction modifier, Gap-134 (danegaptide), between 45 and 60 weeks, increased Cx43 expression and phosphorylation on serine 368 and prevented Cx43 delocalization. Furthermore, we found that Gap-134 prevented fibrosis despite the persistence of the conduction defects and the TGF- β canonical pathway activation.

In conclusion, the present study demonstrates that the age-dependent decrease of Cx43 expression is involved in the ventricular fibrotic process occurring in *Scn5a*^{+/-} mice. Finally, our study suggests that gap junction modifier, such as Gap-134, could be an effective anti-fibrotic agent in the context of age-dependent fibrosis in progressive cardiac conduction disease.

1. Introduction

Chronic fibroproliferative diseases have been proposed to contribute to nearly 45% of the mortality in developed countries [1]. Among those, many cardiovascular diseases are associated with the development of excessive cardiac fibrosis, which contributes to their pathophysiological mechanisms [2]. This is most likely related to the crucial role played by the extracellular matrix in cardiac structure and function. However, despite its significant impact in cardiac diseases, fibrosis has become a focus for therapeutic development only recently [2]. There is thus a need for identifying novel therapeutic targets.

In the past, we have shown that the ~50% reduction of Na_v1.5 expression in *Scn5a* heterozygous knockout (*Scn5a*^{+/-}) mice, which exhibit conduction defects, is associated with Cx43 expression

remodeling and activation of the TGF- β canonical pathway leading to extensive ventricular fibrosis during ageing [3–5]. In control senescent mice, cardiac interstitial fibrosis onset is also concomitant with Na_v1.5 and Cx43 down-regulation and cellular localization remodeling in ventricles [6]. Furthermore, in a heart failure mouse model, structural remodeling was shown to follow electrical remodeling characterized by a ~50% decrease of Na_v1.5 protein expression and a ~70% reduction of Cx43 protein expression and phosphorylation [7]. A reduction of Cx43 and Na_v1.5 expression has also been shown to precede fibrosis in a mouse model of cardiac hypertrophy [8,9]. Finally, based on studies performed with young hypertrophic or senescent conditional heterozygous Cx43 knockout (Cx43^{Cre-ER(T)/fl}) mice, Jansen and co-workers proposed that a reduction of Cx43 expression might be the trigger for fibrosis development [10]. However, this might be more complex.

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Indeed, in a recent article, Valls-Lacalle and co-workers have shown that the enhanced fibrotic response seen after angiotensin II treatment in the same model of Cx43^{Cre-ERT}/fl mice, expressing 50% of normal Cx43 content, is independent on this reduction in Cx43 expression, as it was not apparent on Cx43^{+/-} mice [11]. On the contrary, a further reduction in Cx43 levels after 4-hydroxytamoxifen administration was associated with attenuation in collagen deposition in the angiotensin II-treated group. Similarly, permanent coronary artery ligation has been associated with a reduction in scar area in Cx43^{+/-} mice [12]. These studies highlight the need to take into account all the changes expression and roles of Cx43 (hemichannel, phosphorylation, localization etc.), which can differently participate to fibrosis depending on the context.

Recently, we have shown that young *Scn5a*^{+/-} mice are characterized by a higher Cx43 expression and phosphorylation than wild-type (WT) mice while, after the age of 45 weeks, Cx43 expression and phosphorylation decrease concomitantly with the activation of the TGF- β pathway and fibrosis in both WT and *Scn5a*^{+/-} mice, although the decrease is larger in *Scn5a*^{+/-} mice [3]. These results suggest that connexin 43 downregulation might play a key role in cardiac fibrosis development in this model.

In this context, the aim of this study was to determine whether preventing Cx43 down-regulation with age prevents fibrosis development in *Scn5a*^{+/-} mice. For this purpose, we investigated the effects of a chronic treatment with a gap-junction activator, Gap-134, on fibrosis development in *Scn5a*^{+/-} mice between 45 and 60 weeks of age. Gap-134, also known as (2*S*, 4*R*)-1-(2-aminoacetyl)-4-benzamidopyrrolidine-2-carboxylic acid, Danegaptide, or ZP1609, is a dipeptide analog of the antiarrhythmic hexapeptide, Rotigaptide. Gap-134 was shown to increase Cx43 coupling [13], prevents dephosphorylation of Cx43 [14] and prevent conduction abnormalities in models of cardiac disease [15,16].

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in the animal facility of Nantes University Health Research Institute (*Unité de Thérapeutique Expérimentale*) which has been accredited by the French Ministry of Agriculture. The animal experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH publication No. 86-23, revised 1985) and to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. They were approved by the regional ethic committee [CEEA-Pays de la Loire; approvals CEEA-2009-21 (10 January 2010) and 01630-01(14 May 2014)].

2.2. Experimental groups & in vivo treatment with Gap-134 and GW788388

Wild-type (WT) and *Scn5a*^{+/-} mice (Supplementary Fig. 1) used in the present study were littermates (129/Sv genetic background). They were genotyped by polymerase chain reaction as previously described [17]. At the age of 45 weeks, *Scn5a*^{+/-} mice (male and female) were randomly assigned to the Gap-134- or the solvent (DMSO)-treated groups. They were chronically treated orally (drinking water) with Gap-134 at 5 mg/kg/day, according to the study of Butera and collaborators [18], or with solvent (DMSO), from the age of 45 weeks to the age of 60 weeks, corresponding to the period of fibrosis development [3]. Gap-134 was synthesized by CHEM-Symbiose core facility and dissolved in DMSO. The maximum concentration of DMSO in drinking water was 0.02% in both Gap-134- and DMSO-treated groups. For GW788388, 45-week-old *Scn5a*^{+/-} mice were treated chronically until the age of 60 weeks at a dose of 5 mg/kg/day (in drinking water), as previously described [3]. GW788388 was dissolved in DMSO. The maximum

concentration of DMSO in drinking water was 0.2% in both GW788388- and placebo-treated groups.

2.3. Electrocardiography

Mice were anaesthetized for ECG recording with etomidate (25 mg/kg i.p.). Body temperature was maintained at 37 °C with a heating pad (Harvard Apparatus, USA). Six-lead ECG was recorded with 25-gauge subcutaneous electrodes on a computer through an analog-digital converter (IOX 1.585, EMKA Technologies) for monitoring and off-line analysis (ECG Auto v3.2.0.2, EMKA Technologies). ECG parameters were analyzed on lead I as previously described [4].

2.4. Morphological and histological analyses

Sixty-week-old mice were euthanized by cervical dislocation and lungs, liver, kidneys, and heart were isolated. After excision, the organs were rinsed in phosphate buffered saline and weighted. The hearts were rinsed in saline solution, fixed in 4% paraformaldehyde, embedded in paraffin, and transverse sectioned at 7- μ m intervals. Section were stained with picosirius red as previously described [3]. Sections were mounted in QPath Coverquick 3000 (Labonord SAS, France) and examined with a phase contrast or polarized (to distinguish collagen I from collagen III) light microscope (Nikon Eclipse E-600 microscope with NIS-Elements BR v4.10 Software, Nikon, Japan). Quantitative assessment of fibrosis was determined based upon the extent of patchy and interstitial fibrosis. For each heart, 2 regions (middle and base) were observed and at least 20 pictures were obtained in each region. Automatic analysis (Threshold: Otsu, 65-255; Analyze particles: 0-infinity) was performed using ImageJ software 1.45b (NIH Software).

2.5. Immunohistochemistry

Sections of hearts were permeabilized (1% TritonX-100) and blocked using 0.1% bovine serum albumin (BSA) for 1 min and 1% BSA for 2 h. Slices were incubated with primary antibody targeted against Cx43 (Sigma Aldrich, Ref 6219; 1:250) overnight. Then, slices were rinsed in 0.025% Triton X-100 and incubated with the ad-hoc secondary horseradish fluorochrome antibody (Alexa Fluor[®]568; Ref A11061; 1:1000). After a wash in 0.025% TritonX-100 twice, slices were mounted with ProLong[®] Gold (P36934, Life Technologies). Slices were examined with a fluorescence microscope (Nikon Eclipse E-600 microscope with NIS-Elements BR v4.10 Software, Nikon, Japan). Analysis was performed in subendocardial and subepicardial zones of left ventricular sections using ImageJ software 1.45b (NIH Software).

2.6. Ventricular fibroblasts isolation and culture

Ventricular fibroblasts were isolated from 45-week-old wild-type or *Scn5a*^{+/-} mice, without treatment, by using the classical Langendorff technique. Ten minutes after being heparinized, mice were sacrificed and their heart quickly excised and dropped in a cold perfusion solution containing (in mM): NaCl, 120; KCl, 5.4; MgSO₄, 2.45; NaH₂PO₄, 1.2; HEPES, 1.2; glucose, 5.6; 2,3-butanedione 2-monoxime (BDM), 10; taurine, 5; pH 7.4 with NaOH. After cannulation of the aorta, the heart was perfused for 1 min in a Langendorff system (37 °C) with the same solution supplemented with 1 mM of CaCl₂, followed by 5 min of perfusion with a solution without CaCl₂. Then, the heart was perfused with a low-CaCl₂ solution (12.5 μ M) containing 28 U of type 14 protease (Sigma[®] P5147) and 8500 U of type 2 collagenase (Worthington[®] LS00477) for 12–16 min. Then the digested heart was cut below the atria, and the ventricles were recovered in the digestion solution and gently triturated. Then a "stop" solution [perfusion solution with 10% of fetal calf serum (FCS) and 12.5 μ M of CaCl₂] was added to digested heart and cardiomyocytes sedimented during 10 min and the supernatant with cardiac fibroblasts was collected in a 50 mL conical tube

and centrifuged at $500 \times g$ for 10 min at room temperature. After centrifugation, the supernatant was collected and re-centrifuged at $1000 \times g$ for 10 min. The two cell pellets were re-suspended together in 50 mL of DMEM medium (Dulbecco's modified Eagle's medium with 4.5 g/L of D-glucose, 4 mM of L-glutamin, and 1 mM of pyruvate; Gibco® Ref 41966-094) supplemented with 10% of FCS, 1% of antibiotics (Penicillin, Streptomycin) and 2 mM of glutamin. Then 10 mL of the cell suspension were seeded in 100-mm plastic cell culture dishes (Nunc®, P7741) and incubated at 37 °C with 5% CO₂. After 24 h, the cell cultures were washed with 1 × phosphate-buffered saline (PBS Gibco® Ref 14190-094) and fresh supplemented DMEM medium was added. Non-adherent myocytes were removed by changing the culture medium. Every two days, cultures were rinsed once with PBS and fresh medium was added.

2.7. Triton solubility and western-blot

Protein samples were prepared from the left ventricle free walls and from ventricular fibroblasts at 8 days of culture. After euthanasia, the hearts were quickly removed and tissues were snap-frozen in liquid nitrogen. Frozen samples and whole cell lysates were homogenized in ice-cold 1% Triton X-100 buffer (in mM: NaCl, 100; Tris-HCl, 50; EGTA, 1; Na₃VO₄, 1; NaF, 50; phenylmethylsulfonyl fluoride, 1 (Roche Applied Science); and protease inhibitor mixture (Sigma P8640; 1:100); pH 7.4). Samples were sonicated, and centrifuged at $15,000 \times g$ for 15 min at 4 °C. The supernatant generated the Triton-soluble fraction (free non-junctional Cx43). The pellet resulting from the centrifugation was mixed with 20% sodium dodecyl sulfate (SDS) buffer (in mM: Tris-HCl, 200; EDTA, 19.8; pH 6.8) to generate the Triton-insoluble fraction (aggregated junctional Cx43) [19]. Protein concentration of both fractions were determined using the Pierce™ BCA Protein Assay Kit. For cell lysates, only the supernatant was used. Samples were prepared with 40 µg of total protein, 2 µL of NuPAGE Sample Reducing Agent (Invitrogen), 5 µL of NuPAGE LDS Sample Buffer (Invitrogen) and boiled during 5 min. Samples were run on 10% or 4–15% Mini-PROTEAN® TGX Stain-Free™ Precast Gels (Bio-rad) and transferred on Trans-Blot® Turbo™ Nitrocellulose Transfer Packs (Bio-rad). Membranes were blocked (using 5% non-fat milk) and incubated with primary antibodies targeted against Cx43 (Sigma Aldrich Ref 6219; 1: 10,000), NP-Cx43 (Invitrogen Ref 138300; 1:500), P-Cx43 Ser368 (Cell Signaling Ref 35115; 1:1000), Na_v1.5 (Cell Signaling Ref 14421S; 1:1000), TGF-β (Cell Signaling Ref 3711S; 1:500), Smad-2 (Millipore Ref 07-408; 1:1000), P-Smad-2 (Cell Signaling Ref 8828S; 1:1000), CTGF (Santa Cruz Ref 1439 ; 1: 500), α-SMA (Sigma-Aldrich Ref A5228; 1:1000), Periostin (Abcam Ref ab14041; 1: 500), Collagen Type I (Cell Signaling Ref 84336; 1:500), MMP-2 (Santa Cruz Ref sc-13595; 1:1000), MMP-9 (Santa Cruz Ref sc-10737; 1:1000), TIMP-1 (Santa Cruz Ref sc-5538; 1:250), TIMP-2 (Santa Cruz Ref sc-5539; 1:250), TIMP-3 (Santa Cruz Ref sc-30075; 1:250), TIMP-4 (Santa Cruz Ref sc-9375; 1:250) or ZO-1 (Invitrogen Ref 339100; 1:500). Then, membranes were incubated with the ad-hoc secondary horseradish peroxidase antibody (sc-2055, sc-2054 and sc-2922 Santa Cruz; 1:5000). Incubation was followed by detection using chemiluminescence (Clarity™ Western ECL Substrate, ChemiDoc™ MP System Bio-rad). Each Western-blot was made in duplicate and quantification was performed with Image Lab™ Software (Bio-rad) and normalized to total protein amount (stain-free method) in the corresponding lane. All original western blots from which Figs. 2, 3, 5 and supplementary Figs. 1–4 are shown in supplementary Figs. 5–11.

2.8. Impedance-based xCELLigence proliferation assay

The xCELLigence system (impedance-based real time cell analyzer - RTCA Roche, ACEA Biosciences Inc.™) was used to study in real time cardiac fibroblast proliferation [20], according to the manufacturer's instructions. At 5 days of culture, cardiac fibroblasts were washed with PBS 1X (without calcium and magnesium) and isolated with 2.5% of

trypsin-EDTA during 5 min at 37 °C. One milliliter of culture medium was added to stop the trypsin action and recover the fibroblasts. Then, cardiac fibroblasts number was quantified with Malassez cell. For xCELLigence proliferation assay, the background impedance of culture medium in a E-plate View (ACEA Biosciences Inc.™ - 16 wells or 96 wells) was measured first with 100 µL of culture medium added to each well. Then fibroblasts were seeded into the E-plate at a density of 5000 cells/well. The plate was set on the RTCA station, in an incubator at 37 °C (5% CO₂). Proliferation was monitored every 15 min for up to 70 h by the RTCA Analyzer, and culture medium was changed every day during the time of the experiment. Data were analyzed with the RTCA Software 1.2.1. and proliferation was quantified between 20 and 25 h.

2.9. In vitro treatments of cardiac fibroblasts with GW788388 and Gap-134

For xCELLigence assays, fibroblasts were chronically treated with either GW788388 (20 µM) diluted in DMSO (final concentration of 0.0003% [21]) from the beginning of the xCELLigence experiment (day 5 of culture) or Gap-134 (1 µM) diluted in DMSO (final concentration of 0.0003% [22]) from the beginning of cell culture (5 days before seeding in e-plates). Fibroblasts treated with, respectively, 0.0003% and 0.0003% of DMSO were used as controls. Each condition was performed in duplicate. For Western-blot, cardiac fibroblasts were chronically treated with Gap-134 from the beginning of cell culture until day 8, when the proteins were isolated.

2.10. Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with Prism6 (GraphPad Software, Inc., USA). Statistical significance was determined with Student paired t-test, Mann-Whitney rank sum test and one- or two-way analysis of variance with a Bonferroni post-hoc test for multiple comparisons, when appropriate. A value of $p \leq 0.05$ was considered significant.

3. Results

3.1. Fibrosis development is associated with Cx43 remodeling

We have previously shown that in *Scn5a*^{+/-} mice, the decrease in Cx43 expression and phosphorylation at the age of 45 weeks, after an initial up-regulation up to 30 weeks of age, precedes fibrosis development between 45 and 60 weeks of age [3]. Present results confirm the presence of extensive ventricular fibrosis in 60-week-old *Scn5a*^{+/-} mice when compared to WT mice (Fig. 1a). Since fibrosis expands mostly in subendocardium (Fig. 1a), we investigated Cx43 localization and distribution in subendocardium and subepicardium of the left ventricle. As shown in Fig. 1b, 60-week-old WT mice have a higher Cx43 density in subepicardium than in subendocardium. This expression gradient differs from that observed in young WT mice in which Cx43 expression is larger in subendocardium than in subepicardium (Supplementary Fig. 2), as previously shown [23]. *Scn5a*^{+/-} showed the same expression pattern (Fig. 1c). However, when WT and *Scn5a*^{+/-} hearts are compared, Cx43 density is lower in both regions in the transgenic animals (Fig. 1d). We also observed that *Scn5a*^{+/-} hearts had smaller Cx43 plaques than WT mice in both areas. However, there was no difference in Cx43 plaques number between *Scn5a*^{+/-} and WT mice (Fig. 1d). As zonula occludens-1 (ZO-1) regulates the organization of gap-junction plaques, we also investigated Cx43-ZO-1 interaction. Cx43-ZO-1 interaction was almost doubled in *Scn5a*^{+/-} mice (Supplementary Fig. 3). Since plaques are preferentially localized in the intercalated discs, these results are in favour of a disorganization of the Cx43 at the intercalated disc. In addition, fibrosis development seemed mostly located where Cx43 expression was highly decreased in *Scn5a*^{+/-} mice.

In order to evaluate the hypothesis that inhibiting the age-

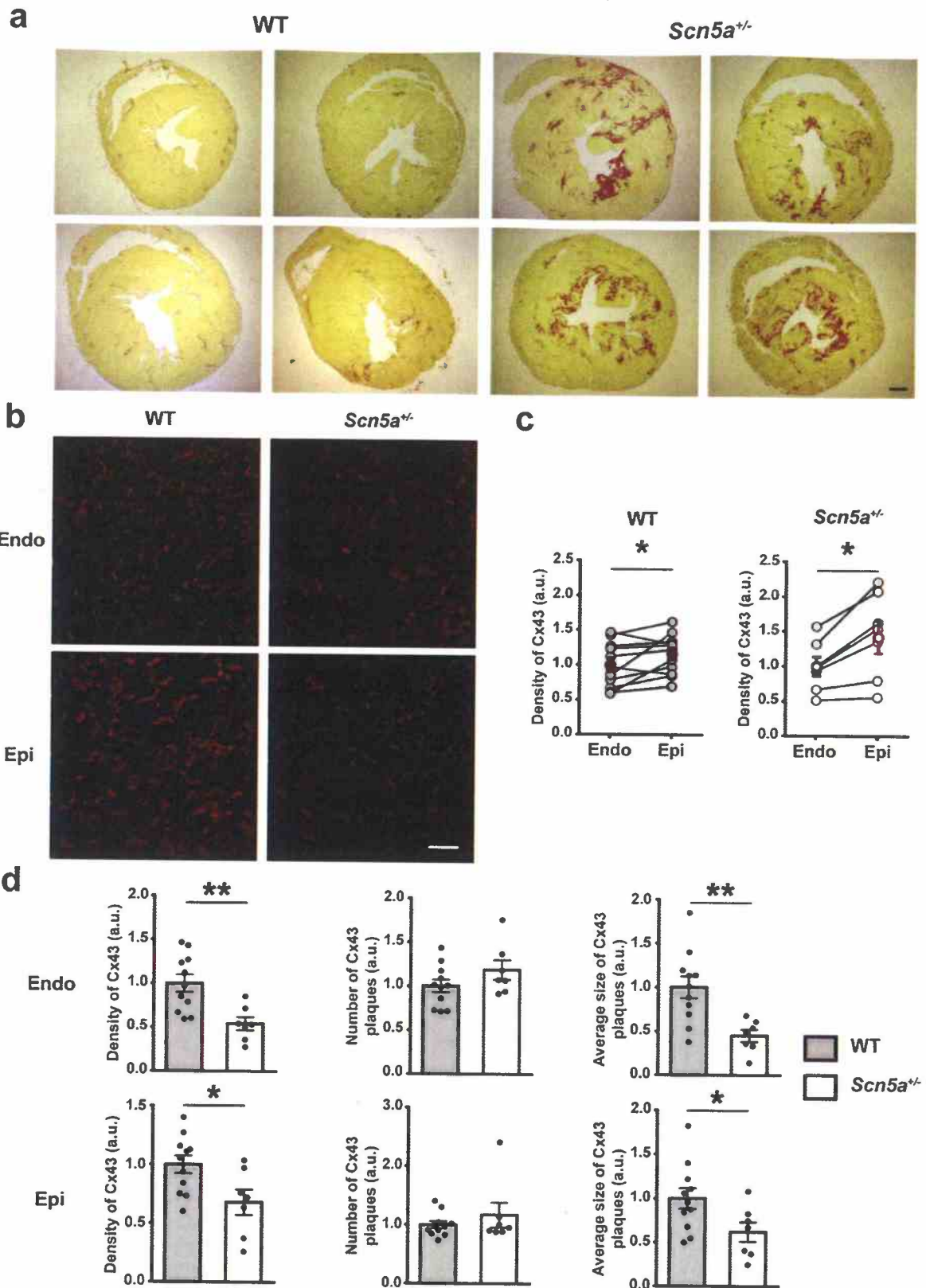


Fig. 1. Fibrosis and alteration of connexin43 (Cx43) localization in 60-week-old *Scn5a*^{+/-} mice.

(a) Representative transverse heart sections (left panels; scale bar: 500 μ m) stained with picosirius red from 60 week-old wild-type (WT) and *Scn5a*^{+/-} mice. (b) Representative immunostainings of Cx43 in subendocardial (Endo) and subepicardial (Epi) zones of left ventricular sections from 60-week-old WT or *Scn5a*^{+/-} mice (scale bar: 50 μ m). (c) Cx43 density in subendocardial (Endo) and subepicardial (Epi) zones in WT and *Scn5a*^{+/-} mice ($n = 10$ and $n = 7$ for WT and *Scn5a*^{+/-} mice, respectively) Student paired *t*-test; * $P \leq 0.05$. (d) Cx43 density, number and average size of Cx43 plaques in *Scn5a*^{+/-} mice compared to WT mice ($n = 10$ and $n = 7$ for WT and *Scn5a*^{+/-} mice, respectively). Mann-Whitney rank sum test; * $P \leq 0.05$ and ** $P \leq 0.01$.

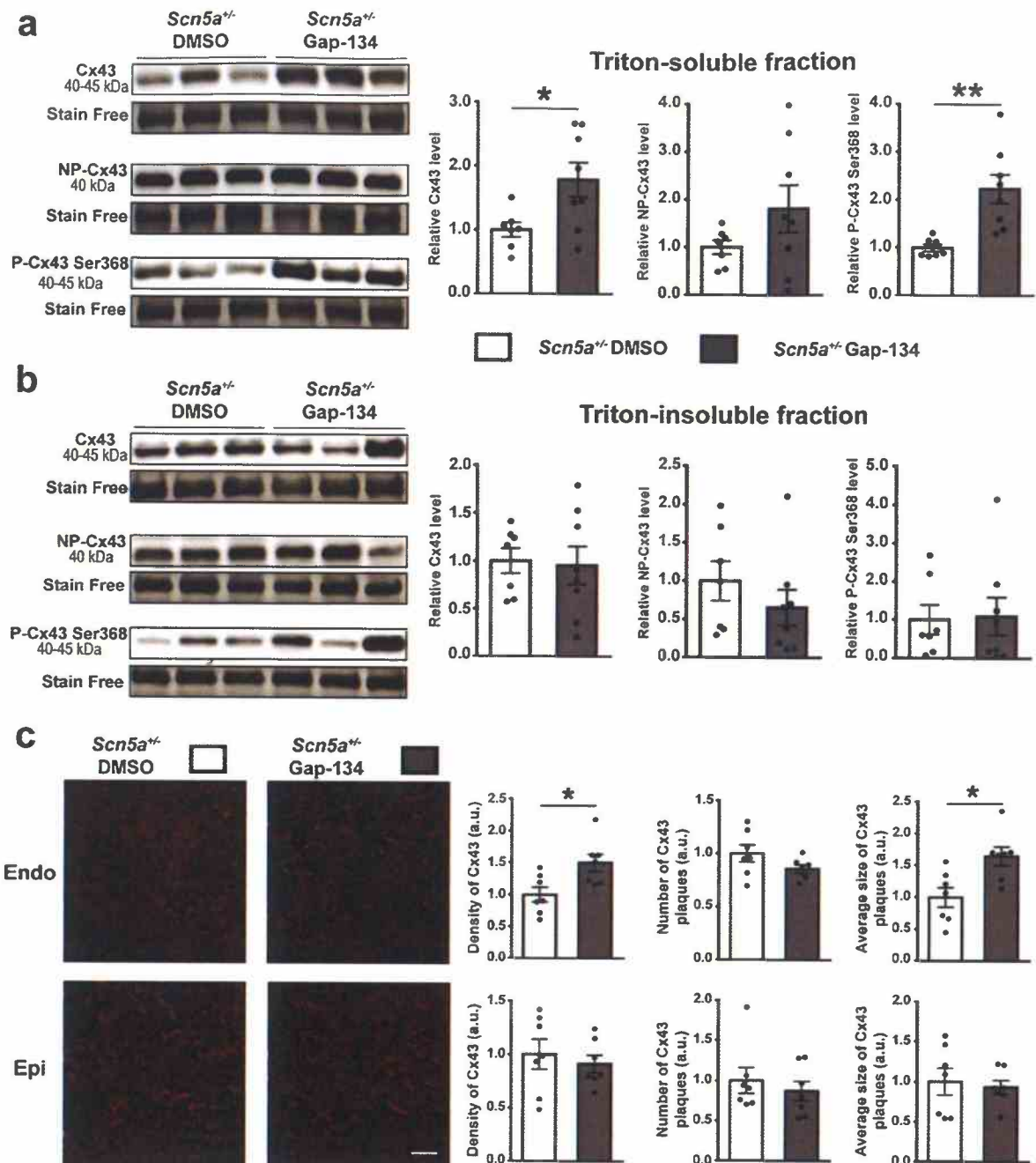


Fig. 2. Effect of chronic treatment with Gap-134 on ventricular expression, phosphorylation and localization of connexin 43 (Cx43) in *Scn5a*^{+/-} mice. (a, b) Representative western blots and Cx43, non-phosphorylated Cx43 (NP-Cx43) or phosphorylated Cx43 on Ser368 (P-Cx43 Ser368) average density relative to corresponding total lane densities from the stain-free blot in Triton-soluble (a) and Triton-insoluble (b) fraction of left ventricle from 60-week-old *Scn5a*^{+/-} mice chronically treated orally with 0.02% DMSO or Gap-134 (5 mg/kg/day) from the age of 45 weeks to the age of 60 weeks. Average values in DMSO-treated group are arbitrarily set at 1 ($n = 7$ and $n = 8$ for DMSO- and Gap-134-treated groups, respectively). (c) Representative immunostainings of Cx43 in subendocardial (Endo) and subepicardial (Epi) zones of left ventricle sections from 60-week-old DMSO- or Gap-134-treated *Scn5a*^{+/-} mice (scale bar: 50 μ m) and mean Cx43 density, Cx43 plaques number and average plaque size ($n = 7$ per group). Mann-Whitney rank sum test * $P \leq 0.05$.

dependent decrease of Cx43 expression prevents fibrosis development in *Scn5a*^{+/-} mice, we chronically treated *Scn5a*^{+/-} mice with Gap-134, an activator of Cx43 expression and function, at the age of 45 weeks, *i.e.*, when Cx43 expression is similar to the level of WT mice and fibrosis absent.

3.2. Gap-134 treatment prevents the decrease of Cx43 expression and increases phosphorylation on Ser368

We observed no change in body and organ weight or morphological index after a treatment for 15 weeks with Gap-134 (Supplementary Table 1). We investigated the Gap-134 effect on Cx43 expression and phosphorylation in Triton-soluble and -insoluble cardiac fractions. It is admitted that the soluble fraction corresponds to un-aggregated

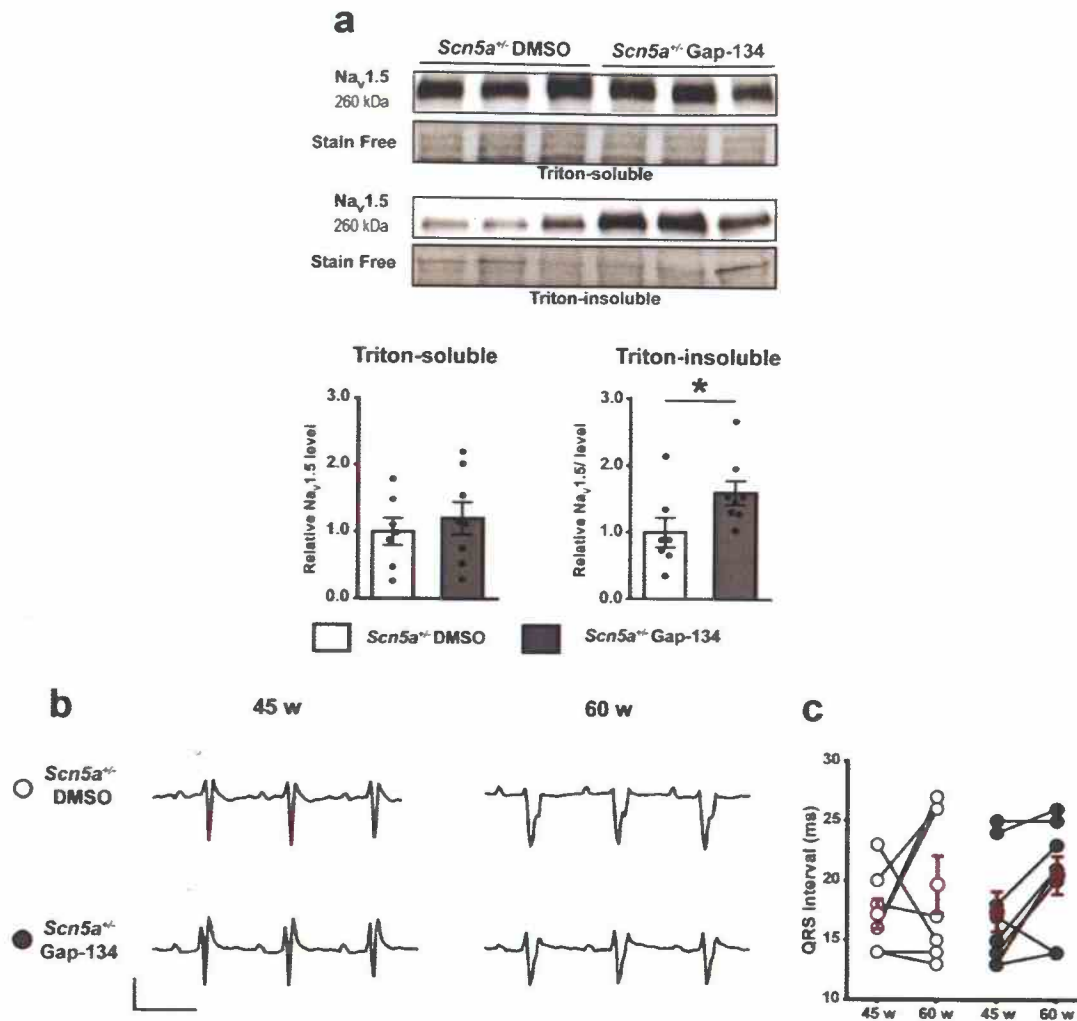


Fig. 3. Cardiac conduction after 15 weeks of treatment with Gap-134 in *Scn5a*^{+/-} mice.

(a) Representative western blots and Na_v1.5 density relative to corresponding total lane density from the stain-free blot in Triton-soluble and Triton-insoluble fractions of left ventricle from in 60-week-old *Scn5a*^{+/-} mice chronically treated orally with 0.02% DMSO or Gap-134 (5 mg/kg/day) from the age of 45 weeks to the age of 60 weeks. Average values in DMSO-treated group are arbitrarily set at 1 ($n = 7$ and $n = 8$ for DMSO- and Gap-134-treated group, respectively). Mann-Whitney rank sum test; * $P \leq 0.05$. (b) Representative lead-I ECGs of *Scn5a*^{+/-} mice before (45-week-old) and after (60-week-old) DMSO- or Gap-134-treatment. Scales bars: 0.5 mV and 100 ms. (c) QRS complex duration in 45 and 60-week-old DMSO- (open circles) or Gap-134- (filled circles) treated *Scn5a*^{+/-} mice ($n = 7$ per group). Two-way ANOVA with a Bonferroni post-hoc test.

proteins (free Cx43) in cell membranes [24,25]. As shown in Fig. 2a, the free-Cx43 fraction density was almost doubled after 15 weeks of treatment. The density of phosphorylated free Cx43 on Ser368 was also highly increased whereas non-phosphorylated (NP) free-Cx43 density was not significantly changed. The Triton-insoluble fraction is supposed to be enriched with Cx43 junctional plaques of the plasma membrane [25,26]. Unlike the free fraction, density and phosphorylation of aggregated Cx43 did not change (Fig. 2b). Interestingly, the increase of free Cx43 was already observed after only 3 weeks of treatment without any change of Ser368 phosphorylation (Supplementary Figs. 4 and 5). Finally, we investigated the Cx43 distribution in subendocardial and subepicardial areas of DMSO- or Gap-134-treated *Scn5a*^{+/-} hearts. As shown in Fig. 2c, Gap-134 treatment increased the Cx43 density and the plaques average size but did not change the number of plaques in the subendocardium. On the other hand, Gap-134 treatment had no effect in the subepicardium. Altogether, these results suggest that Gap-134 treatment significantly increased total Cx43 expression, preferentially as free proteins, with a major increase of the phosphorylated un-aggregated Cx43 form and, possibly, a normalization of Cx43 plaques in the subendocardium only.

3.3. Gap-134 modifies Na_v1.5 localization

As Cx43 plaques and Na_v1.5 interact in the perinexus area of the intercalated discs [27], we also investigated Gap-134 effects on Na_v1.5 in both Triton-soluble and -insoluble fractions. Na_v1.5 density increased in the Triton-insoluble fraction only, in the Gap-134-treated group compared to the DMSO-treated group (Fig. 3a). These results suggest that Gap-134 affects the Na_v1.5 multi-protein complex located close to junctional plaques of Cx43.

However, these effects were not associated with a significant impact on ventricular conduction. Indeed, we investigated the cardiac conduction after 15 weeks of treatment with Gap-134 in *Scn5a*^{+/-} mice at the ECG level (Supplementary Table 2). As shown in Fig. 3b, c, Gap-134 treatment did not improve ventricular conduction. The same absence of effect on conduction was observed after an acute application of Gap-134 for 20 min (Supplementary Fig. 4c) or a treatment for 3 weeks (Supplementary Fig. 5b).

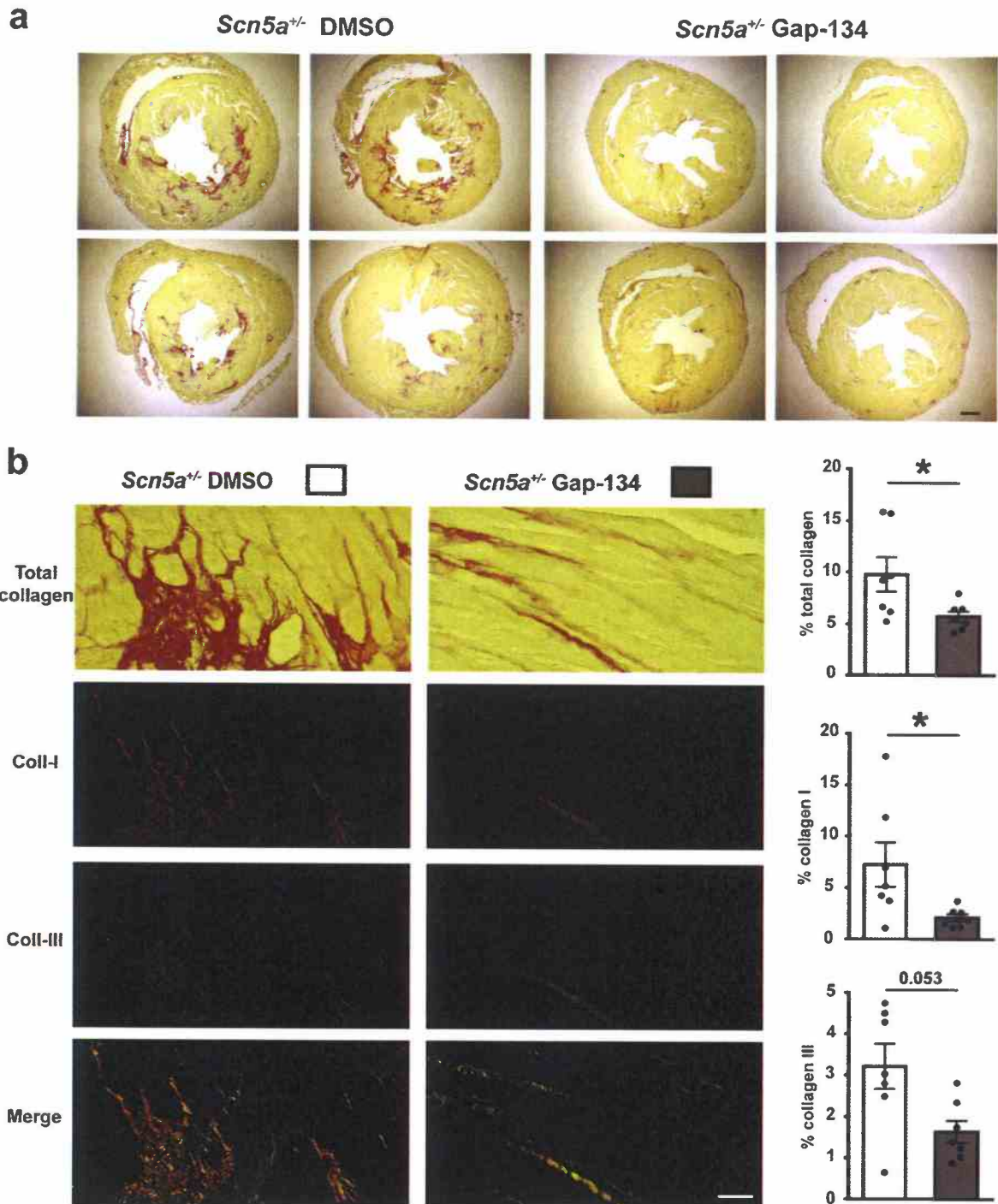


Fig. 4. Effect of chronic treatment with Gap-134 on fibrosis development in *Scn5a*^{+/-} mice.

(a) Representative transversal ventricle sections stained with picrosirius red and obtained from 60 week-old *Scn5a*^{+/-} mice chronically treated orally with 0.02% DMSO or Gap-134 (5 mg/kg/day) from the age of 45 weeks to the age of 60 weeks (scale bar: 500 μ m). (b) Left. Representative left ventricle sections stained with picrosirius red (left panels) in phase contrast (top) and polarized light to quantify collagen I (Coll-I, in red) and III (Coll-III, in green); scale bar: 50 μ m). Right. Total collagen, collagen I and collagen III (expressed as% collagen per histological section) for DMSO- and Gap-134-treated *Scn5a*^{+/-} mice ($n = 7$ per group). Mann-Whitney rank sum test * $P \leq 0.05$.

3.4. Gap-134 treatment prevents fibrosis development in *Scn5a*^{+/-} mice

Nevertheless, Fig. 4 shows that *Scn5a*^{+/-} mice treated during 15 weeks with Gap-134 did not develop any fibrosis at the age of 60 weeks, in contrast to what was observed in vehicle-treated mice. Different types of collagen are implicated in fibrosis, collagen III regulating the

fibrillogenesis of collagen I [28]. Accretion of both collagens I and III in the cardiac tissue at high level is pathological. As expected, a major decrease of collagen I production was observed in Gap-134-treated mice (Fig. 4b). Collagen III production was also reduced, though not quite significantly. Thus, these results show that Gap-134 prevents fibrosis development in *Scn5a*^{+/-} mice.

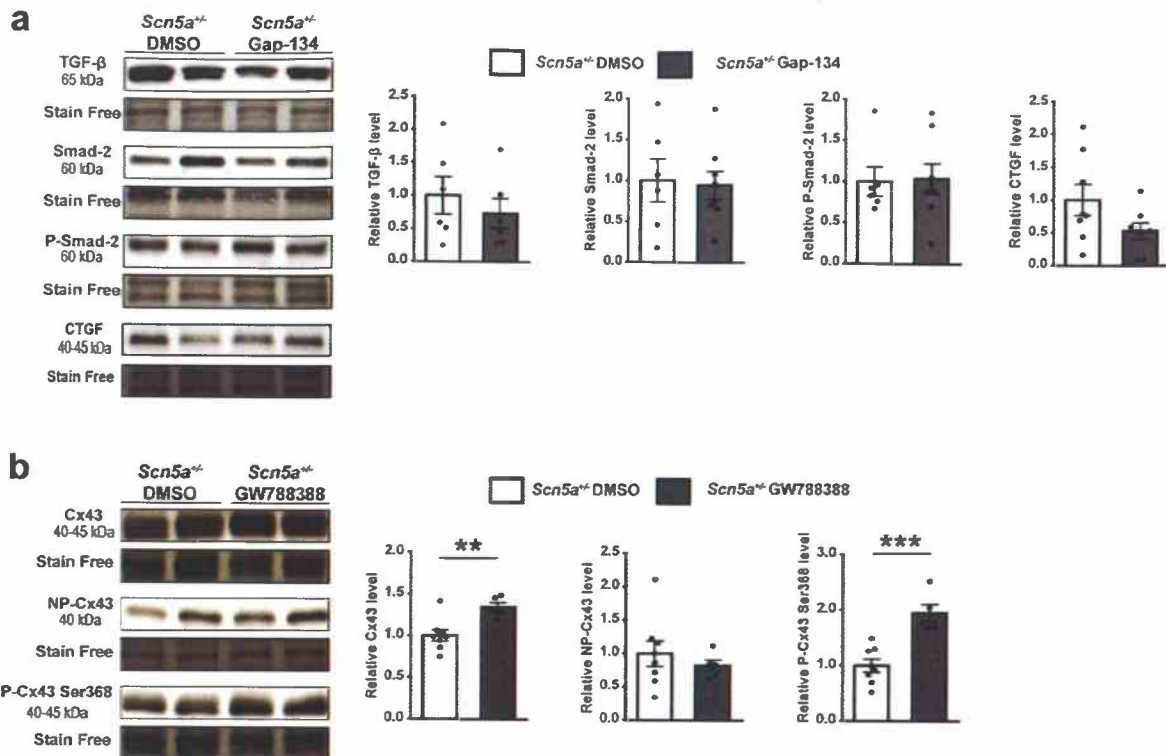


Fig. 5. Effect of chronic treatment with Gap-134 on TGF- β pathway expression and effect of chronic inhibition of TGF- β receptor on connexin 43 (Cx43) expression and phosphorylation in *Scn5a*^{+/-} mice.

(a) Representative western blots and Transforming Growth Factor β (TGF- β), phospho-Smad2 (P-Smad2) total Smad2 ($n = 6$ per group) and Connective Tissue Growth Factor (CTGF; $n = 8$ per group) density relative to corresponding total lane density from the stain-free blot in Triton-soluble fraction of left ventricle from 60-week-old *Scn5a*^{+/-} mice chronically treated orally with 0.02% DMSO or Gap-134 (5 mg/kg/day) from the age of 45 weeks to the age of 60 weeks. Average values in DMSO-treated group are arbitrarily set at 1. (b) Representative western blots and Cx43 non-phosphorylated Cx43 (NP-Cx43) and phosphorylated Cx43 on Ser368 (P-Cx43 Ser368) density relative to corresponding total lane density from the stain-free blot in Triton-soluble fraction of left ventricle from 60-week-old *Scn5a*^{+/-} mice chronically treated orally with 0.2% DMSO or GW788388 (5 mg/kg/day) from the age of 45 weeks to the age of 60 weeks. Average values in DMSO-treated group are arbitrarily set at 1 ($n = 8$ and $n = 5$ for DMSO- and GW788388-treated groups, respectively). Mann-Whitney rank sum test $**P \leq 0.01$ and $***P \leq 0.001$.

3.5. Gap-134 prevents fibrosis independently of the TGF- β canonical pathway

We showed previously that fibrosis development in *Scn5a*^{+/-} mice involves the activation of the TGF- β /Smad-2 canonical pathway [3]. In the same study, we showed that inhibition of TGF- β 1 receptors with GW788388 prevents fibrosis development and Cx43 expression decrease. To determine the involvement of this pathway in the Gap-134 effects on fibrosis, we investigated TGF- β and Smad2 expression and activity after Gap-134 treatment. As shown in Fig. 5a, treatment during 15 weeks with Gap-134 did not change either TGF- β and Smad-2 expression or Smad-2 phosphorylation when compared to the vehicle-treated group. Gap-134 also did not alter the expression of CTGF, which we had shown to be overexpressed in *Scn5a*^{+/-} mice compared to WT mice in our previous study [3]. Furthermore, no change of the expression level of MMP2, MMP9 or their inhibitors (TIMPs) was observed after Gap-134 treatment (Supplementary Fig. 6). Unlike GW788388, Gap-134 prevented fibrosis but not by inhibiting the TGF- β pathway in *Scn5a*^{+/-} mice. However, inhibition of the TGF- β pathway had the same effects as Gap-134, *i.e.*, increased density of free-Cx43 fraction as well as Cx43 Ser368 phosphorylation (Fig. 5b). These last results strongly suggest that activation of the TGF- β pathway reduces Cx43 expression and phosphorylation and alters its localization and that Gap-134 acts on fibrosis, downstream the TGF- β /Smad-2 canonical pathway, by directly targeting Cx43 expression and phosphorylation.

3.6. *Scn5a*^{+/-} mouse cardiac fibroblasts proliferate faster than WT fibroblasts, a defect prevented by GW788388 or Gap-134 treatment

Cardiac fibroblasts activation is known to modify extracellular matrix and promote fibrosis in various cardiac diseases. This activation is characterized by fibroblasts proliferation, differentiation and an excessive production of collagen type I and III [29]. As shown in Fig. 6a, cardiac fibroblasts from 45-week-old *Scn5a*^{+/-} mice proliferated faster than fibroblasts from WT mice. To determine the involvement of TGF- β and Cx43 in cardiac fibroblast proliferation, these cells were treated by GW788388 or Gap-134. As shown in Fig. 6b-c, whatever the compound, the rate of proliferation of *Scn5a*^{+/-} mouse fibroblasts decreased significantly and returned to the rate of proliferation of WT fibroblasts. We then investigated if lower *Scn5a* expression could alter the differentiation potential of fibroblasts into myofibroblasts and, if so, whether Gap-134 could have an impact on this process. As shown in Fig. 6d, the expression of differentiation markers (α -SMA and periostin) did not differ between WT and *Scn5a*^{+/-} fibroblasts, suggesting that, at least in our experimental conditions, the differentiation capacity of fibroblasts isolated from *Scn5a*^{+/-} mice was not altered. Accordingly, the expression of collagen I was similar in both genotypes. In addition, the expression of TGF- β and CTGF was unchanged in *Scn5a*^{+/-} fibroblasts compared to WT fibroblasts (Fig. 6e). These results suggest that the lower *Scn5a* expression has an impact on fibroblast proliferation but not differentiation. Treating the fibroblasts from *Scn5a*^{+/-} mice with Gap-134 did not modify differentiation properties.

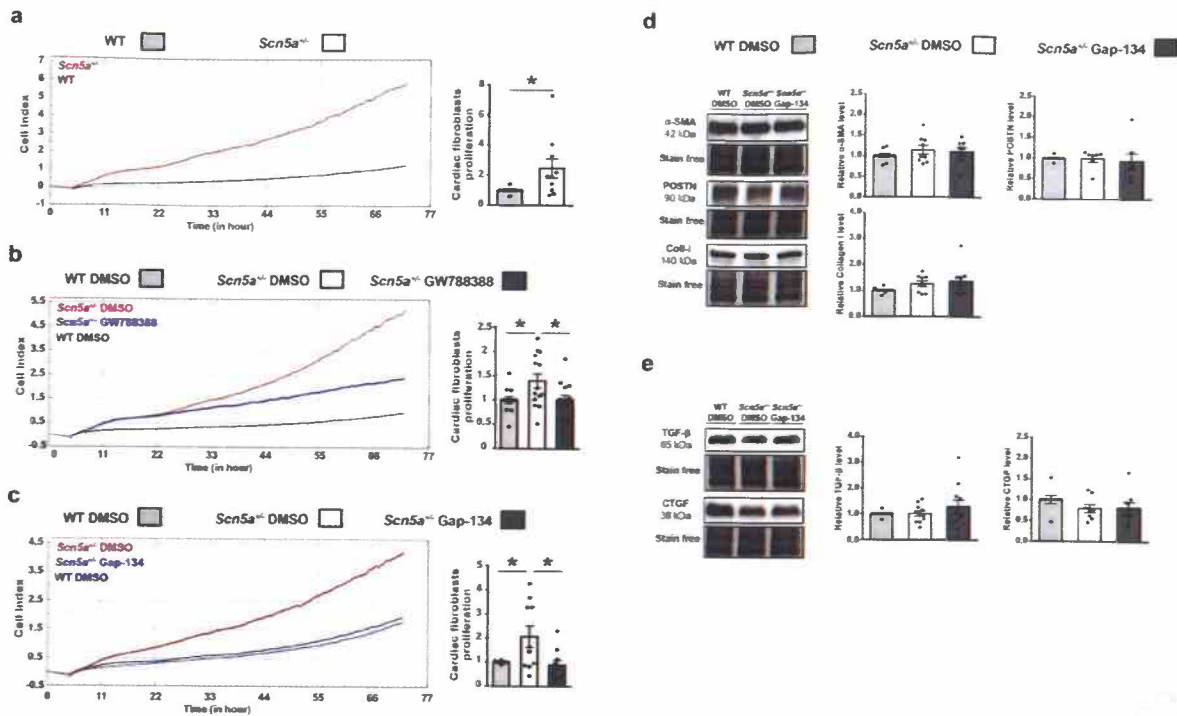


Fig. 6. Increased proliferation rate of cardiac fibroblasts from $Scn5a^{+/-}$ mice, and effect of GW788388 and Gap-134 on proliferation of $Scn5a^{+/-}$ cardiac fibroblasts. (a) Representative xCELLigence results (5000 cells/well) and proliferation rates for cardiac fibroblasts from 45-week-old wild-type (WT; $n = 8$) and $Scn5a^{+/-}$ ($n = 10$) mice. Mann–Whitney rank sum test $*P \leq 0.05$. (b–c) Representative xCELLigence recordings and proliferation rates of $Scn5a^{+/-}$ cardiac fibroblasts treated with (b) 20 μ M of GW788388 or vehicle (DMSO) from the beginning of the xCELLigence experiment (day 5 of culture; $n = 13$ WT mice; $n = 14$ $Scn5a^{+/-}$ mice) or (c) 1 μ M of Gap-134 from the beginning of cell culture (5 days before seeding in e-plates; $n = 10$ WT mice; $n = 10$ $Scn5a^{+/-}$ mice). Data from WT fibroblasts treated with vehicle are provided for comparison. One-way ANOVA $*P \leq 0.05$. (d–e) Representative western blots and average densities of (d) α -Smooth Muscle Actin (α -SMA; $n = 10$ WT mice; $n = 9$ $Scn5a^{+/-}$ mice for both DMSO and Gap-134), Periostin (POSTN; $n = 8$ WT mice; $n = 7$ $Scn5a^{+/-}$ mice for both DMSO and Gap-134), Collagen I (Coll-I; $n = 9$ per group), and (e) Transforming Growth Factor β (TGF- β ; $n = 11$ per group) and Connective Tissue Growth Factor (CTGF; $n = 8$ per group). Average densities are given as relative to corresponding total lane densities from the stain-free blot of ventricular fibroblasts (8 days of culture) treated with 1 μ M of Gap-134 or DMSO from the beginning of cell culture. Ventricular fibroblasts are from 45-week-old $Scn5a^{+/-}$ mice or WT mice.

4. Discussion

In previous studies, we have shown that $Scn5a$ heterozygous knockout mice develop extensive ventricular fibrosis after the activation of TGF- β canonical pathway at around 45 weeks of age [3,4]. We have also shown that Cx43 expression and phosphorylation were higher in 20–30-week-old $Scn5a^{+/-}$ mice than in wild-type littermates, and decreased concomitantly with the activation of TGF- β pathway and fibrosis onset [3], suggesting that Cx43 may be implicated in the fibrosis process in these mice.

In the present study, we show that (i) 60-week-old $Scn5a^{+/-}$ mice exhibit abnormal cardiac expression and localization of Cx43; (ii) inhibiting the TGF- β pathway with GW788388 between 45 and 60 weeks of age upregulates Cx43 expression and prevents fibrosis; (iii) increasing Cx43 expression and phosphorylation with a chronic treatment with Gap-134 between 45 and 60 weeks of age prevents fibrosis; (iv) cardiac fibroblasts in 45-week-old $Scn5a^{+/-}$ mice present an abnormal proliferation, prevented by GW788388 and Gap-134 treatments. Altogether, these results highlight the role of Cx43 in fibrosis in $Scn5a^{+/-}$ mice and the anti-fibrotic potential of Gap-134.

Down-regulation of Cx43 has often been proposed to be involved in the development of fibrosis during senescence [6] or in pathological conditions [7,8]. Indeed, numerous models characterized by the development of ventricular fibrosis also exhibit altered Cx43 expression and/or phosphorylation [30]. In some models, including ours, Cx43 down-regulation was found to precede fibrosis onset [3,9,10] but other studies have denied this role of Cx43 [11,12]. However, preventing fibrosis with inhibitors of the renin-angiotensin-aldosterone system

[31–33] or of the TGF- β receptor has been shown to be associated with a prevention of Cx43 down-regulation [3] and dephosphorylation (present study). Altogether, these studies suggest that Cx43 down-regulation is involved in fibrosis development and our study is the first to show that preventing Cx43 down-regulation and dephosphorylation does indeed prevent fibrosis despite the persistence of the primary disease, *i.e.*, conduction defects, and despite the upstream activation of the profibrotic TGF- β canonical pathway.

How Cx43 dysregulation may trigger fibrosis remains unknown. The present study also shows an increased proliferative activity of fibroblasts isolated from $Scn5a^{+/-}$ mouse ventricles, consistent with an activation of fibroblasts at 45 weeks, but no change in differentiation capacity. The proliferative phenotype of fibroblasts may correspond to a proto-myofibroblast phenotype [34], which is consistent with our *in vivo* studies since at 45 weeks, *i.e.*, at the beginning of tissue remodeling, fibrosis is not yet visible. At this age, the higher cardiac expression of TGF- β in $Scn5a^{+/-}$ mice is unlikely due to fibroblast activity, based on our *in vitro* studies, but most likely participates to the activation of fibroblasts. Indeed, we showed that blocking TGF- β receptors decreases fibroblasts proliferation, which supports the importance of the TGF- β pathway in this disease [3].

Interestingly, fibroblast proliferation was also blocked in the presence of Gap-134, *i.e.*, with Cx43 activation, revealing the potential role of Cx43 in cardiac fibroblast activity. Especially, cardiac fibroblasts can interact with their environment *via* Cx43 forming hemichannels or gap junctions. Hemichannels in fibroblasts participate to the secretion of factors such as ATP which can activate fibroblasts and recruit inflammatory cells such as monocytes [35,36]. Interestingly, Gap-134 has

been shown to prevent hemichannel opening [18], which may explain, at least partly, its effect of proliferation.

Fibroblasts also have the ability to establish communication with cardiomyocytes through gap junctions involving Cx43 [37,38]. This coupling between cardiomyocytes and fibroblasts could potentially be involved in the triggering of increased collagen production and/or stabilization although the molecular mechanism involved remains to be identified.

Furthermore, Gap-134 and GW788388, which both prevent fibrosis development in *Scn5a*^{+/-} mice, also increase Cx43 phosphorylation on serine 368. Interestingly, a decrease in Cx43 serine 368 phosphorylation level was proposed to be linked to the development of fibrosis in calcineurin-induced murine cardiac hypertrophy [9], suggesting that this serine is involved in fibrosis development. Also the increase in Cx43 serine 368 phosphorylation has been shown to prevent hemichannel opening [39]. Gap-134 has been shown to have the same effect [18]. As mentioned above, opening of Cx43 hemichannels favors inflammation and fibroblasts activation that may amplify collagen production by cardiac fibroblasts [40]. Thereafter, it will be interesting to distinguish the roles of Cx43 Ser368 phosphorylation induced by Gap-134 on gap junction communication and hemichannel activity. Indeed, concerning gap junction ion permeability, Ser368 phosphorylation has been shown to have complex and contradictory effects leading to either a decrease the gap-junction ion conductance [41], or inversely, maintained maintenance of conduction during metabolic stress in cardiomyocyte monolayer [42]. In view of these discrepancies, the functional consequences of Ser 368 phosphorylation remain uncertain in *Scn5a*^{+/-} mice.

Rotigaptide and Gap-134 have been shown to increase cell-to-cell electrical coupling *in vitro* [43]. At the atrial level, Rotigaptide has been shown to modestly (10–20%) improve conduction velocity and reduce atrial fibrillation inducibility in various models of atrial disease [15,44,45]. In our study, Gap-134 did not improve ventricular conduction, as shown by its absence of effect on QRS interval. This is consistent with what has been observed in Cx43 knockout mice. Indeed, heterozygous Cx43 knockout mice exhibit little, if any, reduction of ventricular conduction velocity and reducing Cx43 expression by 90% results in only 50% decrease in conduction velocity [46,47], showing that strong reductions in Cx43 expression are required for affecting conduction velocity. Therefore, the Gap-134-induced up-regulation of Cx43 expression may not be sufficient to improve conduction in the context of a strong reduction of conduction velocity due to *Scn5a* haplo-insufficiency. Nevertheless, we have previously shown that at the age of 80 weeks, the occurrence of spontaneous ventricular tachyarrhythmias in 50% of the *Scn5a*^{+/-} mice was correlated with the amount of fibrosis [48]. Therefore, limiting fibrosis with Gap-134 may be beneficial to prevent arrhythmias.

To the best of our knowledge, this study is the first to investigate the effects of a chronic treatment with Gap-134 on cardiac fibrosis. Rotigaptide and Gap-134 have been shown to prevent impairment of atrial conduction associated with atrial fibrillation [15,44,45]. In these previous studies, only acute or short-term treatments have been tested. To date, an increase in Cx43 expression has been shown only in culture after 24 h incubation with Gap-134 [49]. In the present study, we observed that chronic exposure to Gap-134 prevented the decrease in Cx43 expression and phosphorylation during ageing and fibrosis development in *Scn5a*^{+/-} mice. We propose that this anti-fibrotic effect of Gap-134 is, in part, directly linked to cardiac fibroblast activation blockade. These results not only confirm the Cx43 implication in fibrotic process in *Scn5a*^{+/-} mice but also pave the way to new therapeutic approaches for treating fibrosis related not only to cardiac conduction diseases, but also to other cardiac diseases [30].

In conclusion, the present study demonstrates that a chronic treatment with Gap-134 prevents ventricular fibrosis occurring in *Scn5a*^{+/-} mice after the age of 45 weeks, suggesting that Cx43 activators could be effective as anti-fibrotic agents.

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Author contributions

JL, HLM, IB, FC and MD conceived the research, obtained the funding and assessed the results. JP, CC, MS, AH, AC, AT, AB, AD performed the experiments and analyzed the data. JP, CC, IB, FC and MD wrote the manuscript. All authors reviewed and corrected the manuscript.

Declaration of Competing Interest

The authors indicated no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.phrs.2020.104922>.

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