Arsenic-induced interstitial myocardial fibrosis reveals a new insight into drug-induced long QT syndrome

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Aims
Arsenic trioxide (ATO), an effective therapeutic agent for acute promyelocytic leukaemia, can cause sudden cardiac death due to long QT syndrome (LQTS). The present study was designed to determine whether ATO could induce cardiac fibrosis and explore whether cardiac fibroblasts (CFs) are involved in the development of LQTS by ATO.

Methods and results
ATO treatment of guinea pigs caused substantial interstitial myocardial fibrosis and LQTS, which was accompanied by an increase in transforming growth factor β1 (TGF-β1) secretion and a decrease in ether-α-go-go-related gene (HERG) and inward rectifying potassium channel (IK1) subunit Kir2.1 protein levels. ATO promoted collagen production and TGF-β1 expression and secretion in cultured CFs. Whole-cell patch clamp and western blotting showed that treatment with TGF-β1 markedly reduced HERG and IK1 current densities and downregulated HERG and Kir2.1 protein expression in HEK293 cells stably transfected with the human recombinant HERG channel and in cardiomyocytes (CMs). These changes were completely reversed by treatment with the protein kinase A (PKA) antagonist, H89. CM and CF co-cultures showed that ATO significantly increased TGF-β1 levels in the culture medium, whereas markedly reduced HERG and Kir2.1 protein levels were observed in CMs compared with ATO-treated CMs not co-cultured with CFs. Finally, in vivo administration of LY364947, a pharmacological antagonist of TGF-β signalling, dramatically prevented interstitial fibrosis and LQTS and abolished aberrant expression of TGF-β1, HERG, and Kir2.1 in ATO-treated guinea pigs.

Conclusion
ATO-induced TGF-β1 secretion from CFs aggravates QT prolongation, suggesting that modulation of TGF-β signalling may provide a novel strategy for the treatment of drug-induced LQTS.

Keywords
Long QT syndrome • Cardiac fibrosis • Arsenic trioxide • HERG channel • TGF-β1

1. Introduction
Arsenic trioxide (As2O3, ATO) offers a very effective treatment for patients with acute promyelocytic leukaemia (APL),1 but its clinical use is hampered by its cardiotoxicity, including QT prolongation, torsade de pointes, and sudden cardiac death.2 The mechanism of ATO-induced long QT syndrome (LQTS) might be direct inhibition of the repolarizing K+ current (IKr) carried by the K+ channel subunit encoded by the human ether-α-go-go-related gene (HERG), which has been proved to be a common mechanism for drug-acquired LQTS.3 Intriguingly, some in vitro studies revealed that the HERG channel was not sensitized to direct ATO stimuli. For example, short-term exposure to a clinically relevant peak concentration (3–5 μM) of ATO for 3 h did not reduce the currents when HERG was stably expressed in HEK293 cells.4 Accordingly, action potential duration (APD) was not affected by short-term application of 3 μM ATO in...
isolated ventricular myocytes of guinea pigs, and only slightly prolonged under an extremely high concentration (50 μM ATO) for 30 min. Although recent evidence indicates that drug-disrupted HERG trafficking represents a mechanism for drug-acquired LQTS, HERG protein level was substantially decreased (by >70%) in vivo in a guinea pig model of ATO-induced cardiomyopathy (Figure 5F). Similarly, current densities of the cardiac inward rectifier current (I_K1) did not change upon long-term exposure to ATO in vitro, but our lab recently demonstrated that I_K1 was reduced in ATO-treated rats.

Most notably, Edge et al. revealed that pathological alterations were not only found in cardiomyocytes (CMs), but also observed in myocardial interstitium in patients with ATO therapy. In a study reported by Wu et al., ATO induced liver fibrosis in mice. Cardiac fibroblasts (CFs), the predominant cell type in the heart, have been implicated in the initiation and maintenance of arrhythmias via affecting cardiac electrical propagation by interacting with CMs. The role of cardiac fibrosis in atrial fibrillation has been intensively studied, but the relationship between cardiac fibrosis and LQTS remains unclear. Transforming growth factor-β1 (TGF-β1), a multifunctional cytokine mainly secreted by fibroblasts, mediates the signalling pathway to regulate matrix deposition and collagen production during the development of cardiac fibrosis. TGF-β1 signal-transduction pathways are initiated by binding of TGF-β1 to membrane-bound heteromeric receptor kinases (TGFBR1 and TGFBR2) that transduce intracellular signals via both Smad and non-Smad pathways. While these data suggest that ATO has both proarrhythmic and proproliferotic effects, it is unclear whether these two actions are interrelated. This prompted us to propose that ATO enhanced TGF-β1 secretion from CFs, and that the secreted TGF-β1 activates downstream components in the signalling pathway in CFs, leading to fibrosis on one hand and causing changes in ion channel expression and function in CMs leading to LQTS on the other. This study was designed to test this hypothesis.

2. Methods

(For details on other methods, see Supplementary material online.) All animal experiments were approved by the Animal Experiments Committee of the Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health).

2.1 Animals

Adult guinea pigs (250–350 g) were provided by the Experimental Animal Center of Harbin Medical University (Grade II). Food and water were freely available throughout the experiments. The guinea pigs were anaesthetized with sodium pentobarbionate (40 mg/kg, i.p.) and xylazine (12.5 mg/kg, i.p.). The adequacy of anaesthesia was monitored by the absence of withdrawal reflex to tail pinch. The animals were randomly divided into three groups (n = 6 for each group): control (Ctrl), ATO (1 mg/kg, i.p.), and LY364947 (1 mg/kg, an antagonist of TGF-β signalling) + ATO (1 mg/kg) groups. ATO was administered intravenously two hours after LY364947 i.p. injection, and thereafter every other day for one week. Control animals were given an equal volume of saline.

2.2 Electrocardiogram (ECG) recordings

Three-channel ECG was recorded for off-line analysis. The ECG was recorded before and after drug infusion for two hours, and the heart-rate corrected QT interval (QTc) was calculated with the Bazett formula: QTc = QT/(RR)^1/2, an accepted method for correcting QT interval in guinea pig. QT interval was measured from the beginning of the QRS complex to the end of the T wave, which was defined as the return to the TP baseline (between the end of the T wave and the following P wave). RR is the time between the two R waves in ECG.

2.3 Masson staining

The heart was quickly dissected and immersed in 10% neutral buffered formalin. After 24 h it was stained with Masson’s trichrome to detect the fibrotic areas. The extent of interstitial fibrotic areas was calculated with image analysis software (Image-Pro Plus v4.0; Meida Cybernetics, Bethesda, MD, USA) as previously described.

2.4 Transmission electron microscopy

In brief, samples were immersion-fixed overnight in phosphate-buffered 2.5% glutaraldehyde (pH7.4), postfixed for 1 h with 1% osmium tetroxide in 0.1 M cacodylate buffer, and dehydrated with a graded ethanol series. The samples were embedded in Epon medium. Ultrathin sections (60–70 nm) were stained with uranyl acetate and lead citrate, and examined with a JEM 1200 electron microscope (JEOL Ltd, Tokyo, Japan).

2.5 Isolation and co-culture of CMs and CFs

Neonatal rat CMs and CFs were obtained from 1–2-day-old neonatal Wistar rats, as previously described. Animals were anaesthetized using 4–5% isoflurane-inhalation anaesthesia. Adequate anaesthesia was assured by the absence of reflexes, prior to rapid heart excision.

2.6 Protein kinase A (PKA) activity assay

Kinase activity of PKA was assayed with a ProFluor® PKA Assay (Promega, Madison, WI, USA) according to the manufacturer’s recommended protocol.

2.7 Quantification of TGF-β1 secretion

Cytokine ELISA kits (R&D Systems, Minneapolis, MN, USA) were used for quantification of TGF-β1 in the media. All analytical steps were performed according to the manufacturer’s recommended protocol.

2.8 Measurement of collagen content

Total collagen content was determined by using a colorimetric reaction with picrosirius red, as described previously. Briefly, cells (2 × 10^5) after various treatments (at 85% confluence) were lysed, and the lysate (100 μL) was dehydrated and stained with picrosirius red in saturated picric acid (0.1% w/v) in a 96-well plate. The dye was solubilized, and absorbance read at 540 nm. Readings were converted to protein units using a linear calibration curve generated from standards (Vitrogen 100; Angiotech Biomaterials, Palo Alto, CA, USA) and normalized to the wet weight of each cell sample (1.2–1.5 mg).

2.9 Stable HERG-expressing cell line

HERG current recordings were performed on HEK293 cells that stably expressed the wide-type HERG gene. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) foetal bovine serum and 200 μM geneticin (G418, Gibco) at 37°C in a humidified 5% CO₂ atmosphere.

2.10 Immunocytochemistry

The anti-HERG antibody was the same as that used for western blot analysis (see below). The cells were examined under an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).
2.11 Isolation of ventricular myocytes in guinea pigs

Briefly, hearts were quickly removed from guinea pigs, washed in cool, oxygenated Tyrode’s solution (NaCl 126 mM, KCl 5.4 mM, HEPES 10 mM, NaH₂PO₄ 0.33 mM, MgCl₂ 1.0 mM, CaCl₂ 1.8 mM and glucose 10 mM; pH adjusted to 7.4 with NaOH), mounted on a Langendorff perfusion apparatus, and retrogradely perfused via the aorta.

2.12 Western blotting analysis

Protein samples were extracted from cells or whole-heart tissue as previously described. The membranes were incubated with the following antibodies: anti-TGF-β1 (1:500, rabbit polyclonal; Cell Signalling Technology, Beverly, MA, USA), anti-HERG (1:500, rabbit polyclonal; Alomone labs, Jerusalem, Israel), or anti-Kir2.1 (1:500, rabbit polyclonal; Alomone Labs). Goat anti-rabbit (1:4000, Alexa Fluor 700 conjugated; Molecular Probes/Life technologies) was used as the secondary antibody.

2.13 Patch-clamp recording

Currents were recorded in the whole-cell voltage-clamp mode, and action potential duration (APD) was measured in the current-clamp mode with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). The details are described in our previous studies.

2.14 Statistics

Data are presented as mean ± SEM. Differences were evaluated by Student t-test analysis (P < 0.05).

3. Results

3.1 ATO induces cardiac fibrosis in guinea pigs

The effects of ATO (1 mg/kg) on cardiac fibrosis were assessed in guinea pigs. Masson staining analysis demonstrated substantial structural alterations in both right and left ventricles of guinea pigs treated with ATO, relative to control animals. In control specimens, rod-shaped cardiac muscles were well aligned and surrounded by little interstitial tissue. In contrast, sections of ventricular tissue from ATO-treated guinea pigs showed a large proportion of connective tissue that had accumulated between bundles of fibres. Moreover, normal collagenous structures had a parallel arrangement of relatively broad bands of collagen fibres, whereas the abnormal interstitial fibrosis consisted of haphazardly arranged, disorganized, fine collagen fibres (Figure 1A). Accordingly, electron microscopy examination showed numerous CFs that were proliferating diffusely and an abundance of collagen deposition secreted in CFs of ATO-treated guinea pigs (Figure 1B). Upregulation of TGF-β1 expression in ATO-treated guinea pigs as determined by western blotting. Data were normalized to GAPDH and are presented relative to Ctrl guinea pigs. *P < 0.05 vs. Ctrl, n = 3 each group.

Figure 1 ATO induces cardiac fibrosis and TGF-β1 overexpression in the ventricular tissue of guinea pigs. (A) Microscope images showing fibrotic tissues (blue) of Masson’s trichrome-stained sections. Bar graph shows percentage cross-sectional area of fibrous tissue relative to whole ventricle. The images from three slides of each group were analysed using Image Pro Plus software. Five areas of 0.3 × 0.4 mm² and 2 mm apart were analysed in each slide. *P < 0.001 vs. Ctrl; n = 5 guinea pigs for each group. (B) Characteristic changes in CFs after ATO incubation detected by electron microscopy. (I) Control: green arrow indicates normal CF; (II) ATO treated: abnormal proliferation in CFs; (III) ATO treated: red arrow indicates increase of collagen production in CFs. (C) Upregulation of TGF-β1 expression in ATO-treated guinea pigs as determined by western blotting. Data were normalized to GAPDH and are presented relative to Ctrl guinea pigs. *P < 0.05 vs. Ctrl, n = 3 each group.
ATO-treated guinea pigs (Figure 1B). It is known that TGF-β is expressed in the heart and results in production of collagens. In order to investigate whether TGF-β plays a role in ATO-stimulated collagen production and cardiac fibrosis, we measured the expression level of TGF-β protein in ventricular tissue. Western blot analysis revealed significant upregulation of TGF-β in the ventricular myocardium of ATO-treated guinea pigs (Figure 1C).

3.2 ATO enhances collagen production and promotes expression and secretion of TGF-β1 in rat neonatal CFs

CFs are known to synthesize collagens via autocrine/paracrine action of TGF-β1, and are thus responsible for tissue fibrosis/remodeling. Therefore, we evaluated the effects of ATO on collagen production and TGF-β1 protein expression in cultured rat neonatal CFs. Consistent with the results from in vivo ATO-treated guinea pigs, western blot analysis revealed significant upregulation of TGF-β1 protein in ATO-treated CFs, relative to non-treated control cells (Figure 2A). Collagen content was also significantly higher with ATO treatment (0.5 or 5 μM), compared with non-treated control (Figure 2B). The collagen-production-stimulating effect of ATO was concentration-dependent. LY364947 (3 μM, a TGF-β signalling blocker) nearly abolished the elevation of collagen content induced by 5 μM ATO (grey bar; Figure 2B). Moreover, when CFs or CMs were exposed to 0.5 μM ATO for 24, 48, and 72 h, TGF-β1 in the culture medium was increased in a time-dependent manner (Figure 2C). The level of TGF-β1 in culture medium of CFs after 72 h ATO treatment reached 3.82 ± 0.78 ng/mL, which was significantly higher than that in culture medium of CMs (0.32 ± 0.16 ng/mL). Furthermore, an MTT assay showed that ATO at 0.5 μM for 72 h had no significant effect on CM viability (Supplementary material online, Figure S1). These results suggest that low dose and chronic ATO did not exert toxic effects on CMs, but promoted collagen deposition in CFs and TGF-β1 secretion from CFs.

3.3 TGF-β1 inhibits HERG and I_K1 currents and downregulates HERG and Kir2.1 protein levels

In order to eliminate the deviation induced by the HERG K⁺ current rundown, we observed the acute effect of TGF-β1 on HERG K⁺ current with a parallel control. We used TGF-β1 at 5 ng/mL, as the concentration of secreted TGF-β1 in the cultured medium of CFs exposed to chronic ATO treatment was 3.8 ± 0.78 ng/mL. In HEK293 cells, representative recordings showed that TGF-β1 significantly reduced HERG K⁺ currents (by 52%) after 24 h of TGF-β1 treatment. The data for step and tail currents are shown in Figure 3A. Western blot and immunocytochemistry analyses revealed that TGF-β1 inhibited HERG protein expression in both HEK293 cells (Figure 3B and C) and neonatal rat CMs (Figure 3G). Moreover, we also examined the effects of TGF-β1 on I_K1 current density and Kir2.1 protein level in CMs isolated from guinea pigs and neonatal rats.
respectively, and found that TGF-β1 also significantly reduced the density of \( I_{K1} \) and downregulated Kir2.1 protein level (Figure 3D and E). Previous studies have demonstrated that activation of PKA is able to regulate HERG and Kir2.1 protein synthesis and inhibit HERG and \( I_{K1} \) currents.\(^{17–19}\) Our data show that forskolin (10 \( \mu M \)) increased PKA activity to a greater extent (1.7-fold greater) in HEK293 cells 24 h after exposure to TGF-β1 than in control cells without TGF-β1 treatment (Figure 3F). Furthermore, TGF-β1-induced downregulation of HERG and Kir2.1 proteins and TGF-β1-suppressed HERG and \( I_{K1} \) currents were prevented by PKA antagonist H89 (10 \( \mu M \)), suggesting a role of PKA in mediating the TGF-β1 regulation of HERG and \( I_{K1} \) (Figure 3A, B, D and E). In addition, the action potential duration to 90% full repolarization (APD\(_{90}\)) was prolonged by TGF-β1 treatment for 24 h (Figure 3H). Taken together, the results suggest that exogenous TGF-β1 is able to prolong APD\(_{90}\) possibly through inhibiting HERG and \( I_{K1} \).

### 3.4 Cardiac fibroblasts contribute to downregulation of HERG and Kir2.1 proteins in cardiomyocytes by ATO

To understand how the fibrotic signal in CFs could affect the electrical activities in CMs, we conducted the following experiments: we first compared the effects of ATO on HERG and Kir2.1 expression between monoculture of CMs and co-culture of CMs with CFs. ATO downregulated HERG and Kir2.1 protein levels in CMs monoculture (Figure 4A and B), and this downregulation was considerably exacerbated in the CM/CF co-culture. Correspondingly, ATO

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**Figure 3** Inhibition of HERG and \( I_{K1} \) currents and expression of \( K^+ \) channel subunits carrying these currents by TGF-β1. (A) Representative HERG current traces in control (Ctrl), in the presence of 5 ng/mL TGF-β1 alone, or with co-application of H89 (+H89) for 24 h. Normalized I–V relationships measured at the end of depolarizing steps (middle panel) and tail currents (right panel) in the absence or presence of 5 ng/mL TGF-β1/H89 (mean values, ≥8 cells for each condition). (B) Inhibitory effects of TGF-β1 on HERG protein expression measured by western blot analysis in stable HERG-expressing HEK293 cells. Cells were untreated or treated with 5 ng/mL TGF-β1 or 10 \( \mu M \) H89 + 5 ng/mL TGF-β1. Data were normalized to β-actin (n = 5). (C) Immunocytochemistry showed reduced HERG protein expression with TGF-β1 incubation. (D) Whole-cell patch-clamp recording of \( I_{K1} \) in guinea pig CMs treated with 5 ng/mL TGF-β1 for 24 h. Left: raw traces; right: I–V curves (mean values from ≥8 cells for each group). The density of \( I_{K1} \) decreased with TGF-β1. Co-application of H89 attenuated the effect of TGF-β1. (E) Inhibitory effect of TGF-β1 on Kir2.1 protein in neonatal rat CMs determined by western blot analysis. Data were normalized to GAPDH (n = 5). (F) PKA was activated by TGF-β1 in HEK293 cells. Forskolin (10 \( \mu M \)) was used as an internal control for PKA activation (n = 5). (G) Inhibitory effect of TGF-β1 on HERG protein expression determined by western blot analysis in cultured neonatal rat CMs. Data were normalized to β-actin (n = 5). (H) TGF-β1 (5 ng/mL) acute treatment in vitro prolonged action potential duration of ventricular CMs isolated from guinea pig. APD\(_{90}\) values are shown in the bar graph (n = 6). *\( P < 0.05 \) vs. Ctrl, #\( P < 0.05 \) vs. TGF-β1 treated cells.
elevated TGF-β1 content to a significantly greater degree in the culture medium from CM/CF co-culture than in monocultures of either CMs or CFs alone (Figure 4C). Notably, as expected, increase of TGF-β1 level in the culture medium and downregulation of HERG and Kir2.1 protein in ATO treated co-culture cells were totally prevented by exogenous LY364947 (3 µM).

### 3.5 Inhibition of TGF-β signalling pathway prevents QT prolongation in guinea pigs

Consistent with previous reports, our ECG results show that i.v. administration of ATO prolonged QTc. QTc gradually extended through the 7 days after ATO administration, from 320 ± 23 ms (control) to 396 ± 24 ms (7th day after ATO treatment) (Figure 5D). PR intervals and QRS were also increased in ATO-treated guinea pigs (Figure 5E), which suggests that fibrosis may change these and alter conduction, and thereby induce arrhythmia via reentry. Western blot analysis was used to measure the changes in HERG and Kir2.1 protein levels in the ventricular tissues of guinea pigs with an antibody directed against the N terminus (anti-N) of HERG protein. The anti-N antibodies recognized two species of HERG protein (155 and 135 kDa), which were similar in size to mature glycosylated and immature core-glycosylated HERG fractions. The levels of both bands of HERG protein were significantly decreased in the ATO-treated model (Figure 5F). Similarly, Kir2.1 protein (the main K⁺ channel subunit that carries I_K1) was also downregulated by ATO in guinea pigs (Figure 5G).

To explore whether the above findings have any pharmacological implications, guinea pigs were injected with 1 mg/kg LY364947 (TβR-I inhibitor) and then subjected to ATO injection. Masson

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**Figure 4** Expression of HERG and Kir2.1 proteins and secretion of TGF-β1 in co-culture experiments of neonatal rat CFs and CMs. CMs were co-cultured with CFs or cultured alone, and with ATO (0.5 µM) or ATO (0.5 µM) + LY364947 (3 µM; LY) for 72 h. HERG (A) and Kir2.1 (B) expression, and TGF-β1 secretion (C) detected by western blot and ELISA assays, respectively. Cultured neonatal rat CFs contribute to ATO-induced downregulation of HERG and Kir2.1 proteins in CMs via paracrine TGF-β1, but this effect was reversed by LY364947. Data are mean ± SEM (n = 3). *P < 0.05 vs. CM Ctrl, #P < 0.05 vs. co-culture cells treated with ATO stimuli; unpaired Student’s t-test.
staining and electron microscopy showed that production of collagen from fibroblasts was increased markedly in the ATO-treated guinea pigs. Pretreatment with LY364947 significantly reduced the content of collagen in ATO-treated guinea pigs, suggesting LY364947 dramatically prevents development of cardiac fibrosis induced by ATO (Figure 5A and B). ECG recording showed LY364947 also abolished ATO-induced QT prolongation in guinea pigs (Figure 5D). Western blotting revealed that increase in TGF-β1 protein expression (Figure 5C) and decreases in HERG and Kir2.1 protein levels (Figure 5F and G) caused by ATO were completely prevented by LY364947. These results suggest that TGF-β signalling plays a key role in ATO-induced cardiac fibrosis and LQTS.

4. Discussion

Previous studies in cardiotoxicology have focused only on the direct toxic effect of ATO on CMs, while generally ignoring other cells types of the heart; CFs account for about 75% of all cardiac cells. The findings of our present study in a guinea pig model indicate that ATO induces cardiac fibrosis and promotes TGF-β1 secretion and collagen production in CFs, which in turn confers an increased risk of adverse cardiac electrical events. More importantly, our present study also indicates that ATO stimulates TGF-β1 secretion from CFs, exerts a paracrine inhibitory effect on HGRG and I_{K1} currents, and downregulates HERG and Kir2.1 protein levels in CMs, which subsequently contributes to the development of QT prolongation.

In ATO-treated APL cases, patients undergoing induction therapy receive daily infusions of ATO until achieving bone marrow remission, or for a maximum of 60 days. The median daily dose of ATO in a pilot study was 0.15 mg/kg (range, 0.06–0.2 mg/kg), within the clinically relevant concentrations being 0.1–1.5 μM. We have previously demonstrated that only a high dose of ATO (>2 μM) directly induced apoptosis and increased ROS production in myocytes. In the present study, a low concentration (0.5 μM ATO) for 72 h chronically enhanced TGF-β1 secretion, corresponding with induction of collagen production, but did not exhibit its cytotoxic effect on myocytes (Supplementary material online, Figure 1S). Epidemiologic evidence has shown that chronic exposure to arsenic for the treatment of APL is associated with mortality from heart disease. Our study suggests that chronic interstitial myocardial fibrosis is one of the mechanisms in the induction of cardiac toxicity for long term ATO therapy or chronic arsenic exposure. A series of experiments also indicated that CFs affect the function of CMs. Electrical interaction between the two cell types can principally occur through capacitive coupling of their cell membranes and via gap junctions, but indirect effects of CFs on CMs have been less extensively investigated. TGF-β1 secreted from fibroblasts has been established as an important cytokine intimately involved in tissue fibrosis. So far, only a few studies have investigated whether TGF-β1 affects the electrophysiological properties of the heart, and these have focused mainly on atrial myocytes. I_{K1} current carried by HERG K<sup>+</sup> channel proteins is a critical determinant of cardiac repolarization in humans. It is generally believed that the main cause of LQTS is direct blockage of the HERG channel. In addition to HERG currents, L-type calcium channel currents (I_{Ca,L}), and I_{K1} are also important for cardiac repolarization. We found that acute (5 ng/mL) treatment with TGF-β1 prolonged APD in isolated
ventricular myocytes from guinea pigs (Figure 4H), and that this was associated with inhibition of HERG and \( I_{\text{Kr}} \) currents. Most notably, a very recent study has indicated that in cardiomyocytes exposed to 200 pM TGF-β1 for 1–2 days, the densities of inward rectifier (\( I_{\text{IK}} \)) and outward sustained (\( I_{\text{Ks}} \)) currents, but not of outward transient (\( I_{\text{Kt}} \)) currents, were decreased (by \( \sim 50\% \)). Moreover, a study by Avila et al. also revealed that TGF-β1 exhibits selective inhibition of the \( I_{\text{ICS-L}} \) channel from atrial but not ventricular myocytes. Another study suggested that TGF-β1 reduces the time to peak and the rate of decay of intracellular Ca\(^{2+}\) transients, in the absence of significant alterations to \( I_{\text{ICS-L}} \) in ventricular myocytes. Together, these results suggest that \( I_{\text{IK}} \) and \( I_{\text{ICS-L}} \) currents might not be involved in the ventricular proarrhythmic effects of TGF-β1. Here we observed that acute TGF-β1 stimulation for 24 h resulted in a marked decrease in HERG and Kir2.1 proteins, and that this was prevented by PKA antagonist H89, suggesting PKA activation is involved in the regulation of HERG and Kir2.1 protein synthesis. This notion was supported by previous studies; for example, acute TGF-β1 treatment stimulated PKA in mesangial cells and human pulmonary artery smooth muscle cells, and sustained elevation of PKA activity profoundly affected HERG protein abundance, current density, and rate of synthesis. Moreover, PKA immunolocalized with synapse associated protein-97 (SAP97) possibly through interaction with A-kinase anchor protein, AKA79, in CMs quenched Kir2.1 protein expression.

We performed co-culture experiments and found that elevation of the TGF-β1 level in co-culture medium was concomitant with reduction in HERG and Kir2.1 protein levels upon ATO treatment, whereas aberrant expression of both potassium channel proteins was abolished by LY364947 (TβRI inhibitor) in vitro. This does not eliminate the possibility that a small autocrine response to TGF-β1 from CMs influenced potassium channel expression, but at least it indicates that the paracrine function of CFs contributes to electrical remodeling induced by ATO. More importantly, a recent study showed suppression of TGF-β1/Smad activation inhibited arsenic-induced liver injury in rats. Thus, we also evaluated whether inhibition of TGF-β signalling was able to produce protective effects against cardiac fibrosis and QT prolongation induced by ATO. We also used in vivo pretreatment of the TβRI inhibitor LY364947, which has been shown to decrease cardiac fibrosis and attenuate QT prolongation. Correspondingly, aberrant regulation of TGF-β1 and HERG and Kir2.1 expression was reversed in ATO-treated guinea pigs by LY364947 pretreatment, suggesting that ATO-induced TGF-β1 secretion downregulates ventricular HERG and Kir2.1 expression and may therefore contribute to the in vivo cardiac electrical remodeling. In a report by Oka et al., the inhibition of endogenous TGF-β1 signalling by LY364947 in vivo accelerates lymphpangiogenesis and inhibits TGF-β1 protein expression in a mouse model of chronic peritonitis. Although inhibition of the TGF-β signalling pathway abrogated ATO’s action on the electrical remodeling, the present study does not answer the question as to how ATO regulated TGF-β1 expression and secretion. However, Zhang et al. demonstrated that promyelocytic leukemia protein (PML) is a direct binding target of arsenic trioxide. PML is a critical regulator of TGF-β signalling. These studies suggest that PML is probably involved in regulating ATO-induced TGF-β1 synthesis, but this issue remains to be elucidated.

We did not observe torsade de points arrhythmia or any early or delayed afterdepolarization in our model, and we did not include additional functional studies in naive myocytes or isolated heart to study potential arrhythmogenesis. In addition to QT prolongation, ATO also affected the QRS complex in our model, which is consistent with the report of Ragh et al. These data indicate that in addition to QTs, ATO may also be able to cause other types of arrhythmias associated with conduction delay. The present investigation was limited to the abnormal QT prolongation and does not provide insight into the mechanisms of ATO-induced cardiac conduction slowing, into which future studies are warranted.

In summary, the present study provides compelling experimental evidence that ATO induces cardiac fibrosis and that CFs are involved in ATO-induced development of LQTS, through, at least in part, the paracrine secretion of TGF-β1 by CFs, after ATO stimuli inhibit HERG and Kir2.1 protein in CMs. These results aid our understanding of the mechanisms of drug-acquired LQTS and chronic cardiotoxicity induced by ATO, and modulation of TGF-β signalling as a potential approach for the prevention or therapy of drug-induced LQTS.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

### Conflict of interest: none declared.

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