Insights into sick sinus syndrome from an inducible mouse model

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Aims
Sick sinus syndrome is a generalized abnormality of cardiac impulse formation and is responsible for a large proportion of pacemaker implantations. Although the exact aetiology is not known, it is widely accepted that age-dependent degenerative fibrosis of nodal tissue is the most common cause. Despite its importance, an animal model for sick sinus syndrome is lacking. We attempted to generate a mouse model phenocopying the pathohistological changes as well as the characteristic arrhythmic manifestations of this syndrome.

Methods and results
We crossed two genetically engineered mouse lines, ROSA-eGFP-DTA and HCN4-KiT-Cre, to achieve an inducible deletion of cells specifically in the cardiac pacemaking and conduction system. This deletion resulted in a degenerative fibrosis of nodal tissue, which accurately reflects the pathohistological findings in human sick sinus syndrome. The extent of the sino-atrial fibrosis could be controlled by varying the dosage of the inducing substance, tamoxifen. A high-dose protocol resulted in the complete ablation of all sino-atrial cells as demonstrated by histochemical analysis and quantitative reverse transcriptase–polymerase chain reaction. The animals developed a variety of arrhythmias, including progressive bradycardia, sinus pauses, supraventricular and ventricular tachycardia and chronotropic incompetence. Remarkably, the complete destruction of the primary pacemaker centre resulted in only a small increase in mortality.

Conclusion
This study describes the generation and analysis of an inducible mouse model which closely reflects the pathophysiological characteristics of sick sinus syndrome. The model, with the ability to control the extent of nodal cell ablation and fibrosis, offers new insights into sick sinus syndrome and other cardiac conduction diseases.

Keywords
Sick sinus syndrome • Sino-atrial node • Arrhythmia • Transgenic animal • Site-specific recombination

1. Introduction
Sick sinus syndrome is characterized by a generalized malfunction of the cardiac conduction system. The syndrome occurs in roughly one of every 600 cardiac patients older than 65 years and accounts for around 50% of all pacemaker implantations. Thus, every year around 400 000 patients are diagnosed for sick sinus syndrome and treated with a pacemaker. The average patient who suffers from this disease is 68 years old, with a medical history of concomitant heart disease. The aetiology of sick sinus syndrome is not completely understood, but histopathological findings suggest that a degenerative fibrosis is the main cause of the abnormal cardiac automaticity and conduction. With ageing, nodal tissue and the surrounding myocardium are gradually replaced by fibrous stroma. This process ends in a complete fibrosis of the sinus node and also affects adjacent parts of the conduction system, such as the atrioventricular (AV) node or bundle of His. The ECG of sick sinus patients comprises a collection of various arrhythmias, including inappropriate sinus bradycardia, sinus arrest/sinus pause, sino-atrial exit block, atrial flutter, atrial fibrillation and atrial tachycardia. Conduction disturbance of the atrioventricular node can result in an AV block of any degree. In late stages, the incidence of ventricular tachycardia or fibrillation increases, thereby augmenting the risk for sudden cardiac death. Despite the importance of sick sinus syndrome, an adequate animal model mimicking the pathohistological changes of the conduction system and exhibiting the various cardiac arrhythmias...
is lacking. To create such a model, we developed a genetic system capable of ablating cardiac pacemaker cells in a temporally specific manner by crossing two genetically engineered mouse lines. The first is the HCN4-KiT-Cre line,\(^9\) which enables temporally controlled Cre recombination exclusively in cells of the primary cardiac conduction system. The second is the ROSA26-eGFP-DTA line,\(^10\) which facilitates diphtheria toxin expression after Cre-mediated excision of a floxed stop sequence. The thorough analysis of the resulting double-transgenic animals revealed that these mice develop a degenerative fibrosis of nodal tissue together with a broad range of electrophysiological abnormalities typical of sick sinus patients.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and was approved by the local regulatory authority (Regierung von Mittelfranken, \#54-2531.31-8/07).

2.1 β-Galactosidase (LacZ) staining

LacZ staining was done as described\(^11\) using HCN4-KiT-Cre animals crossed with ROSA26 reporter (R26R)\(^12\) mice.

2.2 Tamoxifen treatment

Diphtheria toxin A (DTA) mediated cell ablation in double-transgenic mice was induced by i.p. administration of tamoxifen (40 mg/kg; Sigma). Tamoxifen was freshly dissolved in miglyol oil (Caelo) at a concentration of 10 mg/mL.

2.3 Masson's trichrome staining and immunohistochemistry

Hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, embedded in paraffin and cut into 10 μm sections. Consecutive sections were stained either with Masson's trichrome (Sigma HT15) for detection of fibrosis or with an anti-hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) antibody (Alomone Lab) for labelling sino-atrial node cells. For immunolabelling, endogenous peroxidase activity was quenched and antigen retrieval was done as described.\(^13\) Images were acquired using a Zeiss LSM 5 Pascal confocal microscope. Control stainings omitting the primary antibody gave no signal.

2.4 RNA isolation from sino-atrial node tissue

Mice were killed by cervical dislocation. Hearts were quickly removed and placed in ice-cold phosphate-buffered saline, pH 7.4, containing 4 mM EDTA. The right atrium, including the superior vena cava, was isolated in 10 μm sections. Consecutive sections were stained with Masson's trichrome (Sigma HT15) for detection of fibrosis or with an anti-hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) antibody (Alomone Lab) for labelling sino-atrial node cells. For immunolabelling, endogenous peroxidase activity was quenched and antigen retrieval was done as described.\(^13\) Images were acquired using a Zeiss LSM 5 Pascal confocal microscope. Control stainings omitting the primary antibody gave no signal.

2.5 Quantitative reverse transcriptase–polymerase chain reaction

One-tube reverse transcriptase–polymerase chain reaction (RT–PCR) was performed using a Quantitect Probe RT-PCR Kit (Qiagen). Expression of genes was determined by TaqMan assays on an ABI Prism 7900. Gene specific primers and probes were purchased from Applied Biosystems (Darmstadt, Germany). For each RT-PCR, the threshold cycle (C\(_t\)) was defined as the cycle at which the fluorescence exceeds 10 times the standard deviation of the mean baseline emission for cycles 3–10, was determined. The C\(_t\) value of each gene was normalized to GAPDH according to the following formula: \(\Delta C_t = C_t(\text{examined gene}) - C_t(\text{GAPDH})\). Values were averaged and then used for the \(2^{-\Delta Ct} \times 100\) calculation. TaqMan assays are used in Supplementary Methods.

2.6 ECG recordings in anaesthetized mice

Adult mice were anaesthetized by inhalation of isoflurane. Body temperature was maintained at 36–37°C using an infrared light source controlled by a rectal temperature probe. For signal detection, needle electrodes were inserted through the skin. The ECG signals were amplified with a differential amplifier (Animal Bio Amp ML136, AD Instruments) and were collected using a Power Lab B30 (AD Instruments) data acquisition system. Data were analysed using Chart 5 Pro ECG and HRV analysing software (AD Instruments). The Q–T interval was corrected for heart rate using the formula \(QT_c = Q/T \sqrt{RR/T}/100\), where QT\(_c\) is corrected QT interval and RR is RR interval.

2.7 ECG recording in conscious mice

Telemetric ECG recordings were performed as described\(^14\) with radio-transmitters (Data Sciences International, St Paul, MN, USA) implanted intraperitoneally. Details are given in Supplementary Methods.

2.8 Electrophysiological analyses in the isolated heart

Isolated, Langendorff-perfused mouse hearts were studied using previously published techniques.\(^15\) In brief, an octapolar murine electrophysiology catheter was inserted into the right atrium and right ventricle. Atrial and ventricular electrograms, a tissue bath ECG, and monophasic action potentials from left and right ventricle were simultaneously recorded. Analysis was done in mice 9–14 days after tamoxifen treatment. Details are given in Supplementary Methods.

2.9 Analysis of the incidence of arrhythmias

Analysis of the occurrence of specific arrhythmias was done during long-term ECG recordings. Data were sampled for 20 s every 10 min, resulting in 144 rhythm strips per day. All rhythm strips were examined for arrhythmias by two investigators blind for genotype. If one (or more) arrhythmia was detected in the 20 s recording, one event was counted. For calculation of the relative occurrence, all counted events per day (i.e. all arrhythmia-positive rhythm strips) were divided by all examined rhythm strips per day and multiplied by 100. If a specific type of arrhythmia was present in more than 30 rhythm strips per day for more than two consecutive days and until the end of the recording period (day 17), counting was stopped and defined as an arbitrary maximum arrhythmia level.

2.10 Statistical analysis

All experiments and primary analyses were blinded. Data are presented as means ± SEM. Statistical analysis was done using Student's t-test for paired or unpaired data using Bonferroni's correction as appropriate. A P-value of < 0.05 was considered statistically significant.

3. Results

3.1 Generation of mice with inducible deletion of cardiac pacemaking cells

To generate an animal model for sick sinus syndrome, we crossed ROSA26-eGFP-DTA (DTA) and HCN4-KiT-Cre (KiT) mice. In the case of the latter strain, the tamoxifen-inducible CreERT2 recombinase\(^16\) is knocked into the HCN4 gene locus, enabling Cre-mediated recombination in cardiac pacemaker cells in a temporally controlled manner.\(^9\) In the double-transgenic animals, Cre-mediated excision of a floxed ‘stop’ region allows the conditional expression of diphtheria toxin A,\(^10\) leading to selective death of Cre-expressing cells (Figure 1A).
general, we used a high-dose tamoxifen induction protocol (40 mg/kg for 5 consecutive days) to achieve complete activation of all Cre-expressing cells. This treatment led to death of approximately 20% of the binary (DTA/KiT) mice over a period of 60 days, compared with no deaths in control animals (Figure 1B). Typically, death occurred suddenly, with no ante-mortem indication of illness. The macroscopic examination of the hearts of the transgenic animals that died suddenly, as well as others that were killed, revealed no structural abnormalities (Figure 1C; see Supplementary material online, Figure S1B). In addition, we calculated the heart/body weight ratio of tamoxifen-treated animals and found no difference between DTA/KiT (5.4 ± 0.37 mg/g, n = 24) and DTA/Ctr mice (5.7 ± 0.14 mg/g, n = 16, n.s., Figure 1D). This indicates that the DTA-mediated ablation of pacemaker cells did not result in cardiac hypertrophy or heart failure.

3.2 Sino-atrial cell deletion and fibrosis is dependent on the dose of tamoxifen

One reason for using an inducible genetic model was the consideration that the extent of cardiac cell loss and the severity of
pathological changes in the cardiac conduction system could be controlled by the dosage of tamoxifen. We tested this hypothesis by histological analysis of the primary pacemaker centre of DTA/KiT mice that received different induction protocols. Every treatment group received an equal daily dose of tamoxifen (40 mg/kg), with the first group injected only on day 1 (T1), the second treated for two consecutive days (T2) and so on up to treatment for 5 days (T5, high-dose protocol). Injection of a single dose of tamoxifen resulted in moderate ablation of sino-atrial node cells as shown by immunostaining for HCN4, which represents a sino-atrial node cell-specific marker17–19 (Figure 2A, left panels). Successively higher doses resulted in a progressive reduction of HCN4-positive cells. Finally, specimens from mice treated with the high-dose protocol showed a complete ablation of virtually all sino-atrial node cells. We calculated this gradual decrease in living nodal cells by quantification of HCN4 mRNA and found a strong inverse correlation ($r = 0.925$) between the applied dose of tamoxifen and HCN4 transcript levels (Figure 2B). Hence, in the system developed here the extent of sino-atrial node cell loss can be tightly controlled. The deletion of pacemaker cells was accompanied by a destructive fibrosis of nodal tissue (Figure 2A, right panels). Physiologically, the murine sino-atrial node is embedded in a layer of connective tissue, which separates the pacemaker centre from atrial muscle,20 as shown in trichrome-stained sections from control animals (Figure 2A, T0). Sections from the series of induced DTA/KiT mice revealed that the compact node was gradually infiltrated by collagen fibres, with the degree of nodal fibrosis being proportional to the dosage of tamoxifen. After five consecutive treatment days, the sinus node artery, a hallmark of the compact node, was no longer surrounded by densely packed pacemaker cells but encircled by loosely perforated connective tissue (Figure 2D). An acetylcholinesterase staining in DTA/KiT animals (see Supplementary material online, Figure S1A) corroborated the ablation of sino-atrial node cells. In addition, we analysed the expression of other genes that are known to be enriched in the conduction system, including HCN1 and Cav3.1.20 As expected, the transcript levels of these genes were significantly reduced in specimens of DTA/KiT compared with DTA/Ctr mice (Figure 2C). The unchanged mRNA expression of HCN2, a gene which is not enriched in murine nodal tissue,20 confirms that the DTA-mediated ablation is restricted to cells of the cardiac pacemaking system.

3.3 Tamoxifen-treated DTA/KiT mice become bradycardic and show increased heart rate variability and chronotropic incompetence

A strength of our inducible model is that cell deletion and development of arrhythmias can be followed in parallel. ECGs were recorded both in conscious mice by telemetric transmission and under isoflurane anaesthesia. Administration of tamoxifen for five consecutive days was followed by a decrease in heart rate in DTA/KiT mice. Telemetric ECG recordings showed a substantial decline in heart rate during the first week after treatment (Figure 3A). The mean basal heart rate fell from 493 ± 14 (before treatment, $n = 6$) to 275 ± 59 beats/min (7 days after treatment, $n = 6, P < 0.001$), representing a heart rate reduction of about 40%. The bradycardia persisted over the whole observation period in all treated DTA/KiT mice, although the extent of the reduction varied amongst individual animals between 20 and 60%. The heart rate of DTA/Ctr animals did not change significantly (before treatment $482 ± 64$ beats/min; 7 days after treatment $474 ± 97$ beats/min, $n = 4$, n.s.). We noted that during the induction protocol, DAT/Ctr and DTA/KiT mice displayed a slight and transient heart rate reduction, most probably as a consequence of a direct heart rate lowering effect of tamoxifen (Figure 3A).

Recordings of a separate group of anaesthetized mice gave similar results. Determined under isofurane anaesthesia, a heart rate reduction of about 50% was observed in animals treated with the high-dose protocol (DTA/Ctr, $n = 13$; DTA/KiT, $n = 11, P < 0.001$, Supplementary material online, Figure S2). The DTA/KiT mice that were treated with a lower dose of tamoxifen displayed an intermediate phenotype, with a heart rate decrease of about 12% ($n = 4, P < 0.05$ vs. control).

We also observed increased heart rate variability of binary transgenic mice. A quantitative analysis of P–P and R–R interval variability during tamoxifen injection and the following 3 weeks was performed by computing RMSDD values [root-mean square of the difference of successive P–P (or R–R) intervals; Table 1]. These values remained constant in control animals, whereas a progressive increase was seen in DTA/KiT mice. Figure 4A shows an example of an R–R tachogram of a DTA/KiT animal (red) characterized by alternating periods of slow and fast frequencies. The beat-to-beat intervals oscillated between 336 and 142 ms, which reflects a heart rate fluctuating between 179 and 423 beats/min. The histogram indicates that the R–R intervals occurred in a completely irregular pattern, with no preference for a certain interval length (red open columns, Figure 4B). In contrast, control animals displayed only a narrow fluctuation of R–R intervals (black trace and black open columns), indicating a stable heart rhythm. These periods of alternating fast and slow heart rates occurred intermittently and were observed in ECG traces of most DTA/KiT mice (five of seven), but never in control animals (zero of seven). A similar phenotype could be observed in isolated, Langendorff-perfused mouse hearts. Analysis of monophasic action potentials showed alterations of long electrical standstills (sinus pauses) and intermittent rapid atrial activity in DTA/KiT hearts (seven of seven), but in none of the control hearts (zero of five; $P < 0.05$), suggestive of atrial tachycardias and tachycardia–bradycardia syndrome. Representative brady-tachyarrhythmias are depicted in Figure 4C.

Chronotropic incompetence during exercise is another symptom that often occurs in sick sinus patients;21,22 therefore, we next examined the heart rate response to β-adrenergic stimulation (Figure 3B). Administration of isoprenaline to control animals caused a significant heart rate increase of $199 ± 34$ beats/min ($n = 4, P < 0.01$), whereas the heart rate of DTA/KiT mice increased by only $69 ± 39$ beats/min ($n = 6$, n.s.). We observed some variability in the response of DTA/KiT mice, as the majority of these animals (four of six) showed no or only a minimal heart rate response upon isoprenaline injection, whereas a few (two of six) reacted with a relative increase in heart rate that was even higher than the average of control animals (see Supplementary material online, Figure S3). However, in all DTA/KiT mice the absolute value of the maximally stimulated heart rate remained low ($447 ± 33$ beats/min). In addition, we performed an analysis of the heart response to various drugs implicated in heart rate regulation (see Supplementary material online, Figure S4). We found a significant heart rate deceleration in both animal groups in response to the A1 adenosine receptor agonist 2-chloro-N-cyclopentyladenosine (CCPA) (0.3 mg/kg), indicating that down-regulation of the heart rate via this signal transduction pathway is intact in our mouse model. In addition, the parasympathetic system seems not to be up-regulated in DTA/KiT animals.
3.4 Arrhythmias in DTA/KiT mice

The ECG of DTA/KiT animals indicated AV nodal conductance disturbances. On the last two tamoxifen treatment days (T4 and T5), the P–R interval of binary transgenic mice started to get longer, and during the following days the impulse propagation through the AV node slowed down further (Figure 5A). The progressive prolongation of the P–R interval tended to result in complete isolated contraction of atria and ventricles (see Supplementary material online, Figure S5A). A complete heart block was observed some weeks after treatment in several DTA/KiT animals. A lower induction protocol (T1 and T2) also resulted in some prolongation of the P–R interval, but to a much lesser extent (see Supplementary material online, Figure S5B). To analyse the AV conduction defect in more detail, we performed an intracardiac electrophysiology study and documented complete AV block, which appeared supra-Hisian, with normal H–V intervals (Figure 5B). The activation time from the atrial pacing site to the ventricular recording sites was measurable in control mice (48 ± 3 ms, n = 5), but maximal due to complete AV block in all examined DTA/KiT hearts (n = 7). Other ECG parameters, including P duration and QRS, Q–T, QTc and J–T intervals, were not affected by the DTA-mediated cell deletion (see Supplementary material online, Figure S5C).

**Figure 2** Tamoxifen-controlled ablation of sino-atrial node cells. (A) Analysis of the SN region after different tamoxifen treatment protocols. Shown are sections through the SN area of mice that received the following treatment protocols: T0, DTA/Ctr animals injected with tamoxifen for 5 consecutive days; T1, T2, T4, T5, DTA/KiT mice treated once, two, four and five times with tamoxifen, respectively. Left panels, detection of SN cells by immunolabelling HCN4 protein (green). Right panels, adjacent sections were stained with Masson’s trichrome to highlight interstitial fibrosis. Scale bar represents 100 μm. (B) HCN4 gene expression in sino-atrial specimens from differently treated animals (each treatment group T0–T5, n = 3). HCN4 mRNA levels were normalized to expression of GAPDH. The exponential regression curve is indicated by the dashed line (r² = 0.925). (C) Relative expression of HCN1, HCN2, HCN4 and Ca3.1 transcripts in SN specimen from DTA/Ctr (n = 3, open bars) and DTA/KiT mice (n = 3, black bars). Quantitative RT-PCR was performed with TaqMan probes 2 months after tamoxifen treatment and normalized to GAPDH expression. *P < 0.05, ***P < 0.001. (D) Masson’s trichrome-stained section through the SN area of a DTA/KiT mouse after five injections of tamoxifen. RA, right atrium; SN, sino-atrial node; SN-A, sino-atrial node artery; SVC, superior vena cava. Scale bar represents 100 μm.
A detailed analysis of telemetric ECG recordings revealed a variety of additional cardiac rhythm disturbances, such as sino-atrial arrhythmia, sino-atrial pause and supraventricular or ventricular tachycardia (Figure 6). This diversity of conspicuous arrhythmias is a well-known phenomenon of sick sinus syndrome. In order to characterize the frequency and time dependence of arrhythmias, recordings from 2 days before treatment to 17 days after treatment were analysed, and the occurrence of various types of arrhythmia per day was counted (Figure 6). The susceptibility for sino-atrial arrhythmias increased strongly during the five treatment days, which might be caused by the acute ablation of pacemaker cells. After this initial phase, the occurrence of sino-atrial arrhythmias stabilized at a high level for the remaining observation period. This type of arrhythmia was also found in control animals, but only on a few days and only at a low level (Figure 6A). In contrast, abnormalities such as sinus pauses and atrioventricular blocks (second degree) were not observed during the treatment period, but afterwards the incidence of these arrhythmias also increased to a constant high level (Figure 6B and C). Both supraventricular and ventricular tachyarrhythmias were also present in DTA/KiT animals, but these types of rhythm disturbances appeared only to a minor extent (Figure 6D and E). The susceptibility for supraventricular tachycardia (SVT) was elevated in the period between the last treatment days and the following 5 days. After this period, SVT was found only sporadically (Figure 6D). The incidence of ventricular tachycardia was very low (Figure 6E). In contrast to SVT, ventricular tachycardia appeared in the late phase of the observation period (around 2 weeks after the induction protocol), which is in agreement with the increased incidence of ventricular tachycardia in late stages of sick sinus patients.

Table 1: Effects of tamoxifen treatment on P–P and R–R interval variability in DTA/Ctr and DTA/KiT animals over time

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<th>RMSDD (mean ± SEM)</th>
<th>Average (mean ± SEM)</th>
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<td></td>
<td>DTA/Ctr</td>
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<td>P–P variability</td>
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<tr>
<td>P–P interval</td>
<td>n = 4</td>
<td>n = 5</td>
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<tr>
<td>T1-T5</td>
<td>4.65 ± 0.40</td>
<td>5.58 ± 1.72</td>
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<tr>
<td>1 W</td>
<td>4.84 ± 0.68</td>
<td>12.24 ± 1.10</td>
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<tr>
<td>2 W</td>
<td>4.07 ± 0.54</td>
<td>46.61 ± 17.41</td>
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<tr>
<td>3 W</td>
<td>6.81 ± 4.48</td>
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<td>R–R variability</td>
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<td>R–R interval</td>
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<tr>
<td>T1-T5</td>
<td>3.98 ± 0.52</td>
<td>3.76 ± 0.45</td>
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<tr>
<td>1 W</td>
<td>2.62 ± 0.44</td>
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<tr>
<td>2 W</td>
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<tr>
<td>3 W</td>
<td>4.47 ± 4.42</td>
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RMSDD, root-mean square of the difference of successive P–P (or R–R) intervals; W, weeks.
To characterize the mouse model in more detail, atrial and ventricular refractory periods and the inducibility of arrhythmias were examined by using intracardiac electrogram (EGM) recordings. Atrial effective refractory periods were not different between genotypes (control hearts, 17 ± 7 ms, n = 5; DTA/KiT, 18 ± 4 ms, n = 7, P > 0.05). Likewise, ventricular effective refractory periods were not significantly different (control hearts, 21 ± 5 ms, n = 5; DTA/KiT, 18 ± 4 ms, n = 7, P > 0.05). Atrial arrhythmias were rare during programmed stimulation, but were observed during spontaneous beating (Figure 4C). Ventricular stimulation provoked monomorphic and polymorphic tachyarrhythmias in DTA/KiT hearts (not shown).

4. Discussion

4.1 Deletion of the primary pacemaker

In this work, a tamoxifen-inducible genetic system was used to delete cardiac pacemaker cells selectively. In the majority of cases, the induction protocol and the resulting cell deletion was remarkably well tolerated. This was unexpected in view of the complete destruction of the primary pacemaker centre as revealed by histological analyses. The fibrotic changes were dramatic and extended over the full area of the sino-atrial node. Nevertheless, the ECG of these mice displayed a sometimes arrhythmic but otherwise regular-shaped atrial excitation (P wave). The origin of the impulse generation is not known. One possibility is that some cells of the primary pacemaker centre survived as a result of an insufficient 4-OH-tamoxifen concentration or an inadequate expression of the recombinase construct in individual sino-atrial node cells. However, labelling of HCN4 was no longer detected in the high-dose tamoxifen-treated double-transgenic animals. This strongly argues against an insufficient recombinase activity, because HCN4 serves as a very sensitive molecular marker for murine sino-atrial node cells.19,22 Even if some individual cells survived the induction protocol, it is hard to imagine that such sporadic cells surrounded by connective tissue and cell fragments can be
responsible for the impulse propagation to the atria. However, recent studies showed that, in principle, fibroblasts are able to form functional electrical connections between separate cardiomyocytes. This heterogeneous cell coupling may contribute to impulse conduction in our mouse model.

A more likely explanation is a spontaneous excitation in the atrial muscle outside the destroyed pacemaker centre. A recent publication described a unique area in the human atria, which was similar in some respects to right atrium (for example low expression of HCN4), while in other respects it resembled sino-atrial node. This so-called paranodal area showed an intermediate expression pattern of many ion channels, which may contribute to a stronger depolarization compared with the surrounding atria. It may be possible that cells of such a region develop automaticity if the leading pacemaker

Figure 5  Atrioventricular node conduction disturbances. (A) Upper panel, representative ECG traces of a DTA/KiT mouse before (left) and after tamoxifen treatment (right). Dotted lines mark the P–R intervals. Lower panel, P–R intervals of DTA/Ctr (n = 7, open squares) and DTA/KiT mice (n = 6, filled squares) plotted against time. *P < 0.05, **P < 0.01, ***P < 0.001. (B) Intracardiac electrophysiology study of isolated hearts from control and DTA/KiT mice during spontaneous beating and atrial pacing. Asterisks mark potential His bundle depolarizations. Atrioventricular dissociation is apparent during spontaneous rhythm in DTA/KiT hearts. Complete AV block is observed during atrial pacing with a fixed cycle length. Lines 1–4: (1) left atrial monophasic action potential (MAP); (2) octapolar catheter; (3) octapolar catheter in DTA/KiT spontaneous rhythm, otherwise ECG; and (4) ventricular MAP. Scale bars (left) indicate 5 mV and 100 ms, respectively.
is destroyed. However, despite intensive investigation, such a region has not yet been described in the mouse.

Another explanation for the cardiac impulse formation could be related to remodelling processes of atrial myocardium. It is well known that cardiac hypertrophy or chronic atrial fibrillation is accompanied by alteration of gene expression. Cardiac hypertrophy or atrial dilatation did not occur in our animal model, but the damage of the nodal area could lead to remodelling of adjacent atrial myocytes in order to compensate the sino-atrial node function.

Expression analysis of a variety of genes assumed to be involved in spontaneous excitation ($\text{HCN1}$, $\text{HCN2}$, $\text{HCN4}$, $\text{Ca}_{1.3}$, $\text{Ca}_{3.1}$, $\text{Ca}_{3.2}$ and $\text{RYR2}$) in the right atria displayed no significant alterations (data not shown), although the expression of several potassium channel genes ($\text{ERG1}$, $\text{Kir2.1}$, $\text{Kir3.1}$ and $\text{K}_{\text{LQT1}}$) displayed a tendency to decrease (see Supplementary material online, Figure S6). This diminished expression of genes participating in repolarization could contribute to a more depolarized state of individual atrial cells. Distinct atrial myocytes with a higher propensity for spontaneous excitation

Figure 6 Cardiac rhythm abnormalities in freely moving DTA/KiT mice. Left panels, representative ECG recordings from DTA/KiT mice. Observed ECG abnormalities include SN arrhythmia (A), SN pause (B), second degree AV block (C), supraventricular tachycardia (SVT; D) and ventricular tachycardia (VT; E). Right panels, relative occurrence of the respective type of arrhythmia. Averaged values per day from tamoxifen-treated DTA/Ctr ($n = 3$, open squares) and DTA/KiT mice ($n = 3$, filled squares) were plotted against time.
could then be sufficient to drive the atria if the dominant influence of the primary pacemaker is not present any more.

4.2 ECG abnormalities observed in the sick sinus model

The sick sinus model presented here shows various ECG abnormalities that have also been described in human sick sinus patients.1,2,4 – 6 It is estimated that in approximately 16% of patients with sick sinus syndrome the AV node is affected as determined in our mouse model. It is estimated that in approximately 16% of patients with sick sinus syndrome the AV node is affected as determined in our mouse model. However, it cannot be excluded that the extent of the fibrosis differed among DTA/KiT animals, because immunohistochemical analysis revealed a reproducible elimination of all sino-atrial cells in every probe examined. Similar to patients suffering from sick sinus syndrome, the animals of our disease model also displayed inter-individual differences in the degree of the ECG abnormalities. This was particularly obvious for bradycardia and the response to \( \beta \)-adrenergic stimulation. We detected a significant heart rate decrease in all DTA/KiT animals and control hearts, suggesting that the observed ECG changes are not related to a structural defect of the atrial or ventricular myocardium.

Remarkably, the various types of arrhythmia occurred at clearly different time points. Sinus arrhythmia was observed during the third treatment day, suggesting that this early phase was triggered by pacemaker-cell apoptosis rather than by the following fibrosis. In contrast, sino-atrial node pauses and AV blocks (second degree) appeared only after the end of the treatment, implying a contribution of the fibrotic changes to these arrhythmias. Ventricular tachycardia did not occur until the late observation phase. At this time, a complete heart block was partly present, most probably augmenting the susceptibility to ventricular arrhythmias.

Similar to patients suffering from sick sinus syndrome, the animals of our disease model also displayed inter-individual differences in the degree of the ECG abnormalities. This was particularly obvious for bradycardia and the response to \( \beta \)-adrenergic stimulation. We detected a significant heart rate decrease in all DTA/KiT animals during the first week after injection, but the degree of the reduction varied between individuals. It is unlikely that these differences were caused by variable recombination efficiencies between individual animals, because immunohistochemical analysis revealed a reproducible elimination of all sino-atrial cells in every probe examined. However, it cannot be excluded that the extent of the fibrosis differed between individuals, which may result in variable affected heart rates.

The AV conduction time was a remarkably sensitive ECG parameter in our mouse model. It is estimated that in approximately 16% of patients with sick sinus syndrome the AV node is affected as well.2 Atrioventricular blocks of any degree may occur, with progression to a complete heart block (third degree) of around 2.6% per year. Even after one tamoxifen injection, mice displayed a significant prolongation of P–R intervals (see Supplementary material and Figure 5B), and the conduction time was slowed down with each subsequent injection. By the use of a high-dose treatment protocol (T4, T5) mice developed a complete AV block with independent atrial and ventricular excitation after 1–2 weeks. Therefore, depending on the treatment protocol used, our mouse model is suitable for the analysis of AV conduction disturbance of any degree.

4.3 Conclusion

The model presented here faithfully reflects the pathohistological findings and the typical ECG abnormalities reported for human sick sinus syndrome. In contrast to human studies, which are obviously limited, our model allows the cell deletion and the development of arrhythmias to be followed in parallel. The induced cell loss is strictly confined to the cardiac pacemaking and conduction system. Moreover, the extent of the pathological changes in the sino-atrial node can be controlled by the applied dosage of tamoxifen, opening up the possibility of studying sick sinus syndrome at defined stages. The therapy of patients suffering from sick sinus syndrome usually includes implantation of a pacemaker. Recently, there has been much interest in generating biological pacemakers by transducing cells with pacemaking-related genes or by using stem-cell-based approaches.7–31 In contrast to transgenic mice with a sinus node dysfunction resulting from the knockout of various ion channels,3 our model mirrors not only the pacemaking and conduction defect but also the structural changes in the sino-atrial node. Hence, the model presented here could be a useful tool for direct evaluation of the potential of the above approaches as new therapies for sick sinus syndrome.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References


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