Perlecan is critical for heart stability

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Aims Perlecan is a heparansulfate proteoglycan found in basement membranes, cartilage, and several mesenchymal tissues that form during development, tumour growth, and tissue repair. Loss-of-function mutations in the perlecan gene in mice are associated with embryonic lethality caused primarily by cardiac abnormalities probably due to hemopericards. The aim of the present study was to investigate the mechanism underlying the early embryonic lethality and the pathophysiological relevance of perlecan for heart function.

Methods and results Perlecan-deficient murine embryonic stem cells were used to investigate the myofibrillar network and the electrophysiological properties of single cardiomyocytes. The mechanical stability of the developing perlecan-deficient mouse hearts was analysed by microinjecting fluorescent-labelled dextran. Maturation and formation of basement membranes and cell-cell contacts were investigated by electron microscopy, immunohistochemistry, and western blotting. Sarcomere formation and cellular functional properties were unaffected in perlecan-deficient cardiomyocytes. However, the intraventricular dye injection experiments revealed mechanical instability of the early embryonic mouse heart muscle wall before embryonic day 10.5 (E10.5). Accordingly, perlecan-null embryonic hearts contained lower amounts of the critical basement membrane components, collagen IV and laminins. Furthermore, basement membranes were absent in perlecan-null cardiomyocytes whereas adherens junctions formed and matured around E9.5. Infarcted hearts from perlecan heterozygous mice displayed reduced heart function when compared with wild-type hearts.

Conclusion We propose that perlecan plays an important role in maintaining the integrity during cardiac development and is important for heart function in the adult heart after injury.

KEYWORDS Baseline membranes; Extracellular matrix; Infarction; Hemopericard; Ventricular function

1. Introduction

Perlecan is the major proteoglycan of basement membranes.1 Perlecan binds to integrins and dystroglycan, recruits growth factors and modulates their activity, and interacts with other extracellular matrix (ECM) components thereby serving as an important scaffold to stabilize ECM structures such as basement membranes.2 Perlecan is expressed throughout embryogenesis with high levels in the developing cartilage, blood vessels, and heart.3 Loss of the perlecan gene in man and mice revealed a critical role of perlecan for cartilage, heart, and brain development.4,5 Perlecan-deficient mice develop two types of heart defects. Approximately 70–80% of the knock-out embryos die at embryonic day 10.5 (E10.5) of massive blood leakage into the pericardial cavity.4 Morphological analysis of basement membranes revealed defects in E10.5 hearts and brains.4 Since these defects were exclusively seen in the beating heart and in the developing brain, it was proposed that perlecan may play an important role for stabilizing basement membranes against mechanical forces.

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The second heart defect in perlecan-deficient embryos develops after E10.5 and can lead to a transposition of the great arteries.6

After embryogenesis, perlecan expression is restricted to basement membranes. However, during pathological conditions including cancer and tissue damage perlecan is found at high levels in stromal tissues.7 Since perlecan-deficient mice die either during development or at birth, it was so far impossible to investigate the function of perlecan at the adult stage, in particular under conditions of tissue injury and/or repair.

In the present work, we tried to unravel the cause for the defects in the heart muscle wall of perlecan-deficient embryos. These studies revealed that basement membranes lacking perlecan deteriorate in the entire heart, lead to cell–cell detachment in the ventricle and outflow tract and blood leakage into the pericardial cavity. To test a potential role of perlecan after the loss of cell–cell contacts in adult hearts, we generated myocardial infarctions in photometer,35 nL in E9.5/10.5 embryos and 700 nL in E14.5/15.5 embryos. For E9.5 only embryos with intact hearts and good injections were included, for E14.5/15.5 only embryos with beating hearts because of the embryonic lethality of perlecan-null embryos. Microinjection was monitored through a ×5 (E9.5/10.5) or a ×2.5 (E14.5/15.5) objective and an upright microscope (Zeiss) and a three CCD video cameras (AVT Horn, Germany). Videos were digitized with a DV-Master Pro acquisition board, and SpeedRazor and Adobe after-effects software (Dazzle).

2.4 Western blotting and quantitative polymerase chain reaction

For determination of the levels of collagen IV and laminin A5, E9.5 hearts were lysed in SDS–Urea sample buffer (8 M Urea, 2 M Thiourea, 0.2% SDS, 1.5% Triton X-100, 0.05% Tris–HCl, pH 6.8) and separated by SDS-PAGE on a 4–15% gradient Tris–HCl gel (Criterion, Bio-Rad). Proteins from five hearts each were transferred to PVDF membrane and incubated overnight with a rabbit polyclonal antibody against collagen IV (R1041; 1:500; Acris Antibodies) or a rabbit polyclonal antibody against the laminin IHS (1:1000, kindly provided by M. Paulsson). Subsequently, horse radish peroxidase (HRP)-conjugated immunoglobulins (goat anti-rabbit) and Super Signal West Dura Extended Substrate (Pierce Biotechnology) were used. Actin staining was used for loading control. For determination of mRNA of collagen IV, RNA from three E9.5 wild-type and three perlecan-deficient hearts was extracted with trizol and transcribed into cDNA using SUPERSCRIPT III kit (Invitrogen). Quantitative PCR was performed with the Quantitect SYBR Green PCR Kit (Qiagen) and a QI thermocycler (Biorad). Collagen IV was normalized to GAPDH and relative expression was primer efficiency-corrected according to Pfaffl et al.12 The specificity of primers (collagen IV forward: 5′-TTGTGGACCCGCATGACG-3′, reverse: 5′-AATTGCCGATCACTGAG-3′; GAPDH forward: 5′-GTGTTCTTACCCCAATTG-3′, reverse: 5′-CCTGCTCAGTGTCCTGTGCTG-3′) was proven by melting curve analysis and gel electrophoresis.

2.5 Plastic embedding and electron microscopy

For light and electron microscopy of heart tissue, 4% paraformaldehyde immersion-fixed embryos were post-fixed with 2% osmium tetroxide in PBS for 2 h at 4 °C as already reported before. The fixation routine allowed ultrastructural and immunohistochemical analysis of the same hearts. Embryos were block-stained with 1% uranyl acetate at 70% ethanol for 8 h. Afterwards, the specimens were dehydrated, infiltrated, and embedded with araldite and stained with methylene blue. Ultrastructural analysis of basement membranes and cell–cell contacts was performed as described earlier.13

2.6 Calculation of cell–cell contacts

For calculation of the amount of cell–cell contacts, the length of cell–cell interface at the intercalated disc and the overall length of all specialized cell–cell contacts in this interface region were measured on 3–10 electron microscopic pictures at ×20,000 magnification of three hearts per group. Exclusively, disc intercalaires-like structures crossing the whole micrographs were used for the statistical analysis. Total of 9–50 cell–cell contacts per heart were analysed. Then, the ratio between cell–cell contact and whole cell–cell interface at the intercalated disc was calculated.

2.7 Immunohistochemistry and densitometry

PFA fixation and immunohistochemistry on embryoid bodies (EBs), paraffin slices of murine embryos and frozen sections of adult and embryonic hearts was performed as described earlier using mouse anti-α-actinin antibody (1:800), mouse monoclonal antibody against Pan Cadherin (1:500, Sigma), mouse monoclonal antibody against multi-epitope cocktail to desmoplakin 1 and 2 (undiluted, Progen), monoclonal rat anti-perlecan (1:1000, Biotrend), rabbit polyclonal anti-collagen IV (1:500; Acris Antibodies), or laminins...
antibody (1:2000 provided by M. Paulsson) as first antibodies and Cy3 labelled rabbit anti-mouse (1:1000, Biotrend), donkey anti-rat Cy5 (1:200) or donkey anti-rabbit Cy5 (1:400, Jackson Immunoresearch), biotinylated goat anti-mouse (Dako), or goat anti-rat (Amersham) as second antibody. For light microscopy, extravidin HRP (1:150, Amersham) and DAB was used for visualization. Quantification of perlecan–DAB-stained slices of infarcted hearts were performed from five perlecan heterozygous and five wild-type hearts 3–4 weeks after infarction. The background-subtracted (cell-free area) average grey value from five randomly selected areas of each slide was analysed using the ImageJ (NIH) software program.

2.8 Cryoinjury, transplantation of wild-type cardiomyocytes, and in vivo assessment of left ventricular function infarcted mice

Ventricular cardiomyocytes harvested from transgenic α-actin EGFP E16.5 embryos (C57/B16) were transplanted into cryo-lesioned ventricle of C57/B16 wild-type male. The surgical procedure, the cryoinjury (3 × 10s exposure of a liquid N2 cooled copper probe with a diameter of 4–5 mm), and the injection of cells (100,000 diluted in 5 μL or control solution into the lesion) were performed as reported earlier.15,16

Left ventricular function was evaluated from eight wild-type and eight perlecan heterozygous mice 2 weeks after the lesion as reported earlier.17,18 In brief, pressure–volume loops of the left ventricle were recorded with a pressure-impedance catheter (Millar Instruments). Parallel conductance was estimated by injection of 10 μL of 10% NaCl and subtracted; volume calibration was performed with blood. Data were recorded with BioBench Software (National Instruments) and analysed with PVAN software (Millar Instruments) by a blinded investigator.

3. Results

3.1 Embryonic stem cell-derived, perlecan-null cardiomyocytes have normal sarcomeres and electrical activity

Loss of perlecan in nematodes causes severe muscle cell adhesion defects and abnormal sarcomeric architecture in skeletal muscle.19 To test whether similar defects lead to the abnormalities of the developing perlecan-deficient heart, we investigated the development of the contractile apparatus, ion channel expression, and their β-adrenergic and muscarinic modulation using ES cell-derived cardiomyocytes from wild-type and perlecan-null EBs. Figure 1A shows wild-type (left panel) and perlecan-null (right panel) ES cell-derived cardiomyocytes with identical sarcomeric organization and orientation indicating that the formation of the contractile apparatus occurred independent of perlecan. The functional expression of ion channels was normal in perlecan-null cardiomyocytes. Wild-type (data not shown) as well as perlecan-null cardiomyocytes showed K1 and Ca2+ currents when applying voltage ramp protocols (Figure 1D). This was corroborated by comparing the 90% action potential (AP) duration (APD90), a sensitive parameter for the aggregate ion channel expression, in spontaneously beating cardiomyocytes (Figure 1C). As depicted in Figure 1B, perlecan-null cardiomyocytes displayed APs (right panel), which were very similar to control cells (left panel). The muscarinic agonist carbachol (CCh, 1 μM) had a negative chronotropic effect in perlecan-null cardiomyocytes (Figure 1E). Application of the β-adrenergic agonist Isoprenaline (ISO, 1 μM) and subsequently CCh (1 μM) resulted in the typical

Figure 1 Myofibrillogenesis and cardiomyocyte function in wild-type and perlecan-null embryonic stem cell-derived cardiomyocytes. (A) Cluster of wild-type (WT) embryonic stem cell-derived cardiomyocytes (EB, 7 + 9 days) showed sarcomeric structure and cross-striation after immunostaining with an antibody against cardiac-α-actinin (left panel). Comparable differentiation of the sarcomeric apparatus was observed in perlecan-null (KO) embryonic stem cell-derived cardiomyocytes (right panel, EB, 7 + 9 days). (B) Action potentials (APs) from an embryonic stem cell-derived wild-type (left) and perlecan-null (right) cardiomyocyte. (C) Statistic of 90% AP duration (APD90) values. (D) Ramp depolarizations (from −100 to 50 mV, 110 ms) evidenced Ica and Ica expression in an embryonic stem cell-derived perlecan-null cardiomyocyte (7 + 4 days). (E) Effect of the muscarinic agonist carbachol (CCh, right panel) on a representative spontaneously beating perlecan-null cell (7 + 4 days, left panel). (F) Effect of the β-adrenergic agonist Isoprenaline (ISO) and of CCh on Ica in perlecan-null cardiomyocytes. Ica was evoked by 50 ms lasting depolarizing pulses to 0 mV, holding potential −50 mV. Bar = 20 μm.
modulation of the L-type Ca$^{2+}$ current (Figure 1F), indicating intact regulation ($n = 4$) by hormones of the autonomous nervous system. These findings suggest that the increased early embryonic lethality in perlecan-null embryos was not caused by structural or functional defects of cardiomyocytes.

3.2 Early stage perlecan-null embryos display reduced stability of the heart wall

Next we performed dye injection experiments using constant pressure and volume in early stage embryos (E9.5) prior to the onset of blood leakage. While the majority of E10.5 perlecan-null embryos have developed blood leakage into the pericardial cavity (Figure 2A, right panel) and ventricular clefts (Figure 2B), cardiomyocytes in E10.5 wild-type heart build an intact myocardial wall without blood leakage (Figure 2A, left panel). None of the perlecan-null embryos at E9.5 showed blood leakage or abnormalities of the cardiac tissue with light- or electron microscopy (data not shown). To enable microinjections into the heart at this early stage of development, perlecan heterozygous mice were crossed with transgenic mice, in which the human-cardiac-$\alpha$-actin promoter drives the early cardiac expression of EGFP. The intraventricular injection of TRITC-labelled dextran in EGFP-positive wild-type and heterozygous littersmates showed only in 6% of hearts ($n = 33$) leakage of dye into the pericardial cavity (Figure 2C, left panels, Supplementary material online, Video S1). However, 70% ($n = 10$) of perlecan-null embryos presented leakage in the ventricle and outflow tract during the injection (Figure 2C, right panels, Figure 2D, see Supplementary material online, Video S2). Since 30% of perlecan-null embryos survived this critical stage of development, we investigated whether the instability of the myocardial wall persisted in E14.5/E15.5 embryos by injecting dye into the beating hearts. Neither perlecan-null ($n = 8$) nor wild-type and perlecan heterozygous (+/–) ($n = 44$) embryos revealed seepage of the injected dye (Figure 2E). Moreover, even after repetitive injections causing pronounced distensions of hearts, leakage of the dye was neither visible in perlecan-null ($n = 4$) nor in perlecan (+/–) ($n = 19$) embryos (data not shown). To further define the critical time period of embryonic lethality, perlecan-null embryos without hemopericards at E10.5 were functionally analysed by employing the microinjection technique ($n = 3$, data not shown). None of these hearts showed leakage into the pericardial cavity indicating that hemopericards and subsequent death of perlecan-null embryos occurred after E9.5 but before E10.5.

3.3 Basement membranes of perlecan-null hearts are defective

The mechanism leading to the heart muscle defects in perlecan-null embryos occurs between E9.5 and 10.5, which coincides with the assembly of basement membranes and outflow tracts (OFT) were visible (middle and lower panels). In wild-type embryos no trans-passage of dye was observed (left panels), whereas in the perlecan-null embryos leakage into the pericardial cavity was seen (right panels). The white arrow indicates the site of the leak. (D and E) Statistical analysis of dye injection experiments in wild-type and perlecan heterozygous (+/–) and null (–/–) E9.5 (D) and E14.5/15.5 (E) hearts.

Figure 2. Morphology of the myocardial wall and the pericardial cavity in a perlecan-null E10.5 embryo and microinjection of TRITC-labelled Dextran into E9.5 and E14.5/15.5 embryonic ventricles. (A) In the whole mount picture accumulation of blood in the pericardial cavity and around the heart anlage can be observed in perlecan-null (right panel) but not in wild-type (left panel) hearts. (B) Structural analysis in embryos showed a loss of cell–cell contacts leading to discontinuity of the heart wall in a perlecan-null heart. The dashed lines indicate a representative trans-myocardial defect. Note the red blood cells in the pericardial cavity (PC). Endo, endocardium; Epi, epicardium. Bar B = 15 µm. (C) Microinjection of the TRITC-labelled Dextran into EGFP-positive E9.5 hearts. Filling of ventricles (V), atria (A),
around cardiomyocytes. Immunostaining detected perlecan in and adjacent to cardiomyocytes at E9.5 (arrows, Figure 3A) as well as E14.5 (Figure 3B). In contrast, at the adult stage, perlecan staining was restricted to the basement membranes (Figure 6A). To determine the morphological integrity and the functional relevance of the basement membranes in wild-type and perlecan-null embryos, electron microscopy was performed after dye injection experiments. E9.5 wild-type hearts showed clearly visible basement membranes surrounding the cardiomyocytes (Figure 3C, arrows), whereas E9.5 perlecan-null embryos showed no or only rudimentary basement membranes (Figure 3D, arrow heads). Furthermore, basement membranes were either completely absent or rudimentary in E14.5 and E15.5 perlecan-null hearts (Figure 3F) and strikingly differed from that typically found in wild-type hearts at this stage (Figure 3E). The expression of key basement membrane components was determined by western blots of collagen IV and laminins in early embryonic hearts (E9.5). Both proteins were reduced in perlecan-deficient compared with wild-type hearts (Figure 4C and D). We also tested the mRNA level for collagen IV with quantitative PCR and found that it was almost not changed in perlecan-deficient hearts (79 ± 3% of wild-type hearts). The quantitative differences in protein expression also translated into qualitative basement membrane alterations in perlecan-deficient embryonic hearts [collagen IV staining in E9.5 (Figure 4A) and E14.5 (Figure 4B) hearts] suggesting that the lack of perlecan affects the occurrence of a homogeneous and mature basement membrane. These findings indicate that perlecan controls either formation of basement membranes around cardiomyocytes or their maintenance. This implies that the basement membrane disintegrate during mechanical stress triggered by muscle contraction and/or increased blood pressure in the developing embryo (see also Costell et al.4).

Despite severe basement membrane defects at E14.5/E15.5, perlecan-deficient hearts withstood the dye injection pressure. To test whether myocardial cell–cell contacts contributed to the stability of perlecan-deficient hearts, detailed ultrastructural and immunohistochemical analysis was performed using the same protocols described earlier.19,21 The mechanically relevant cell–cell contacts including small fascia adherens and desmosomes interrupted by intercellular spaces were established at E9.5 in both
Cardiomyocytes are not yet fully established. A similar situation re-occurs in the adult heart after myocardial infarction, as wasting of cardiomyocytes causes loss of structural integrity of the ECM and of cell–cell contacts. To determine the function of perlecan during cardiac injury and to elucidate its potential role for the stability of the infarcted heart we generated cryoinfarcts in mouse hearts. This myocardial lesion model was chosen as it results in loss of cell–cell contacts and in contrast to the coronary artery ligation in highly reproducible lesion sizes without pronounced adverse remodelling shortly after the injury. This type of lesion is preferable to aortic banding, as the latter causes hypertrophy and gain of cell–cell contacts. We monitored perlecan deposition in the injured area with and without injecting murine embryonic cardiomyocytes, which express large amounts of perlecan. As can be seen in Figure 6A, faint perlecan immunoreactivity was detected in the intact adult murine myocardium only around cardiomyocytes due to the basement membranes. Within the scar 6 days after cryoinjury, the amount of perlecan was found to be slightly increased and the distribution more homogeneous. Injection of embryonic cardiomyocytes into the infarct led to a strong up-regulation of perlecan within the scar 6 days after the operation. A more detailed analysis of transplanted cardiomyocytes (EGFP fluorescence) and perlecan expression (immunofluorescence) revealed prominent perlecan staining around the engrafted cardiomyocytes, whereas lower perlecan staining was found in the infarcted areas devoid of transplanted cardiomyocytes 7 days after the injury.

To assess the functional relevance of perlecan deposition for the injured heart wall, we tried to transplant perlecan-null cardiomyocytes harvested from E9.5 mice. These experiments failed because we could not obtain enough cardiomyocytes during isolation.

3.4 Perlecan plays an important role for left ventricular function after heart injury

Wild-type and in perlecan-null embryos (Figure 5A and B). At E14.5/E15.5 cell–cell contacts between cardiomyocytes were more mature and became more extended in wild-type and perlecan-null hearts, providing increased mechanical stability to the tissue (Figure 5C and D). The development-dependent maturation of cell–cell contacts was further corroborated by analysing the expression of N-cadherin (Figure 5E–H) and desmoplakin (data not shown), which are main components of the fascia adherens and the desmosomes, respectively. Whereas in E9.5 hearts both proteins were expressed in a spot-like fashion (Figure 5E and F), at later stages they showed a homogeneous expression pattern (Figure 5G and H). This indicates that at both the stages of development wild-type as well as perlecan-null hearts develop normal cell–cell contacts (Figure 5E–H). We assessed this aspect also quantitatively by measuring the ratio of cell–cell contacts and whole cell–cell interface at intercalated discs in electron micrographs (see also Section 2). We found that this ratio was similar in wild-type and perlecan-null embryos at early (wild-type: 15.9 ± 0.6%, perlecan-null: 16.4 ± 0.5%, P > 0.5) and late stage (wild-type: 20.7 ± 4.4%, perlecan-null: 22.4 ± 4.7%, P > 0.5). Altogether our findings suggest that in perlecan-null hearts, the mechanical instability observed at late stage (Figure 2C and D) cannot be explained by altered cell–cell contacts but by the lack of perlecan itself. In addition, we propose that the mechanical stability observed at late stage (Figure 2E) is ensured by the formation and maturation of cell–cell contacts (see also Figure 7).
viable cardiomyocytes from the very few (only 20% of perlecan-null embryos live until E16.5) perlecan-null hearts. Instead, we generated cryolesions without cell transplantation in adult female wild-type and perlecan heterozygous mice with the assumption that perlecan synthesis is insufficient in heterozygous animals under conditions of loss of cell–cell contacts. To assess the perlecan content in these hearts we performed densitometric analysis of perlecan-DAB-stained sections after the injury. The values from the native region showed a tendency but were not significantly \( P = 0.08 \) different between wild-type \((12.9 \pm 1.2, n = 5)\) and perlecan heterozygous animals \((10.0 \pm 0.7, n = 5)\). However, we found significantly \( P = 0.046 \) less perlecan in the scar region of perlecan heterozygous \(8.1 \pm 0.8\) compared with wild-type animals \(14.0 \pm 2.3\). Functional assessment of left ventricular function with a pressure–volume catheter 2 weeks after infarction showed that stroke volume \( P = 0.002 \) and ejection fraction \( P = 0.003 \) were significantly lower in the perlecan heterozygous than in wild-type mice (Figure 6F), implying compromised left ventricular function. Heart rate, end-diastolic and end-systolic pressures, and end-diastolic volumes were unchanged (Figure 6F).

4. Discussion

In a previous study, we showed that the constitutive ablation of the perlecan gene in mice leads to heart defects in ~70% of embryos at E10.5 characterized by blood leakage into the pericardial cavity, the development of hemopericards, and arrest of heart function.\(^4\) In the present paper, we investigated the cause for these heart defects and tested the role of perlecan during repair of damaged heart tissue. We found that at the onset of heart development perlecan is an adhesive substrate for cardiomyocytes and an essential player for maintaining the basement membrane surrounding cardiomyocytes. At later stages of embryonic development, heart stability is achieved by the formation and maturation of cell–cell contacts. Interestingly, infarcted adult heart tissue displays a similar requirement of perlecan for stabilization of the damaged heart muscle wall.

In the adult heart the disci intercalares composed of desmosomes and adherens junctions are the most important structure that provides mechanical stability.\(^24\) Between E9 and E10, however, we found only random and immature cell–cell contacts between the relatively loosely aligned cardiomyocytes, indicating that other mechanisms must exist to stabilize the developing heart. Cell–cell contacts are not responsible for the mechanical instability induced by perlecan deficiency, because neither qualitative nor quantitative differences were detected in perlecan-null compared with wild-type hearts at both early and later embryonic stages. We therefore propose that adhesion of cardiomyocytes to ECM proteins including perlecan, laminin, and collagen IV ensure mechanical stability until the cell–cell contacts have formed and matured. Differentiating cardiomyocytes deposit basement membrane components to promote its assembly. Perlecan-deficient cardiomyocytes are, at the ultrastructural level, not surrounded by a typical basement membrane after mechanical stress although laminin and collagen IV are expressed. Importantly, lack of basement membranes in perlecan-deficient
hearts is most likely due to changes in the stability of key basement components like collagen IV and laminins that are reduced in the knock-out hearts. The intact basement membranes in perlecan-null skin or kidneys point to an essential role of perlecan for maintaining mechanically stressed basement membranes rather than for their formation. This was unequivocally confirmed using pressure-controlled dye injections into the beating heart tubes of wild-type and perlecan-null mice revealing dye leakage in perlecan-deficient hearts already at E9.5 before morphological changes or hemopericards could be detected. Although the end-diastolic volume at E9.5 is unknown, several arguments suggest that the instability of perlecan-null hearts is unrelated to technical issues. First, we injected a volume of 35 nL dye, which is far below the volume of 160 nL reported for E10.5 hearts. Second, the heart tube was not overextended upon dye injection and wild-type hearts of the same developmental stage tolerated the dye injections without leakage. Finally, in accordance with our microinjection data we have shown earlier that around 70% of the E10.5 perlecan-deficient embryos died of hemopericards.4

The important contribution of the cell–cell contacts to heart stability is supported by reports of mice deficient in plakoglobin, a component of desmosomal contacts and N-cadherin, a component of adherens junctions. Plakoglobin-deficient mice die, however, after E10.5 as well as after E10.5.20,21 N-cadherin-deficient mice show an abnormal heart morphogenesis but no signs of reduced stability of the early embryonic heart, as injections of ink into ventricles did not result in transmural leakage.22 This suggests that the heart muscle-related stability becomes relevant at a later time point during development. Although the basement membranes of hearts were still defective in E14.5/E15.5 perlecan-deficient embryos, the physiological maturation of cell–cell contacts during development explains the improved stability of the heart wall (see also Figure 7). In fact, even repetitive injections of dye (700 nL) at volumes similar to the end-diastolic volume (570 nL25) never caused dye leakage in perlecan-deficient embryos. Interestingly, in E14.5 perlecan-deficient embryos we noticed delayed ejection of the dye (data not shown), which can nicely be explained with a report showing that hearts of perlecan-null embryos develop malformations of the outflow tract.6

Mutations of unc-52, the nematode homologue of perlecan, lead to a disruption of sarcomeres and detachment of body wall muscles.19 Furthermore, perlecan has recently been shown to play a role during skeletal muscle myogenesis.28 Because of differences between nematode unc-52 and mouse perlecan domain structures19 as well as skeletal and cardiac muscle, we have analysed perlecan-null ES cell-derived cardiomyocytes. These cells display intact sarcomeric organization, normal ion channel expression, and electrical function. Unlike β1 integrin-null cardiomyocytes,29 the perlecan-deficient cells established normal intracellular signalling cascades in the form of β-adrenergic and muscarinic hormonal modulation (Figure 1E and F). Heart infarction causes the demise of cardiomyocytes and loss of cell–cell contacts. In the cryoinfarction model, relatively shortly after the lesion is a particularly vulnerable phase of scar formation, because the original structure is lost and the invading fibroblasts have not yet deposited ECM such as collagen.30 We noticed that at this critical stage perlecan distribution had switched from basement membrane localization (Figure 6A) to a more diffuse

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**Figure 7** Schematic representation of the experiments, the results thereof and the proposed interpretation/hypothesis.
pattern (Figure 6B). We therefore assume that the ECM and in particular perlecan could, similar to the early embryonic heart, play a critical role for heart stability by acting as a 'molecular glue'. The relevance of the ECM for heart stability is based on earlier studies from our laboratory, where we noticed a clear improvement of left ventricular function by the engraftment of electrically not coupling skeletal myoblasts and/or relatively low numbers of embryonic or ES cell-derived cardiomyocytes. It is unlikely that the grafted cells and/or neo-vascularization actively enhance left ventricular function. Rather, passive ECM-related effects appear to underlie the observed improvement of function because perlecan content is strongly up-regulated in the areas surrounding the transplanted embryonic cardiomyocytes. These findings are corroborated by an earlier study, where increased perlecan content was reported in areas containing reversibly damaged cardiomyocytes. We have shown that transplanted embryonic cardiomyocytes up-regulate and release perlecan into the extracellular space (Figure 6C and D). We tried to further prove this by transplanting perlecan-null cardiomyocytes into infarcted heart tissue, however, this approach failed because sufficient numbers of perlecan-deficient cardiomyocytes could not be harvested from perlecan-null embryos. Therefore, we generated cryoinjuries without cell transplantation in wild-type and perlecan heterozygous mice reasoning that perlecan synthesis in the perlecan heterozygous mice may be reduced compared with wild-type animals under conditions of increased demand. This could be confirmed with densitometric analysis of perlecan stained infarcts. Left ventricular function measurements with a pressure-volume catheter by a blinded investigator showed that end-diastolic pressure and volume did not differ, ruling out prominent differences in diastolic function. Instead, perlecan heterozygous mice suffered from a significantly lower stroke volume and ejection fraction than wild-type animals. This appears not to be due to differences in the active force of contraction because end-systolic pressure (Figure 6) and dP/dt max (data not shown) were not significantly different (indicating similar cell survival post-injury). Therefore, the most likely mechanism underlying the reduced stroke volume in perlecan heterozygous mice are changes in the passive properties of the scar, such as reduced stiffness, possibly associated with paradox movement during systole (see also Figure 7). These data suggest that cellular cardiomyoplasty may, at least in part, work through stabilization of the infarcted heart wall by ECM proteins such as perlecan leading to improved left ventricular function.

We conclude that perlecan plays a key role in maintaining the physical integrity during early embryonic development and in the adult heart after injury.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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