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Research Paper

Limitations of the MRL mouse as a model for cardiac regeneration

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Abstract

Objective Myocardial repair following injury in mammals is restricted such that damaged areas are replaced by scar tissue, impairing cardiac function. MRL mice exhibit exceptional regenerative healing in an ear punch wound model. Some myocardial repair with restoration of heart function has also been reported following cryoinjury. Increased cardiomyocyte proliferation and a foetal liver stem cell population were implicated. We investigated molecular mechanisms facilitating myocardial repair in MRL mice to identify potential therapeutic targets in non-regenerative species.

Methods Expressions of specific cell-cycle regulators that might account for regeneration (CDKs 1, 2, 4 and 6; cyclins A, E, D1 and B1; p21, p27 and E2F5) were compared by immunoblotting in MRL and control C57BL/6 ventricles during development. Flow cytometry was used to investigate stem cell populations in livers from foetal mice, and infarct sizes were compared in coronary artery-ligated and sham-treated MRL and C57BL/6 adult mice.

Key findings No differences in the expressions of cell cycle regulators were observed between the two strains. Expressions of CD34+Sca1+ckit-, CD34+Sca1+ckit+ and CD34+Sca1-ckit+ increased in livers from C57BL/6 vs MRL mice. No differences were observed in infarct sizes, levels of fibrosis, Ki67 staining or cardiac function between MRL and C57BL/6 mice.

Conclusions No intrinsic differences were observed in cell cycle control molecules or stem cell populations between MRL and control C57BL mouse hearts. Pathophysiologically relevant ischaemic injury is not repaired more efficiently in MRL myocardium, questioning the use of the MRL mouse as a reliable model for cardiac regeneration in response to pathophysiologically relevant forms of injury.

Keywords cell cycle; heart; MRL mouse; myocardial infarction; stem cell

Introduction

Myocardial repair in mammals, e.g. following a myocardial infarction, is compromised, as terminally differentiated adult ventricular cardiomyocytes demonstrate very limited capacity for cell division. The majority of damaged cells therefore cannot be replaced by new cardiomyocytes following injury and a scar is formed.[1–3] Large infarcts can produce significant scar tissue, resulting in compensatory cardiac remodelling, a decrease in cardiac function and possibly heart failure and death. Approaches for stimulating myocardial repair and regeneration following infarct rather than allowing scar formation are therefore desirable. Several avenues have been investigated to facilitate repair and regeneration of the adult heart including cell therapy, stem cell mobilisation and gene therapy.[4–7]

Amphibians and zebrafish display exceptional myocardial regenerative capabilities following injury, demonstrating cardiomyocyte division and complete regeneration without formation of scar tissue.[8–11] This regenerative capacity is not seen in mammals, where response to injury generally is repair with scarring.[12] However, the MRL/MpJ (MRL) mouse is a unique strain that displays some regenerative capacity reminiscent of amphibians. Following a through-and-through ear punch, MRL mice can completely regenerate the damaged area, leading to normal tissue architecture and cartilage replacement.[13,14] The

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MRL ear hole closes by formation of a blastema-like structure similar to that observed in amphibians following injury, leading to a regenerative response in multiple tissues.[12,13] As such, this mouse provides a useful model for studying enhanced regeneration in mammals.[15]

Interestingly, some studies in MRL mice have demonstrated enhanced regeneration in the myocardium.[16] Thus, MRL mice display extensive regeneration of cardiac tissue with restoration of heart function following cryoinjury to the right ventricle in comparison to control C57BL/6 mice.[16–18] Initiation of cardiomyocyte proliferation and the presence of a novel stem cell population in the foetal livers of MRL mice have been implicated in this regenerative response.[16,17,19] These initial findings suggest that it might be possible to repair the mammalian myocardium following injury without the formation of a scar. This would provide significant clinical improvement for human infarct patients.[19,20] Contrary to these early findings, other studies using different injury models have failed to observe myocardial regeneration in MRL mice and have reported scars and impaired cardiac function.[15,20,21]

Since the majority of clinical myocardial infarctions affect the left ventricle and are the result of a period of myocardial ischaemia caused by occlusion of a coronary artery by a thrombus,[22] identification of the mechanisms underlying cardiac regeneration in MRL mice might reveal potential therapeutic targets that could be manipulated in humans to enable myocardial regeneration following infarct. The purpose of this study was to identify the mechanisms facilitating myocardial repair in MRL mice compared to non-regenerative control mice, C57BL/6, focusing on possible intrinsic differences in cell cycle control and the previously reported novel foetal-liver stem-cell population.[16,17] In parallel, we wished to confirm that regenerative capacity exists in MRL mouse hearts when using a more clinically relevant left ventricular injury model of permanent coronary artery occlusion.

Materials and Methods

Materials

Anti-Ki67, anti-CDC 2 and anti-cyclin B1 were obtained from Abcam (Cambridge, UK) and anti-p21 was from Merck Biosciences (Nottingham, UK). FITC-conjugated CD34 antibody was obtained from eBiosciences (San Diego, CA, USA). PE-conjugated Ly-6A/E (Sca-1), APC-conjugated CD117 (c-kit) antibodies were supplied by BD Pharmingen (Oxford, UK). All other primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse-radish peroxidase-conjugated secondary antibodies were purchased from DakoCytomation (Ely, UK) and AlexaFluor 488 anti-rat and anti-rabbit secondary antibodies were from Molecular Probes (Eugene, OR, USA). Mounting medium was supplied by Vector Laboratories Ltd (Peterborough, UK). Nitrocellulose membrane was from GE Healthcare and PVDF membranes and chemiluminescence detection kits were from Millipore (Billerica, MA, USA). All other chemicals and biochemicals were from Sigma-Aldrich Company (Dorset, UK) or Fisher Scientific UK (Loughborough, UK).

Mice

MRL/MpJ and C57BL/6 control mice were obtained from the Jackson Laboratory (ME, USA). All experimental procedures using animals were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986, approved by the ethical committees of University of Reading and King's College London and were carried out in accordance with guidelines of local animal care committees.

Immunoblot analysis

Proteins (40 μg) were extracted from the hearts of MRL and C57BL/6 mice, separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose or PVDF membranes (depending on the size of the protein of interest) as previously described.[23] Following blocking in 5% BSA in Tris-buffered saline (TBS)/0.1% Tween 20, membranes were incubated overnight at 4°C in primary antibody diluted in blocking buffer. We investigated the expression of positive regulators of the cell cycle (CDC 2, CDK 2, CDK 4, CDK 6, cyclins A, E, B1 and D1), negative regulators of the cell cycle (p21, p27 and E2F5) and phosphorylation of histone H3 at serine 10 or serine 28, which correlates with chromosome decondensation during mitosis. The antibodies were used in the following dilutions: CDC 2, 1 : 1000; CDK 2, 1 : 500; CDK 4, 1 : 200; CDK 6, 1 : 3000; cyclin A, 1 : 500; cyclin E, 1 : 100; cyclin D1, 1 : 100; cyclin B1, 1 : 100; p21, 1 : 500; p27, 1 : 100; E2F5, 1 : 250; histone H3 phospho-serine 10, 1 : 500; histone H3 phospho-serine 28, 1 : 100; and GAPDH, 1 : 40 000. Membranes were washed and incubated in goat anti-rabbit or goat anti-mouse-horseradish peroxidase-conjugated antibody (1 : 4000 diluted in blocking buffer) for 1 h. Finally, membranes were washed and positive immunoreactive bands detected by chemiluminescence. Densitometry was carried out using Quantity One® version 4.0.2 (Bio-Rad, Hemel-Hempstead, UK).

Flow cytometry

Flow cytometry was used to evaluate three known stem cell markers, CD34 (marker of haematopoietic precursors), c-kit (receptor for stem cell factor) and Sca-1 (expressed on multipotent haematopoietic stem cells) in MRL and C57BL/6 foetal livers.[6,24] A single cell suspension of unfixed, healthy foetal liver cells (embryonic day 16 to 18) was prepared from MRL and C57BL/6 mice by gently passing liver tissue from four to nine foetuses from separate litters back and forth through a 21-gauge needle followed by filtration through a 40 μm nylon mesh. Cells from three independent cell preparations were suspended in PBS containing 2% (v/v) foetal calf serum stained with FITC-conjugated CD34, PE-conjugated Sca-1 and APC-conjugated c-kit antibodies at a concentration of 1 μg per 10^6 cells for 45 min at room temperature. Control cells were incubated with the appropriate directly-conjugated isotype controls (Pharmingen, Oxford, UK). Cells were washed and analysed using a FACSCalibur flow cytometer (Becton Dickinson, UK).

Surgical procedures

Permanent left anterior descending (LAD) coronary artery ligation was performed on both MRL and C57BL/6 mice

producing a severe transmural injury as previously described.[22] A combination of ketamine (10 mg/ml) and medetomidine (20 μg/ml) was used for general anaesthesia and analgesia, and administered intraperitoneally at 10 μl/g mouse body weight. Control mice received a sham procedure (no ligation). Surgery was carried out on male mice aged 8–10 weeks.

Analysis of cardiac function

Four weeks after surgery, mice were anaesthetised and left ventricular function assessed using a miniature conductance micromanometer (1.4 F, SPR 716) (Millar Instruments, Texas, USA). The conductance catheter was inserted via the carotid artery and measurements of both pressure and volume collected as previously reported.[25]

Planimetry and histology

Following functional analysis, hearts were arrested in diastole by the injection of 15% (w/v) KCl. Hearts then were fixed by retrograde infusion with 10% (v/v) formaldehyde using the Langendorff perfusion system. Hearts were embedded in 5% (w/v) agarose and sectioned into 700 μm sections with a vibratome.[22] The infarct zone was paler than healthy tissue, and the LV thickness was reduced. Planimetry was performed using SigmaScan software. Histological analysis of vibratome sections was carried out on formaldehyde-fixed paraffin-embedded sections cut to a thickness of 5 μm and stained with Masson's trichrome stain enabling visualisation of collagen deposition within the section. Serial images of the left ventricle were captured and the percentage of blue staining, denoting collagen, vs red staining, denoting muscle and cytoplasm, was calculated using the computer program, KS-300 version 3.0. (Carl Zeiss, New York, USA)

Immunohistochemistry

Fixed foetal and adult heart tissues (LAD-ligated and sham-operated) were embedded and cryosectioned at a thickness of 4 μm. Sections were permeabilised (0.5% triton-X-100 in PBS) for 15 min, washed and endogenous peroxidases quenched with 0.3% (v/v) H_2O_2 in 0.3% (v/v) goat serum in PBS for 5 min. Sections were washed, blocked in 5% goat serum for 1 h and incubated in Ki67 antibody (1 : 200) overnight at 4°C. Following washing, sections were incubated in biotinylated secondary antibody and visualised using ABC and DAB substrate staining (Vector Laboratories Ltd, Peterborough, UK). Sections were counterstained with Eosin (1% (w/v) in 70% ethanol) and mounted.

Statistical analysis

All results are presented as means ± standard error of the mean. Data were analysed by non-parametric one-way ANOVA (Mann–Whitney or Kruskal–Wallis where appropriate); $P < 0.05$ was considered significant.

Results

Initially, we confirmed the regenerative capacity of MRL mice following injury to the ear, which has previously been reported.[13,14] MRL and C57BL/6 control mice were given through-and-through ear punch wounds that were monitored for 4 weeks following injury. We verified previous studies and observed complete closure of the ear-hole wound in MRL mice within 4 weeks. Tissue regeneration was not observed in C57BL/6 control animals ($n = 3$; data not shown).

MRL mouse heart retains normal cell cycle characteristics during development and growth

Previously published data has demonstrated the ability of MRL mice to undergo myocardial repair following cryoinjury to the right ventricle,[16] suggesting that cardiomyocytes in this strain are capable of re-entering the cell cycle and proliferating in response to injury. To determine whether this results from an intrinsic difference in cell-cycle control, the expression of cell-cycle regulators in MRL and C57BL/6 mice was assessed in ventricular tissues at different stages of development, during which cardiomyocytes become withdrawn from the cell cycle and lose the ability to proliferate.[26,27] Adult ventricular tissue was separated into left ventricle plus septum and right ventricle to investigate any differences between the two sides of the heart that might be responsible for the previously reported healing following cryoinjury to the right ventricle.[16,17] Representative immunoblots are shown in Figure 1. Interestingly, no discernable difference was observed in the levels of any of the cell cycle regulators in MRL or C57BL/6 heart tissues ($n = 3$), suggesting that cell-cycle control during development is intrinsically the same between the two strains. This was valid for ventricular tissues obtained from foetal hearts at embryonic day 18 (E18), neonatal hearts at 1 (P1) and 6 (P6) days after birth, and adult hearts. Furthermore, no differences in cell cycle molecule expression were noted between the left and right ventricles of adult MRL and C57BL/6 mice.

MRL mice do not express increased stem cell markers in foetal liver tissue

Previous studies (e.g.[17]) have suggested that E18 MRL mouse livers possess a novel stem cell population that is responsible for the regenerative potential in this strain. The presence of potential stem cell populations, determined by the markers CD34, c-kit and Sca-1, was compared in foetal liver tissues from MRL and C57BL/6 mice. Flow cytometry was used to determine subpopulations of cells expressing different combinations of the stem cell markers and the results are summarised in Table 1 ($n = 3$). Three subpopulations of CD34 positive cells, namely CD34+ Sca1+ c-kit-, CD34+ Sca1+ c-kit+, and CD34+ Sca1- c-kit+, were significantly increased in C57BL/6 mice compared to MRL. All other potential subpopulations of stem cells remained at comparable levels between strains.

MRL mice show no regeneration of left ventricle following coronary artery ligation

Regeneration of the myocardium has been reported in the *right* ventricle of MRL mice following cryoinjury.[16] In the present study, we determined whether a similar regenerative potential could be achieved in the left ventricle of these mice following a more pathophysiological form of myocardial infarction. Thus, we induced a severe transmural injury in MRL

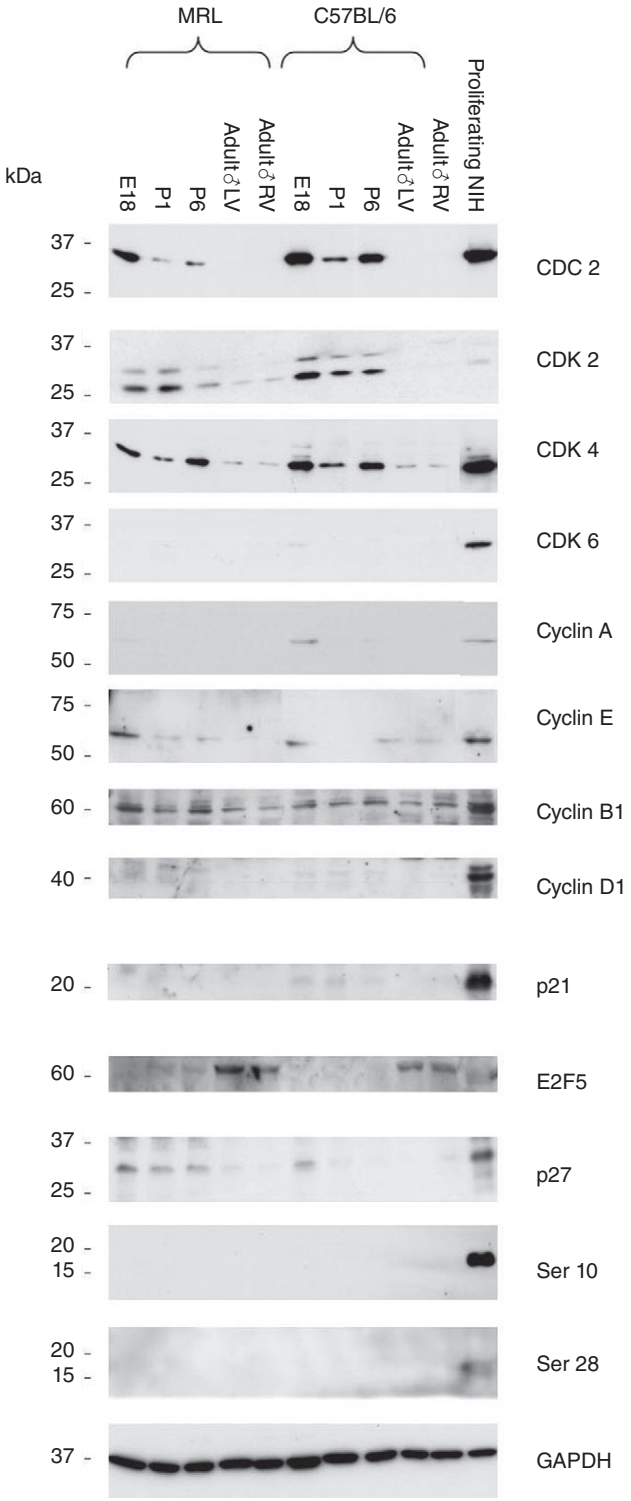

Figure 1 Changes in cell cycle proteins during development in MRL and C57BL/6 mice. Protein expressions of CDC 2, CDK 2, CDK 4, CDK 6, cyclin A, cyclin E, cyclin B1, cyclin D1, E2F5, p21, p27, histone H3 phosphoserine 10 and histone H3 phosphoserine 28 in total ventricular tissues isolated from E18, P1 and P6 MRL and C57BL/6 mouse hearts and in both left and right ventricular tissues from adult male animals. GAPDH was used to ensure equal protein loading. Proliferating mouse fibroblast cells (NIH 3T3) were included as a positive control, confirming that the proteins detected were cell-cycle regulated. Representative blots from three separate experiments carried out on sample sets taken from different litters are shown.

Table 1 Comparison of foetal liver stem cell populations in MRL and C57BL/6 mice

	MRL (% total cells ± SEM)	**C57BL** (% total cells ± SEM)
CD34+ Sca1+ ckit-**	0.26 ± 0.02	1.27 ± 0.10
CD34+ Sca1+ ckit+**	0.15 ± 0.009	0.45 ± 0.02
CD34+ Sca1- ckit-	2.35 ± 0.18	2.47 ± 0.31
CD34+ Sca1- ckit+**	0.74 ± 0.009	1.50 ± 0.08
CD34- Sca1+ ckit-	0.00 ± 0.03	0.67 ± 0.57
CD34- Sca1+ ckit+	0.02 ± 0.003	0.06 ± 0.02
CD34- Sca1- ckit+	1.51 ± 0.06	1.44 ± 0.10

Unfixed foetal liver cell suspensions were prepared from MRL and C57BL/6 mice, stained and analysed using flow cytometry to evaluate the presence of stem cell populations. Antibodies against CD34, Sca1 and c-kit were used as markers to distinguish between different stem cell subpopulations and flow cytometric profiles were gated to identify the number of cells expressing each combination of markers (seven subgroups). The table quantifies the number of cells in each subgroup as a percentage of the total number of cells present in the foetal liver preparation (±standard error of the mean; $n = 3$). ** denotes populations that are significantly different ($P < 0.01$) in MRL and C57BL/6 mice.

and C57BL/6 mice using permanent ligation of the LAD coronary artery.[22]

LAD ligation was performed in eight C57 mice and ten MRL mice. Sham operation was performed in seven MRL mice. At the end of 4 weeks, five, six and five animals were alive, respectively, and all showed a similar rate of survival (62.5, 60 and 71%, respectively). Technical difficulties at the time of contractility assessment resulted in complete data collection only in three animals in the C57 group and five animals in the MRL group. Furthermore, two MRL LAD animals did not have myocardial infarction and were excluded.

All animals receiving sham surgery displayed a normal, healthy left ventricular myocardium 4 weeks after surgery (Figure 2a). However, severe myocardial thinning was seen in both MRL and C57BL/6 mice 4 weeks after LAD occlusion (Figure 2a). Surprisingly, MRL and C57BL/6 mice showed comparable infarct sizes in the *left* ventricle following injury (Figure 2b), with similar infarct thicknesses (Figure 2c, $n = 3$). Furthermore, both mouse strains showed equivalent levels of compensatory hypertrophic growth following surgery, as measured by thickness of the left ventricle opposite the infarcted area (Figure 3, $n = 3$). *In vivo*, cardiac function (assessed as cardiac contractility, pressure and volume) was evaluated in all hearts using a conductance catheter 4 weeks after surgery; the results are summarised in Table 2 ($n = 3$). No differences in heart function were seen between MRL and C57BL/6 mice, suggesting that MRL mice showed comparable infarct sizes, hypertrophic growth and cardiac function to control, non-regenerative mice following LAD ligation. MRL sham-operated animals had normal cardiac function (data not shown).

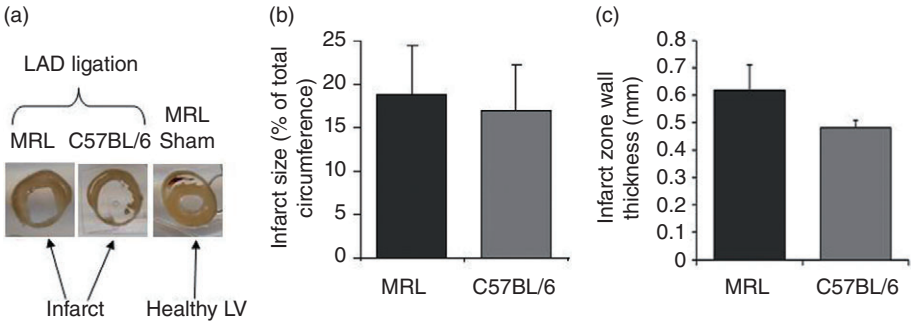

Figure 2 (a) Comparison of infarcts produced by LAD ligation of MRL and C57BL/6 mice. (b) MRL and C57BL/6 mice showed comparable infarct sizes and (c) thicknesses following LAD ligation ($n = 3$). No changes were observed in sham animals.

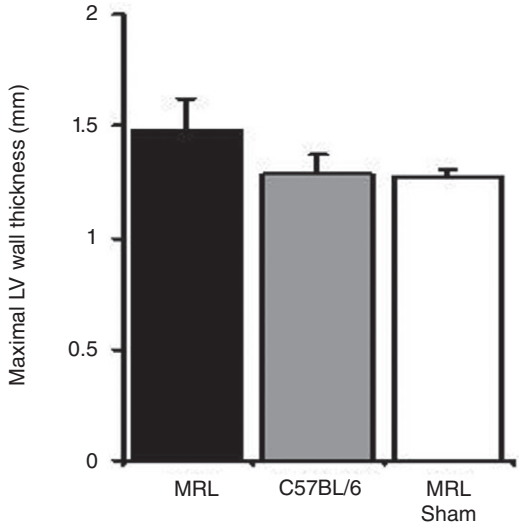

Figure 3 Evaluation of hypertrophic growth following LAD ligation. Maximal left ventricular wall thickness (opposite the infarcted zone) was measured after 4 weeks to evaluate the extent of hypertrophy in MRL and C57BL/6 mice following LAD ligation in comparison to sham ($n = 3$).

Table 2 Assessment of cardiac function following coronary artery occlusion

	MRL infarct $n = 3$	C57 infarct $n = 3$	*P*
Heart rate (/min)	420 ± 53.8	394 ± 49.5	0.86
Stroke volume (µl)	18.38 ± 6.97	19.41 ± 6.25	0.43
Cardiac output (µl/min)	7006 ± 2004	8168 ± 3142	0.56
Ejection fraction (%)	38.5 ± 10.2	44.7 ± 18.4	0.47
Maximum pressure (mmHg)	93.5 ± 8.59	89.2 ± 8.24	0.47
Minimum pressure (mmHg)	4.24 ± 3.05	3.48 ± 2.91	0.98
Minimum volume (µl)	37.7 ± 12.1	43.0 ± 32.4	0.96
Maximum volume (µl)	56.2 ± 16.4	62.2 ± 32.0	0.91
dP/dt_{max} (mmHg/s)	5410 ± 694	5710 ± 1500	0.75
dP/dt_{min} (mmHg/s)	–4540 ± 903	–4470 ± 1280	0.72
Tau (Glantz) (ms)	16 ± 3.45	19.9 ± 8.52	0.88
Arterial elastance (mmHg/µl)	6.84 ± 1.83	5.35 ± 1.53	0.59
Stroke work (mmHg/µl)	1404 ± 587	1498 ± 603	0.56
Maximal elastance (mmHg/µl)	8.09 ± 2.86	4.55 ± 1.73	0.53
EDPVR (mmHg/µl)	1.70 ± 0.66	1.67 ± 1.10	0.95
ESPVR (mmHg/µl)	46.8 ± 18.2	34.2 ± 16.5	0.4
PRSW (mmHg/µl)	150 ± 37	154 ± 71	0.5

A conductance catheter was used to evaluate cardiac contractility and function in MRL and C57BL/6 mice 4 weeks after surgery ($n = 3$). dP/dt_{max}, maximum derivative of change in systolic pressure over time; dP/dt_{min}, minimum derivative of change in systolic pressure over time; EDPVR, end-diastolic pressure volume relationship; ESPVR, end-systolic pressure volume relationship; PRSW, preload recruitable stroke work.

Collagen and connective tissue were visualised and compared in sham and infarcted hearts using Masson's trichrome stain (representative images shown in Figure 4a). The percentage collagen (blue) staining vs muscle and cytoplasm (red) staining was calculated for the mid-ventricular level of the left ventricle, encompassing both the infarcted and non-infarcted regions to eliminate bias. Comparison of the percentage of collagen present in the left ventricle demonstrates a similar level of fibrosis in the myocardium of MRL and C57BL/6 mice (Figure 4b, $n = 3$). Collagen/infarct size ratios were also similar (data not shown). This data confirms that comparable levels of collagen deposition are found in the scar tissue of MRL and C57BL/6 mice following injury to the left ventricle following LAD ligation.

Finally, the presence of the proliferation marker, Ki67, was evaluated in frozen sections of infarcted heart tissues from MRL and C57BL/6 mice (Figure 5). DAB staining was used to visualise nuclei expressing the Ki67 antigen (shown in brown). The presence of Ki67 positive nuclei in the infarct border zone was used to determine whether MRL possessed an increased proliferative potential following myocardial injury in comparison to C57BL/6 mice. As a positive control, foetal heart sections were stained for Ki67 and showed the presence of Ki67 positive nuclei (Figure 5a). MRL and C57BL/6 infarcted mouse hearts (Figure 5c and 5d, respectively) showed no discernable difference in Ki67 staining in the border zone of the infarct, with both strains demonstrating comparable numbers of positively stained nuclei to the sham-operated control (Figure 5b).

Discussion

MRL mice have previously been shown to demonstrate significant regenerative capabilities in a range of tissues, including the heart following myocardial injury, although the mechanism of repair is not currently understood. In this

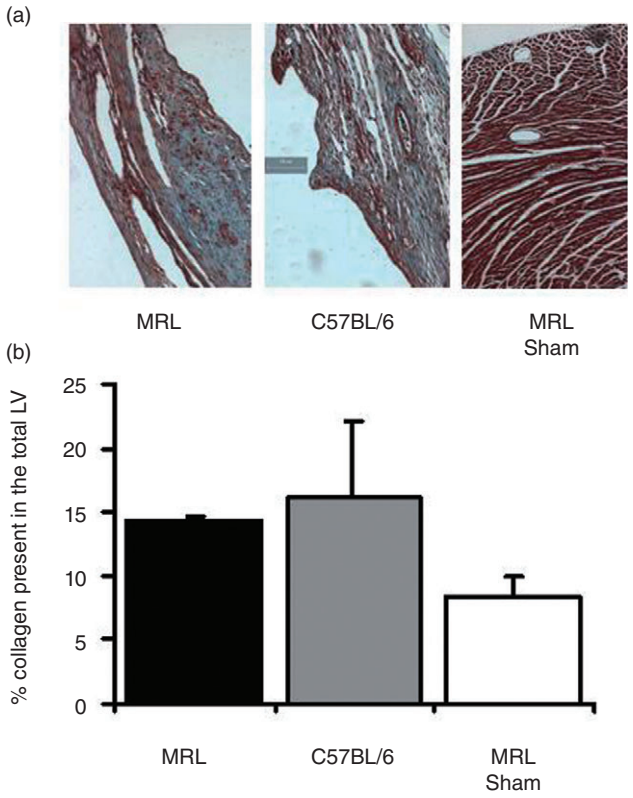

Figure 4 Comparison of myocardial fibrosis following myocardial infarction in MRL and C57BL/6 mice. (a) Trichrome Masson staining of paraffin-embedded heart sections demonstrates the extent of collagen deposition (blue) compared to muscle/cytoplasm (red) in MRL and C57BL/6 following LAD ligation and sham. (b) The percentage of collagen present in the mid-ventricular level of the left ventricle was quantified as the percentage of blue vs red staining ($n = 3$).

study we have investigated whether or not there are differences in specific cell-cycle regulators in myocardial tissues of MRL and C57BL/6 mice during development that might explain potential differences in myocardial growth and/or regenerative capacity between these strains. Interestingly, no intrinsic differences in expression of these molecules were observed. The possibility of a novel stem cell population being present in the livers of foetal MRL mice was explored and, surprisingly, we found an increase in three stem cell subpopulations in control C57BL/6 mice compared to MRL mice. Finally, we examined the regenerative capacity of MRL mouse hearts following a pathophysiologically relevant injury model and observed similar infarct sizes, levels of myocardial scarring and impaired cardiac function as in control, C57BL/6 mice.

Is increased cardiomyocyte proliferation responsible for MRL heart regeneration?

A previous study by Leferovich *et al*. suggested that repair of cryoinjured right ventricles in MRL mice resulted from cardiomyocyte proliferation.[16] We investigated the potential for intrinsic differences in cell-cycle control, during development and following injury, between MRL and non-regenerative C57BL/6 mouse hearts and demonstrated that both strains express similar levels of these molecules. This suggests that if surviving cardiomyocytes in MRL mice are capable of re-initiating cell division post-injury to repopulate the damaged zone with new myocytes then they are stimulated to do so *de novo* rather than as a result of activation of special intrinsic properties of their cell cycle machinery. An alternative explanation might be that a small subpopulation of myocardial cells exists that exhibits stem cell properties with different cell-cycle control characteristics that are not quantitatively detectable with our methods. It is feasible that such cells become activated only following injury. Since the right ventricle is reported to regenerate following cryoinjury,[16,17] whilst the left ventricle does not,[15,20,21] the fact that we could detect no discernible differences between right and left ventricular tissues additionally supports the hypothesis that any regenerative capacity in MRL cardiomyocytes does not reside in the cell-cycle machinery associated with the differentiated cardiomyocyte population.

Foetal liver stem cells

Another scenario for cardiomyocyte regeneration in MRL mice involves proliferation and subsequent differentiation from stem cells. It has been reported that livers of foetal MRL mice might hold a novel stem cell population capable of transferring the phenotype of cardiac regeneration (but not ear wound closure) by foetal liver cell transfusion into a non-regenerative mouse strain 30–45 days prior to injury.[17] In this study, we characterised cells in foetal liver using the classical stem cell markers CD34, c-kit and Sca-1.[6,24] It was expected that MRL livers might possess more cells expressing stem cell markers or, in respect of the lack of cardiac regeneration observed in MRL mice, that C57BL/6 foetal liver cells might demonstrate a similar profile to MRL mice. Surprisingly, we found an increase in three stem cell subpopulations in C57BL/6 mice as opposed to expression in MRL. Consistent with this observation, a recent publication has reported that pluripotent stem cell lines from mouse strains with different genetic backgrounds (129 and C57BL/6 mice) exhibit grossly different gene expression profiles.[28] As such, inherent strain-dependent differences in stem cell profiles cannot be eliminated and this might be responsible for the differences we observed between stem cells obtained from MRL and C57BL/6 livers. A further possibility includes the fact that MRL liver stem cells might be more efficient in terms of integrating and regenerating within the young myocardium. However, in the MRL foetal liver cell transplant study[17] no quantitative contribution of Y-chromosome cells to repair the injured right ventricle was reported by day 30 and only a few cells were observed with weak troponin-I co-staining. In a previous study by this same group,[16] significant repair of the right ventricle was observed by day 15 post-injury, suggesting that the Y-chromosome liver cells in their transplant study[17] contributed predominantly to the connective tissue pool, rather than to the production of new cardiomyocytes. Interestingly, there is an increasing body of evidence supporting the fact that the control of cardiomyocyte proliferation is not intrinsic to the cardiomyocytes themselves, but that signalling from fibroblasts in the connective tissue is responsible.[29]

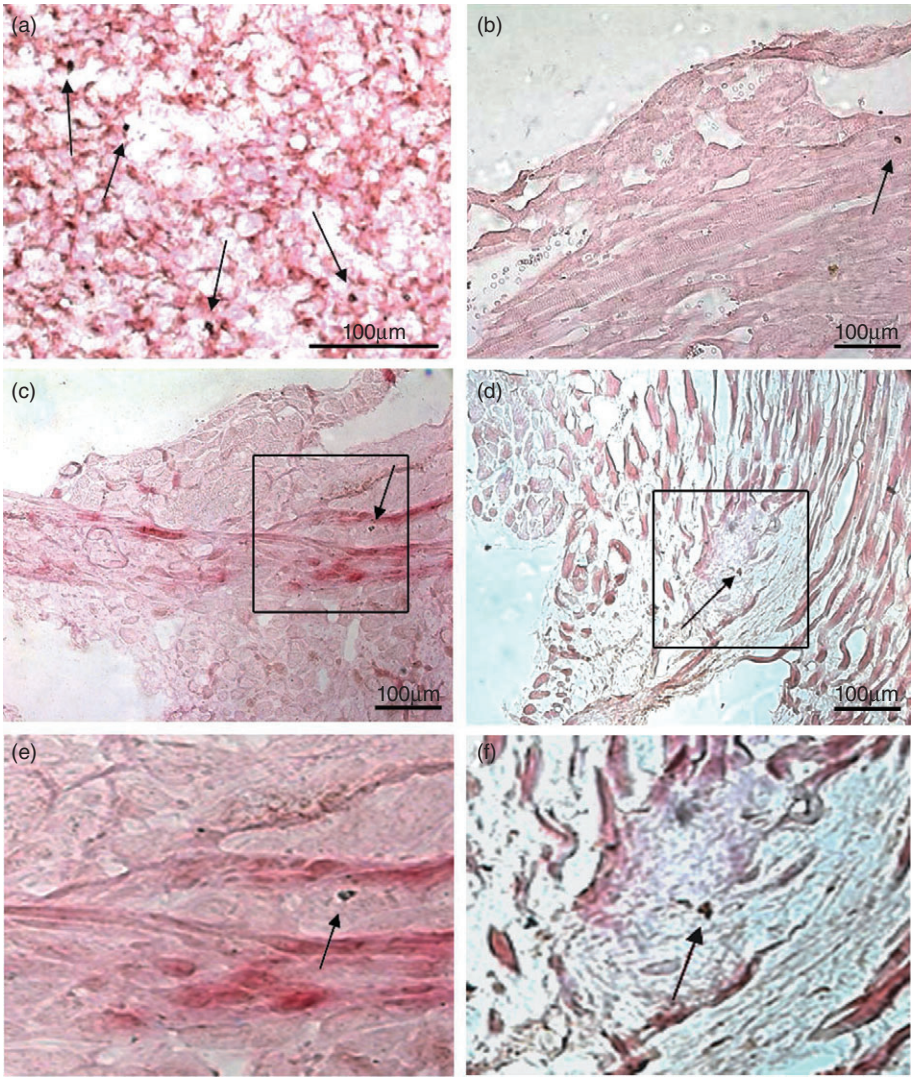

Figure 5 Comparison of Ki67 staining in MRL and C57BL/6 infarcted hearts. Cryosectioned C57BL/6 (c) and MRL (d) infarcted hearts (4 µm) were stained with Ki67 (brown nuclei) to identify the number of proliferating cells within the infarct border zone (infarct located to the left) following LAD ligation. Arrows denote Ki67-stained nuclei. Boxed regions in panels (c) and (d) have been enlarged further to show Ki67-positive nuclei in the border zones of C57BL/6 (e) and MRL (f) infarcts. Staining also was carried out in foetal heart (E18) tissue as a positive control (a) and sham MRL tissue to evaluate non-infarcted adult tissue (b). Sections were counterstained with eosin. Scale bars indicate 100 µm.

Proliferation of cardiomyocytes in MRL mice

Earlier studies have suggested that MRL heart regeneration following cryoprobe injury might be due to an increase in cardiomyocyte proliferation as determined by BrdU staining in heart sections.[16,19] Unfortunately, BrdU measurements do not reliably demonstrate that cardiomyocytes (nor indeed any cells) actually undergo cell division, as it is a measure of DNA synthesis – which can reflect DNA repair, apoptosis, polyploidy and/or myocyte hypertrophy – and not mitosis. Ki-67 expression more accurately represents cells that are in a proliferative state[30] and we therefore investigated Ki67 expression in the border zones of MRL and C57BL/6 LAD infarcts and observed comparably low numbers of stained cardiomyocyte nuclei representative of a very low myocyte proliferation rate. Similar results have been reported by others, who also failed to find cells that were positive for either BrdU and/or cardiac markers.[31,32] Only two studies have clearly documented that the proliferation marker BrdU was actually present in cardiomyocytes, as opposed to fibroblasts or endothelial cells. Thus, Naseem *et al*.[15] showed 23 vs 38% BrdU/troponin I-positive cardiomyocytes in the border region of LAD-ligated MRL mice. In contrast, α-sarcomeric actinin/BrdU positive cells accounted for less than 1% of BrdU-positive cells.[19]

The left ventricle does not regenerate following ischemic injury in MRL mice

In 2001, Leferovich and co-workers reported that myocardial regeneration could occur naturally in a mammalian system, namely the MRL mouse, following cryoinjury of the right

ventricle.[16] In this study, we examined the regenerative capacity of MRL mice following a more pathophysiologically relevant injury model: permanent LAD occlusion leading to infarction of the left ventricle, similar to the injury received in the majority of human myocardial infarctions. Both MRL and C57BL/6 showed the same outcome in terms of clinically insignificant repair of heart function, not warranting further experimentation. We did not observe differences in cardiac function between the two strains 28 days after injury, but Naseem and colleagues[15] have described better functional outcomes (survival) at 60 days in MRL mice with LAD ligation compared to C57BL/6.

It remains unclear why our data and that of others[19–21,31,32] failed to demonstrate myocardial regeneration whilst other studies apparently do.[15–17] The reason for this discrepancy might relate to the severity of injury that affects the extent of damage to the affected cells, the vasculature, and the degree of inflammation, all of which ultimately impact on the actual regenerative process. There is evidence to suggest that cryoprobe and ischaemic injury models do not cause comparable severities of injury, with permanent LAD occlusion leading to a severe transmural injury, in contrast to cryoprobe damage, which can lead to a superficial injury. In addition to the effects of the size of the cryoprobe and the length of time it is applied to the heart, it is difficult to regulate the extent of damage caused by a cryoprobe, as the pressure with which it is applied to the myocardium cannot be standardised. Thus, MRL mice show evidence of myocardial regeneration in response to a superficial injury but are unable to demonstrate complete regeneration following a more severe injury sustained from a transmural cryoinjury or LAD ligation.[15] Interestingly, the regenerative capacity of the MRL ear hole is also affected by the injury method, with the thumb punch causing a cruder wound leading to better repair than after a sharp clinical biopsy.[33] Finally, individual models of ischaemic and cryoinjury have dramatically different effects on the vasculature and circulation. Thus, cryoprobe injuries might cause less damage to the vasculature allowing for the delivery of circulating factors/cells. On the other hand, LAD ligation results in permanent obstruction of a coronary artery, blocking the blood flow to an area of the myocardium and preventing any circulating factors that might improve regeneration. However, a 45 min ischaemia–reperfusion injury to the left ventricle of MRL hearts resulted in scar tissue at levels comparable to control animals,[21] suggesting that circulatory effects are not the reason for the contradictory findings.

Injury location also has not been consistent in studies examining MRL cardiac regeneration. As previously highlighted, the original manuscripts documenting myocardial regeneration in MRL mice involved causing injury to the right ventricle[16,17] whereas subsequent research has focused on the more clinically relevant injury location of the left ventricle.[15,20,21] As shown in Figure 1, expression of cell-cycle regulators appears to be consistent between the different sides of the heart, suggesting that, at the level of cell cycle control, the two chambers of the heart are analogous. However, the right ventricle is much thinner than the left, so the fresh warm blood on the luminal side might provide thermal protection to adjacent cells. This is supported by the localisation of the regenerated myocardium on the luminal side (Figure 2d in [16]). Thus, a comparable cryoprobe injury actually causes a significantly greater area of damage in the left ventricle. Indeed, Naseem *et al*.[15] showed that a mild (5 s) cryoinjury to the left ventricle allowed regeneration of the myocardium, whilst a longer (15 s) cryoinjury prevented extensive regeneration.

It has been hypothesised that some residual evolutionary regenerative capabilities might be retained by MRL mice, which are able to regenerate superficial wounds in certain areas of the body.[12] Consistent with this idea, we have confirmed the ability of MRL mice to regenerate ear-hole wounds, a trait not observed in other strains of mice. MRL mice also have been reported to show enhanced regeneration in response to injuries to the cornea and tips of the digits.[34,35] However, additional studies have demonstrated no difference in wound healing following dorsal skin wounds and to lesions in the cortex of the brain.[36–38] Our study has clearly highlighted that the regenerative capacity of the MRL mouse heart is not as clearly defined as previously suggested.

Conclusions

The early observation that cryoinjury to the right ventricle of MRL mice was repaired in these animals[16] led to great interest in the field of cardiovascular research, raising the possibility that regeneration of the mammalian myocardium, particularly human hearts, might become clinically feasible. Subsequently, a number of studies have been published that have provided conflicting findings[15,19–21] and our study has confirmed that MRL mice do not show clinically significant repair of a severe ischaemic myocardial injury. Whilst we cannot discount the possibility that (i) injury methods, (ii) the severity of injury, and/or (iii) the genetic background of animals might stimulate alternative cellular responses resulting in the regeneration previously reported in some MRL mouse hearts, we do not believe that there is sufficient evidence to support continued use of this strain as a model for cardiac regeneration.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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