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Assessment of DNA synthesis in Islet-1$^+$ cells in the adult murine heart

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ABSTRACT

Rationale: Islet-1 positive (Islet-1$^+$) cardiac progenitor cells give rise to the right ventricle, atria and outflow tract during murine cardiac development. In the adult heart Islet-1 expression is limited to parasympathetic neurons, few cardiomyocytes, smooth muscle cells, within the proximal aorta and pulmonary artery and sinoatrial node cells. Its role in these cells is unknown. Here we tested the hypothesis that Islet-1$^+$ cells retain proliferative activity and may therefore play a role in regenerating specialized regions in the heart. *Methods and results:* DNA synthesis was analyzed by the incorporation of tritiated thymidine (^{3}H-thymidine) in Isl-1-nLacZ mice, a transgenic model with an insertion of a nuclear beta-galactosidase in the Islet-1 locus. Mice received daily injections of ^{3}H-thymidine for 5 days. DNA synthesis was visualized throughout the heart by dipping autoradiography of cryosections. Colocalization of an nLacZ-signal and silver grains would indicate DNA synthesis in Islet-1$^+$ cells. Whereas Islet$^-$ non-myocyte nuclei were regularly marked by accumulation of silver grains, colocalization with nLacZ-signals was not detected in >25,000 cells analyzed. *Conclusions:* Islet-1$^+$ cells are quiescent in the adult heart, suggesting that, under normal conditions, even pacemaking cells do not proliferate at higher rates than normal cardiac myocytes.

1. Introduction

Cardiovascular diseases are the leading cause of mortality worldwide. Congestive heart failure accounts for almost half of these cases. It often results from the heart's inability to regenerate after the loss of working myocardium e.g. in the case of myocardial infarction. Until recently the heart has been regarded as a postmitotic organ incapable of regeneration. However, recent publications have shown consistently that a small number of cardiomyocytes are generated in the adult mammalian heart [1–3]. At present the source of these newly generated cardiomyocytes in the mammalian heart is unknown. Whereas some studies suggested the differentiation of cardiac progenitors to cardiomyocytes [1], other studies proposed that new cardiomyocytes are derived from the proliferation of pre-existing cardiomyocytes [4]. At present this question remains unsolved.

Several cardiac progenitor cell populations have been described in the embryonic and adult heart [5,6]. The transcription factor Islet-1 marks a cardiac progenitor population that gives rise to the right ventricle, atria and outflow tract during cardiac development [7]. Interestingly, these cells can differentiate to the different cell types that form an adult heart (cardiomyocytes, smooth muscle cells, and endothelial cells) [8,9]. We have recently described three Islet-1$^+$ structures in the adult murine heart [10]. Islet-1 was expressed in parasympathetic neurons, smooth muscle cells, few cardiomyocytes within the proximal aorta and pulmonary artery and many in the sinoatrial node. Although we did not find evidence for an Islet-1$^+$ progenitor cell population in the adult heart, the defined localization of Islet-1 argues for a certain biological role of Islet-1. During development Islet-1 is required for cell proliferation [7]. Additionally it has been shown that sinoatrial node cells, just like ventricular cardiomyocytes, proliferate in the embryonic heart [11,12]. Therefore the aim of this study was to investigate whether Islet-1$^+$ cells proliferate in the adult heart under physiological conditions.

The evaluation of proliferation of a certain cell population within tissue sections of the heart is difficult [13] and these difficulties might account for some of the conflicting findings in the field of cardiac regeneration [14]. For this reason we combined two approaches that allow for sensitive and robust detection of

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Islet-1 positivity and DNA synthesis. The use of the Isl-1-nLacZ model allowed for a sensitive and mostly unambiguous identification of Islet-1$^+$ cells, autoradiographic visualization of incorporated tritiated thymidine (^{3}H-thymidine) for the unambiguous evaluation of DNA synthesis.

2. Materials and methods

2.1. Animals

The investigation conforms to the guide for the care and use of laboratory animals published by the NIH (Publication No. 85-23, revised 1985) and was approved by the local authorities (BGV, Freie und Hansestadt Hamburg: 10/11). Isl1-nLacZ-mice were generated and characterized as described previously [15]. Investigations were done in heterozygous Isl1-nLacZ-mice on a mixed Blackswiss and C57BL/6J background.

2.2. Preparation of heart sections for autoradiography

Assessment of DNA-synthesis was performed following a protocol adapted from Soonpaa and Field [16]. Isl-1-nLacZ mice received daily injections of ^{3}H-thymidine (7.4 MBq (200 μCi) at 532.8 GBq/mmol i.p., Perkin Elmer) for 5 days. Mice were sacrificed 4 h after the last ^{3}H-thymidine injection by cervical dislocation. Hearts were perfusion fixed with 4% formaldehyde in situ, removed and washed in 0.9% NaCl. For Bromo-chloro-indolyl-galactopyranoside (X-Gal)-staining hearts were incubated in a staining solution containing potassium ferrocyanide (5 mM), potassium ferricyanide (5 mM), $MgCl_2$ (2 mM), 0.02% NP-40, 0.01% sodium deoxycholate, Tris pH 7.4 (20 mM) and 1 mg/ml X-Gal in phosphate buffered saline for 2 h at 37 °C.

Hearts were then embedded in a sucrose (30%)/Tissue-Tek (Sakura) mixture (1:1). Serial cryosections (10 μm) of the heart were performed in a frontal axis. All sections were collected on Superfrost Plus-slides (Fisher Scientific). Sections were postfixed in 4% formaldehyde for 10 min at room temperature and washed in 0.9% NaCl before autoradiography. Hematoxylin and eosin-staining (H&E) was performed on some sections to allow for an overview and nuclear identification after autoradiography. The vast majority of sections were not counterstained after autoradiography to facilitate the detection of silver grains.

2.3. Autoradiography

Slides were dipped in a photographic emulsion (Ilford L.4, Polyscience, diluted 1:1 in water) and placed in an opaque box for 7 days at 4 °C. Afterwards slides were developed in Kodak D-19 for 5 min and fixed in 30% sodium thiosulfate for 5 min. Slides were then washed in PBS and dehydrated by ascending ethanol series.

Table 1
DNA synthesis in nLacZ$^+$ cells.

	nLacZ$^+$ cells (n = 3 mice)	nLacZ$^+$ and silver grain$^+$ cells
Sinoatrial node	6500	0
Pulmonary artery/aorta	19,000	0
Cardiac ganglia	300	0

2.4. Cell counting

X-Gal$^+$ structures were identified with an ×10 objective. These structures were further analyzed in higher magnification with an ×40 objective. Cells showing a clear blue nuclear signal were counted (nLacZ$^+$). Pictures were taken with an Axioskop 2 microscope driven by the Axiovison software (Zeiss).

3. Results

DNA synthesis was assessed in Isl-1nLAcZ-mice (n = 3, 12 weeks of age) by the incorporation of tritiated thymidine. These animals received daily injections of ^{3}H-thymidine for 5 days. Hearts were harvested and X-Gal stained 4 h after the last injection. nLacZ$^+$ cells could be detected in cardiac ganglia, the proximal aorta and pulmonary artery and the sinoatrial node in all animals after X-Gal staining (Fig. 1).

No nLacZ$^+$ cells could be identified in the ventricular myocardium (n = 3, 500 sections per heart). We analyzed ~1500 (480–540 per animal) sections containing ~25,000 nLacZ$^+$ cells in total (Table 1). Most nLacZ$^+$ cells were localized in the proximal aorta and pulmonary artery (~19,000 cells in total) and only ~300 nLacZ$^+$ in parasympathetic neurons. DNA synthesis in small non-muscle cells (most likely fibroblasts; >50 cells per section) resulted in superimposed silver grains after autoradiography throughout the heart (Fig. 2A), serving as a positive control. To ensure that theses silver grains really superimposed nuclei, we performed H&E counterstaining on some slides (Fig. 2B).

Colocalization of the nLacZ signal and silver grains would indicate DNA synthesis in Islet-1$^+$ cells. Although we could identify DNA synthesis in nuclei adjacent to nLacZ$^+$ nuclei (Fig. 2C), we never detected a cell that showed colocalization of nLacZ and silver grains (Table 1).

4. Discussion

Precise assessment of DNA synthesis of a certain cell population in tissue sections is difficult and error-prone [13]. This is especially

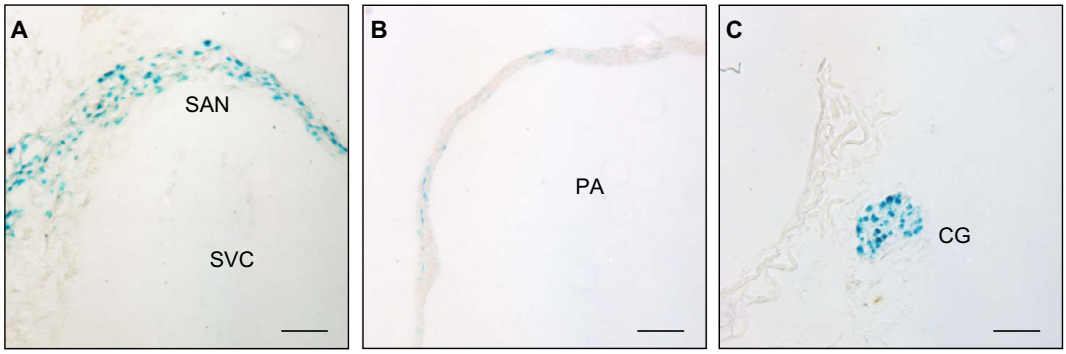

Fig. 1. nLacZ$^+$ structures in an Isl-1-nLacZ adult mouse heart. nLAcZ$^+$ cells in the (A) sinoatrial, (B) pulmonary artery and (C) cardiac ganglion of a 12 weeks-old Isl-1-nLacZ-mouse heart after X-Gal staining. CG: cardiac ganglion, PA: pulmonary artery, SAN: sinoatrial node, SVC: superior vena cava. Scale bars: 100 μm.

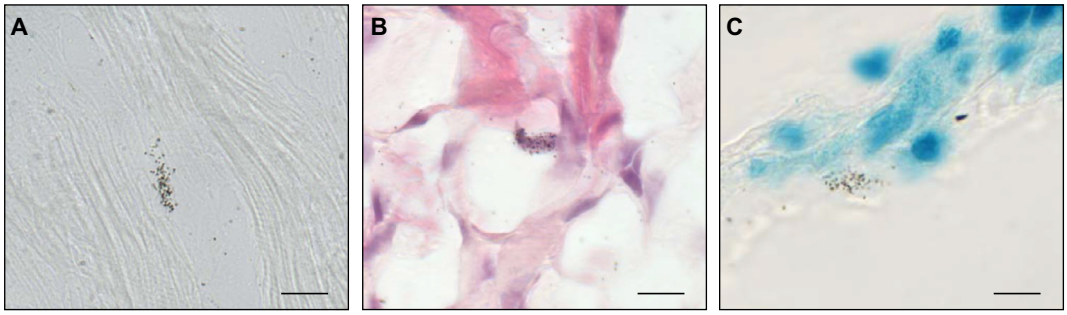

Fig. 2. Autoradiography of sections from Isl-1-nLacZ adult mouse hearts. (A) Labeled nLAcZ$^-$ cell in the ventricular myocardium without counterstaining. (B) Labeled nLacZ$^-$ cell after H&E counterstaining. (C) Unlabeled nLacZ$^+$ cells and labeled nLacZ$^-$ cell in the proximal aorta. Scale bars: 10 μm.

true for cells with a low proliferation index. We therefore aimed at combining two methods that, on the one hand, allowed for an unambiguous detection of single Islet-1$^+$ cells and on the other hand permit a clear detection of DNA synthesis. The incorporation of tritiated thymidine is an well established method to investigate DNA synthesis in vivo and has been first combined with a transgenic model that expresses a nuclear β-galactosidase by Soonpaa and Field to investigate the proliferation of cardiomyocytes in the adult heart [17]. Isl-1nLacZ mice showed a blue nuclear signal after X-gal staining that allowed for an easy identification of positive cells and autoradiography resulted in clearly identifiable silver grains superimposing nuclei that had incorporated ^{3}H-thymidine. When analyzing ~25,000 cells we could not identify a single cell that showed a colocalization of nLacZ and silver grains, resulting in a labeling index of <0.00004%. These findings are in agreement with a former publication that failed to detect Ki67-positivity in cells that stained positive with an antibody against Islet-1$^+$ cells [18]. If one assumes that our experiments cover 5 days of the life of a mouse, the DNA synthesis rate per year was <365/ 5 × 0.00004% = <0.003%.

Islet-1 is required for the proliferation of cardiac progenitors in the embryonic heart, and genetic inactivation of the Islet-1 gene led to a significant reduction in proliferation in the usually Islet-1$^+$ pharyngeal endoderm and splanchnic mesoderm during development [7]. We have recently identified Islet-1$^+$ sinoatrial node cells, smooth muscle cells and parasympathetic neurons and few cardiomyocytes in the adult murine heart. This distinct localization of Islet$^+$ cells argued for a specific biological role. Particularly, the essential role of the few pacemaking cells for cardiac biology raised the hypothesis that the Islet-1$^+$ cell population in the sinoatrial node could serve a regenerative role. In this regard, it is interesting that the Islet-1$^+$ subpopulation of sinoatrial node cells seems responsible for pacemaker activity in the zebrafish [19] and zebrafish embryos lacking Islet-1 showed bradycardia. This fits to data from Hoffmann et al. who identified Islet-1 as a transcriptional target of Shox2. The lack of Shox2 also caused bradycardia in zebrafish, which could be rescued by the overexpression of Islet-1, demonstrating an important role of Islet-1 in the adult sinoatrial node. Our data is, to the best of our knowledge, the first study that uses a specific transgenic approach to evaluate the proliferative activity of adult pacemaking cells and suggests that even these specialized cells, which play a crucial role for life, do not proliferate at a rate which exceeds the generally assumed <1% per year. Early work from Rumyantsev described the induction of cell cycle activity in the conduction system after myocardial injury [reviewed in [20]]. Our study did not address the question whether Islet-1$^+$ cells proliferate after myocardial injury. The fact that we did not observe any major changes regarding localization and number of Islet-1$^+$ cells after myocardial infarction in a previous study [10] argues against a high proliferative activity after injury. However, the question whether Islet-1$^+$ sinoatrial node cells possess the capacity to proliferate after injury is interesting and has to be evaluated in further studies. In conclusion our data suggest that the transcription factor Islet-1 plays an as yet undefined role in the adult heart that differs from that during development.

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Disclosure

None.

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