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Identification of interventricular septum precursor cells in the mouse embryo

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Abstract

Little is known about the formation of the interventricular septum (IVS), a central event during cardiogenesis. Here, we describe a novel population of myocardial progenitor cells in the primitive ventricle of the mouse embryo, which is characterized by expression of lysozyme M (lysM). Using LysM-Cre mice we show that lysozyme expressing cells give rise to the IVS and to a part of the left ventricular free wall, demonstrating that these heart regions are developmentally related. LysM+ precursors are not of hematopoietic origin and develop in the absence of transcription factors that regulate lysozyme expression in macrophages. LysM-deficient mice lack an overt cardiac phenotype, perhaps due to compensation by the related lysozyme P, which we also found to be expressed in the developing heart. Direct visualization of lysM expression, using LysM-EGFP knock-in mice, showed that ventricular septation is initiated at embryonic day 9 by the movement of myocardial trabeculae from the primitive ventricle towards the bulbo-ventricular groove and revealed the dynamics of IVS formation at later stages. Our studies predict that LysM-Cre mice will be useful to inactivate genes in the developing IVS.
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Keywords: Lysozyme; Heart development; Interventricular septation; Lineage tracing; Heart morphogenesis; Cardiomyocytes

Introduction

The heart is the first organ that forms during mammalian development, satisfying the oxygen and nutrients needs of the growing embryo. The mature heart consists of two atria and two ventricles, the latter of which are separated by the interventricular septum (IVS). Ventricular septal defects are among the most common congenital heart lesions (Vaughan and Basson, 2000). Nevertheless, the knowledge about the origin of the IVS as well as of the dynamics and the control of its development remains sparse.

The first morphological evidence of cardiogenesis is the cardiac crescent, which forms in the mouse embryo at about embryonic day 7.5 (E7.5) from anterior mesoderm (Buckingham et al., 2005). Shortly thereafter the cardiac crescent fuses to give rise to the linear heart tube, which starts beating around E8, when extra-embryonic and embryonic vascular networks amalgamate (McGrath et al., 2003). The heart tube undergoes complex remodeling processes during midgestation to form the four-chambered adult organ. During the initial remodeling steps between E8.5 and E10.5, the cardiac chambers become evident as a consequence of the forward looping of the posterior parts of the heart tube and the thickening of the myocardium (Buckingham et al., 2005). The chambers then get physically separated by the growth of the septae and valves, thereby allowing for directed flow of the blood stream. The developing heart is subdivided by the formation of the IVS, which is located between the left and right ventricles. IVS formation in the mouse occurs approximately between E11 and E12.5 and involves the recruitment of myocardial cells as well as of non-muscular mesenchymal cells (Kaufman and Bard, 1999).

Dye injection experiments in chicken embryos showed that cardiomyocytes in the IVS are derived from the region of the bulbo-ventricular groove (de la Cruz et al., 1997). This structure demarcates the separation between the embryonic left and right ventricle and becomes detectable at about E10 in the mouse embryo. The analysis of staged chicken embryos suggested that the IVS forms by the coalescence of individual muscle fiber bundles – so-called trabeculae – present in the primitive left ventricle (Ben-Shachar et al., 1985). However, this study did not

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address where IVS precursors reside prior to formation of the bulbo-ventricular groove. Elegant lineage tracing experiments in the mouse indicate that the myocardium of the IVS has a dual origin, being derived partly from the right and partly from the left ventricular primordium (Meilhac et al., 2004). A number of genes have been implicated in IVS formation. Thus, mice with targeted deletions of the transcription factors Tbx5 (Takeuchi et al., 2003) and SRF (Parlakian et al., 2004) as well as of the chemokine receptor CXCR4 (Tachibana et al., 1998; Zou et al., 1998) and its ligand SDF-1 (Nagasawa et al., 1996) all have ventricular septal defects. However, because specific gene ablation in the IVS is not yet possible it cannot be ruled out that the observed phenotypes are secondary to one of the many cardiac and systemic abnormalities in these animals.

Lysozyme is a bacteriolytic enzyme that is part of the innate immune system. Mice have two closely related lysozyme genes: LysM (*Lyzs*, called *lysM* in the following), which is expressed in macrophages and granulocytes (Cross et al., 1988) as well as type II alveolar epithelial cells in the lung (Rehm et al., 1991). And lysP (*Lzp-s*, called here *lysP*), which is expressed in the Paneth cells of the small intestine (Cross et al., 1988). Here, we report the surprising finding that lysM is also expressed transiently in a novel population of myocardial precursors of the IVS. The study of a mouse model with a knock-in of *egfp* into the endogenous *lysM* locus (Faust et al., 2000) allowed us to directly visualize the dynamics of IVS formation. In addition, we traced the developmental fate of lysM+ myocardial cells by Cre/loxP technology. Our study shows that IVS precursors are determined before septation is initiated and that the muscular compartment of the IVS is derived from at least two types of progenitor cells. In addition, our work suggests that cardiac-specific mechanisms of lysozyme regulation exist and that this enzyme has hitherto unknown functions in the heart.

Materials and methods

Mice

LysM ancestry (Ye et al., 2003) and LysM-EGFP (Faust et al., 2000) mice were used as homozygotes for the LysM-Cre and LysM-EGFP alleles, respectively. Vav ancestry mice (Stadtfeld and Graf, 2005) were heterozygous for vav-Cre. LysM ancestry mice were homozygous for ROSA26R-YFP or ROSA26R-lacZ (Soriano, 1999; Srinivas et al., 2001), respectively. Lysozyme ancestry mice lacking PU.1 were obtained by crossing them for two generations to animals heterozygous for a germ line deletion of PU.1 (Ye et al., 2005). Similarly, LysM-EGFP mice lacking C/EBPβ were generated by crossed them with C/EBPβ mutant mice (Sterneck et al., 1997). All genotyping was performed as previously described (see above references). Mice were bred and maintained in accordance with guidelines from the Institute for Animal Studies of the Albert Einstein College of Medicine.

X-gal and EGFP whole mounts

For X-gal whole mounts of adult hearts, mice were anesthetized with isoflurane and perfused into the left ventricle with PBS followed by 1.5% paraformaldehyde (PFA, Electron Microscope Science), before hearts were dissected and fixed in 1.5% PFA overnight at 4°C. Fixed hearts were incubated in X-gal staining solution containing 1 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-βD-galactoside, FisherBiotech) in 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 0.02% NP40 in PBS, pH 7.4 for 12–24 h in the dark at 37°C. Imaging was done using a Nikon SMZ 1500 dissection microscope equipped with an Insight FireWire camera and SPOT imaging software (Diagnostic Instruments). To render hearts transparent they were dehydrated through a methanol series followed by incubation in a 2:1 mixture of benzyl benzoate: benzyl alcohol (Rentschler et al., 2001).

For EGFP whole mounts of the developing heart embryos were obtained from timed matings with the noon of the day of the vaginal plug designated embryonic day 0.5 (E0.5). The developmental stage was verified by counting somite pairs (between E8.5 and E11.5) or by other morphological criteria (Downs and Davies, 1993). Embryonic hearts were isolated with microdissection tweezers (Roboz Surgical) by clipping the distal part of the outflow tract and the two horns of the sinus venosus. For EGFP visualization, hearts were mounted in a drop of PBS and imaged with a Nikon Eclipse E600 microscope equipped with a FITC filter (Chroma #41001) and a MagnaFire (Optronics, Goleta, CA) camera. Image processing was done in Adobe Photoshop.

Preparation and analysis of frozen sections

Anesthetized adult mice were perfused into the left ventricle with PBS followed by 4% PFA. Dissected hearts were fixed in 1.5% PFA containing 30% sucrose for 1 h at 4°C, then they were cut in half and incubated for another 8–12 h in the fixation solution. Embryos and newborn mice were fixed as a whole for 6 h to overnight, depending on the developmental stage. For embryos older than E12.5, the head was removed to allow better penetration of the fixative. Tissues were embedded in O.C.T. compound (Sakura, Torrance, CA) and submerged in 2-methylbutane at −80°C. 10-μm sections were cut using a Leica CM1900 cryostat, transferred onto pre-cleaned glass slides (Superfrost/Plus, Fisher Scientific) and stored at −80°C.

Immunofluorescence was performed using the M.O.M. basic kit (Vector Labs, Burlingame, CA) following the manufacturer's instructions with the additional inclusion of 0.3% Triton-100 in all solutions and of 3% BSA and 5% goat serum in the blocking solution. Antibodies used were mouse-monoclonal anti α-actinin (Sigma) followed by an Alexa Fluor 546®-conjugated goat anti-mouse antibody (Invitrogen) as well as APC-conjugated rat monoclonal antibodies against mouse CD31, CD45, Mac-1, Gr1 (Pharmingen) and F4/80 (Caltag, Burlingame, CA). GFP and YFP signals were sometimes amplified using an Alexa Fluor 488®-conjugated rabbit anti-GFP antibody (Invitrogen). DAPI (0.4 μg/ml) was included to visualize nuclei. Sections were observed under a Nikon Eclipse E600 microscope equipped with filters to visualize DAPI (Chroma #31000), GFP or Alexa Fluor® 488 (Chroma #41001), YFP (Chroma #41028), Alexa Fluor® 546 (Chroma #41002c) and APC (Chroma #41013). Images were taken using a MagnaFire camera (Optronics) and analyzed with Adobe Photoshop software. For the calculation of the proportion of reporter labeled cardiomyocytes in vav ancestry mice it was assumed that the adult murine heart contains a total of 1.9×10^6 cardiomyocytes (Brodsky et al., 1985). Sections containing 10,000 to 50,000 cardiomyocytes were analyzed per vav ancestry mouse.

Real-time PCR analysis

Hearts were dissected from E9.5 (19–24 somite pairs) LysM-EGFP and C57BL/6 embryos and common atrial chamber, outflow tract and large parts of the bulbus cordis were dissected away. Repeated washing steps removed contaminating maternal and embryonic blood cells. RNA was extracted from pools of 3–5 hearts using the QIAGEN RNeasy Micro Kit following the manufacturers' instructions. Reverse transcription was done with the iSelect cDNA Synthesis Kit (Bio-Rad) and real-time PCR reactions were performed in triplicates with an Opticon2 (MJ Research) using AmpliTag Gold (Roche) and SybrGreen (Applied Biosystems). The input cDNA amount for each reaction was equivalent to 1/20 to 1/10 of one dissected embryonic heart. The following primer pairs were used: 5′-GGAATGGCTGGCTACTATGG-3′ and 5′-TGCTCTCGTGCTGAGCTAAA-3′ (lysM), 5′-ATGGCTACCGTGGTGT-CAAG-3′ and 5-CGGTCTCCACGGTTGTAGTT-3′ (lysP), and 5′-GACGGC-CAGGTCATCACTATTG-3′ and 5′-AGGAAGGCTGGAAAAGAGCC-3′ (β-actin). The PCR efficiency with all primer pairs was >90%. Specificity of lysM and lysP detection was tested by digestion of the PCR products with HaeIII, which only cuts lysM.

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