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Downregulation of atrial markers during cardiac chamber morphogenesis is irreversible in murine embryos

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SUMMARY

Vertebrate cardiogenesis is a complex process involving multiple, distinct tissue types which interact to form a four-chambered heart. Molecules have been identified whose expression patterns co-segregate with the maturation of the atrial and ventricular muscle cell lineages. It is not currently known what role intrinsic events versus external influences play in cardiac chamber morphogenesis. We developed novel, fluorescent-based, myocardial, cellular transplantation systems in order to study these questions in murine embryos and report the irreversible nature of chamber specification with respect to the downregulation of atrial myosin light chain 2 (MLC-2a) and alpha myosin heavy chain (α-MHC). Grafting ventricular cells into the atrial chamber does not result in upregulation of MLC-2a expression in ventricular cells. Additionally, wild-type ventricular muscle cells grafted into the wild-type background appropriately downregulate MLC-2a and α-MHC. Finally, grafting of RXRα gene-deficient ventricular muscle cells into the ventricular chambers of wild-type embryos does not rescue the persistent expression of MLC-2a, providing further evidence that ventricular chamber maturation is an early event. These studies provide a new approach for the mechanistic dissection of critical signaling events during cardiac chamber growth, maturation and morphogenesis in the mouse, and should find utility with other approaches of cellular transplantation in murine embryos. These experiments document the irreversible nature of the downregulation of atrial markers after the onset of cardiogenesis during ventricular chamber morphogenesis and temporally define the response of cardiac muscle cells to signals regulating chamber specification.

Key words: Ventricular chamber, Myosin light chain, Cardiac morphogenesis, RXRα, Heart, Mouse

INTRODUCTION

Atrial and ventricular muscle cell lineages display distinct electrophysiological (Maltsev et al., 1994; Davies et al., 1996; Chuck et al., 1997; Thomas et al., 1997), biochemical (Zeller et al., 1987; Seidman et al., 1988; Argentin et al., 1994) and contractile properties (Parker-Thorneburg et al., 1992; Gulick et al., 1997), which are largely dependent on the expression of distinct subsets of chamber-specific genes during the course of cardiogenesis (Lyons, 1994; Fishman and Chien, 1997; Fishman and Olsen, 1997). Relatively little is known regarding the positional and molecular cues that regulate this panel of proteins. Although the initial diversification of atrial and ventricular lineages occurs shortly after gastrulation, a large number of chamber-specific genes acquire a regionally restricted pattern of expression relatively late during cardiac development. In certain cases, these changes are observed post-septation or in the neonatal period (Edmondson et al., 1994; Lyons, 1994; Fishman and Chien, 1997; Fishman and Olsen, 1997). Thus, while the initial commitment to atrial and ventricular lineages occurs relatively early during vertebrate cardiogenesis, a series of maturational steps within the ventricular muscle lineage must occur for appropriate chamber development.

Subsequent steps, such as the expansion of the compact layer of the ventricular chamber, formation of the spongy myocardium, initiation of trabeculation and cues for the appropriate onset of conotruncal and endocardial cushion formation, are critical in the maintenance of normal cardiac morphogenesis. Mutations in specific signaling pathways that impair these various steps of ventricular chamber morphogenesis can result in embryonic death. Recent studies using gene-targeted approaches of specific retinoid receptors in mice have now provided direct evidence that retinoids are required for normal cardiogenesis (Kastner et al., 1994; Sucov et al., 1994, 1996). Retinoid X receptor alpha (RXRα) gene-deficient mice display a persistent atrial-like phenotype that is characterized by a lack of expansion of the compact zone, abnormal ventricular trabeculation and persistent expression of an atrial marker myosin light chain 2 (MLC-2a), which is normally downregulated during the course of murine

cardiogenesis (Sucov et al., 1994; Dyson et al., 1995; Ruiz-Lozano et al., 1998). These studies imply that the downregulation of this atrial marker may be a critical step during ventricular chamber maturation and morphogenesis.

These observations lead to the question of whether the maturational step from an atrial to ventricular phenotype is mediated via irreversible or non-irreversible pathways. Irreversible regulation suggests phenotypic stability in the face of altered environmental challenges. Reversible regulation suggests a phenotypic alteration in response to modified external conditions. A variety of lines of evidence support the importance of paracrine pathways for the activation of ventricular trabeculation. For example, neuregulin-dependent signaling pathways elicited from the endocardium activate erbB2 and erbB4 receptors in the myocardial target cells, leading to the onset of trabeculation (Gassmann et al., 1995; Lee et al., 1995). In addition, a large body of work suggests that the expression of atrial genes in the ventricular chamber can be significantly modulated by differential mechanical stimuli, such as workload and mechanical loading in the adult context (Chien et al., 1993; Ross, 1997). Lastly, exogenously produced growth factors have been shown to influence the cellular phenotype of cardiac myocytes (Pinset et al., 1988; Florini et al., 1996; Navarro et al., 1997) further strengthening the hypothesis that external conditions may play a role in cardiac muscle cell maturation and determination. To approach this problem, we designed methods for transplantation of wild-type and mutant cardiac muscle cells into embryonic hearts of either wild-type or RXRα gene-deficient, murine embryos. We show that, in both a short-term, ex utero, whole-mouse culture system and a long-term, in utero culture system, the embryos display an appropriate spatial and temporal regulation of ventricular chamber maturation and morphogenesis as assessed by the onset of trabeculation, expansion of compact zone and the downregulation of the atrial markers MLC-2a and α-MHC.

These studies document that the downregulation of MLC-2a and α-MHC are irreversible when introduced after the onset of septation. Grafting ventricular cells into the atrial chamber does not result in upregulation of MLC-2a expression in ventricular cells. Additionally, wild-type ventricular muscle cells grafted into the wild-type background appropriately downregulate MLC-2a and α-MHC. Finally, grafting of RXRα gene-deficient ventricular muscle cells into the ventricular chambers of wild-type embryos does not rescue the persistent expression of MLC-2a, providing further evidence that this step of ventricular chamber maturation is an early event. These studies provide a new approach for the mechanistic dissection of critical signaling events during ventricular chamber growth, maturation and morphogenesis in the mouse, and should find utility with other approaches of cellular transplantation in murine embryos. They also provide an assay system in which rescue of the RXRα-deficient and other embryonic lethal phenotypes may eventually be examined in the context of cell transplantation. Finally, these experiments document the irreversible nature of the downregulation of atrial markers after the onset of cardiogenesis during ventricular chamber morphogenesis and suggest that these steps reflect intrinsic signals arising within ventricular muscle cells themselves during the course of cardiac development.

MATERIALS AND METHODS

Breeding and identification of RXRα gene-targeted mice

C57/Bl6 RXRα gene-deficient mutant mice were maintained in colonies and bred as previously described (Sucov et al., 1994). Genotype of individual animals was determined by the polymerase chain reaction (PCR) analysis of DNA extracted from the tail or hind limb (Dyson et al., 1995).

Ex utero embryo culture

Preparation of rat serum was performed as described (Cockroft, 1990). Whole mouse embryos were cultured according to the method of Sturm and Tam (Sturm and Tam, 1993). Timed pregnant female mice were killed by cervical dislocation, the uterus removed and rinsed in PBS (7.52 g K_2HPO_4, 1.32 g NaH_2PO_4*H_2O, 7.2 g NaCl, q.s. 1 l H_2O, pH 7.35; all reagents unless otherwise noted from Sigma Biochemicals, St Louis, MO) to remove residual blood. The uterus was then transferred to PB1 media (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 8.04 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.33 mM sodium pyruvate, 1 g/L glucose, 0.01 g phenol red, 100 ml/ml streptomycin, 100 IU/ml penicillin, pH 7.35) for removal of embryos. Embryos between 10.5 and 11.5 days post coitum (E) were dissected from the uterus and the decidual mass removed. Riechert's membrane, the visceral yolk sac and amnion were dissected away from the uterus but left attached, so as not to disrupt the vessels connecting the embryo to the yolk sac or the umbilical vessels from the embryo to the placenta. Embryos were then transferred to the pre-equilibrated media in continuously gassed (95% O_2, 5% CO_2), roller culture bottles and incubated for 24-36 hours.

In utero embryo culture

In utero manipulations were performed according to a variation of methods initially described by Muneoka et al. (1986). Briefly, mothers were anesthetized with an intraperitoneal injection of 0.2 ml/10 g body weight Avertin (2.5 g 2,2,2-tribromoethanol, 5 ml 2-methyl butanol and 195 ml distilled, sterile H_2O). A midline laparotomy and antimesenteric hysterotomy were performed to expose the embryos. Embryos for synchronous ex utero culture were removed with a sterile cotton applicator at the fetal-maternal placental junction and immediately placed in the rotating culture system. For in utero grafting experiments, the two embryos most proximal to the cervix on each uterine horn were removed using sterile cotton swabs with complete hemostasis achieved through a combination of direct pressure, electrocautery and pig fibrinogen. The distal portion of the uterine wall was subsequently incised and the embryos positioned with sterile, saline-soaked, cotton fragments. Embryonic manipulations were performed as described below. The abdominal cavity was copiously irrigated with sterile saline, embryonic membranes closed with 10-0 monofilament nylon (all sutures, Ethicon, Cincinnati, OH) and the uterus closed with interrupted 7-0 PDS. The abdomen was closed with a running 5-0 Vicryl and the mother allowed to recover in a warm area.

Preparation and injection of transplanted cell populations

Three populations of cells were used as hosts and grafts: wild-type atrial, wild-type ventricular and RXRα gene-deficient ventricular cells. Pregnant (products of a heterozygous cross) RXRα heterozygous mice at E10.5 were killed by cervical dislocation and embryonic hearts removed; simultaneously, the yolk sac was used for PCR analysis. Not less than 40 atria or 40 ventricles were used for any individual experiment. The hearts were dissected (Fig. 3A) such that the outflow tract and atrioventricular cushions were discarded while the atria and left ventricular chambers were placed in individual tubes containing 100 μl of 1 mg/ml collagenase and 0.5 mg/ml pancreatin (Life Technologies, Gaithersburg, MD). Tissue fragments were incubated at 37°C for 20 minutes with occasional, gentle

pipetting until the tissue was fully dissociated. Cells were then briefly pelleted at low speed (800 ***g***), rinsed twice in ADS buffer (Kubalak et al., 1996), and incubated in a 37°C, 5% CO_2 incubator until identification by PCR. PCR was performed on DNA prepared from yolk sac fragments with RXRα and neomycin-specific oligonucleotide primers as previously described (Dyson et al., 1995).

After genotyping, cells from wild-type or gene-deficient chambers were separately pooled in 300 mM glucose/PBS containing 10 mM Cell-Tracker DiI (Molecular Probes, Eugene, OR) and incubated at 37°C for 20 minutes followed by 10 minutes at 4°C. Finally, the cells were washed once in 300 mM glucose/PBS and twice in 50% rat serum/50% Weymouth's media to remove residual dye. Crafted 6 mm side-hole, pulled glass micropipettes were made on a Model 730 pipette puller (David Kopf, Tujunga, CA) and fashioned on a model MF-79 Narishige microforge (Leica/Narishige Instruments, Sea Cliff, NY) and attached to a MX-110-R 4 axis, manual, micromanipulator (Newport Instruments, Newport, CA) via electrode holders. Cells were raised in 5-10 μl of the pre-equilibrated media from the rotating culture bottle and aspirated into the injection needle. For ex utero injections, the host embryo was removed from the culture apparatus and placed in a right decubitus position, thus exposing the left ventricle. Approximately 50 labeled cells were injected into the wall of the left ventricle and the embryo under a 4-20× Zeiss SV-6 stereomicroscope (Carl Zeiss, Inc., Thornwood, NY) and returned to the culture apparatus for 24-36 hours. Cells injected in utero differed only in that the yolk sac was closed as described above with great care being taken throughout the procedure to preserve the uteroplacental junction.

Co-culture of CMAC-Blue and DiI-labeled cells was performed with cells isolated in an identical fashion. After labeling, the blue and red cell populations were inoculated at opposite sides of a single well of a 1 cm^2, 2-well tissue-culture slide (Falcon, Cockeysville, MD) and allowed to grow for 2.5 days. The cells were washed briefly with PBS and fixed for 5 minutes in 4% paraformaldehyde/PBS. The wells were removed from the plastic slide and coverslipped before confocal microscopy as described below.

Cryosectioning and immunohistochemistry

Grafted embryos were perfusion fixed with 4% paraformaldehyde/PBS through the left and right ventricular walls before incubation in the same fixative for 2 hours at 4°C. Embryos were embedded in 1:1::OCT:Aquamount on a solid block of dry ice. Sequential, 14 μm cryosections were cut on a Fridgocut 2800E (Leica, Deerfield, IL) and transferred to Superfrost-Plus slides (Fisher, Pittsburgh, PA). Four-color fluorescent immunohistochemistry was performed as described below. All immunoreagents and serums were obtained from Jackson Research (West Grove, PA) except where otherwise noted. Tissue sections were dried at room temperature for 1 hour before rinsing in PBS for 10 minutes. Sections were neutralized in 50 mM NH_4Cl/PBS for 5 minutes, then washed three times in PBS for 5 minutes each. Tissue was simultaneously permeabilized and blocked by incubation in PBT (1.0% Triton X-100/PBS), 3% bovine serum albumin (BSA) and 10% normal goat serum (NGS) for 25 minutes. This solution was changed directly to the primary antibody solution containing 3% BSA, 5% NGS in PBT for 60 minutes followed by 4× 5 minute washes in PBT. This was followed by a brief blocking period of 10 minutes in 5% normal donkey serum/PBT after which the secondary antibodies were applied in the same buffer. Slides were prepared for confocal microscopy by rinsing three times in PBT, once in distilled water and mounted using Gelvitol with 2.5% (w/v) DABCO. Primary and secondary antibody preparations were as follows: rabbit anti-MLC-2a (Kubalak et al., 1994) (1:500, detected with FITC donkey anti-rabbit at 1:50), rabbit anti-alpha MHC (Wessels et al., 1991) (1:10, detected with FITC donkey anti-rabbit at 1:50), mouse anti-MF20 (Bader et al., 1982; Developmental Studies Hybridoma Bank) (1:50, detected with Cy5 donkey anti-mouse at 1:200), DiI (Molecular Probes, Eugene, OR) and 75 ng/ml DAPI. Slides were imaged with a Bio-Rad MRC1000 laser scanning confocal system (Bio-Rad, Inc., Hercules, CA) on a Nikon Axiophot inverted microscope (Nikon Inc., Melville, NY). Image analysis was performed on a Power Macintosh 7200/90 (Apple Computer, Cupertino, CA) with Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA) and NIH Image 1.60 (http://rsb.info.gov/nih-image), and composed in QuarkXPress 3.32 (Quark, Inc., Denver, CO).

RESULTS

Cultured murine embryos display a normal temporal and spatial pattern of ventricular chamber morphogenesis between E11.0 and E12.5

Mammalian embryo culture (New and Coppola, 1970; New and Cockcroft, 1978; Sturm and Tam, 1993) is a valuable technique for examining development ex utero. Advantages include continuous observation of development, facile surgical manipulation, elimination of maternal factors and direct exposure of animals to agents unaffected by host metabolism (Naruse et al., 1997). Alternatively, one can perform in utero manipulations with the benefits of assured temporal fidelity and inclusion of maternal factors, but with the impediment of technical difficulty. We utilized both approaches for region-specific, cardiac tissue manipulations. First, we confirmed the fidelity of temporal events in murine cardiogenesis both in utero and ex utero. Timed pregnant mice at E11.0 were anesthetized and the gravid uterus exposed. Through an antimesenteric hysterotomy the two embryos adjacent to the cervix removed at the maternal-fetal placental junction. The explanted embryos were further dissected as described in the Methods section and placed in the rotating culture system. The mother was closed, recovered and carried the remainder of her litter. After 36 hours, the embryos were simultaneously removed from culture and their littermates removed from the mother. The in utero and ex utero groups were then perfusion fixed and photographed as described.

Fig. 1 shows representative examples of synchronous in utero and ex utero murine embryos. The overall size of post-culture embryos were similar with 7 mm crown-rump lengths. There was some collapse of the 4th ventricle as well as tail shortening in the ex utero cultured embryos though the overall structure remains similar (Fig. 1A). These differences have been previously documented (Cockcroft, 1976). The gross limb morphology (Fig. 1B) is also comparable with initiation of interdigitary troughs. These studies support normal growth of the murine embryo in the whole mouse culture system.

Cardiac muscle cell transplantation in cultured murine embryos

Cellular transplantation has been previously utilized in a number of systems to address questions of cellular autonomy (Artinger et al., 1992; Sommer et al., 1997). Detailed quail-chick (LeDouarin, 1969, 1985) and chick-mouse (Fontaine-Perus et al., 1995) chimeric grafting experiments have been performed examining the developmental potential of transplanted cells, however, analysis of molecular programs of grafted, gene-targeted cells or chamber-specific derivatives in the heart has not to our knowledge been reported.

Our questions involved the immunohistochemical phenotype of grafted cells, therefore we needed to label cells such that they could be monitored as being derived from either specific chambers or genetic backgrounds. Rather than time-intensively

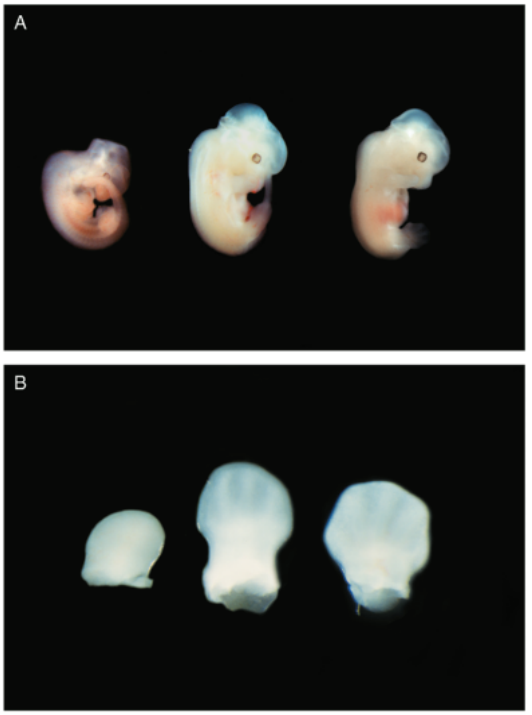


Fig. 1. Synchronous development of in vivo and ex utero cultured murine embryos. (A) Pregnant dams were operated upon at E11.5 and a fraction of the embryos were removed and either immediately fixed (left) or placed in culture. After 24 hours at E12.5, the remaining in utero embryos were removed and fixed (center) simultaneous with the ex utero cultured embryos (right.) Equal crown-rump lengths and overall morphology indicate similar development [10×, original magnification]. (B) Limbs removed from the embryos above were mounted, cleared and fixed. Significant growth of both the in utero (center) and ex utero (right) cultured embryos compared to the pre-culture (left) has occurred. Note the similar advancement of the interdigitary troughs between the in utero (right) and ex utero (right) embryos [20×, original magnification].

crossing gene-targeted mice into a line of mice with a constitutively expressed cellular marker such as β-galactosidase, we chose to tag cells with a series of lipophilic dyes derived from DiI. Though used extensively in neuronal tracing experiments, we confirmed DiI was stable in myocardial cells through multiple cell divisions as well as being non-transferable between adjacent cell populations.

Fig. 2 shows an experiment in which two populations of murine embryonic ventricular myocytes were labeled separately with either the red fluorescent lipophilic dye, Cell-Tracker DiI, resulting in the specked red pattern, or the blue fluorescent lipophilic dye, CMAC-blue. Labeled cells were inoculated on opposite sides of the tissue culture chamber and allowed to grow for 2.5 days. Although intensely labeled, the myocytes do not transfer dye to closely apposed cells (see arrows) indicating that the dye is a non-transferable marker for myocyte tracing experiments and stable through multiple cell divisions.

We then asked if one could unambiguously identify the labeled transplanted cells in the context of the intact heart. Fig. 3A-D (summarized in Fig. 3E) outline the protocol used to isolate embryonic murine myocytes and inject them into individual cardiac chambers. One can selectively dissect individual chambers from either wild-type or RXRα gene-deficient embryos. The RXRα gene-deficient hearts are globular in shape with persistent MLC-2a expression (Fig. 3A, lower left panel), while wild-type hearts are more conical in shape with some residual levels of MLC-2a expression in the right ventricle (Fig 3A, upper left panel). After microdissection, dissociation, and labeling, cells can be injected into the atrial wall (Fig. 3B) or the ventricular wall of either wild-type (Fig. 3C) or gene-deficient (Fig. 3D) ventricles. An example is shown in Fig. 4, where DiI-labeled cells were injected into the right ventricular wall of cultured, murine embryos. When viewed under dual fluorescence and light microscopy, these cells are unambiguously identified in the right ventricular (RV) wall of the heart (Fig. 4C).

To better define the three-dimensional localization of the grafted cells, additional embryos were injected with DiI-labeled, ventricular myocytes into the ventricular wall, cultured, hearts dissected free and subjected to confocal microscopy. Fig. 5 shows the red, DiI-labeled ventricular myocytes grafted into the right ventricular apex, immediately lateral to the interventricular groove (IVG). The stereopair of this region demonstrates DiI-labeled, heterotopic myocytes deeply embedded within the ventricular wall, yet they do not transfer dye to adjacent cells. Thus, in vivo grafting of fluorescently labeled cardiac myocytes allows unambiguous identification of transplanted cells.

Normal MLC-2a downregulation following ventricular muscle cell transplantation in the atrial chambers of wild-type murine embryos

Given our ability to identify grafted cells in vivo, we asked the question if transplantation of heterotopic ventricular myocytes results in an irreversible regulation of the chamber-specific muscle markers. We isolated cells from the immature (MLC-2a-expressing) ventricular chamber by microdissection, injected the labeled cells into the wall of the atrial chamber, cultured the embryos in vitro for 36 hours and immunostained sections of the grafted heart for the atrial marker MLC-2a (Fig. 3A,B; Table 1). Fig. 6 shows a confocal microscopic image of these DiI-labeled ventricular myocytes injected into the thin

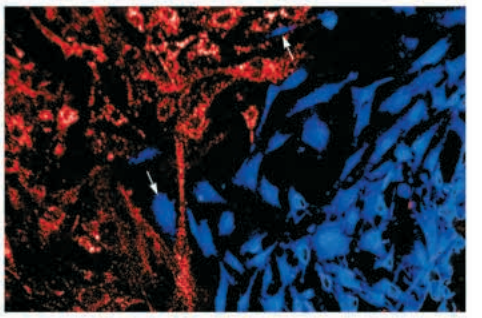
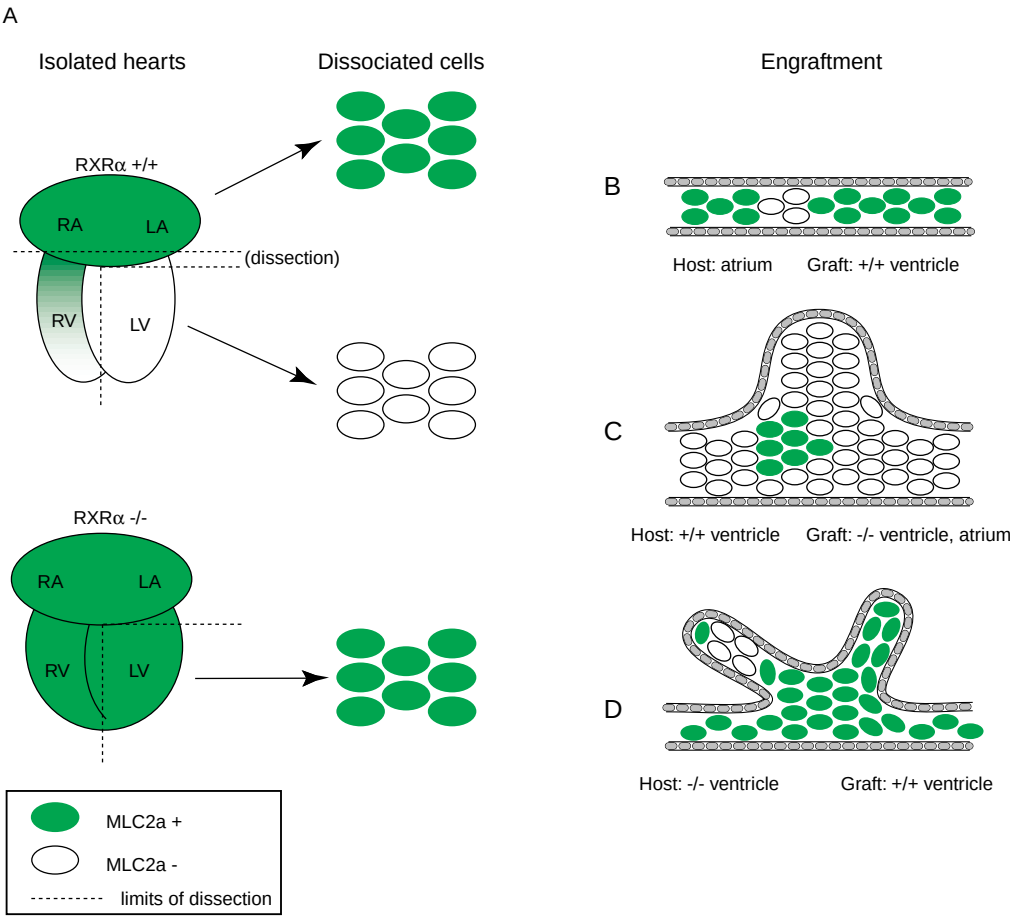


Fig. 2. Non-transfer of dye between co-cultured CMAC and DiI-labeled cells [40×, original magnification]. Ventricular myocytes from E11.5 murine embryos were dissociated, purified, labeled with either CMAC (blue) or DiI (red) and plated at opposite sides of a tissue culture dish. After 36 hours, they were fixed and analyzed by confocal microscopy. Note the close apposition between CMAC and DiI-labeled cells indicated by white arrows, though no transfer of blue dye to red cells nor red dye to blue cells is seen.



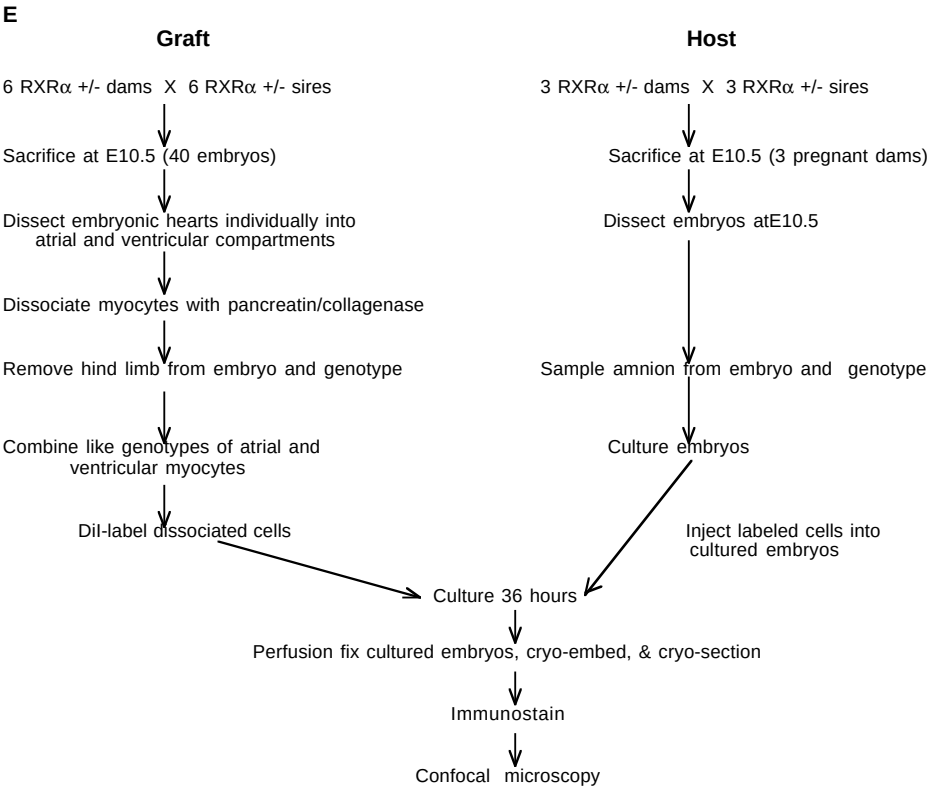


Fig. 3. Grafting protocol. (A) Cells were isolated from either wild-type (conical in shape) or gene-deficient hearts (globular in shape). Both RXRα wild-type (+/+) and gene-deficient (–/–) hearts strongly express MLC-2a in the atrium; however, only RXRα$^{-/-}$ hearts strongly express MLC-2a in the ventricle. Wild-type ventricular cells were engrafted into the atrial wall (B), wild-type atrial cells or gene-deficient ventricular cells into the the wild-type ventricular wall (C), or wild-type ventricular cells into the gene-deficient ventricle (D). MLC-2a-positive cells (atrium, right ventricle and gene-deficient left ventricle) are indicated by green ovals while the MLC-2a-negative staining cells (wild-type left ventricle) are indicated by open ovals. Note the schematic morphology of three host chambers: atrium, thin with no trabeculae; wild-type ventricle, thick compact zone with projecting trabeculae; gene-deficient ventricle, thin compact zone with attenuated, disorganized trabeculae. (E) Outline of grafting protocol.

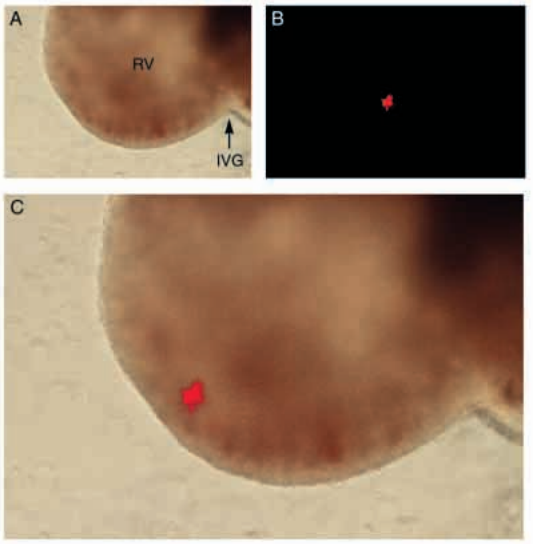


Fig. 4. Transplantation of DiI-labeled embryonic murine ventricular myocytes into the lateral, right ventricular wall of E11.5 ex utero cultured murine embryos. Ventricular myocytes from E11.5 murine embryos were dissociated, purified, labeled with DiI (red) and injected into the lateral, right ventricular wall of E11.5 ex utero cultured murine embryos. After 36 hours, they were harvested, fixed and analyzed by confocal microscopy. (A) [20×, original magnification] light microscope image of the right ventricular apex (RV) of a heart microdissected from an E13.0 murine embryo. (B) [20×, original magnification] epifluorescence microscope image of the same right ventricular apex (RV) demonstrates the brilliant fluorescence of DiI-labeled embryonic murine ventricular myocytes in vivo. (C) Co-localization by digital superposition of the transplanted cells in the lateral wall of the right ventricle. The graft consists of approximately 10 cells and does not loose its tracking signal (DiI) upon transplantation and culture ex utero for 36 hours.

atrial wall. In this experiment, identification of DiI-labeled, transplanted cells was unambiguous, demonstrating high levels of MLC-2a protein only in the atrial chamber host cells, not in the transplanted, ventricular cells. This indicates that despite an environment of MLC-2a expression (the atrium), ventricular myocytes appropriately downregulate MLC-2a indicating that the ventricular program of MLC-2a downregulation is irreversible with respect to spatial relocalization of myocytes from the ventricle to the atrium. Additionally, atrial myocytes immediately adjacent to the grafted ventricular cells maintain their expression of MLC-2a indicating that the transplanted microenvironment does not alter the atrial muscle expression program of the host chamber.

Table 1. MLC-2a-expressing cells in grafted cells

Host chamber RXRα genotype	Graft cell RXRα genotype		
	$A^{+/+}$	$V^{+/+}$	$V^{-/-}$
$A^{+/+}$	9/9 (100%)	1/11 (9%)	n.d.
$V^{+/+}$	33/35 (94%)	0/43 (0%)	27/31 (97%)
$V^{-/-}$	n.d.	1/25 (4%)	10/10 (100%)

All parities comparisons resulted in $P<0.05$ using Fisher's Exact Test. Total number of scored cells from 21 animals = 164.

Normal, persistent MLC-2a and α-MHC expression following atrial myocyte transplantation into ventricular chambers of wild-type murine embryos both ex utero and in utero

Ventricular myocytes do not revert to the primordial atrial phenotype when transplanted into atrial walls. We then asked if atrial myocytes differentiate light-chain isoforms into the ventricular phenotype when transplanted into the ventricular wall? Additionally, we asked if maternal factors or increased engraftment time could influence these decisions? We isolated atrial cells at (E10.5) by microdissection and dissociation, injected the DiI-labeled cells into the thick ventricular wall of the 'undifferentiated' ventricular chamber (persistently expressing MLC-2a at E10.5), and immunostained sections of the grafted heart for the atrial marker MLC-2a (Figs 3B, 7A-D). The grafted cells maintained their expression of MLC-2a despite an environment of MLC-2a downregulation suggesting that maintenance of atrial MLC-2a expression (like ventricular downregulation) was also irreversible.

We then asked if either circulating factors in the pregnant mother or extension of the culture time could affect the outcome of this experiment. We therefore developed an in utero cardiac grafting procedure, which allows injection of cells into the embryonic heart from E10.0-14.0. Directed injection before E10.0 is limited by thick decidua preventing visualization of the target tissue; injection after E14.0 is limited by the now thickened thoracic cage. Pregnant dams containing the injected embryos are reared for variable time until E19.0-20.0 at which point the animals can be delivered by Cesarean section; this extends the culture period of engrafted cells indefinitely, limited only by the durability of the cell marker. We injected cells at E10.5 and harvested embryos at E16.0. Fig. 8A-D shows a confocal microscopic image of DiI-labeled atrial myocytes injected into the wild-type ventricular wall of murine embryos harvested at E16.0. Grafted atrial cells continued to express high levels of both MLC-2a protein despite the 6 day culture period and exposure to maternal factors in utero. Additionally, we examined the expression of a second structural protein marker, which is normally expressed in all cardiac chambers until approximately E11.5 at which point its expression is downregulated in the ventricle and outflow tract. Like MLC-2a, α-MHC expression is maintained in the grafted, atrial cells again indicating that the atrial program of both MLC-2a and α-MHC downregulation is irreversible with respect to relocalization of myocytes from the atrium to the ventricle (Fig. 9A-D). Again, cells immediately adjacent to the grafted cells express neither MLC-2a nor α-MHC confirming that the transplanted microenvironment does not affect the expression program of the host chamber.

RXRα gene-deficient embryos harbor a defect of chamber specification at the level of MLC-2a protein

Chamber specification takes place early during cardiogenesis with ventricular markers restricted to the caudal portion of the primitive murine heart tube as early as E7.5 (O'Brien et al., 1993). This is in distinction to atrial markers, which are widely expressed in the primordial heart (Zeller et al., 1987; Kubalak et al., 1994; Lyons, 1994). Thus, restriction of atrial markers appears to be a relatively late event in cardiogenesis as compared to ventricular specification. However, mutations of

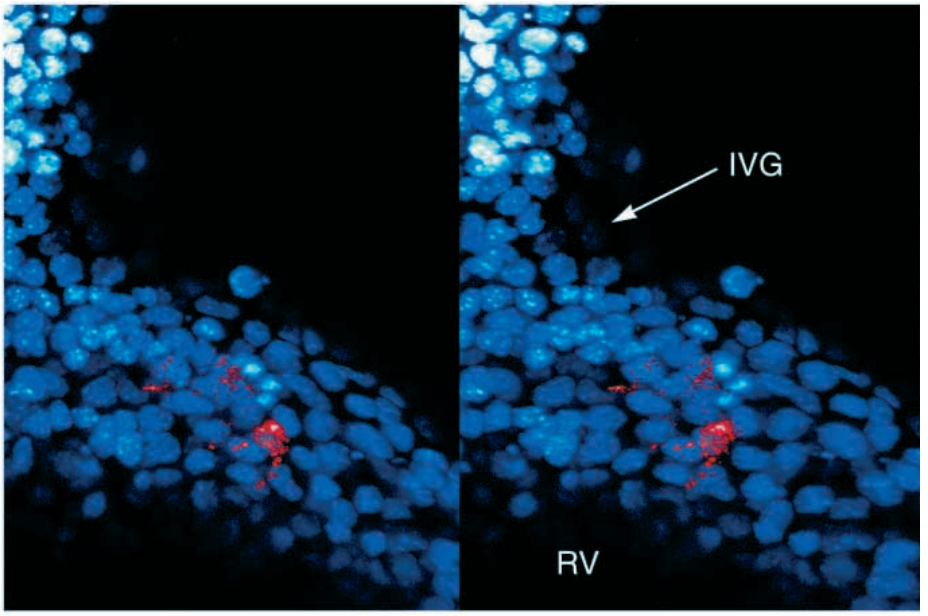


Fig. 5. Confocal, stereoimage of DiI-labeled embryonic murine ventricular myocytes injected into the medial, apical, right ventricular wall of E11.5 ex utero cultured murine embryos [20×, original magnification]. Ventricular myocytes from E11.5 murine embryos were dissociated, purified, labeled with DiI (red) and injected into the right ventricular apex immediately lateral to the interventricular groove (IVG) of E11.5 ex utero cultured murine embryos. After 36 hours, they were harvested, fixed, stained with DAPI (blue) and whole, microdissected hearts analyzed by confocal microscopy. Stereoimage clearly demonstrates the deep engraftment of the four transplanted (red) cells within the wall of the right ventricle. Note the unlabeled DAPI nucleus sandwiched between the two DiI-labeled grafted cells with prominent tails pointing towards 7 and 9 o'clock immediately inferior to the three bright nuclei. Right ventricular cavity is at the lower left-hand corner of each frame (RV.)

the RXRα receptor gene result in dysregulation of this pathway in that the atrial gene MLC-2a is constitutively expressed in the ventricle. This is a specific rather than generalized defect of muscle gene expression since MLC-2v is appropriately expressed both spatially and temporally in mutant animals (Sucov et al., 1994; Gruber et al., 1996). In addition to the previously characterized cardiac developmental defects in RXRα gene-deficient mice, there also exist visceral abnormalities (Kastner et al., 1994; Sucov et al., 1994; P. J. G. et al., unpublished observation). Given the inductive nature of diffusable factors such as BMP-2 and BMP-4 (Schulteiss et al., 1995) or HGF (Uehara et al., 1995; Ohmici et al., 1998) and the critical role molecules of the neurotrophin family play in the presumed paracrine regulation of trabeculation (Gassmann et al., 1995; Lee et al., 1995), one can reasonably postulate that molecules derived from non-cardiac tissues may be responsible for portions of the cardiac phenotype. Previously, we and others have demonstrated that RXRα was not required for embryonic viability acting in a non-irreversible fashion (Tran and Sucov, 1998; Chen et al., 1998). We therefore asked the question if we could apply our system of cellular grafting to further define the timing of RXRα responsiveness.

We carried out experiments transplanting 'atrialized' RXRα gene-deficient ventricular cells to the left ventricles of wild-

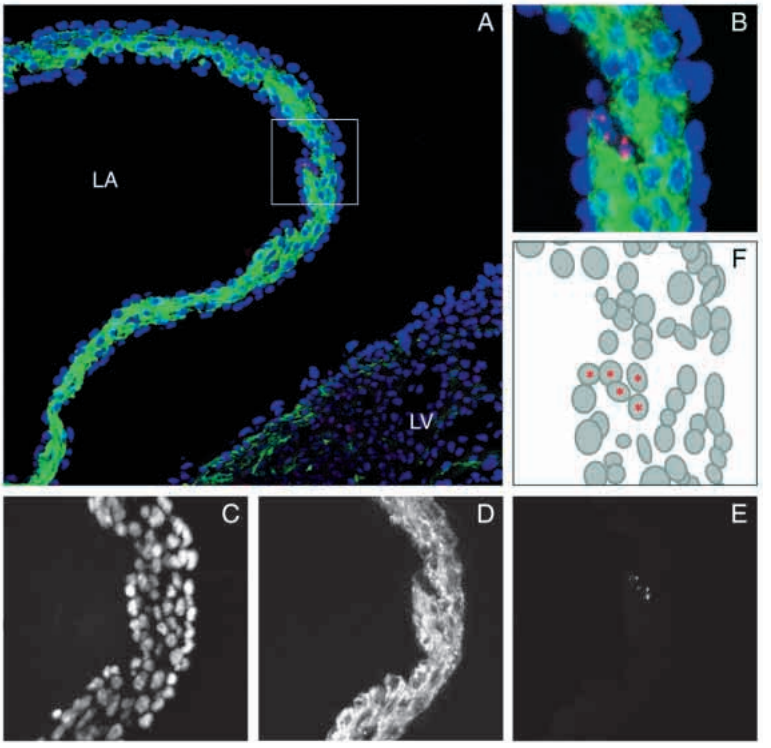


Fig. 6. Confocal, pseudocolor analysis of DiI-labeled embryonic, murine ventricular myocytes injected into the left atrial wall (LA) of E10.5 ex utero cultured murine embryos. (A) [20×, original magnification] Ventricular myocytes from E10.5 murine embryos were dissociated, purified, labeled with DiI (red) and injected into the left atrium of E10.5 ex utero cultured murine embryos. After 36 hours, they were harvested, cryosectioned, immunostained with rabbit anti-MLC-2a (detected with an FITC-conjugated, donkey anti-rabbit secondary antibody, green) stained with DAPI (blue) and analyzed by confocal microscopy. Ventricular myocytes at E13.0 do not normally express MLC-2a (see DAPI identified cells immediately superior and lateral to the 'LV' label) while atrial myocytes at all ages strongly stain for MLC-2a. (B) [60×, original magnification] The atrially engrafted ventricular myocytes (labeled red) do not stain for MLC-2a (green) indicating that the expression of this chamber-specific marker (MLC-2a) is irreversible with respect to chamber relocalization from the ventricle to the atrium and cannot be induced by the atrial environment. Original greyscale confocal images [60×, original magnification] of (C) DAPI, (D) MLC-2a, and (E) DiI channels. (F) Schematic of atrial wall in panel B. Grey ovals represent all cell types; red asterisks indicate DiI-positive grafted cells.

type mice (Figs 3D, 10A-E). The spatial relocalization of RXRα gene-deficient ventricular cells into a wild-type left ventricular environment at E10.5 did not alter the expression of MLC-2a of either transplanted cells or their surrounding host cells. Even in the context of chamber dysmorphogenesis, the atrial program of the ventricular myocyte is unchanged.

Conversely, wild-type ventricular myocytes were isolated and injected into the ventricular wall RXRα gene-deficient mice (Figs 3E, 11A-F). Fig. 11A shows the localization of the grafted cells (red) incorporated into a trabeculae of the ventricular wall of the mutant ventricle. The wall is characteristic of RXRα gene-deficient mice with a thin compact zone, aberrant trabecular organization (compare with the wild-type left ventricular trabeculae of Figs 7A,B or 8B), and persistent (though less than that of the atrium) expression of MLC-2a. Fig. 11B shows a four-color, confocal image of the grafted ventricle. An antibody to myosin heavy chain (MF-20) was used as an marker of the myocyte lineage independent of MLC-2a and demonstrates that grafted wild-type ventricular cells (a), are myocardial cells (MF-20-positive, magenta) yet MLC-2a-negative compared to the adjacent RXRα gene-deficient cells (b)which stain both MF-20 and MLC-2a-positive.

Thus, after the onset of trabeculation and septation (E11.0), cardiac muscle cell differentiation is maintained through multiple developmental stages (E10.5-16.0), genotypes (wild-type and RXRα gene-deficient), tissue types (atrium and ventricle) and structural markers (MLC-2a and α-MHC).

DISCUSSION

Cardiac muscle cell transplantation in cultured mouse embryos helps define embryonic mechanisms responsible for cardiac morphogenesis

Transplantation studies identifying developmental mechanisms during embryonic development have been a major experimental advantage of avian-based systems (LeDourarin, 1969, 1985). In addition to classical quail-chick studies, recent experiments have employed the transplantation of murine cells into the chick as an additional model system (Fontaine-Perus et al., 1995). Adapting these approaches in order to develop a completely murine based system has several potential advantages, including the wealth of knowledge of complex stages of murine cardiac morphogenesis, the wide availability of mouse mutants displaying defects at multiple stages of cardiac development, and the ability to both spatially and temporally genetically manipulate the organism (Rajewsky et al., 1996; Chen and Chien, 1998; Fishman, 1998).

We describe two approaches to examine embryonic mechanisms responsible for cardiac development in which cardiac muscle cells are transplanted into either cultured mouse embryos in vitro or embryos in utero. Cultured mouse embryos allow the continuous examination of cardiac development across optimal time windows for morphogenesis. We and others have documented the fidelity of the culture system. However, likely as a result of placental failure, accurate development in vitro is limited to a maximum of 48 hours and takes place outside the influence of maternal factors. Additionally, there are some characteristics of cultured embryos that commonly differ such as tail bud length and limb webs (New and Coppola, 1970; Cockcroft, 1976; New and Cockcroft, 1978). We therefore developed a second system of in utero cardiac muscle cell transplantation that allows growth of manipulated embryos to full gestation with delivery by Cesarean section. Previously, we developed techniques to allow the in vivo monitoring of cardiac function in living murine embryos with placental circulation intact (Dyson et al., 1995; Kubalak et al., 1996). Utilizing an analogous approach, we have now been able to deliver a wide variety of genes via adenoviral vectors (Gruber et al.,

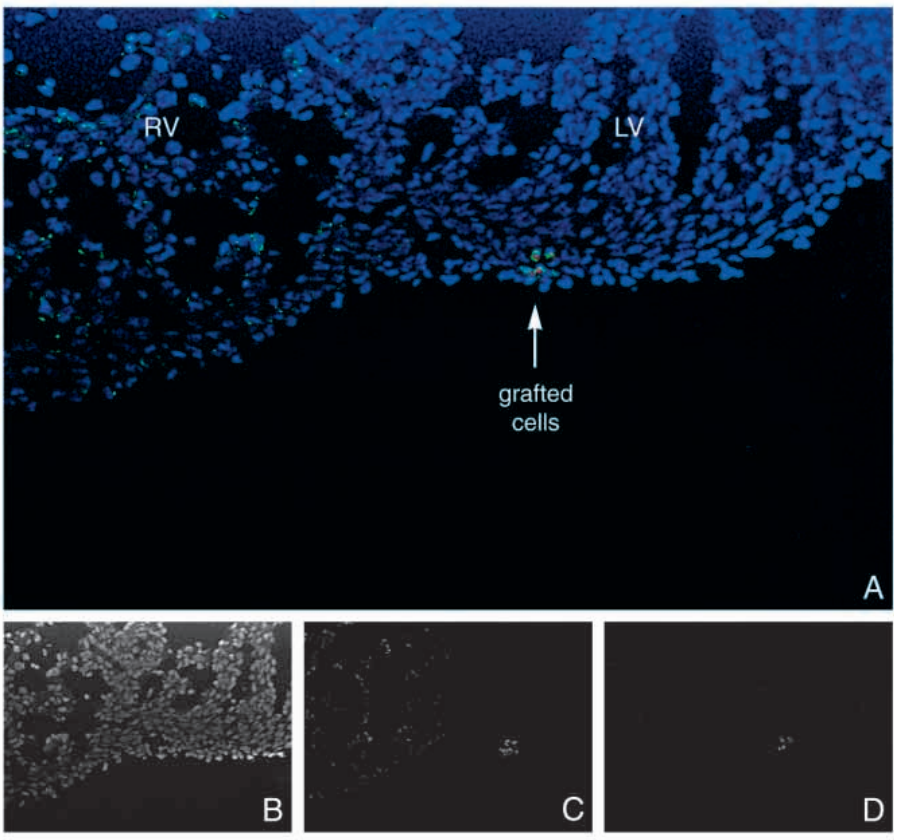


Fig. 7. (A) DiI-labeled, embryonic murine atrial myocytes injected into the left ventricular wall (LV) of E11.5 ex utero cultured murine embryos [10×, original magnification]. Ventricular myocytes from E11.5 murine embryos were dissociated, purified, labeled with DiI (red) and injected into the left ventricular wall of E11.5 ex utero cultured murine embryos [40×, original magnification; boxed area of upper panel]. After 36 hours, they were harvested, cryosectioned, and immunostained with rabbit anti-MLC-2a (detected with an FITC-conjugated, donkey anti-rabbit secondary antibody, green), stained with DAPI (blue) and analyzed by confocal microscopy. The grafted atrial cells stain MLC-2a-positive indicating they maintain an atrialized phenotype despite a ventricular environment. Original confocal images of (B) DAPI, (C) MLC-2a and (D) DiI channels.

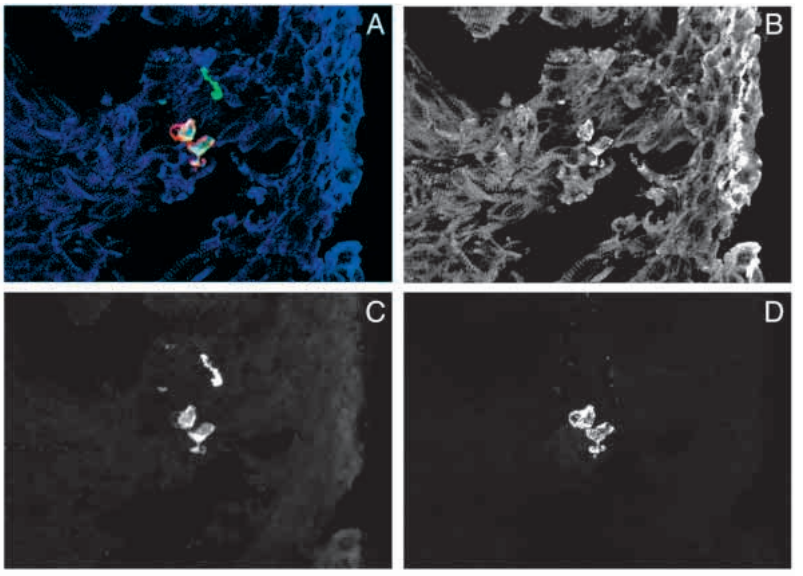


Fig. 8. DiI-labeled embryonic murine atrial myocytes injected into the left ventricular wall of E10.5 in utero murine embryos [60×, original magnification]. Atrial myocytes from E10.5 murine embryos were dissociated, purified, labeled with DiI (red) and injected into the left ventricular apex of E10.5 cultured murine embryos in utero. After 144 hours, they were harvested at E16.5, cryosectioned, immunostained with rabbit anti-MLC-2a (detected with an FITC-conjugated, donkey anti-rabbit secondary antibody, green) and mouse anti-MF20 (detected with an Texas red-conjugated, donkey anti-mouse secondary antibody, blue pseudocolor), and analyzed by confocal microscopy. In the composite image (A), three atrial myocytes in the ventricular wall continue to express (C) the atrial marker MLC-2a and (B) the muscle marker MF–20; (D) two of these are intensely labeled with DiI; one is weakly labeled, upper right.

unpublished data) and cells to both cultured and in utero murine embryos. Coupled with our previous documentation of the ability to culture ventricular and atrial cells from single embryos of either wild-type, transgenic or gene-deficient backgrounds (Lee et al., 1994), we are now able to deliver small numbers of these cells via direct injection into various cardiac compartments in cultured murine embryos. The cells are fluorescently tracked by the co-localization of the lipophilic dye DiI, and the phenotype of the cell assayed with immunological markers.

Though we and others have use the DiI series of lipophilic dyes to trace cell populations either by marking tissues in vivo (Lumsden et al., 1991) or via early embryo culture (Tam et al., 1997) for ease of labeling and intense fluorescence, there are clear disadvantages. The dye eventually fades, which limited our in utero experiments to 6 days and prevented identification of DiI-labeled fluorescent cells after delivery of the embryos by Cesarean section. Longer lineage tracing has been accomplished by the use of radiographic tracers (Johnson, 1966) and more recently by a laser-activated caged dye technique in zebrafish (Serbedzija et al., 1998). However, we required rapid labeling and single-cell resolution for which these techniques are less-well suited.

The present studies document the feasibility of embryonic, murine myocardial transplantation to study developmental mechanisms responsible for cardiac muscle cell defects from the onset of cardiogenesis to birth. They also represent an important adjunctive approach to experiments that rely on the generation of mosaic embryos which contain cells that harbor a double allelic genotypes. In this type of study, the opportunity for examining environmental rescue as well as examining the effects of wild-type cells in mutant backgrounds that may display aberrant gain-of-function of a particular gene defect is readily feasible. The purely environmental change of growing embryos in ‘wild-type’ rat serum in contrast to serum of the heterozygous dam argues against issues of transplacental rescue seen in other studies such as the TGF-β1 knockout (Lettario et al., 1994). Indeed, all native, murine maternal factors are eliminated in the cultured murine embryo system. Since cultured myocardial cells can be readily manipulated genetically with either viral vectors or through transient transfection approaches, these studies allow the opportunity for multiple, intersecting approaches in examining rescue of null genotypes. Taken together, these studies have led to the characterization of a new approach to examine developmental mechanisms in genetically manipulated murine models displaying cardiac developmental defects. This has been clearly shown in the wild-type adult context (Soonpaa et al., 1994); however, this is the first report of such transplantation in the developing mouse heart.

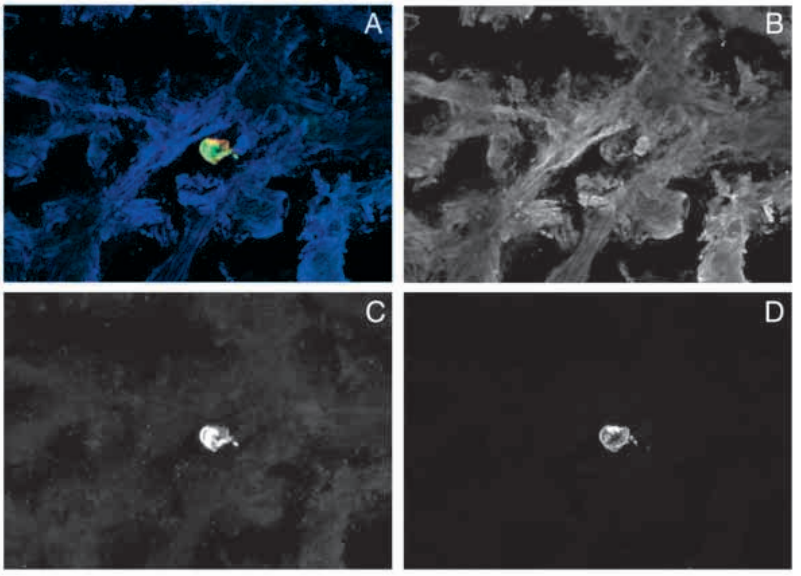


Fig. 9. DiI-labeled embryonic murine atrial myocytes injected into the left ventricular apex of E10.5 in utero murine embryos [60×, original magnification]. Atrial myocytes were injected, cultured and prepared in a manner similar to Fig. 8. In the composite image (A), an atrial myocytes in the ventricular wall continues to express (C) the atrial marker α-MHC (green) and (B) the muscle marker MF–20 (blue); (D) DiI channel (red).

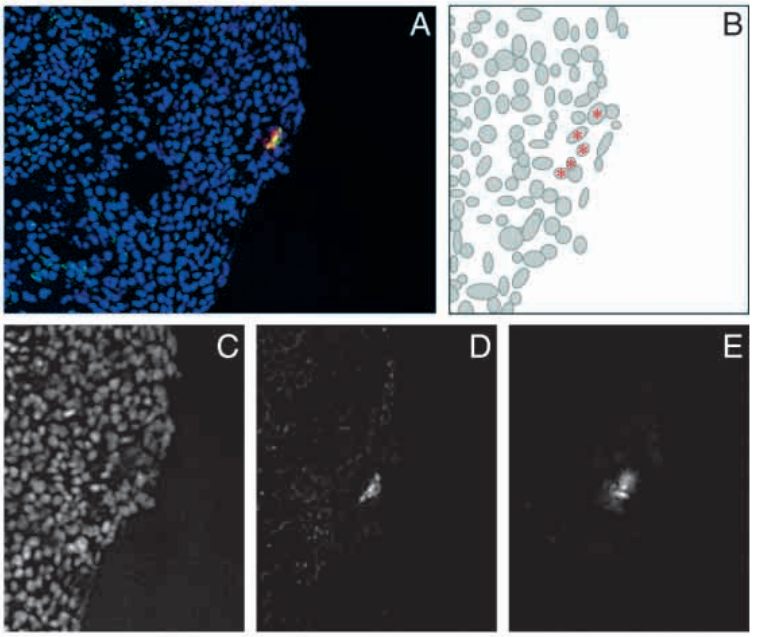


Fig. 10. (A) Confocal analysis of DiI-labeled embryonic murine RXRα$^{-/-}$ ventricular myocytes injected into the left ventricular apex of E11.5 ex utero cultured RXRα$^{+/+}$ murine embryos [40×, original magnification]. Ventricular myocytes from E11.5 RXRα$^{-/-}$ murine embryos were dissociated, purified, labeled with DiI (red) and injected near the interventricular septum of E11.5 ex utero cultured RXRα$^{+/+}$ murine embryos. After 36 hours, they were harvested, cryosectioned, immunostained with rabbit anti-MLC-2a (detected with an FITC-conjugated, donkey anti-rabbit secondary antibody, green) stained with DAPI (blue), and analyzed by confocal microscopy. Left ventricular myocytes at E13.0 do not normally express MLC-2a while RXRα$^{-/-}$ ventricular myocytes maintain this atrial characteristic of persistent MLC-2a expression. Right ventricular myocytes continue to express low levels of MLC-2a. (B) Schematic with grey ovals drawn from DAPI channel; red asterisks indicate DiI-labeled cells. (C) DAPI, (D) MLC-2a and (E) DiI channels.

Ventricular maturation is irreversible

The maintenance of chamber morphogenesis is dependent upon the expression of a panel of chamber-specific genes that must be controlled both spatially and temporally during the course of cardiogenesis. For example, the earliest marker of ventricular specification, ventricular myosin light chain-2 (MLC-2v), is found only in the ventricle and its expression appears to be primarily regulated through a combinatorial pathway via two distinct regulatory elements, HF-1 and MEF2 (Lee et al., 1994; Zou et al., 1995). In this manner, the expression of MLC-2v occurs primarily through positive regulation of this muscle-specific marker. In addition to this early specification, there is also a panel of genes that are co-expressed in both the atrial and ventricular chambers early during cardiac development and subsequently are downregulated in the ventricular compartment. Extinguishing these markers in the ventricular chamber is a critical event during the maturational steps of cardiogenesis. For example, the ANF gene is expressed in both the atrial and ventricular chambers and is subsequently downregulated in the ventricular chamber, displaying primarily atrial expression in the postnatal state (Argentin et al., 1994). The re-induction of this atrial marker in the ventricular chamber can occur in response to environmental cues, such as mechanical, hormonal or neuronal stimuli (Knowlton et al., 1995). We have recently described the atrial counterpart of the MLC-2v gene, MLC-2a, which is initially expressed throughout the heart tube and subsequently restricted later during the maturational stages of expansion of the compact zone, trabeculation and septation events during

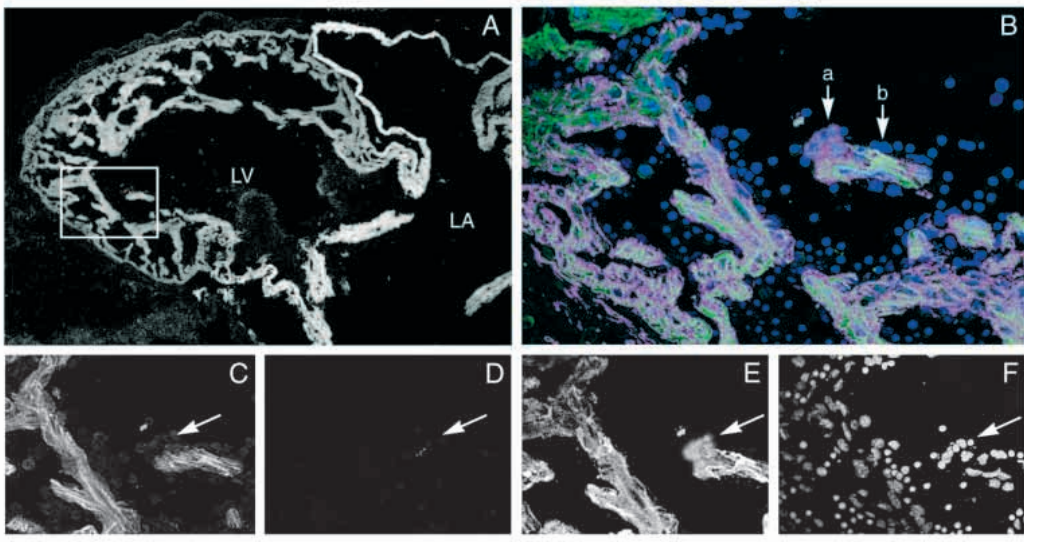


Fig. 11. (A) DiI-labeled, embryonic murine RXRα$^{+/+}$ ventricular myocytes injected into the left ventricular wall (LV) of E11.5 ex utero cultured RXRα$^{-/-}$ murine embryos[10×, original magnification]. (B) Ventricular myocytes from E11.5 RXRα$^{+/+}$ murine embryos were dissociated, purified, labeled with DiI (red) and injected into the left ventricular wall of E11.5 RXRα$^{-/-}$ ex utero cultured murine embryos [40×, original magnification; boxed area in A]. After 36 hours, they were harvested, cryosectioned, immunostained with rabbit anti-MLC-2a (detected with an FITC-conjugated, donkey anti-rabbit secondary antibody, green) and mouse anti-MF-20 antibodies (detected with a Cy5-conjugated, donkey anti-mouse secondary antibody, magenta), stained with DAPI (blue), and analyzed by confocal microscopy. The five grafted +/+ cells (a) are incorporated in a trabecular projection and stain MF-20-positive (indicating they are myocardial cells) yet (b) MLC-2a-negative (indicating they have appropriately downregulated MLC-2a). Original confocal images of (C) MLC-2a, (D) DiI, (E) Cy5 and (F) DAPI channels.

ventricular chamber morphogenesis (Kubalak et al., 1994). The downregulation of MLC-2a coincides with the acquisition of these morphogenic features of the ventricular chamber and serves as a molecular marker for these events. Following its downregulation in the ventricular chamber between E11.0 and E12.0, MLC-2a expression remains at negligible levels in the left ventricular chamber throughout the normal growth and development of the heart. The right ventricular chamber maintains a low level of MLC-2a expression between that of the atrium (strongly positive) and the left ventricle (off).

The current study employs MLC-2a as a marker to examine whether the maturation of ventricular muscle cells is an irreversible event. Certain atrial markers can be turned on by external environmental hemodynamic stimuli, as well as hormonal stimuli (Rockman et al., 1991); specific steps of ventricular trabeculation can also be regulated by paracrine stimuli emanating from the endocardium (Gassmann et al., 1995; Lee et al., 1995). Thus, the possibility exists that control of these events and the downregulation of MLC-2a may be primarily related to environmental cues, as opposed to being an independent, inherent, irreversible function. Consequently, the question arises as to which steps during maturation of ventricular muscle lineages are truly irreversible. Utilizing the cardiac muscle cell transplantation approach of ventricular cells into the atrial chamber, we document that the downregulation of MLC-2a appears to be an event independent of the cell's environment after E10.5. There appear to be intrinsic irreversible-based differences between atrial and ventricular muscle cell lineages. Taken together, these studies document that control of MLC-2a downregulation as an index of the maturation of ventricular muscle cell lineages is an irreversible event and support the utility of this as a model system to study the process of ventricular maturation.

Ventricular myocytes are RXRα unresponsive after E11.5

Recent studies have implicated retinoid-dependent pathways as playing a critical role in cardiac chamber maturation and morphogenesis. RXRα gene-deficient embryos display defects in ventricular trabeculation, expansion of the ventricular compact zone, and embryonic heart failure and lethality at E14.5 (Kastner et al., 1994; Sucov et al., 1994; Dyson et al., 1995; Gruber et al., 1996). RXRα gene-deficient embryos also display a persistent, aberrant expression of an atrial marker (MLC-2a) in the ventricular compartment, suggesting a maturational defect and the atrialization of the RXRα gene-deficient ventricular myocardium. Using MLC-2a expression as a molecular marker, we have employed two independent approaches to determine if the defect in ventricular maturation in the RXRα gene-deficient ventricular cells is reversible after E10.5. Utilizing the cardiac muscle cell transplantation assay in cultured murine embryos, we have documented the inability of a wild-type environment to rescue the persistent MLC-2a expression in RXRα gene-deficient ventricular muscle cells. Transplantation of wild-type ventricular cells into the ventricular chamber of RXRα gene-deficient embryos results in the appropriate downregulation of MLC-2a in the transplanted cells.

Recently we and others have demonstrated the non-cell-autonomous requirement for RXRα in ventricular development. Chimera analysis (Tran and Sucov, 1998) demonstrated that there is an RXRα requirement outside the cardiac myocyte compartment after the blastocyst stage. Since RXRα wild-type signals at E11.5 are not sufficient to rescue RXRα gene-deficient cells at E11.5 but are sufficient at the blastocyst stage, this suggests the blastocyst can respond appropriately to the non-cell-autonomous signal, but E11.5 cells cannot. Therefore, although the blastocyst is competent to respond to RXRα-dependent, non-cell-autonomous signals, programs that exist within the cardiac myocyte at E10.5 do not allow them to do so. Ventricular myocyte-restricted targeting of RXRα (Chen et al., 1998) unequivocally demonstrated that the removal of RXRα in the cardiac myocyte at E8.5 did not affect the phenotype of the cell. However, introduction of RXRα-deficient cells at E11.5 does not result in a normal cellular phenotype, implying that there is a transition in the cardiac myocyte between E8.5 and E11.5 that eliminates cellular responsiveness to RXRα-dependent signals, thus defining the temporal window for vitamin A signaling in the developing mouse heart.

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REFERENCES

Argentin, S., Ardati, A., Lihrmann, I., Robitaille, L., Drouin, J. and Nemer, M. (1994). Developmental and stage-specific regulation of atrial natriuretic factor gene transcription in cardiac cells. *Mol. Cell. Biol.* **14**, 777-790.

Artinger, K. B. and Bronner-Fraser, M. (1992). Notocord grafts do not suppress formation of neural crest cells or comissural neurons. *Development* **116**, 877-886.

Bader, D., Masaki, T. and Fischman, D. A. (1982). Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and ex utero. *J. Cell. Biol.* **95**, 763-770.

Chen, J., Kubalak, S. W. and Chien, K. R. (1998). Ventricular muscle-restricted gene targeting of the RXRα gene reveals a non-cell autonomous requirement in cardiac chamber morphogenesis. *Development* **125**, 1943-1949.

Chien, K. R., Zhu, H., Knowlton, K. U., Miller-Hance, W., van Bilsen, M., O'Brien, T. X. and Evans, S. M. (1993). Transcriptional regulation during cardiac growth and development. *Ann. Rev. Physiol.* **55**, 77-95.

Chuck, E. T. and Watanabe, M. (1997). Differential expression of PSA-NCAM and ANK-1 epitopes in the developing cardiac conduction system of the chick. *Developmental Dynamics* **209**, 182-195.

Cockroft, D. L. (1976). Comparison of *in vitro* and *in vivo* development of rat fetuses. *Dev. Biol.* **48**, 163-172.

Cockroft, D. L. (1990). Dissection and culture of post-implantation embryos. In *Post-implantation Mammalian Embryos: a Practical Approach*. pp. 15-40. Oxford, UK: IRL Press.

Davies, M. P., An, R. H., Doevendans, P., Kubalak, S. W., Chien, K. R. and Kass, R. S. (1996). Developmental changes in the ionic channel activity in the embryonic murine heart. *Circ. Res.* **78**, 15-25.

Dyson, E., Sucov, H. M., Kubalak, S. W., Schmid-Schoënbein, G., DeLano, F. A., Evans, R. M., Ross, J., Jr. and Chien, K. R. (1995). Atrial-like phenotype is associated with embryonic ventricular failure in retinoid X receptor α gene-deficient mice. *Proc. Natl. Acad. Sci., USA* **92**, 7386-7390.

Edmondson, D. G., Lyons, G. E., Martin, J. F. and Olson, E. N. (1994). Mef2 expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120**, 1251-1263.

Fishman, G. I. (1998). Timing is everything in life. Conditional transgene expression in the cardiovascular system. *Circ. Res.* **82**, 837-844.

Fishman, M. C. and Chien, K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-2177.

Fishman, M. C. and Olsen, E. N. (1997). Parsing the heart: genetic modules for organ assembly. *Cell* **91**, 153-156.

Florini, J. R., Ewton, D. Z. and Coolican, S. A. (1996). Growth hormone and the insulin-like growth factors system in myogenesis. *Endocr. Rev.* **17**, 481-518.

Fontaine-Perus, J., Jarno, V., Fournier le Ray, C., Li, Z. and Paulin, D. (1995). Mouse chick chimera: a new model to study the in ovo developmental potentialities of mammalian somites. *Development* **121,** 1705-1718.

Gassmann, M., Casagranda, F., Orioli, D., H, S., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394.

Gruber, P. J., Kubalak, S. W., Pexieder, T., Sucov, H. M., Evans, R. M. and Chien, K. R. (1996). RXRα deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J. Clin. Invest.* **98**, 1332-1343.

Gulick, J., Hewitt, T.E., Klevitsky, R., Buck, S. H., Moss, R. L. and Robbins, J. (1997). Transgenic remodeling of the myosin light chains in the mammalian heart. *Circ. Res.* **80**, 655-664.

Johnson, M. C. (1966). A radiographic study of the migration and fate of cranial neural crest cells in the chick embryo. *Anat. Rec.* **156**, 130-143.

Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dolle, P. and Chambon, P. (1994). Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**, 987-1003.

Knowlton, K. U., Rockman, H. A., Itani, M., Vovan, A., Seidman, C. E. and Chien, K. R. (1995). Divergent pathways mediate the induction of ANF transgenes in neonatal and hypertrophic ventricular myocardium. *J. Clin. Invest.* **96**, 1311-1318.

Kubalak, S. W., Doevendans, P. A., Rockman, H. A., Hunter, J. J., Tanaka, N., Ross, J. and Chien, K. R. (1996). Molecular analysis of cardiac muscle diseases via mouse genetics. *Methods in Molecular Genetics* **8**, 470-487.

Kubalak, S. W., Miller-Hance, W. C., O'Brien, T. X., Dyson, E. and Chien, K. R. (1994). Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. *J. Biol. Chem.* **269**, 16961-16970.

LeDouarin, N. M. (1969). Particularités du noyau interphasique chez la caille japonaise (Coturnix coturnix japonica). *Bull. Biol. Fr. Belg.* **103**, 435-452.

LeDouarin, N. M. (1985). Interspecific cell markers and cell lineage in birds. *Phil. Trans. Royal Soc. Lond. B: Biol. Sci.* **312**, 153-162.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398.

Lee, K. J., Hickey, R., Zhu, H. and Chien, K. R. (1994). Positive regulatory elements (HF-1a and HF-1b) and a novel negative regulatory element (HF-3) mediate ventricular muscle-specific expression of myosin light-chain 2-luciferase fusion genes in transgenic mice. *Mol. Cell. Biol.* **14**, 1220-1229.

Lettario, J. J., Geiser, A. G., Kulkarni, A. B., Roche, N. S., Sporn, M. B. and Roberts, A. B. (1994). Maternal rescue of TGF-β1 null mice. *Science* **264**, 1936-1938.

Lumsden, A., Sprawnson, N. and Graham, A. (1991). Segmental origin and migration of neural crest in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.

Lyons, G. E. (1994). In situ analysis of the cardiac muscle gene program during embryogenesis. *Trends Cardiovasc. Med.* **4**, 70-77.

Maltsev, V. A., Wobus, A. M., Rohwedel, J., Bader, M. and Hescheler, J. (1994). Cardiomyocytes differentiated ex utero from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ. Res.* **75**, 233-244.

Muneoka, K., Wanek, N. and Bryant, S. V. (1986). Mouse embryos develop normally *ex utero*. *J. Exp. Zool.* **239**, 289-293.

Naruse, I., Keino, H. and Taniguchi, M. (1997). Surgical manipulation of mammalian embryos *in vitro*. *Int. J. Dev. Biol.* **41**, 195-198.

Navarro, M., Barenton, B., Garandel, V., Schneckenberger, J. and Bernardi, H. (1997). Insulin-like growth factor I (IGF-I) receptor over-expression abolishes the IGF requirement for differentiation and induces a ligand-dependent transformed phenotype in C2 inducible myoblasts. *Endocrinology* **138**, 5210-5219.

New, D.A.T., and Coppola, P.T. (1970). Effects of different oxygen concentrations on the development of rat embryos in culture. *J. Reprod. Fert.* **21**, 109-118.

New, D.A.T. and Cockroft, D.L. (1978). A rotating bottle culture method with continuous replacement of the gas phase. *Experientia* **35**, 138-140.

O'Brien, T. X., Lee, K. J. and Chien, K. R. (1993). Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proc. Natl. Acad. Sci., USA* **90**, 5157-5161.

Ohmici, H., Koshimizu, U., Matsumoto, K. and Nakamura, T. (1998). Hepatocyte growth factor (HGF) acts as a mesenchyme-derived morphogenic factor during fetal lung development. *Development* **125**, 1315-1324.

Parker-Thorneburg, J., Bauer, B., Palermo, J. and Robbins, J. (1992).Structural and developmental analysis of two linked myosin heavy chain genes. *Dev. Biol.* **150,** 99-107.

Pinset, C., Montarras, D., Chenevert, J., Minty, A., Barton, P., Laurent, C. and Gros, F. (1988). Control of myogenesis in the mouse C2 cell line by medium composition and by insulin: characterization of permissive and inducible C2 myoblasts. *Differentiation* **38**, 28-34.

Rajewsky, K., Gu, H., Kuhn, R., Betz, U. A. K., Muller, W., Roes, J. and Schwenk, F. (1996). Conditional gene targeting. *J. Clin. Invest.* **98,** 600-603.

Rockman, H. A., Ross, R. S., Harris, A. N., Knowlton, K. U., Steinhelper, M. E., Field, L. J., Ross, J. J. and Chien, K. R. (1991). Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. *Proc. Natl. Acad. Sci., USA* **88,** 8277-8281.

Ross, J., Jr. (1997). On variations in the cardiac hypertrophic response to pressure overload *Circulation* **95,** 1349-1351.

Ruiz-Lozano, P., Smith, S. M., Perkins, G., Kubalak, S. W., Boss, G. R., Sucov, H. M., Evans, R. M. and Chien, K. R.(1998). Energy deprivation and a deficiency in downstream metabolic target genes during the onset of embryonic heart failure in RXRα$^{-/-}$ embryos. *Development* **125**, 533-544.

Schultheiss, T. M., Xydas, S. and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior ectoderm. *Development* **121,** 4203-4214.

Seidman, C. E., Wong, D. W., Jarcho, J. A., Bloch, K. D. and Seidman, J. G. (1988). Cis-acting sequences that modulate atrial natriuretic factor gene expression. *Proc. Natl. Acad. Sci., USA* **85,** 4104-4108.

Serbedzija, G. N., Chen, J. N. and Fishman, M. C. (1998). Regulation in the heart field of zebrafish. *Devlopment* **125**, 1095-1101.

Sommer, C., Bele, S. and Kiessling, M. (1997). Expression of cerebellar specific glutamate and GABA receptor subunits in heterotopic cerebellar grafts. *Dev. Brain Res.* **102,** 225-230.

Soonpaa, M. H., Koh, G. Y., Klug, M. G. and Field, L. J. (1994). Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science* **264,** 98-101.

Sturm, K. and Tam, P. P. L. (1993). Isolation and culture of whole post-implantation embryos and germ layer derivatives. *Methods in Enzymology* **225,** 164-190.

Sucov, H. M., Dyson, E., Gumeringer, C. L., Price, J., Chien, K. R. and Evans, R. M. (1994). RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Devel.* **8,** 1007-1018.

Sucov, H. M., Luo, J., Gruber, P. J., Kubalak, S. W., Dyson, E., Gumeringer, C. L., Lee, R. Y., Moles, S. A., Chien, K. R., Giguere, V. and Evans, R. M. (1996).The molecular genetics of retinoic acid receptors: cardiovascular and limb development. *Bioch. Soc. Symposia* **62,** 143-156.

Tam, P. P. L., Parameswaran, M., Kinder, S. and Weinberger, R. P. (1997). The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation. *Development* **124**, 1631-1642.

Tran, C. M. and Sucov, H. M. (1998). The RXRα gene functions in a non-cell-autonomous manner during mouse cardiac morphogenesis. *Development* **125**, ?

Thomas, S. A., Schuessler, R. B., Berul, C. I., Beardslee, M. A., Beyer, E. C., Mendelsohn, M. E. and Saffitz, J. E. (1997). Disparate effects of deficient expression of Connexin43 on atrial and ventricular conduction. Evidence for chamber-specific molecular determinants of conduction. *Circulation* **97**, 686-691.

Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T. and Kitamura, N. (1995).Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* **373,** 702-705.

Wessels, A., Vermuelen, J. L. M., Viragh, S., Kalman, F., Lamers, W. H. and Moorman, A. F. M. (1991).Spatial distribution of 'tissue specific' antigens in the developing human heart and skeletal muscle. II. An immunological analysis of myosin heavy chain isoform expression patterns in the embryonic heart. *Anat. Rec.* **229,** 355-368.

Zeller, R., Bloch, K. D., Williams, B. S., Arceci, R. J. and Seidman, C. E. (1987).Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes Dev.* **1,** 693-698.

Zou, Y. and Chien, K. R. (1995).EFIA/YB-1 is a component of cardiac HF-1A binding activity and positively regulates transcription of the myosin light-chain 2v gene. *Mol. Cell. Biol.* **15,** 2972-2982.