Development 125, 1943-1949 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV4993

Ventricular muscle-restricted targeting of the RXRα gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis

Ju Chen[1], Steven W. Kubalak[2] and Kenneth R. Chien[1,*]

[1]Department of Medicine and Center for Molecular Genetics, University of California, San Diego, School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093, California, USA
[2]Medical University of South Carolina, Charleston, South Carolina, USA
*Author for correspondence (e-mail: kchien@ucsd.edu)

Accepted 10 March; published on WWW 22 April 1998

SUMMARY

Mouse embryos lacking the retinoic acid receptor gene RXRα die in midgestation from hypoplastic development of the myocardium of the ventricular chambers and consequent cardiac failure. In this study, we address the issue of whether the *RXRα* gene is required in the cardiomyocyte lineage by generating mice that harbor a ventricular restricted deficiency in RXRα at the earliest stages of ventricular chamber specification. We first created a conditional ('floxed') allele of *RXRα* by flanking a required exon of the gene with loxP recombination sequences. To achieve ventricular myocardium-specific gene targeting, and to avoid potential transgenic artifacts, we employed a knock-in strategy to place cre recombinase coding sequences into the myosin light chain 2v (*MLC2v*) genomic locus, a gene which in the heart is expressed exclusively in ventricular cardiomyocytes at the earliest stages of ventricular specification. Crossing the MLC2v-cre allele with the floxed *RXRα* gene resulted in embryos in which approximately 80% of the ventricular cardiomyocytes lacked RXRα function, and yet which displayed a completely normal phenotype, without evidence of the wide spectrum of congenital heart disease phenotype seen in RXRa$^{-/-}$ embryos, and normal adult viability. We conclude that the RXRα mutant phenotype is not cell autonomous for the cardiomyocyte lineage, and suggest that RXRα functions in a neighboring compartment of the developing heart to generate a signal that is required for ventricular cardiomyocyte development and chamber maturation.

Key words: RXRα, Gene targeting, cre/lox, Cardiogenesis, Mouse, Muscle, Retinoic acid

INTRODUCTION

Normal cardiac morphogenesis is essential for fetal growth and viability. Heart development involves the commitment of mesoderm derived progenitor cells to the cardiomyocyte and endocardial cell lineages, followed by the axial specification of domains of the primitive heart tube to outflow, ventricular or atrial segments (for review, see Fishman and Chien, 1997; Olson and Srivastava, 1996). Subsequently, looping of the heart tube brings these specified domains to their approximate respective positions, thereby allowing each compartment to undergo further maturation and morphogenesis in a coordinated manner. In the ventricular chamber, proliferation of cardiomyocytes in the compact zone forms a thickened chamber wall which is required to meet the needs of the developing embryo. Concomitantly, endocardial cells in the vicinity of the outflow tract and the atrio-ventricular (AV) junction undergo a mesenchymal transition resulting in the development of the conotruncal and AV cushions, thereby separating the heart into four distinct chambers. Current paradigms suggest that the triggers for cushion development might originate from specialized ventricular muscle cells located in positionally restricted locations in the developing heart (Eisenberg and Markwald, 1995).

A variety of gene-targeted mouse model systems have been utilized to genetically dissect signals which are critical for various steps of cardiac morphogenesis (Rossant, 1996; Fishman and Chien, 1997). One of the most extensively studied models has been based upon studies of vitamin A signaling pathways. The vitamin A derivative retinoic acid (RA) comprises a collection of molecules that trigger and modulate complex morphogenic events during vertebrate development and adult physiological homeostasis. A total of six receptors (RARα, RARβ and RARγ and RXRα, RXRβ and RXRγ) which are members of the nuclear receptor family (Evans, 1988) transduce the activities of RA. All of the six receptors have been mutated in the mouse (Kastner et al., 1995). Mutation of the *RXRα* gene (Sucov et al., 1994; Kastner et al., 1994) results in a profound cardiac defect that is manifested as midgestational lethality around E15.5. RXRα$^{-/-}$ embryos have a normal-sized ventricular chamber wall up to E11.5, but thereafter fail to undergo compact zone expansion, leading to an underdeveloped, thin-walled and hypoplastic ventricle. Direct physiological measurement has demonstrated that the RXRα$^{-/-}$ lethal phenotype is the result of severely diminished

cardiac performance and consequent embryonic heart failure (Dyson et al., 1995). Mice heterozygous and homozygous for the mutant *RXRα* allele also display a wide spectrum of morphological defects that are phenocopies of forms of congenital heart disease, including double outlet right ventricle, pulmonary artery stenosis, cleft mitral leaflet, persistent truncus arteriosus and aorticopulmonary window (Gruber et al., 1996).

These ventricular chamber phenotypes might reflect a primary deficiency of RXRα in the ventricular muscle cell lineage, However, it cannot be assumed that the spatial requirement for RXRα in embryonic development is indeed localized within cardiomyocytes. Almost all cell types, including cardiomyocytes, respond to RA signaling (Zhou et al., 1995; Sucov et al., 1990) and RXRα is broadly expressed during early and midgestation (Mangelsdorf et al., 1992; Dolle et al., 1994). Thus, it is possible that another lineage (neural crest, endocardial, mesenchymal) might respond to a RA signal and, in a paracrine fashion, influence maturation and expansion of the myocardium, and also emit positional cues for appropriate cushion and outflow tract development.

Accordingly, we have undertaken a direct investigation of this issue by developing a strategy to generate embryos that harbor a ventricular-restricted deficiency in RXRα at the earliest stages of ventricular chamber specification. A conditional allele of *RXRα*, in which an essential exon is flanked by loxP recombination sequences (Marth, 1996), was first established in the mouse germline. To mutate this allele exclusively in ventricular muscle cell lineages in the early embryonic heart, we have employed a knock-in strategy to insert cre recombinase coding sequences into the genomic locus of the myosin light chain 2v (*MLC2v*) gene (Chen et al., 1998), which is the earliest ventricular-restricted marker during mammalian cardiogenesis (O'Brien et al., 1993). Mice homozygous for the floxed *RXRα* allele and carrying the MLC2v-cre knock-in gene demonstrate conditional mutation of the *RXRα* gene exclusively in the ventricular chamber of the heart as early as E8.75, with an efficiency approximately 80%. Nonetheless, such mice have normal embryonic viability without evidence of any of the multiple cardiac morphogenic defects observed in the complete RXRα-deficient embryos. These data suggest that *RXRα* gene function is not required in the ventricular cardiomyocyte lineage and that ventricular muscle cell expansion in the chamber wall is a non-cell-autonomous process. This same conclusion has been reached by a completely independent approach based on chimeric embryo analysis, as described in the accompanying paper (Tran and Sucov, 1998). Thus, an RA- and RXRα-dependent signal that is essential for ventricular chamber and cushion morphogenesis must be generated in a neighboring non-muscle compartment of the developing heart.

MATERIALS AND METHODS

Targeting vector and generation of the mouse in which the RXRα gene is flanked by two loxP sites.

The organization of the mouse *RXRα* genomic locus has been previously described (Sucov et al., 1994; Kastner et al., 1995). To construct the targeting vector, a 1 kb *Xba*I-*Eco*RI fragment containing the *RXRα* gene exon 4 was cloned into the *Bam*HI site of pflox (a gift from Dr Jamey Marth). Adjacent 3 kb *Bam*HI-*Xba*I and 5 kb *Eco*RI-*Bam*HI fragments were then inserted into *Xho*I and *Xba*I sites of pflox, respectively. The targeting construct was linearized with *Sal*I and electropolated into J1 ES cells. G418-resistant clones were expanded and genomic DNAs were isolated from each clone. The ES cells that were positive for homologous recombination were identified by Southern blotting analysis with probes A and B, which correspond to the gene regions depicted in Fig. 1A. Four positive clones containing all three loxP sites were transiently transfected with the cre-encoding plasmid pmc-cre (a gift from Dr Jamey Marth) and selected with gancyclovir as described (Gu et al., 1994). Colonies surviving the selection were picked and deletion events were identified by Southern blotting analysis with probe B (Fig. 1). Five independent type II deletion ES cell lines were injected into C57/B6 blastocysts; four yielded chimeras and one of them transmitted the type II deletion into germline. Mice carrying cre cDNA in the endogenous *MLC2v* locus were generated as described (Chen et al., 1998). All of the mice used in this aspect of the study are derived from 129/Sv crossed with Black Swiss strains. The protamine-cre line of mice was generously provided by Dr Stephan O'Gorman (Salk Institute, La Jolla, CA). Identification of mouse embryonic genotype was performed as previously described (Ruiz-Lozano et al., 1998).

DNA preparation and analysis

DNA was purified from cells or tissues by overnight lysis at 55˚C in tail buffer containing proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. 0.5 μg of genomic DNA was used for each PCR reaction. Primers used for the MLC2v-cre mice are described elsewhere (Chen et al., 1998); primers for the *RXRα* flox allele were: sense primer P1 (ACCAAGCACATCTGTGCTATCT) located in exon 3, antisense primer P2 (CAACTGTATAC-CCCATAGTGTT) located in intron 3, and antisense primer P3 (ATGAAACTGCAAGTGGCCTTGA) located in intron 4. For Southern blotting, 10 μg of DNA was used for each restriction enzyme digestion and the Southern transfer was performed by TURBOBLOTTER (Schleicher & Schuell). Hybridization was done with QuikHyb (Stratagene).

Purification of cardiomyocytes

Adult ventricular cells were isolated by retrograde perfusion with collagenase (Wolska and Solaro, 1996). Briefly, after 25 minutes of perfusion, ventricular cells were obtained by removing the lower 75% of the heart, the tissue was minced with scissors and the tissue suspension digested in collagenase medium for another 25 minutes. Cardiomyocytes were purified by two sequential 6% BSA gradients (Wolska and Solaro, 1996).

Microdissection and scanning EM

Midgestation embryos were dissected and processed for SEM as described previously (Gruber et al., 1996). The parietal and septal segments of the right ventricle and the parietal segment of the left ventricle were isolated and processed through ethanol dehydration, critical point dried and sputter coated. Primary magnification of scanning EM micrographs was ×80.

RESULTS

Generation of the RXRα floxed allele

To generate an *RXRα* floxed allele, a targeting construct was designed, as shown in Fig. 1, in which a loxP site was introduced into the third intron of the *RXRα* gene and a combined neo/TK cassette flanked by loxP sites introduced into the fourth intron. The fourth exon encodes most of the RXRα DNA-binding domain and is essential for RXRα function. This construct was electroporated into J1 ES cells; 30

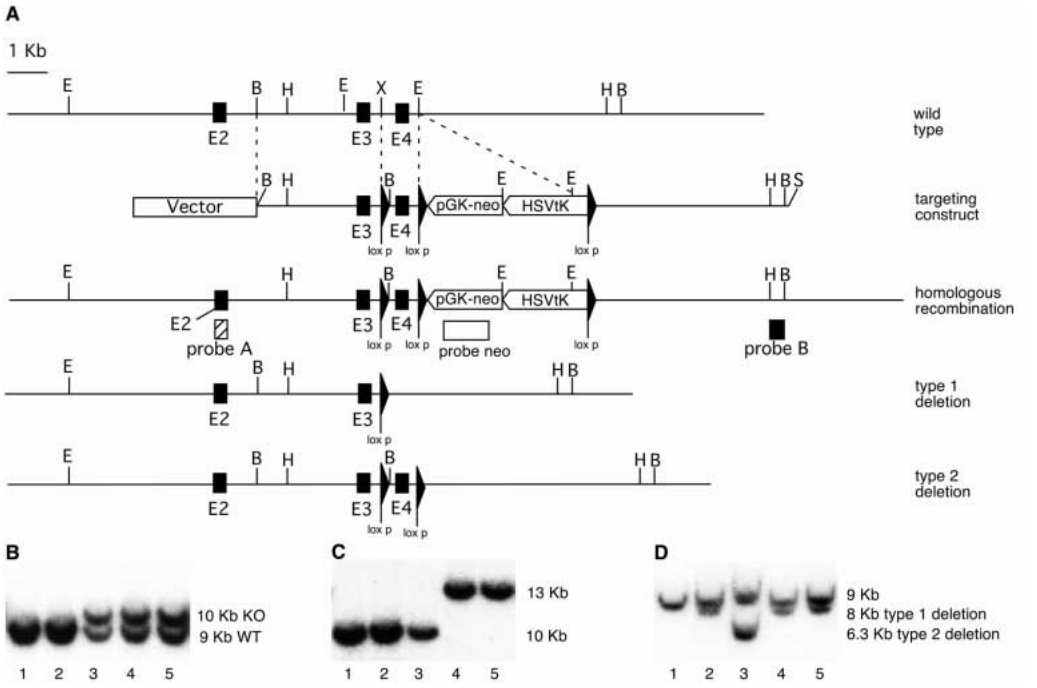

Fig. 1. Generation of ES cells and mice in which RXRα gene exon 4 is flanked by loxP sites. A) Restriction map of the mouse RXRα locus, the targeting construct, the homologous recombinant allele, and the type I and type II deletions generated by transient transfection of cre expression plasmid. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; X, *Xba*I. (B,C) Southern blot analysis of homologous recombination in ES cells. (B) DNAs were isolated from ES cells and digested with *Eco*RI and probed with probe A. The 10 kb and 9 kb bands represent the targeted and wild-type alleles, respectively. (C) DNAs from five positive recombinant ES cells were digested with *Bam*HI and probed with probe neo. The 13 kb band indicates that upstream homologous recombination occurred 5′ of the loxP site in intron 3 of the targeting construct and the 10 kb band indicates that recombination occurred in the region flanked by the two loxP sites. (D) Cre-mediated deletion in ES cells. Genomic DNAs were isolated from ES cells that had been transiently transfected by cre expression plasmid and then selected in gancyclovir. 9 kb, 8 kb and 6.3 kb bands represent wild type, type I deletion and type II deletion, respectively.

of 70 G418-resistant clones represented homologous recombination events, in which half of these carried the 5′-most loxP site. Clones containing all three loxP sites were then transiently transfected with a cre-encoding expression plasmid and grown in the presence of gancyclovir. In approximately 10% of the resulting colonies, a partial recombination event (i.e., type 2 deletion in Fig. 1A) had occurred in which the neo/TK cassette was deleted, but with retention of exon 4 and of loxP sites in the third and fourth introns. We refer to the type 2 recombined allele as the floxed *RXRα* allele, or as RXRαflox. Complete (type 1) deletion of the sequences between the 5′- and 3′-most loxP sites was seen in 80% of the colonies and the remaining 10% of selected clones represented chromosomal loss or deletion of the entire RXRα locus in a cre-independent process. ES cells containing the floxed RXRα allele were introduced into chimeric animals, and the allele established by germline transmission. Because the loxP sequences lie within introns and are only 34 bp in length, the floxed allele is predicted to function normally. Indeed, homozygous animals carrying two floxed RXRα alleles are viable, breed normally and are indistinguishable from wild-type littermates.

To establish that the RXRα floxed allele was capable of generating a phenotype similar to the conventional gene targeted RXRα-/- mice, we employed mice that express cre recombinase in the male germline under the control of the protamine gene (O'Gorman et al., 1997). Recombination occurs in spermatocytes, such that the recombined allele is incorporated into the fertilized egg and can be stably propagated into successive generations. Embryos homozygous for the floxed-out allele did not survive past E14.5 and exhibited ventricular failure (data not shown), consistent with our previous studies of the RXRα complete null genotype. Thus, the RXRα floxed allele was capable of being conditionally mutated to a true null phenotype in vivo via cre-lox strategies, supporting the utility of these mice for tissue-restricted ablation studies.

Ventricular muscle cell lineage restricted gene targeting via knock-in of cre recombinase into the endogenous MLC-2v locus

The myosin light chain 2v (*MLC2v*) gene is expressed bilaterally in the embryo in the cardiac primordia (around E7.5 in the mouse), and is restricted to ventricular precursors and definitive ventricular tissue from the linear heart tube stage throughout the entire postnatal time window (O'Brien et al., 1993). MLC2v is also expressed in slow skeletal muscle, but is absent in fast twitch muscle. Because of the restricted cardiac expression of this gene to the ventricles at the earliest stages of ventricular specification, we chose to express cre recombinase under the control of *MLC2v* regulatory elements. However, in transgenic animals, the *MLC2v* promoter only approximates the expression pattern of

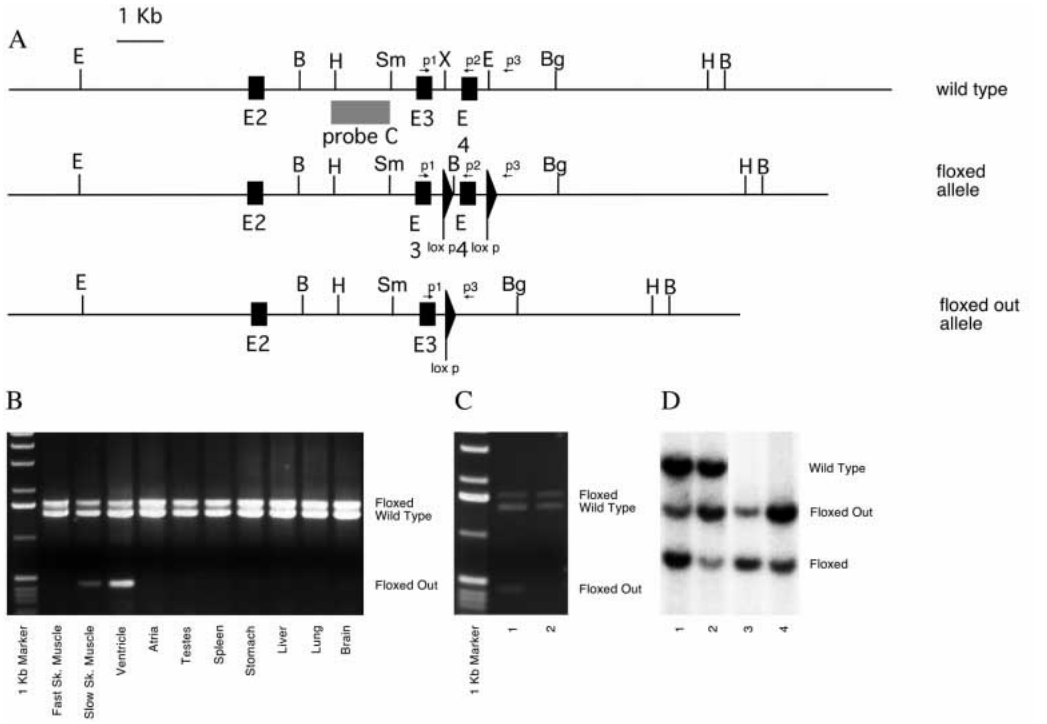

Fig. 2. Specificity and efficiency of cre-mediated recombination in vivo. (A) Restriction map of wild type, 'floxed' and 'floxed out' allele of the RXRα gene. Symbols are as in Fig. 1. (B) Tissue specificity of cre-mediated RXRα deletion. PCR analysis of genomic DNAs isolated from various tissues of MLC2vCreKI/+, RXRαflox/+ adult mice. (C) Time course of cre-mediated RXRα deletion. PCR analysis of genomic DNA isolated from E8.5 heart tube of MLC2vCreKI/+, RXRαflox/+ (lane 1) and MLC2v$^{+/+}$, RXRαflox/+ (lane 2) embryos. (D) Efficiency of cre-mediated RXRα deletion. Genomic DNAs were digested with *Bam*HI and *Bgl*II and probed with probe C. Genomic DNA was isolated from adult mouse ventricle (lanes 1 and 2) or purified ventricular cardiomyocytes (lanes 3 and 4). The genotypes for the samples are MLC2vCreKI/+, RXRαflox/+ (lane 1 and 3) and MLC2vCreKI/+, RXRαflox/flox (lane 2 and 4).

the endogenous gene, displaying a strong bias to right ventricular expression and without expression in slow twitch muscle (Ross et al., 1996).

To avoid these transgenic artifacts, we therefore introduced cre recombinase coding sequences into the chromosomal *MLC2v* gene by homologous recombination ('knock-in'), and have stably propagated this allele (designated 'MLC2vcreKI') through the mouse germline (Chen et al., 1998). Mice that are heterozygous for this allele are normal, display no morphogenic defects and express normal levels of MLC2v protein, although this allele is embryonic lethal when homozygous because MLC2v is an essential component of the cardiomyocyte contractile apparatus (Chen et al., 1998). In all of the studies that follow, the MLC2vcreKI allele was present in embryos and animals as a heterozygous locus.

High efficiency, ventricular restricted targeting of the RXRα floxed allele

To determine the specificity of cre/lox recombination, genomic DNA was isolated from various tissues of adult [MLC2vCreKi/+, RXRαflox/+] animals and analyzed by PCR to detect the presence of the deleted allele. As shown in Fig. 2B, the deletion only occurs in cardiac ventricular and slow skeletal muscle, which matches the expression pattern of the endogenous MLC2v gene. No recombination was evident in atrial muscle or in non-muscle tissues. To determine the time at which recombination occurred during heart development, genomic DNA was isolated from heart tissue of [MLC2vCrekI/+, RXRαflox/+] embryos at E8.75 and analyzed by PCR. As shown in Fig. 2C, recombination is already clearly evident at this stage.

Adult ventricular tissue from adult [MLC-2v cre KI/+, RXRα flox/+] animals was extracted, and genomic DNA digested and probed under quantitative conditions to visualize the non-recombined floxed allele and the recombined floxed-out allele. As shown in Fig. 2D, lane 1, 20% of the alleles in this sample have undergone recombination. Only 14% of adult mouse ventricular cells are cardiomyocytes, and 90-95% of these are binucleated (Soonpaa et al., 1996), thereby indicating that 24% of the DNA content of ventricular tissue originates from cardiomyocytes. The recombination of 20% of the alleles in the sample indicates that approximately 80% of the cardiomyocytes have undergone recombination. When cardiomyocytes were isolated as a partially purified single cell preparation, the ratio of recombined to non-recombined alleles was approximately 80% (lane 2). Since there is still substantial non-myocyte contamination in this preparation, this number represents a lower estimate of the efficiency of cre-mediated recombination in the cardiomyocyte population utilizing this MLC2v-cre knock-in strategy.

Normal ventricular morphogenesis in RXRα flox/flox, MLC2vCreKI/+ mice

[RXRα flox/+, MLC2vCreKI/+] mice were crossed to RXRα

Table 1. Offspring of crosses between MLC2v-creKI/+, RXRαflox/+ and RXRαflox/flox mice

	RXRαflox/flox	RXRαflox/+
MLC2v wild type	16	13
MLC2v-creKI/+	14	18

Offspring of matings between mice that were heterozygous for both the MLC2vcreKI knock-in allele and for the floxed RXRα allele with partners that were homozygous for the RXRαflox allele (RXRαflox/flox) were genotyped.

flox/flox partners to generate [RXRα flox/flox, MLC2vCreKI/+] offspring. Pups of this genotype were born normally, were externally indistinguishable from littermates of other genotypes, were recovered at Mendelian frequency (Table 1) and grew to adulthood without signs of neonatal malfunction or dysfunction. A detailed SEM analysis of mid-gestation fetal hearts failed to reveal any anatomical defects in the ventricular chamber wall or in other compartments of the developing heart in which the RXRα gene mutation has consequences (i.e., outflow tract, conotruncus, AV cushion, vascular morphogenesis, etc.; Table 2). Isolation and analysis of genomic DNA from [RXRαflox/flox, MLC2vCreKI/+] adult heart tissue (Fig. 2D, lane 3) or purified adult cardiomyocytes (lane 4) indicated that approximately 80% of the floxed RXRα alleles in cardiomyocytes had undergone recombination. Thus, absence of RXRα in cardiomyocytes does not impair normal fetal ventricular morphogenesis.

DISCUSSION

Ventricular lineage-restricted gene targeting via a knock-in strategy of cre recombinase into the endogenous MLC-2v locus

Cardiogenesis is a complex, integrative developmental process that requires spatially and temporally restricted cues for cardiac mesodermal specification, lineage diversification and subsequent stages of chamber maturation and morphogenesis (Fishman and Chien, 1997). Given the requirement for the interaction of multiple tissue compartments (myocyte, endocardial, mesenchymal, and neural crest) during normal cardiac morphogenesis, it is not surprising that a growing number of gene targeted models have been reported which display defects at specific stages of cardiac morphogenesis (Rossant, 1996; Fishman and Chien, 1997). In most cases, it has been difficult to gain mechanistic insight into the specific pathways that these genes guide. This difficulty has been compounded by the expression of many of these genes in multiple embryonic tissues and compartments during embryogenesis. Clearly, further insight into the mechanisms by which these genes interact with other upstream and downstream pathways will first require a clear understanding as to the spatial and temporal requirements for these factors during the complex course of mammalian cardiogenesis (Rossant and Nagy, 1995). As such, the development of strategies to achieve cardiac- and chamber-restricted gene targeting at the earliest stages of heart development will ultimately be required for the genetic dissection of the role of

Table 2. Heart defects in embryos from intercrosses between MLC2vCreKI/+/RXRαflox/+ and MLC2vCreKI/+/RXRαflox/flox mice

		Frequency of defects (%)		
Tissue compartment	Phenotype	MLC2vCreKI/+ / RXRαflox/flox(7)*	MLC2v+/+ / RXRαflox/flox(5)	MLC2v+/+ / RXRα+/+ (18)‡
Aortic sac	Absent aorticopulmonary septum/ persistent truncus arteriosus	0(0)†	0(0)	11(2)
	Incomplete aorticopulmonary septum/ aorticopulmonary window	0(0)	0(0)	28(5)
	Pulmonary artery stenosis	0(0)	0(0)	0(0)
Conotruncus	Absent conotruncal ridges	0(0)	0(0)	66(6)
	Hypoplastic conotruncal ridges	0(0)	0(0)	6(1)
	Hyperplastic conotruncal ridges	0(0)	0(0)	0(0)
	Short conotruncal septum	0(0)	0(0)	28(5)
	Double outlet right ventricle	0(0)	0(0)	17(3)
Atrioventricular canal	Hypoplastic cushions	0(0)	0(0)	67(12)
	Absent fusion	0(0)	0(0)	39(7)
	Incomplete fusion	0(0)	0(0)	33(6)
	Cleft mitral valve	N.D.	N.D.	17(3)
	Cleft tricuspid valve	N.D.	N.D.	17(3)
Myocardium	Hypoplastic compact zone	0(0)	0(0)	94(17)
	Ventricular septal defect	0(0)	0(0)	94(17)
	Disorganized trabeculae	43(3)	20(1)	67(12)
	Dysplastic papillary muscle			
	right ventricular	0(0)	0(0)	37(7)
	left ventricular	N.D.	N.D.	17(3)
	Papyraceous ventricles	0(0)	0(0)	0(0)

Numbers represent data combined from an E13.5 and an E14.5 litter.
*Numbers in parentheses refer to the total number of embryos examined for each genotype.
†Numbers in parentheses refer to the number of embryos harboring a defect in the designated tissue compartment.
N.D. Not determined.
‡Data derived from Gruber et al., 1996.

these individual signaling pathways in the control of specific steps of cardiac morphogenesis.

Toward this end, we have adopted a knock-in strategy to place cre coding sequences into the endogenous *MLC-2v* gene, which is the earliest marker of ventricular specification during mammalian cardiogenesis (for review, see Chien et al., 1993). Recent studies have extensively characterized a specific combinatorial pathway that confers ventricular specification of the *MLC-2v* gene during murine cardiogenesis (Ross et al., 1996). Although the MLC-2v promoter has been extensively characterized in transgenic animals, the utility of a transgenic strategy for expressing cre under the control of the *MLC-2v* promoter is complicated by the lack of uniform expression of the transgene (right > left chamber expression), the relative weakness of the promoter and the patchy nature of expression of the transgene even within the right ventricular chamber. To circumvent this problem, we have adopted a strategy of knock-in of cre recombinase into the genomic *MLC-2v* locus. In this manner, the recombinase is expressed in a temporally and spatially restricted manner that corresponds to the endogenous *MLC-2v* gene. Gene targeting restricted to the ventricular chamber is initiated at least as early as E8.75, and most likely is initiated in the ventricular segment of the bilateral cardiac primordia prior to fusion of the heart tube. *MLC-2v* gene is an abundant transcript in all ventricular muscle cell lineages, even at the earliest stages of ventricular chamber specification, which presumably acts to assist in driving the efficiency of recombination to high levels, as documented in the current study.

Non-cell-autonomous requirement of RXRα for ventricular muscle cell maturation and compact zone expansion

Utilizing MLC2v-cre mice, recombination of the floxed *RXRα* allele occurs by E8.75 and probably occurs at least a day earlier when MLC2v expression is first initiated. Thus, RXRα function is not required in cardiac myocytes that have already become specified to the ventricular lineage. However, while *MLC2v* is the earliest ventricular marker, it is not the first molecular marker of cardiogenesis (Harvey, 1996). Thus, the possibility exists that the requirement for RXRα could be in principle at an earlier developmental stage and simply manifests itself at a much later time as a defect in ventricular maturation. However, in a study of RXRα$^{-/-}$ with wild type chimeric embryos (see accompanying paper by Tran and Sucov, 1998), RXRα-deficient cardiomyocytes, derived from cells that lack RXRα from the blastocyst stage, were shown to develop normally. Thus, by several independent methodologies, the RXRα ventricular deficiency is therefore conclusively demonstrated to be a non-cell-autonomous phenotype.

To determine the lineage in which RXRα function is required for normal cardiac development is obviously important. Heart development involves a number of cell lineages distinct from the cardiomyocyte lineage. These include the endocardium and epicardium (cell layers lining the inside and outside of the myocardium, respectively), and cells migrating from the neural crest into the conotruncal region of the heart. All of these cell lineages influence ventricular development and the requirement for RXRα in heart morphogenesis may reside in any one of these lineages. The development of strategies to mutate RXRα specifically in these other compartments, comparable to what we have shown in this paper for the cardiomyocyte population, should be informative in ultimately identifying the specific cell lineages that reflect the RXRα requirement.

RXRα and cushion mesenchyme defects

Formation of distinct cardiac chambers requires septation of the left and right atrial and ventricular chambers. In the outflow tract, the proper formation and maturation of the conotruncal ridges is critical for maintaining the barrier between arterial and venous blood flow high within the ventricular chambers. At the atrial-ventricular junction, the formation of the endocardial cushions are central for septation of the atrial and ventricular chambers, and represent a critical step in formation of the valvular leaflets that maintain appropriate blood flow through the four-chambered heart. In the absence of appropriate formation and maturation of the conotruncal ridges or AV cushions, a wide spectrum of congenital heart phenotypes can ensue, several of which are seen in RXRα-deficient embryos (Gruber et al., 1996).

Previous studies (Rezaee et al., 1993; Wunsch et al., 1994) have suggested that the pathways that drive formation of the conotruncal septum and the atrioventricular valves involve epithelial-mesenchymal transformations, in which the endocardium is induced by myocardium to form mesenchymal cushions that project into the luminal space of the compartments. Although mice completely lacking RXRα display conotruncal and AV deficiencies, these additional defects were not observed in embryos in which RXRα function was eliminated specifically in cardiomyocytes by cre recombination. Thus, RXRα is not implicated in the myocardial component of endocardial induction and subsequent transformation. Our current understanding is that the RXRα requirements that support cushion, conotruncal and ventricular muscle morphogenesis all lie outside of the cardiac myocyte lineage per se. Studies are currently ongoing to utilize the *RXRα* floxed allele mice, and analogous cre knock-in strategies to genes restricted to neural crest, endocardial and cardiac mesenchyme, to precisely define the cell lineages in which RXRα-dependent pathways are localized.

We thank Dr Ronald M Evans and Henry Sucov for providing us the RXRα genomic DNA clones, Dr Stephen O'Gorman for providing the protamine-cre transgenic line, and Dr Jamey Marth for providing cre cDNA and pflox plasmid DNA. This study was supported by National Institutes of Health grants to K. R. C.

REFERENCES

Chen, J., Kubalak, S. W., Minamisawa, S., Price, R. L., Becker, K. D.Hickey, R., Ross, J. and Chien, K. R. (1998). Selective requirement of myosin light chain 2v in embryonic heart function. *J. Biol. Chem.* **273,** 1252-1256..

Chien, K. R., Zhu, H., Knowlton, K. U., Miller, H. W., van, B. M., OBrien, T. X. and Evans, S. M. (1993). Transcriptional regulation during cardiac growth and development. *Annu. Rev. Physiol.* **55,** 77-95.

Dolle, P., Fraulob, V., Kastner, P. and Chambon, P. (1994). Developmental expression of murine retinoid X receptor (RXR) genes. *Mech. Dev.* **45,** 91-104.

Dyson, E., Sucov, H. M., Kubalak, S. W., Schmid-Schonbein, G. W., DeLano, F. A., Evans, R. M., Ross, J. and Chien, K. R. (1995). Atrial-like phenotype is associated with embryonic ventricular failure in RXRα-/- mice. *Proc. Natn. Acad. Sci. USA* **92,** 7386-7390.

Eisenberg L. M. and Markwald R. R. (1995) Molecular regulation of atrioventricular valvuloseptal morphogenesis. *Circ. Res.* **77,** 1-6.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240,** 889-95.

Fishman, M. C. and Chien, K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124,** 2099-117.

Gruber, P. J., Kubalak, S. W., Pexieder, T., Sucov, H. M., Evans, R. M. and Chien, K. R. (1996). RXR alpha deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J. Clin. Invest.* **98,** 1332-43.

Gu, H., Marth, J. D., Orban, P. C., Mossmann, H. and Rajewsky, K. (1994). Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265,** 103-6.

Harvey, R. P. (1996). NK-2 homeobox genes and heart development. *Dev. Biol.* **178,** 203-16.

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.

Kastner, P., Mark, M. and Chambon, P. (1995). Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* **83,** 859-69.

Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A. and Evans, R. M. (1992). Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev.* **6,** 329-44.

Marth, J. D. (1996). Recent advances in gene mutagenesis by site-directed recombination. *J. Clin. Invest.* **97**, 1999-2002.

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,** 5157-61.

O'Gorman, S.,Dagenais, N. A., Qian, M. and Marchuuk, Y. (1997). Protamine-cre recombinase transgenes efficiently recombine target sequences in the male germline of mice, but not in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **94,** 14602-14607.

Olson, E. N. and Srivastava, D. (1996). Molecular pathways controlling heart development. *Science* **272,** 671-6.

Rezaee, M., Isokawa, K., Halligan, N., Markwald, R. R. and Krug, E. L. (1993). Identification of an extracellular 130-kDa protein involved in early cardiac morphogenesis. *J. Biol. Chem.* **268,** 14404-11.

Ross, R. S., Navankasattusas, S., Harvey, R. P. and Chien, K. R. (1996). An HF-1a/HF-1b/MEF-2 combinatorial element confers cardiac ventricular specificity and established an anterior-posterior gradient of expression. *Development* **122,** 1799-809.

Rossant, J. and Nagy, A. (1995). Genomic engineering: the new mouse genetics. *Nature Medicine* **1**, 592-594.

Rossant, J. (1996). Mouse mutants and cardiac development: new molecular insights into cardiogenesis. *Circ. Res.* **78,** 349-353.

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

Soonpaa, M. H., Kim, K. K., Pajak, L., Franklin, M. and Field, L. J. (1996). Cardiomyocyte DNA synthesis and binucleation during murine development. *Amer. J. Physiol.* **271,** H2183-89.

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 Dev.* **8,** 1007-18.

Sucov, H. M., Murakami, K. K. and Evans, R. M. (1990). Characterization of an autoregulated response element in the mouse retinoic acid receptor type beta gene. *Proc. Natl. Acad. Sci. USA* **87,** 5392-6.

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

Wolska, B. M. and Solaro, R. J. 1996. Method for isolation of adult mouse cardiac myocytes for studies of contraction and microfluorimetry. *Amer. J. Physiol.* **271,** H1250-55.

Wunsch, A. M., Little, C. D. and Markwald, R. R. (1994). Cardiac endothelial heterogeneity defines valvular development as demonstrated by the diverse expression of JB3, an antigen of the endocardial cushion tissue. *Dev. Biol.* **165,** 585-601.

Zhou, M. D., Sucov, H. M., Evans, R. M. and Chien, K. R. (1995). Retinoid-dependent pathways suppress myocardial cell hypertrophy. *Proc. Natl. Acad. Sci. USA* **92,** 7391-5.