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The Outflow Tract of the Heart Is Recruited from a Novel Heart-Forming Field

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As classically described, the precardiac mesoderm of the paired heart-forming fields migrate and fuse anteriomedially in the ventral midline to form the first segment of the straight heart tube. This segment ultimately forms the right trabeculated ventricle. Additional segments are added to the caudal end of the first in a sequential fashion from the posteriolateral heart-forming field mesoderm. In this study we report that the final major heart segment, which forms the cardiac outflow tract, does not follow this pattern of embryonic development. The cardiac outlet, consisting of the conus and truncus, does not derive from the paired heart-forming fields, but originates separately from a previously unrecognized source of mesoderm located anterior to the initial primitive heart tube segment. Fate-mapping results show that cells labeled in the mesoderm surrounding the aortic sac and anterior to the primitive right ventricle are incorporated into both the conus and the truncus. Conversely, if cells are labeled in the existing right ventricle no incorporation into the cardiac outlet is observed. Tissue explants microdissected from this anterior mesoderm region are capable of forming beating cardiac muscle *in vitro* when cocultured with explants of the primitive right ventricle. These findings establish the presence of another heart-forming field. This anterior heart-forming field (AHF) consists of mesoderm surrounding the aortic sac immediately anterior to the existing heart tube. This new concept of the heart outlet's embryonic origin provides a new basis for explaining a variety of gene-expression patterns and cardiac defects described in both transgenic animals and human congenital heart disease. © 2001 Academic Press

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INTRODUCTION

A fundamental concept of heart development is that the early heart tube forms from two regions of splanchnic mesoderm called the lateral heart fields located on either side of the embryonic midline. The heart fields migrate ventrally and fuse anteriorly to form the single primitive heart tube composed of inner endocardium and outer myocardium. Numerous cell tracing studies using different kinds of cellular markers have shown that cells labeled within the lateral plate mesoderm at Hamburger and Hamilton (HH) stages 8–12 (Hamburger and Hamilton, 1951) embryo will, with continued development, contribute to most regions or segments of the forming heart. From these studies the spatial boundaries of the heart-forming fields have also been determined and generally accepted (Rawles, 1943; Rosenquist and DeHaan, 1966; Stalsberg and De Haan, 1969).

Less recognized are the results from other detailed mapping studies that suggest the hypothesis that mesodermal precursor cells are restricted to a specific heart segment by the developmental time interval, during which the cells are recruited to the heart lineage. Based on this hypothesis, the developing heart arises from mesoderm that is restricted not only by regional boundaries into the heart lineage but

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also by temporal boundaries that help determine the identity of specific segments (de La Cruz and Markwald, 1998).

The looped embryonic heart tube (HH stage 11) consists of five distinct segments formed in a temporal sequence (Mjaatvedt *et al.,* 1999). The first segment to be formed comprises the entire straight heart tube and is the future trabeculated portion of the right ventricle (segment 1; HH stage 9−). Formation of the first segment is followed by the future trabeculated left ventricle (segment 2; HH stage 9+), the atrioventricular canal (segment 3; HH stage 10+), sinuatrial (segment 4; HH stage 12), and the conotruncus (segment 5; HH stage 11− to stage 22+). Although all five primitive segments are represented in the looped heart, they do not independently give rise to the corresponding heart chambers recognized in the adult. Rather, a single adult chamber arises through a developmental process of remodeling that mediates the interaction and integration of more than one primitive segment (de la Cruz *et al.,* 1977, 1989).

The terms *outlet segment, conotruncus,* or *outflow tract* are used variably to describe the vascular conduit between the embryonic right ventricular segment and the aortic arches. For our studies, we refer to the heart's outlet as the region between the embryonic right ventricle and the aortic arches that includes the conus, truncus, and aortic sac (Pexieder, 1995; Thompson *et al.,* 1985). Both the conus and truncus, but not the aortic sac, have an outer myocardial cell layer during embryogenesis. The conus and truncus also form mesenchymal swellings called "cushions" that project into the lumen. The cushions of the conus ultimately fuse to form the conal or outlet septum that divides the conus into a *potential* outlet for both the right and left ventricle (the future infundibulum and aortic vestibule, respectively). The truncal cushions will differentiate into the valves that guard the exit for each ventricular outlet. Blood from the embryonic truncus continues into a pharyngeal "vascular basket" called the aortic sac that, in turn, connects to each of the pharyngeal arch arteries (Kirby *et al.,* 1997). The aortic sac becomes divided by a septum of neural crest origin into the proximal roots of the aorta and pulmonary arteries (de La Cruz and Markwald, 1998). Malformations in the outlet segment occur at a high frequency in humans and comprise approximately one-third of newborn heart defects (Clark, 1996; Ferencz *et al.,* 1985).

Furthermore, many diverse naturally occurring and targeted gene mutations in mice have exhibited defective phenotypes of the heart outlet at various stages of development (Camenisch *et al.,* 2000; Lin *et al.,* 1997; Lyons *et al.,* 1995; Mjaatvedt *et al.,* 1998). One example is the heart defect (*hdf*) mouse. Analysis of this insertional mutant phenotype indicated that the entire right side of the embryonic heart (first segment and conotruncus) failed to differentiate normally. These observations lead to the hypothesis that the first segment (right ventricle) and fifth segment (conotruncus) are developmentally linked. Two alternative mechanisms of conotruncus formation have been proposed: (1) the conotruncus is derived from the growth of cells existing in the primitive right ventricle; or (2) the primitive right ventricular segment is required to interact with an unrecognized source of cardiac precursor cells to recruit them into the conotruncus. The results of our fate mapping, tissue ablations, and *in vitro* explant assays reported here strongly support the second mechanism.

MATERIALS AND METHODS

Production of Recombinant Adenovirus

Generation and propagation of high titers of the recombinant adenovirus expressing β-galactosidase were performed as described previously using adenoviral genomic DNA tagged with terminal protein (Nakaoka *et al.,* 1997). Expression of the β-galactosidase gene in the recombinant adenovirus is driven by the chimeric CAG promoter [cytomegalovirus enhancer, chicken β-actin promoter, rabbit β-globin poly(A) signal].

Microinjection of Virus and Vital Dyes

For viral microinjections, fertilized White leghorn eggs (viral-free; Spafas) were incubated at 37.5°C in a humidified chamber until the desired developmental stage. After washing the shell with 70% ethanol, the egg was "windowed" by removing a small area of shell and underlying membrane of the egg. Microinjections were made using a micropipette regulated by a picospritzer II pressure regulator. Approximately 5×10^6 plaque-forming units (pfu) (titer: 10^{10}–10^{11} pfu/ml) were delivered to each selected microinjection site in a total 50 nl volume. After microinjections, the embryos were sealed with parafilm and returned to the incubator to allow development to later desired stages.

For vital dye microinjections, fertilized White leghorn eggs (viral-free; Spafas) were incubated at 37.5°C in a humidified chamber until the desired developmental stage. Embryos were removed from the yolk using a small ring of filter paper that was placed on and adhered to the vitilline membrane surrounding the embryo. The vitilline membrane was cut around the outer edge of the paper ring and then removed with the adherent embryo and placed onto the surface of an agar nutrient mixture with the embryo in a ventral position to allow microinjections of Mitotracker red or green fluorescent dyes (Molecular Probes, Eugene, OR). Following microinjections, the embryos were cultured with the endoderm side down on nutrient medium consisting of one part 6% agar in Howard's Ringers solution and three parts hen-egg supernatant (Packard and Jacobson, 1976).

LacZ Staining Methods

Histochemical staining of the mouse embryos for β-galactosidase was conducted as described (Mjaatvedt *et al.,* 1991). Briefly, embryos were fixed in 0.2% glutaraldehyde in PBS with 2 mM $MgCl_2$ and 5 mM EGTA and then rinsed three times in phosphate-buffered saline (PBS) with 2 mM $MgCl_2$, 0.02% NP-40, and 0.01% sodium deoxycholate. Embryos were incubated in 0.1% X-gal (Sigma, St. Louis, MO) in PBS with 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate, 20 mM $K_3Fe(CN)_6$, and 20 mM $K_4Fe(CN)_6$ at 37°C for 12 h and washed in PBS containing 10 mM EDTA. Some β-galactosidase-stained specimens were dehydrated through an ethanol series, embedded into paraplast, and sectioned. Sections were counterstained with nuclear fast red and examined with a Zeiss photomicroscope.

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Embryonic Culture and Ablation of Lateral Heart Field Mesoderm

White leghorn chick eggs (Spafas) were incubated at 37.5°C with humidity. The stage of development was evaluated based on the nomenclature of Hamburger and Hamilton (1951). The embryos were explanted in a paper ring and placed in a dorsal position in a petri dish (35 mm). The embryos were washed with Earl's balanced salt solution (EBSS; Gibco) to remove excess yolk. The cardiogenic mesoderm and the endoderm, as described by Rosenquist and Dehaan (1966), was removed using a fine glass needle. The anterior margin of the foregut was also removed. After the ablation the embryos were transferred to a center-well organ culture dish (Falcon), which contained fresh albumin (1:3) in EBSS without sodium bicarbonate and without neutral red (Sigma). The microdissected embryos were cultured for 24 h in an incubator at 37.5°C, 5% CO_2, and 100% humidity. After the incubation (48–72 h), the embryos were fixed in 3.7% formaldehyde at room temperature for 1 h and immersed in normal goat serum (NGS; 0.75%) and bovine serum albumin (BSA; 1%) overnight. The embryos were then incubated with sarcomeric myosin antibody MF20 (Developmental Hybridoma Studies Bank, IA) for 4 h at room temperature or overnight at 4°C, washed with PBS–Tween 20 (0.1%), and incubated 2 h with goat anti-mouse IgG conjugated with fluorescein (Jackson ImmunoResearch Laboratories, West Grove, PA). The embryos were then extensively washed with PBS–Tween 20 and coverslipped with 50% glycerin in PBS containing DAPCO (Sigma) as an antiquenching agent.

Immunofluorescent Staining of Embryos

Specimens were stained immunohistochemically as previously described, with some modifications (Mjaatvedt *et al.,* 1991). For whole-mount immunostaining, embryos were fixed with 4% paraformaldehyde fixative, rehydrated through a decreasing methanol series to water, equilibrated with PBS, and blocked with 1% bovine serum albumin in PBS (overnight; 4°C). Undiluted MF20 antibody containing cell culture supernatant (or nonantibody-containing supernatant; control) was incubated (4°C) with the embryos overnight. The embryos were then extensively rinsed with PBS and incubated with a diluted (1/100 in PBS) fluorescein- or Cy5-labeled anti-mouse IgG secondary antibody (Cappel Research Products, Durham, NC) overnight (4°C), and rinsed again extensively. For sectioned analysis, specimens were fixed with 4% paraformaldehyde, dehydrated through an ethanol series, infiltrated with xylene, embedded in paraffin, and sectioned. Selected 5- to 10-μm sections were rehydrated with an ethanol series to water and then equilibrated in PBS (10 mM phosphate/140 mM NaCl, pH 7.2). Hydrated sections were blocked with 10% whole goat serum in 1% bovine serum albumin in PBS prior to incubation with primary antibody. The antibody to myosin heavy chain (MF20; Developmental Hybridoma Studies Bank) was diluted 1:100. Cells from quail-derived explants were identified using the quail-specific antibody QCPN (Developmental Hybridoma Studies Bank). The secondary antibody, fluorochrome-labeled rabbit anti-mouse IgG (No. 111-015-003; Jackson ImmunoResearch Laboratories), was diluted 1:100.

Anterior Heart Field Assay

Fertilized White leghorn eggs (viral-free; Spafas) were incubated at 37.5°C in a humidified chamber until developmental HH stages 12–16. Embryos were removed, staged, and further microdissected using tungsten needles to remove small explants of tissue surrounding the aortic sac. Anterior heart field explants consisted of mesoderm alone or mesoderm with the associated ectoderm and endoderm. These were then placed on the surface of hydrated collagen gels (rat tail collagen 1 mg/ml; Collaborative Research, Bedford, MA) saturated with M199 (Gibco; supplemented with penicillin/streptomycin) with or without 1% chicken serum (Gibco), allowed to attach and grow. After 24 h a second explant dissected from the distal outlet of a HH stages 15–16 heart (chicken or quail) was placed at the periphery of the AHF mesodermal outgrowth and incubation was continued for an additional 48 h. A quail-derived mesodermal cell line, QCE-6 (Eisenberg and Bader, 1995), was used as a control for the anterior heart field mesoderm in similar experiments. Confluent monolayers of the QCE-6 cells stably transfected with the marker β-galactosidase were coincubated with an explant dissected from the distal outlet of a HH stages 15–16 chick embryos and incubation continued for an additional 48 h. Explant cultures were then fixed with 4% paraformaldehyde and immunostained in whole mount for sarcomeric myosin heavy chain positive cells (MF20 antibody). Cells from quail-derived explants were identified using the quail-specific antibody QCPN (Developmental Hybridoma Studies Bank). QCE-6 cells were detected using indirect immunofluorescence and a primary antibody recognizing β-galactosidase (Sigma).

Cell Transfection Assays

Transient transfection assays in mouse NIH3T3 cells. The Nkx2.5 expression construct used was the kind gift of Dr. Terrence O'Brien (Medical University of South Carolina). All plasmids used for transfection were purified using the Endo-Free Maxi-Prep kit (Qiagen, Chatsworth, CA). NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Gibco BRL) in a 37.5°C incubator with 5% CO_2. Cells were seeded 24 h prior to transfection in 35-mm dishes at 2×10^5. Cells were transfected using 15 μl Superfect reagent (Qiagen) to 3 μg of DNA per 35-mm dish as previously described (Norris and Kern, 2001). Both expression and reporter plasmids were used at 40% of the final DNA concentration and 20% of an internal control plasmid pSV-βgal (Promega, Madison, WI) was included to allow for normalization. Cells were harvested 24 h posttransfection in 300 μl of 1× reporter lysis buffer (Promega). Luciferase activity was measured as counts per minute using the Monolight 2010 (Analytical Luminescence Laboratory). All transfections were performed in triplicate and repeated a minimum of three times.

RESULTS

To specifically answer the question of conotruncus origin we performed fate-mapping experiments using either a fluorescent cell label called Mitotracker (Molecular Probes) or a replication-deficient adenovirus expressing a β-galactosidase marker. To analyze early conus formation, Mitotracker green was microinjected at HH stage 8 into the mesoderm located approximately 15–20 μm beyond the distal end of the beating straight tubular heart (Fig. 1A). A second label, Mitotracker red, was injected into the middle of the beating straight heart tube (i.e., the region or segment that forms the future trabeculated right ventricle). Incubation was continued to HH stage 10 (prior to overt formation

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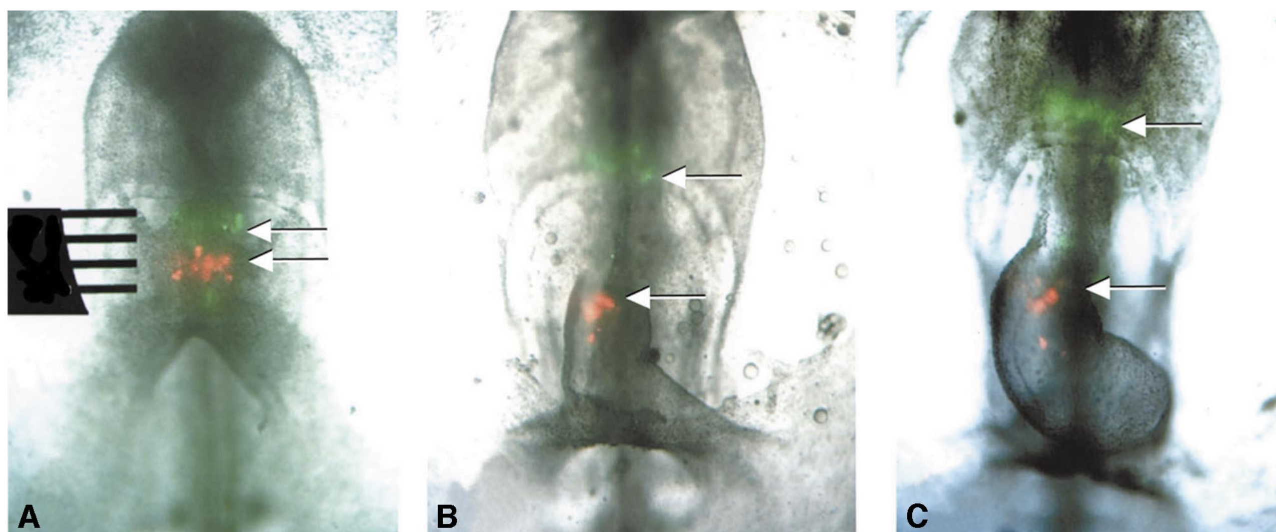

FIG. 1. *In vivo* marking experiments with Mitotracker to analyze conus formation. (A) Mitotracker dye (green spots; arrow) was used to label the anterior mesoderm approximately 15–20 μm beyond the distal edge of the fused heart tube at HH stage 8+ (black horizontal bars are 15 μm apart and apply to all panels). A different Mitotracker dye (red spots; arrow) was used to label the midportion of the definitive heart tube of the same embryo. (B) Positions of the labeled cells after incubation of the embryo to HH stage 10+, shows a significant expansion of tissue between the two markers, corresponding to growth of the preconus segment. (C) Relative positions of the two Mitotracker-labeled cells after continued incubation of the embryo to HH stage 13.

of the conus) or stage 12 (conus clearly seen) (Figs. 1B and 1C), respectively. Between stage 8 and stage 10, the two sets of labels become separated from each other by an intervening mesodermal space of more than 90 μm that exhibited no evidence of MF20 staining (data not shown). Mitotracker red clearly remains within the distal region of the linear heart tube, whereas Mitotracker green tracks with the cephalic mesoderm. The two *in vivo* dyes hold their relative positions through HH stage 13. At that time a definitive [MF20(+)] conus has started to develop in the cephalic mesoderm located between the two sets of markers (Fig. 1C). We interpret these findings to indicate: (1) that the proximal portion of the outlet segment or conus is *not* derived by the distal or cranial extension of the existing linear heart tube (itself a derivative of the paired heart fields), but rather (2) the initial (proximal) portion of the conus is derived from a rapidly elongating band of cephalic undifferentiated mesoderm located immediately anterior/cranial to the distal end of the beating heart tube.

To determine whether the remainder of the conus and the distal portion of the outlet segment (i.e., the truncus) are similarly derived by the recruitment of mesoderm from an anterior source of mesoderm, we performed fate-mapping studies beginning at HH stage 16 or stage 17. At this stage the truncus first appeared and the incubation was continued until stage 22, at which time the truncus was almost fully formed. Both Mitotracker red and a replication-deficient adenovirus expressing β-galactosidase under the control of the chimeric CAG promoter (Nakaoka *et al.*, 1997) were used. Preliminary experiments showed that the CAG promoter allowed detection of the *lacZ* gene as early as 4 h after infection of an embryonic heart. Since the truncus forms over a period of several stages (HH stages 16–22), this adenoviral marker was ideal for fate-mapping studies of the distal portion of the outlet segment.

Two different fate-mapping experiments were performed. First, embryos (HH stage 16) were microinjected *in ovo* with recombinant adenovirus expressing *lacZ* into the right side of the pericardial cavity. This type of microinjection effectively achieved myocardial infection of the entire primitive right ventricular segment and existing conus. In the second set of mapping experiments, embryos (HH stage 17) were microinjected with a fluorescent marker (Mitotracker) into the mesoderm surrounding the existing aortic sac located immediately anterior (cranial) to the distal rim of the beating heart. After infection, the embryos were reincubated for 3 additional days, dissected from extraembryonic tissues, fixed, and processed for whole-mount or section analysis to localize the *in vivo* marker expression patterns. Results presented in Figs. 2A and 2B demonstrate that microinjections of the adenovirus into the pericardial sac labeled only myocardium of the primitive right ventricle and associated conus already present at the time of microinjection, but did not label the developing distal conus or truncus. Conversely, microinjections of markers into the mesoderm surrounding the aortic sac resulted in labeling only of cells in the distal conus and whole truncus heart regions (Fig. 2C). The right ventricle and associated inflow regions remained unlabeled. The results from these two fate-mapping studies supported the hypothesis that the

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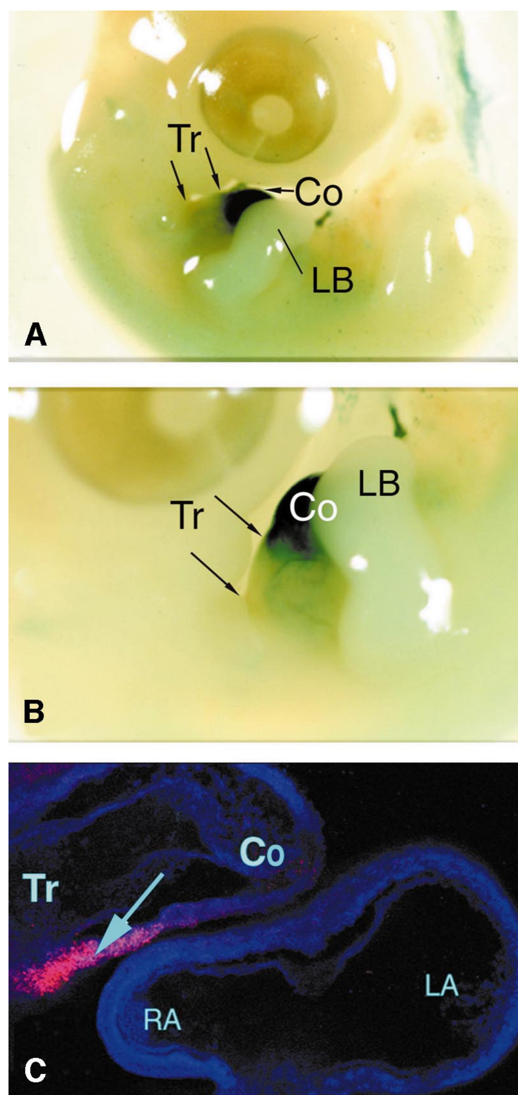

FIG. 2. *In vivo* marking experiments to analyze conus formation. (A) Typical embryo microinjected with *lacZ*-expressing adenovirus at the growing (distal) edge of a HH stage 17 heart (right ventricle and conus), incubated for 3 days, then fixed and stained for β-galactosidase. New growth of the truncus (Tr) is *unlabeled,* showing that it did not arise from the existing labeled heart tube. Co, conus; Tr, truncus; LB, limb bud obscuring the right ventricle. (B) Higher magnification of the embryo in A. (C) Converse of the experiments shown in A and B. Section of stage 16 heart in which the new growth of the truncus is labeled (red) by use of a fate-mapping marker (Mitotracker) microinjected into the putative anterior heart-forming mesoderm located around the aortic sac at HH stage 14. Labeled cells (red) were in the growing conotruncus only (arrow). Tr, truncus; Co, conus; RA, right atrium; LA, left atrium.

growing conus/truncus segment of the outlet is not derived from the preexisting primitive ventricle. Rather the data suggest that this complex region of the heart develops from another source of mesoderm located anterior to the existing heart structures and is not a derivative of the original paired heart-forming fields.

To further test these conclusions, *in vivo* ablation experiments were performed to determine whether cephalic mesoderm contributed to heart development. When the lateral heart fields defined by Rosenquist and deHaan (1966) were carefully and completely excised at stage 8 (i.e., prior to their fusion), no posterior (inlet) heart segments were formed at what normally would have been the venous pole (Fig. 3). However, at the opposite (anterior) pole, a linear but narrow tubular structure did form at stage 11 that resembled a rudimentary conus. Moreover, this putative conotruncal rudiment was contractile and positive for sarcomeric myosin staining, but ended blindly (i.e., no connection with the vascular system). These experiments provide further evidence that the cardiac outlet does not derive from the lateral heart-forming fields but originates from a previously unrecognized source of mesoderm located anterior to the primitive right ventricle.

In the embryo the mesoderm of the anterior to existing heart tube and surrounding the aortic sac normally do not express cardiac muscle markers except at the boundary interface between the existing right primitive ventricle and the undifferentiated anterior mesoderm (Fig. 4). This observation and previous studies of the *hdf* mouse suggest that the developing right ventricular myocardium might participate in actively inducing or recruiting undifferentiated mesoderm into the myocardial lineage.

To test the hypothesis, the ability of this mesoderm to form cardiac muscle was directly examined using an *in vitro* bioassay. In these experiments, mesoderm that is located just anterior to the primitive heart was microdissected from embryos during early stages of conus formation and placed in various culture conditions (Fig. 5). Specifically, mesodermal explants were microdissected from the anterior aspect of dorsal mesocardium proximal to the distal end of the cardiac outlet using embryos at early stages of heart looping (HH stage 12). These explants, consisting only of splanchnic mesoderm (devoid of any beating tissue), were placed on the surface of three-dimensional collagen gels (3 explants/gel; containing medium 199) and incubated at 37.5°C. After 3 days, all of the explants had acquired the ability to contract spontaneously (data not shown). These explants also expressed a cardiac muscle marker, cardiac myosin heavy chain (MHC), as demonstrated by immunofluorescent staining using the MF20 antibody (Han *et al.,* 1992). Explants grown in identical conditions, but without serum, did not acquire the myocardial phenotype (data not shown). Similar results were obtained using explants dissected from the region of the aortic sac of older embryos (HH stages 12–16). These explants consisted of the mesoderm surrounding the aortic sac and any persisting associated ventral endoderm. The results of these experiments demonstrated that mesoderm located anterior to the right primitive ventricle was indeed capable of forming cardiac

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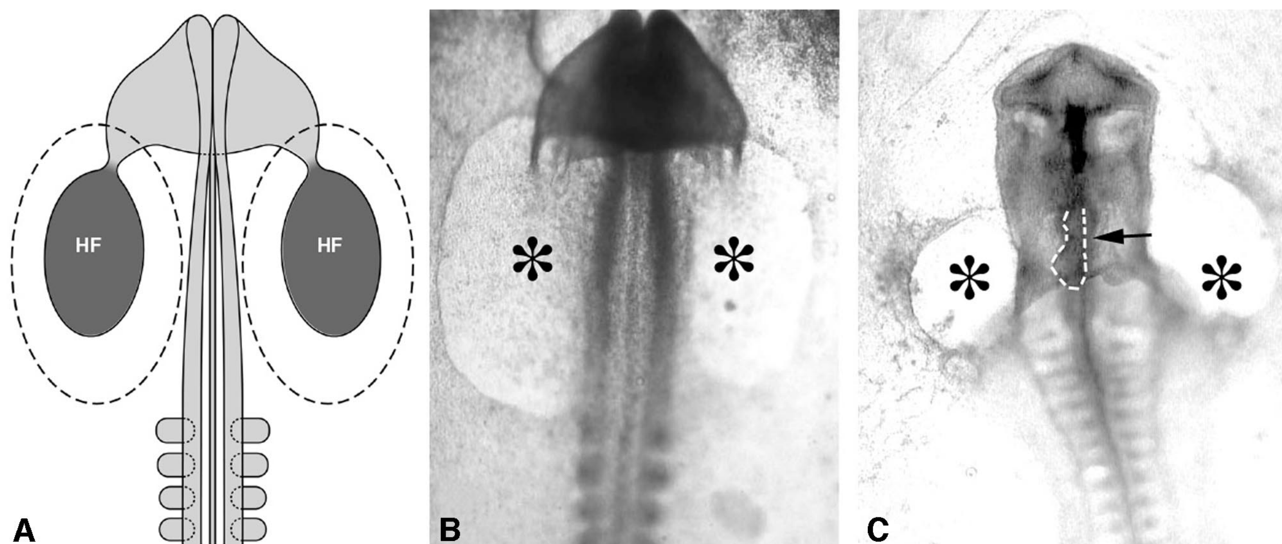

FIG. 3. *In vivo* ablation of the classical heart-forming fields. Ventral view drawing of a HH stage 8+ embryo (A) showing embryonic landmarks and the relationship of the region excised by microdissection (dotted line) to the lateral heart fields (HF) as defined by Rosenquist and DeHaan (1966). Although the lateral heart fields were removed in the stage 8+ embryo (B), the heart outlet is still able to form a beating structure (arrow) with continued incubation of the same embryo to stage 11 (C). Asterisks in both panels denote the region of the ablated heart field mesoderm.

muscle under the same *in vitro* culture conditions (i.e., the presence of serum).

To determine whether the right ventricle actively participates in the recruitment of anterior mesoderm to form an outlet segment, we performed a series of coculture experiments with explants of both the anterior heart field mesoderm and the distal end of the primitive right ventricle. In these experiments, anterior heart field explants dissected free of the right ventricle were placed onto the surface of three-dimensional collagen gels and cultured in a defined medium without serum. Under these conditions, the isolated anterior mesodermal cells grow, but fail to differentiate along the myocardial lineage. If the anterior heart field explants are placed into close coculture with an explant (HH stages 15–16) removed from the distal tip of the primitive right ventricle and proximal conus, cells of the anterior heart field begin to express the cardiac myosin heavy chain marker. A total of 18 AHF explants were coincubated with RV explants and all were determined positive by MF20 positive staining of a minimum of 20 cells. A total of 6 AHF explants were incubated without coculture (controls) and all determined to be negative with less than three positive MF20 cells found within the entire explant. Conversely, cocultures using an explant derived from the left ventricle showed little or no recruitment of cells from the anterior heart field explant (Figs. 5 and 6). This suggests that the primitive heart tube is not uniform in its ability to recruit new cells into the cardiac phenotype. The ability to induce the anterior heart field mesoderm into the cardiac lineage appears to be strongest at the distal tip of the right ventricle but appears to be completely absent in the more posterior segments (e.g., left ventricle) of the primitive heart tube.

To further test the ability of the right ventricular explants to recruit new myocardial cells and control for the possibility that MF20(+) cells were derived directly from the heart explants, we used a continuous cell line, called QCE-6. This cell line was derived from the lateral heart-forming fields of quail embryos (Eisenberg and Bader, 1996) and can form fully contractile cardiomyoctyes, endothelial, or red blood cells when appropriately signaled (Eisenberg and Markwald, 1997). Specifically, coculture experiments were performed with QCE-6 to determine whether the right ventricular explants possessed the appropriate signaling ability to direct cardiomyocyte differentiation from this cellular model of the classical heart fields. The QCE-6 cell line was stably transfected with the β-galactosidase gene for identification of these cells when cocultured with explants of the right primitive ventricle. Results shown in Figs. 7A–7C indicate that the right ventricular explant was capable of recruiting closely adjacent *lacZ*-positive QCE-6 cells into a three-dimensional cluster of cells that expressed the cardiac myosin heavy chain marker. QCE-6 cells more than 2–3 cell diameters away from the myocardium were unaffected, suggesting a short-range inductive event.

A more important direct test of the ability of the right ventricular explants to recruit new myocardial cells from the anterior heart mesoderm was done using a chimeric tissue strategy. We performed the standard anterior heart field assay using the anterior heart field mesoderm from a chick embryo cocultured with a distal outlet heart explant (HH stage 16) derived from a quail embryo. The distribution

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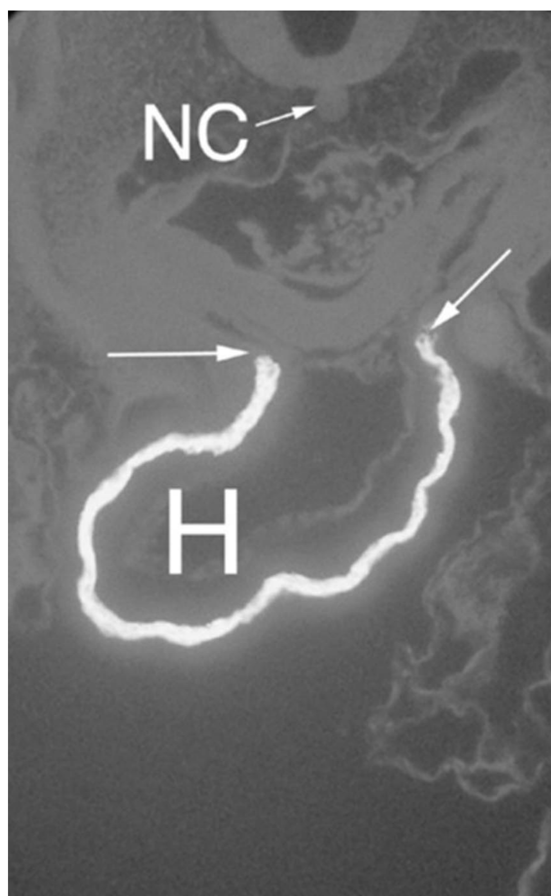

FIG. 4. Transverse section of HH stage 16 embryonic heart immunostained with the MF20 antibody. Arrows indicate the boundary between undifferentiated anterior heart field (AHF) mesoderm and newly formed cardiac myocardial cells expressing myosin heavy chain [MF20(+)]. NC denotes the notochord.

of the quail-derived cells was determined by immunolocalization of the QCPN antibody that recognizes a quail-specific antigen and was compared with MF20 positive staining. Results showed MF20 positive cells in both the AHF mesoderm derived from the chick and in the quail-derived distal outlet heart explant. Immunolocalization of the QCPN antibody was found only in the quail distal heart explant. None of the MF20 positive cells found in the chick derived AHF mesoderm was found to be QCPN positive (Figs. 7D–7G). From these studies, we conclude that the distal rim of the embryonic right ventricle was competent to signal undifferentiated anterior heart field mesoderm cells into the growing distal end of the outlet.

Our previous work has shown that the *hdf* gene (versican) is essential for normal development of the three-dimensional structure of the conotruncus and is highly expressed in this segment (Mjaatvedt *et al.,* 1998; Yamamura *et al.,* 1997). The cardiogenic factor Nkx-2.5 regulates its target genes in a transcriptionally modular fashion (Schwartz and Olson, 1999) and mice lacking this gene possess a lethal phenotype similar to the *hdf* (versican) null mice (Lyons *et al.,* 1995). As a first step toward understanding a potential transcriptional regulatory relationship between the anterior heart field and the formation of conotruncal segment, we tested the potential ability of Nkx-2.5 to control the expression of versican in cell transfection assays. The assays utilized a portion of the versican promoter (−465 to +307 bp) to drive the reporter gene luciferase. Mouse NIH3T3 cells were transfected with the reporter construct and an Nkx-2.5 expression construct. Results showed a 15-fold increase in relative luciferase activity in cells cotransfected with the versican promoter and the Nkx-2.5 transcription factor versus the versican promoter alone (Fig. 8). These results indicate that members of the Nkx gene family, involved in formation of the earliest heart segments, may also regulate other sets of downstream target genes required for addition of the final heart segment.

DISCUSSION

It is generally recognized that the vertebrate heart is derived from a pair of heart-forming fields (Rawles, 1943;

FIG. 5. Anterior heart field (AHF) assay. Fertilized White leghorn eggs were incubated at 37.5°C until developmental HH stages 12–16. Embryos were removed, staged, and further microdissected using tungsten needles to excise small regions of tissue surrounding the aortic sac. Anterior heart field explants consisted of mesoderm alone or mesoderm with the associated ectoderm and endoderm. These were then placed on the surface of hydrated collagen gels saturated with M199, then allowed to attach and grow. After 24 h a second explant from the distal outlet of a HH stages 15–16 heart was placed at the periphery of the AHF mesodermal outgrowth and incubation was continued for an additional 48 h. Explant cultures were then fixed with 4% paraformaldehyde and whole-mount immunostained for sarcomeric myosin heavy chain.

FIG. 6. MF20 immunostaining in the AHF assay. (A) The AHF assay tests the hypothesis that the distal end of the heart tube can recruit undifferentiated cells from the anterior heart field (mesoderm surrounding the aortic sac) to become myocardial [MF20(+)] cells. Explants containing anterior heart field mesoderm were carefully dissected from the aortic sac and placed on the surface of a collagen gel. After 24-h incubation, other explants dissected from the ventricle or distal outlet of HH stage 16 heart were placed in contact with the periphery of the anterior heart field mesoderm. The boundary of the anterior heart field mesoderm monolayer on the gel is delineated with the dotted line. Cocultures were incubated for an additional 48 h, fixed, and immunostained for cardiac myosin (MF20 antibody). Distal outlet explants appeared to recruit MF20(+) cells from the anterior heart field mesoderm near the outlet explants. The ventricular explants did not appear to recruit MF20(+) cells. (B) Higher magnification of the region near the distal outlet explant shown in A. Note the MF20(+) cells in the mesoderm.

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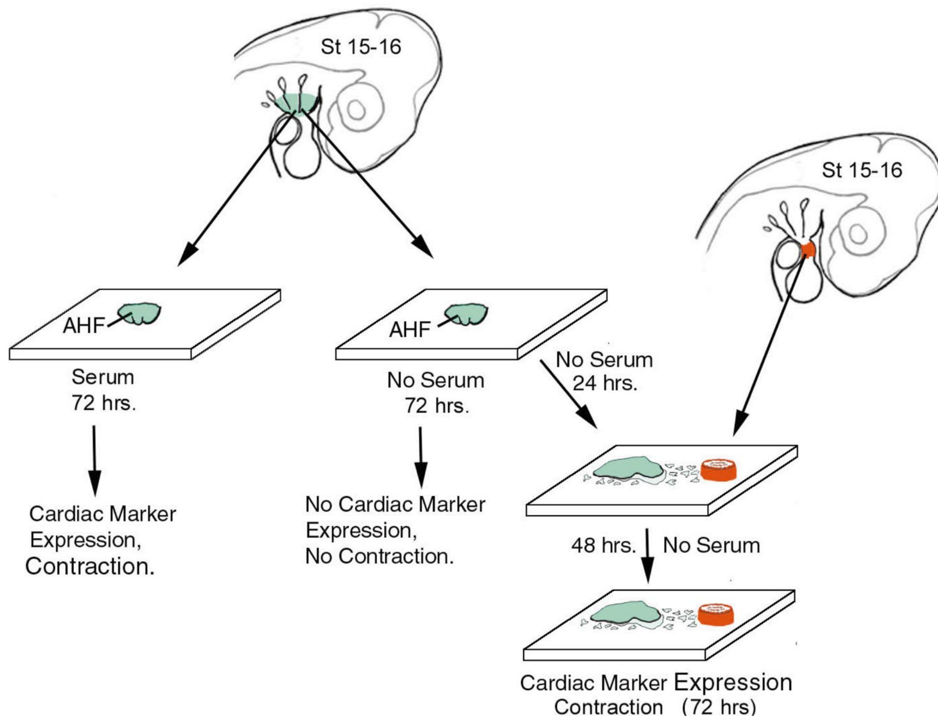

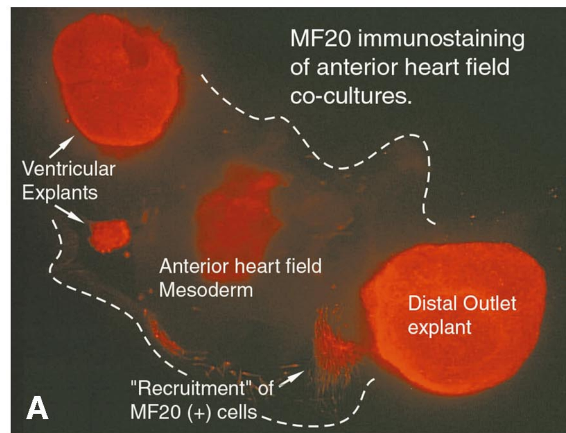

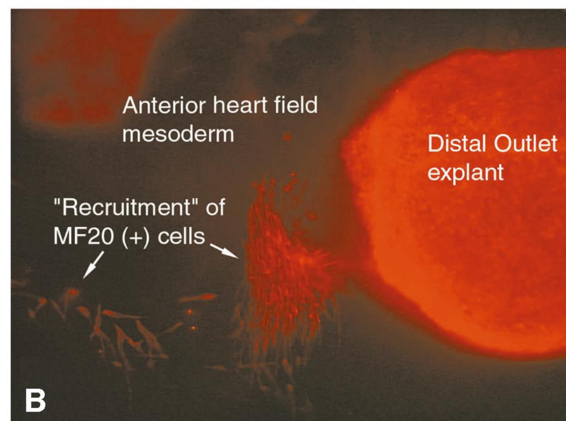

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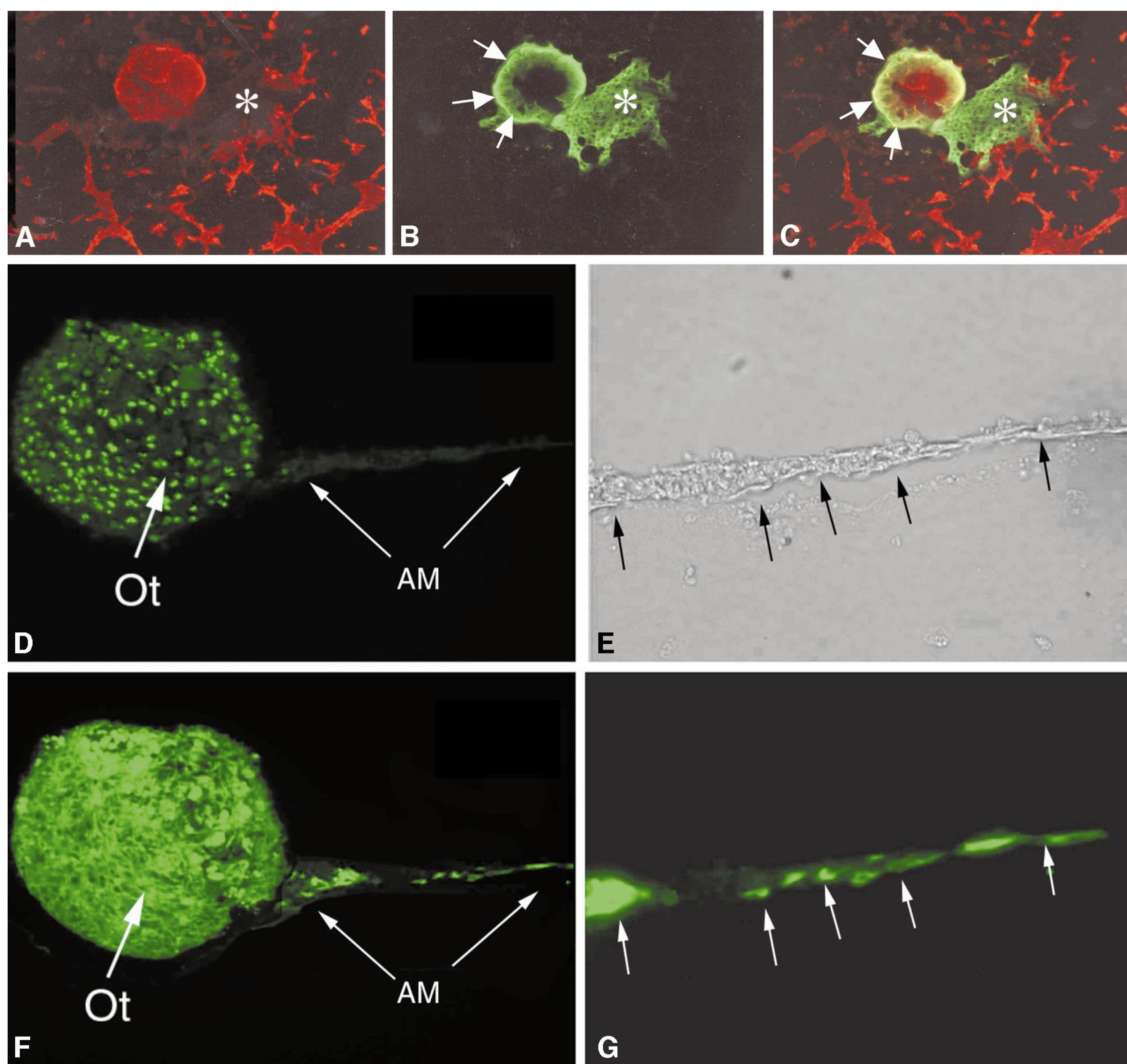

FIG. 7. Mixed coculture AHF experiments confirm the mesoderm as the source of recruited MF20(+) cells. (A–C) Coculture of chick outlet explants (HH stage 16) with the control mesodermal cell line QCE6, which is known to form cardiac muscle under appropriate stimulation. (A) β-Galactosidase expression (red) showing the distribution of the *lacZ*-transfected QCE6 cells cocultured with a chick distal outlet heart explant (marked with an asterisk; HH stage 16). (B) Distribution of MF20(+) cells (green) in the coculture visualized by indirect immunofluorescence. MF20(+) cells are found as expected in the chick heart explant, but also some closely adjacent QCE6 cells (arrow). (C) Co-localization of the MF20(+) (green) and QCE6 (*lacZ*)-positive (red) cells within the coculture. A co-localization of signals (yellow) in cells (arrows) was observed within an aggregate of QCE6 cells that formed adjacent to the outlet heart explant, suggesting a short range interaction of the outlet explant (asterisk) on the QCE6 cells acts to recruit QCE6 cells to the MF20(+) lineage. QCE6 cells without added explants do not express MF20 marker under the culture conditions (data not shown). (*) denotes the site of the OFT explant in all panels. (D, F, G) Immunostained sections of the anterior heart field assay using the anterior heart field mesoderm from a chick embryo (AM) with a distal outlet heart explant (Ot; HH stage 16) derived from a quail embryo. (D) Distribution of the QCPN antibody that recognizes a quail-specific antigen. Note that only the quail-derived cells (Ot) are marked by the QCPN antibody. The mesoderm (AM) is negative for QCPN antibody. (F, G) The anterior heart field mesoderm (AM) is positive for MF20, showing that these cells are differentiating from the explanted mesoderm removed from the AHF of the chick embryo and not from the quail myocardial explant (Ot). (E) Phase-contrast image and (G) are higher magnifications of the cultured mesoderm (AM) shown in D and F, respectively [arrows shown in E and G denote representative MF20(+) cells present in the mesoderm].

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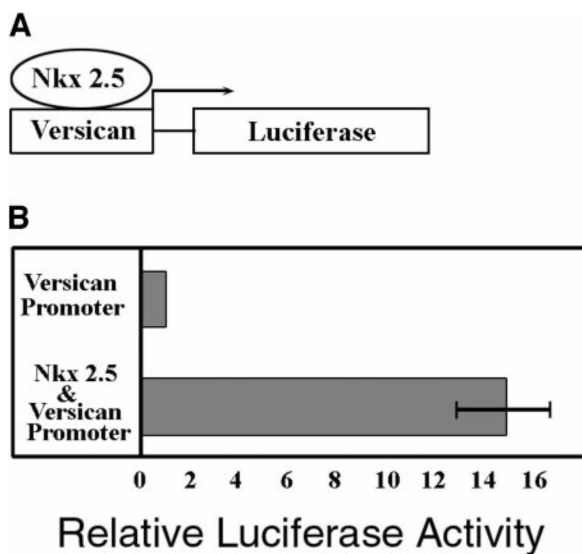

FIG. 8. *In vitro* regulation of the versican promoter by Nkx-2.5. (A) Transient transfection assays of mouse NIH3T3 cells were performed using a portion of the versican promoter (−465 bp to +307 bp) driving luciferase expression. (B) NIH3T3 cells were transfected with the reporter construct and an Nkx-2.5 expression construct. Results showed a 15-fold increase in the relative luciferase activity in cells cotransfected with the versican promoter and the Nkx-2.5 transcription factor. Transfections were performed in triplicate and repeated a minimum of three times.

Rosenquist and DeHaan, 1966). These fields, consisting of mesoderm fated to heart cell lineages (Davis *et al.,* 2000; Mikawa *et al.,* 1992; Schwartz and Olson, 1999), fuse to form sequentially and progressively, over time, the primary heart tube (de la Cruz *et al.,* 1989). However, no marker or *in vivo* label placed within the heart fields or beating heart tube itself has ever been reported to trace, over time, into the outlet segment of the heart (i.e., the conus and truncus regions) (de la Cruz *et al.,* 1977). Rather, if an *in vivo* marker is placed at the distal (beating) end of the straight heart tube at stage 9 or the looped heart tube at stage 12 and development then continued until stage 22, the label remained at the junction of the right ventricle and the conus or at the junction of the conus and the truncus (arterial pole of the heart). These data indirectly suggested to de la Cruz and colleagues that the conus and truncus are not derived from the original heart fields but from mesoderm located anterior (cranial) to the definitive heart tube itself. However, to our knowledge, *cephalic* mesoderm has never been experimentally shown to be cardiomyogenic.

The fate-mapping, ablations, and explant experiments performed in this study not only confirm the *in vivo* labeling studies of de la Cruz but also directly point to a previously unrecognized source of cephalic cardiogenic mesoderm as the progenitor of the conus and truncus regions of the outflow track. The existence of a separate anterior heart field that gives rise to the outlet segment of the heart by inductive recruitment from the existing heart tube provides a new basis for understanding a variety of the outlet-specific gene expression patterns and cardiac defects described in both transgenic animals and in human congenital heart disease. Several targeted gene deletions in mice show pronounced defects of the conotruncal region, even though the gene expression patterns are not confined to this segment. For example, the Nkx-2.5 gene expression pattern is observed initially in the lateral heart-forming fields (E7.5), then in the first fused segments of the heart (E8.5) and, by E12.5, in all segments of the heart (Harvey, 1996; Harvey *et al.,* 1999; Lints *et al.,* 1993). Nevertheless, in the Nkx-2.5 null mouse while the first segments form normally, heart development appears to stop at development of the conus segment.

Similarly, the dHAND/Hand2 gene is expressed in the lateral heart-forming fields and throughout the early segments, but eventually becomes restricted to the conotruncus region (Srivastava *et al.,* 1995). Both the dHAND/Hand2 deleted mouse and the Nkx-2.5 null mouse arrest heart development during embryonic stages of conus formation. In addition, the targeted deletion of MEF2c and a variety of apparently unrelated genes (Camenisch *et al.,* 2000) give rise to similar conotruncal heart defects. These common patterns of defective heart phenotype from apparently unrelated gene perturbations might be more easily understood by data presented in this study that indicate that the conotruncus has a unique embryonic origin. The Nkx-2.5, dHAND/Hand2, and MEF2c genes may work together to regulate downstream target genes that directly influence formation of the conus segment from the anterior heart field. Given that the absence of Nkx-2.5, dHAND, MEF2C and others appear to alter normal development after the formation of the first right ventricular segment, their normal function could be to facilitate the ability of the initial right ventricular segment to signal the anterior heart field formation of the outlet segment.

The target genes of transcription factors such as Nkx-2.5 and dHAND that actually function in the final steps of this mechanism are not known. However, we have found that Nkx-2.5 can directly activate the expression of the *hdf* gene (versican) in NIH3T3 cells (Fig. 8). The *hdf* gene (versican) is one important downstream target that is known to be required for normal outlet segment formation (Mjaatvedt *et al.,* 1998; Yamamura *et al.,* 1997). This regulation of versican expression by Nkx-2.5 during the AHF formation of the conotruncus may explain why the Nkx-2.5 null mice possessed a specific cardiac outlet defect. The *hdf* gene is highly expressed in the conotruncus and its protein product in association with hyaluronan appears to create the three-dimensional structure of the conotruncus. Cells upon expression of the hyaluronate acid synthase 2 (Has2) gene generate hyaluronan. Whether Has2 is also regulated by Nkx-2.5, like the *hdf* gene, is currently under investigation. However, the 5′ regulatory region of the Has2 gene contains

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putative Nkx-2.5 binding sites (our unpublished observations).

The foregut endoderm adjacent to the lateral heart fields appears to play an important inductive role during gastrulation of cardiac mesoderm (Sugi and Lough, 1994). A functional role for endoderm during stages of AHF mesoderm recruitment to cardiac muscle has not been explored extensively in this study. However, we observed that AHF explants containing associated endoderm do not form cardiac muscle under the conditions of the AHF assay. This suggests that endoderm is not alone sufficient to induce AHF mesoderm to form myocardium. This does not preclude a potential role for the endoderm on AHF mesoderm, particularly at the earlier stages of gastrulation.

Recently, supporting data in mammals for the existence of an anterior heart field have come from analysis of an insertional mutation that occurred during transgenic mouse studies on the MLC 1V promoter (Dr. Robert Kelly, personal communication). In one line of mice, the reporter gene (β-galactosidase) appears to be expressed only in mesodermal cells of the putative anterior heart field and the developing conus/truncus. This suggests that cells of the anterior heart field form a unique transcriptional domain represented by the boundaries of β-galactosidase expression in this mouse line. This transgenic mouse line, therefore, may be useful as a cardiosensor mouse (Kelly *et al.,* 1999) for future studies on the murine anterior heart field.

The understanding of the embryonic origin of the heart's outlet, as described here, establishes not only a new paradigm for evaluating a variety of gene and transgene expression patterns (Ross *et al.,* 1996; Srivastava *et al.,* 1995), but also heart outlet defects that have been described in both transgenic animals (Camenisch *et al.,* 2000; Chisaka and Capecchi, 1991; Conway *et al.,* 1997; Farrell *et al.,* 1999; Franz, 1989; Goldmuntz and Emmanuel, 1997; Lin *et al.,* 1997; Lyons *et al.,* 1995; Miyabara *et al.,* 1982; Mjaatvedt *et al.,* 1998; Vuillemin *et al.,* 1991; Yasui *et al.,* 1995) and human (Ferencz *et al.,* 1990) congenital heart disease. For example, because the "cardiac" neural crest plays a pivotal role in the spiral septum development of the cardiac outlet, these cells are commonly implicated as the mechanistic focal point for many different perturbations that result in cardiac outlet defects in mouse and human (Kirby *et al.,* 1983; Kirby and Waldo, 1995). We now know that the neural crest migrates through the anterior heart field and undoubtedly comes into contact with the mesoderm destined to form the conotruncus, as recruited by the existing heart tube. This suggests the possibility that molecular signals between the neural crest and anterior heart field mesoderm may be crucial in the process of forming the spiral septum, which separates the aortic sac into the pulmonary and aortic arteries.

The endocardial cushions of both the conotruncus and atrioventricular regions arise through similar inductive interactions between the outer myocardial layer and inner endothelial lining of the heart. The myocardium in both regions of the heart secretes a molecular signal(s), which traverses the intervening cardiac jelly and triggers the formation of endothelially derived mesenchyme. The cushion mesenchyme invades the underlying cardiac jelly and is ultimately remodeled to form the valve leaflets of both regions. In spite of these basic morphogenetic similarities, various epigenetic and genetic perturbations can differently affect the conotruncus and atrioventricular cushion derivatives. One striking example is the phenotype of the NF-ATc null mouse (Ranger *et al.,* 1998). Although NF-ATc is a transcription factor expressed in endothelial cells throughout the heart, targeted loss of the gene only results in an absence of the pulmonary and aortic valves derived from the conal cushions. The mitral and tricuspid valves, derived from the atrioventricular cushions, are nearly unaffected. The differential response to a lack of NF-ATc in conotruncal versus atrioventricular region endothelial cells may reflect that these different cushions arise from different heart-forming fields.

Our identification of the anterior heart field mesoderm and its importance to formation of the outlet creates new experimental opportunities for understanding the role of the neural crest, anterior heart field mesoderm, endoderm, and cushion mesenchyme in this complex region of the heart. The anterior–posterior gradient of recruitment competence in the heart outlet demonstrated in the AHF assay, was observed to be similar to the anterior–posterior expression pattern of the growth factors BMP2 and BMP4 (Jones *et al.,* 1991; Lyons *et al.,* 1990). This correlation and the phenotype of the BMP4-targeted null mouse (Winnier *et al.,* 1995) lead us to propose that BMPs may function to mediate recruitment of anterior heart field into myocardial cells. This hypothesis is currently under investigation.

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