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Wild-Type Endoderm Abrogates the Ventral Developmental Defects Associated with GATA-4 Deficiency in the Mouse

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GATA-4 knockout mice die by 9.5 days postcoitum and exhibit profound defects in ventral morphogenesis, including abnormal foregut formation and a failure of fusion of the bilateral myocardial primordia. During early mouse development, GATA-4 is expressed in cardiogenic splanchnic mesoderm and associated endoderm, suggesting that the presence of this transcription factor in one or both of these tissue types is essential for ventral development. To distinguish whether GATA-4 expression in mesoderm or endoderm accounts for the phenotype of the knockout mouse, we prepared chimeric mice by injecting *Gata4*−/− ES cells into 8-cell stage *ROSA26*(*Gata4*+/+) embryos. We identified a series of high percentage null chimeras (8–10 days postcoitum) in which *Gata4*+/+ cells were restricted to visceral yolk sac endoderm and small portions of the foregut/hindgut endoderm. Despite an absence of GATA-4 in all other cells of these embryos, there was normal development of the heart, foregut, and surrounding tissues. We conclude that expression of GATA-4 in endoderm rather than cardiogenic mesoderm is required for ventral morphogenesis.

INTRODUCTION

GATA-4 is a zinc finger transcription factor implicated in the regulation of gene expression and differentiation in cardiomyocytes, yolk sac endoderm, and selected other cell types (Arceci *et al.*, 1993). This factor belongs to a family of structurally related proteins that recognize the consensus DNA sequence (A/T)GATA(A/G), present in the promoters or enhancers of a variety of genes (reviewed in Kuo *et al.*, 1997; Molkentin *et al.*, 1997; Narita *et al.*, 1997). GATA-4 is expressed in precardiac splanchnic mesoderm and adjacent endoderm during the active processes of foregut invagination and heart tube formation (Heikinheimo *et al.*, 1994; Kuo *et al.*, 1997; Molkentin *et al.*, 1997). GATA-4 knockout mice die before 9.5 days postcoitum (p.c.) and display severe defects in ventral morphogenesis (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). In these mutants the bilateral myocardial primordia fail to migrate and fuse ventrally, resulting in formation of two dorsolateral heart tubes. In addition, the foregut is disorganized in appearance, the pericardial cavity is absent, and the yolk sac and amnion are displaced dorsally. These findings suggest that expression of GATA-4 in cardiogenic splanchnic mesoderm, associated endoderm, or both of these tissues is essential for ventral patterning. In this study we utilized a chimeric mouse system to demonstrate that expression of GATA-4 in endoderm and not mesoderm is essential for ventral morphogenesis.

METHODS

ROSA26 mice, bearing a β-galactosidase transgene that is ubiquitously expressed throughout development, were obtained from the Jackson Labs. To generate chimeric embryos, male mice homozygous for the *ROSA26* transgene were bred with superovulated C57BL/6J females. Eight-cell stage embryos were harvested at 2.5 days p.c., injected with 4 *Gata4*−/− ES cells (Soudais *et al.*, 1995; Narita *et al.*, 1997), and then implanted into the oviducts of pseudopregnant Swiss–Webster female mice. At the desired gestational ages, the embryos were removed and staged using Kaufman's ''Atlas of Mouse Development.'' Embryos were subjected to whole mount X-gal staining, postfixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5 μm), and then counterstained with H&E (Narita *et al.*, 1997).

RESULTS

Highly chimeric embryos were generated by introducing *Gata4*−/− ES cells into 8-cell stage embryos derived from *ROSA26*(*Gata4*+/+) mice. Embryos and attached yolk sacs

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were harvested between 8 and 10 days p.c. Host-derived (*Gata4*+/+) cells in the chimeric embryos were distinguished from *Gata4*−/− ES cell descendants on the basis of X-gal staining, both in whole mount preparations and in tissue sections (Figs. 1 and 2). Dark-field microscopy was used to enhance visualization of X-gal staining in tissue sections; under dark-field optics, X-gal staining appears bright pink (Fig. 1D), ensuring that even isolated β-galactosidase-positive cells can be recognized. In control experiments, we confirmed that an absence of β-galactosidase activity correlates with GATA-4 deficiency in the *Gata4*−/− ES cell ↔ *ROSA26*(*Gata4*+/+) chimeras (Narita *et al.*, 1997).

We identified chimeric embryos in which all of the visceral yolk sac endoderm cells and a small portion of foregut/hindgut endoderm cells were derived from the *Gata4*+/+ host, while the remaining cells in these embryos were derived exclusively from *Gata4*−/− ES cells (Figs. 1A, 1C, 1D, and 2A–2C). Other than slight growth retardation compared with nonchimeric littermates, these highly chimeric embryos were unremarkable in their gross and microscopic morphology. Despite a complete absence of GATA-4 in all splanchnic mesoderm derivatives, no defects in ventral morphogenesis were evident. The chimeric embryos had a midline, looped heart tube with normal endocardial and myocardial layers (Figs. 1C, 1D, and 2C). At the time of harvest, the hearts in these embryos appeared to beat normally. A pericardial cavity was present in each of these chimeric embryos. The foregut was well organized and correctly positioned dorsal to the heart and ventral to the neural tube (Figs. 1C, 1D, and 2C). Each embryo had a ventral yolk sac and was surrounded by an intact amnion (Figs. 1A, 1C, and 1D). The morphologic features of these chimeras differed markedly from those of GATA-4 knockout embryos, which have few recognizable structures ventral to the notochord (Kuo *et al.*, 1997; Molkentin *et al.*, 1997).

DISCUSSION

GATA-4 knockout mice display profound abnormalities in ventral morphogenesis, including defective heart tube and foregut formation. In contrast, a high percentage of *Gata4*−/− ES cell ↔ *ROSA26*(*Gata4*+/+) chimeras in which *Gata4*+/+ cells populate only endoderm do not exhibit abnormalities in ventral development. We conclude that the presence of wild-type (*Gata4*+/+) cells in primitive (yolk sac) endoderm and/or definitive (gut) endoderm is sufficient to induce normal ventral development in adjoining *Gata4*−/− tissue. These findings support the notion that lateral to ventral folding during mouse embryogenesis involves an interplay between mesoderm and underlying endoderm. Furthermore, our studies and those of other investigators (Kuo *et al.*, 1997; Molkentin *et al.*, 1997) establish that transcription factor GATA-4 plays a critical role in the folding process, presumably by regulating expression of certain genes in endoderm. That endoderm participates in murine cardiac morphogenesis is not unexpected, since endoderm has been shown to influence cardiomyocyte differentiation and heart formation in amphibian and avian systems (Nascone and Mercola, 1996). Proposed mechanisms by which endoderm may influence the differentiation/migration of adjoining precardiac mesoderm include the production of extracellular matrix proteins (e.g., fibronectin) or signaling molecules (e.g., bone morphogenetic proteins, fibroblast growth factors) (Nascone and Mercola, 1996).

The chimera analyses described here do not distinguish whether it is GATA-4 expression in visceral yolk sac endoderm or definitive endoderm that is critical for ventral morphogenesis. However, circumstantial evidence suggests that expression in visceral endoderm rather than definitive endoderm may be essential for proper ventral patterning. During mouse development the visceral endoderm is excluded from the embryo proper by the migration of definitive endoderm cells from the primitive streak (Tam *et al.*, 1993). This displacement by migrating definitive endoderm causes the visceral endoderm to ''gather up'' at the level of the AIP as lateral to ventral folding is initiated. GATA-4 mRNA and protein are expressed in visceral yolk sac endoderm throughout early development, including the portion of the yolk sac endoderm near the AIP (Arceci *et al.*, 1993; Heikinheimo *et al.*, 1994; Kuo *et al.*, 1997; Molkentin *et al.*, 1997). In contrast, GATA-4 message is not readily detected in the definitive endoderm of the invaginated foregut at 9–10 days p.c. (Kuo *et al.*, 1997). Moreover, studies of both GATA-4 knockout mice (Kuo *et al.*, 1997; Molkentin *et al.*, 1997) and *Gata4*−/− ES cell chimeras (this study) indicate that definitive foregut endoderm can form in the absence of GATA-4 expression. Several lines of evidence support a role for GATA-4 in yolk sac endoderm function. In cotransfection experiments, GATA-4 can *trans*-activate the promoters of genes expressed in yolk sac endoderm (Bielinska and Wilson, 1995). *Gata4*−/− ES cells differentiated *in vitro* exhibit defects in visceral endoderm formation and expression of endoderm-specific markers (Soudais *et al.*, 1995). GATA-4 knockout mice have morphologically recognizable visceral yolk sac endoderm, but approximately one-third of GATA-4 knockout embryos do not progress through gastrulation, suggesting that the yolk sac endoderm present in these embryos may be defective in function (Molkentin *et al.*, 1997).

These chimera experiments provide insight into the interactions between endoderm and mesoderm during murine cardiac development. However, as is typical of chimeras derived nearly exclusively from ES cells, highly chimeric *Gata4*−/− ES cell ↔ *ROSA26*(*Gata4*+/+) embryos do not survive to term (Narita *et al.*, 1997). Since our analysis was limited to embryos younger than 10 days p.c., subtle defects in body patterning or organ function may not have been detected. Nevertheless, the striking differences in heart and foregut morphology between the knockout embryos and our highly chimeric embryos provide compelling evidence that the phenotype of *Gata4*−/− mice reflects defects in endoderm rather than in mesoderm.

A recent publication (Srivastava *et al.*, 1997) describing the phenotype of mice deficient in the cardiac basic helix-

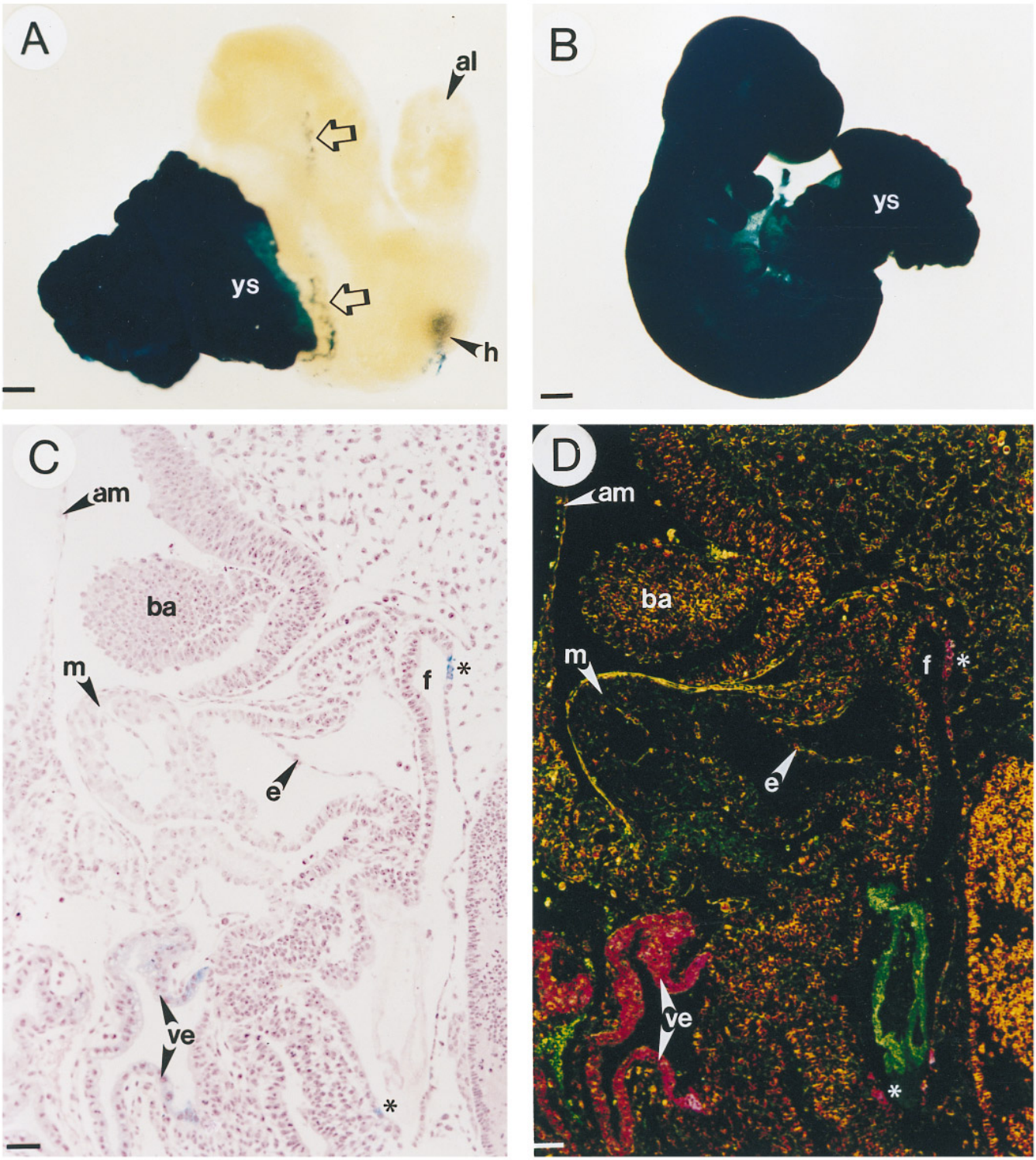

FIG. 1. (A) Whole mount X-gal staining of an 8-day-p.c. "unturned" *Gata4*−/− ES cell ↔ *ROSA26*(*Gata4*+/+) chimeric embryo. β-Galactosidase-positive (*Gata4*+/+) cells can be seen in the yolk sac, foregut endoderm (open arrows), and hindgut endoderm. The remainder of the embryo proper is ES cell derived. Bar, 100 μm. (B) A control nonchimeric 11-day-p.c. *ROSA26*(*Gata4*+/+) animal, demonstrating intense X-gal staining throughout the embryo. Bar, 300 μm. (C and D) Bright- and dark-field views of a sagittal section through the embryo shown in A. Bar, 30 μm. Consistent with the whole mount staining pattern, this tissue section shows that β-galactosidase-positive cells

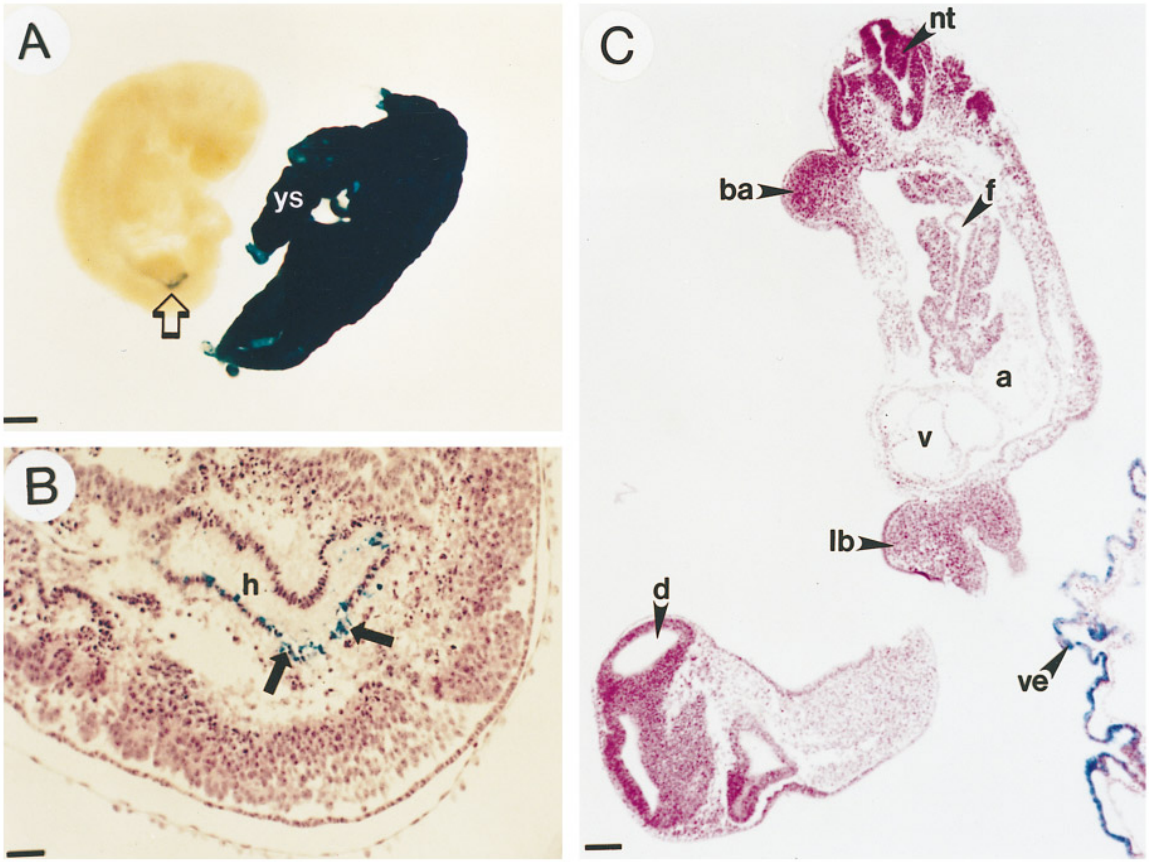

FIG. 2. (A) Whole mount X-gal staining of a 9-day-p.c. ''turned'' *Gata4*−/− ES cell ↔ *ROSA26*(*Gata4*+/+) chimeric embryo and its accompanying yolk sac. β-Galactosidase-positive (*Gata4*+/+) cells can be seen in the yolk sac and hindgut endoderm (open arrow). The remainder of the embryo proper is ES cell derived. Bar, 1 mm. (B) Transverse section through the same embryo shown in A, demonstrating that β-galactosidase-positive cells are limited to the hindgut endoderm (closed arrows). Bar, 50 μm. (C) Transverse section through a different 9-day-p.c. *Gata4*−/− ES cell ↔ *ROSA26*(*Gata4*+/+) chimera. Bar, 100 μm. β-Galactosidase-positive cells are restricted to the visceral yolk sac endoderm. Careful examination of additional sections confirmed that all other cells in the embryo proper, except for a few cells in the hindgut gut endoderm, are derived from *Gata4*−/− ES cells. Again, no morphological abnormalities are evident in this embryo. The neural tube, foregut, and looped heart are all properly positioned. Abbreviations: a, common atrial chamber; ba, branchial arch; d, dilated region of the neural tube in the caudal region of the tail; f, foregut; h, hindgut endoderm; lb, limb bud (left forelimb); nt, neural tube; v, bulboventricular canal; ve, visceral endoderm; ys, yolk sac.

loop-helix protein dHAND provides additional evidence that endodermal, not mesodermal, expression of GATA-4 is essential for normal ventral development. dHAND knockout mice die at 10.5 days p.c. and exhibit an underdeveloped right ventricle and abnormal outflow tract. GATA-4 message is undetectable in the myocardium of dHAND knockout mice but is present in endodermal derivatives. This finding suggests that GATA-4 expression in endoderm and cardiogenic mesoderm may be under the control of different promoters and that GATA-4 expression in myocar-

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are restricted to the visceral yolk sac endoderm and portions of the foregut endoderm (asterisks). Careful examination of serial sections through this embryo confirmed that all other cell types, including splanchnic mesoderm derivatives, are *Gata4*−/− ES cell descendants. Despite an absence of GATA-4 in all mesodermal derivatives, there are no morphological abnormalities in this embryo. The heart tube has formed and is looped. The foregut is normal in appearance. The amnion completely surrounds the embryo and the yolk sac has acquired its proper ventral location. Abbreviations: al, allantois; am, amnion; ba, branchial arch; e, endocardium; f, foregut; h, hindgut; m, myocardium; ve, visceral endoderm; ys, yolk sac.

dium is dependent on dHAND. Despite an absence of GATA-4 expression in cardiogenic mesoderm, dHAND knockout mice exhibit normal fusion of the bilateral myocardial primordia and foregut formation, reinforcing the notion that GATA-4 expression in mesodermal cells is not required for ventral morphogenesis.

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