GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo

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GATA6 belongs to a family of zinc finger transcription factors that play important roles in transducing nuclear events that regulate cellular differentiation and embryonic morphogenesis in vertebrate species. To examine the function of GATA6 during embryonic development, gene targeting was used to generate GATA6-deficient (GATA6−−) ES cells and mice harboring a null mutation in GATA6. Differentiated embryoid bodies derived from GATA6−− ES cells lack a covering layer of visceral endoderm and severely attenuate, or fail to express, genes encoding early and late endodermal markers, including HNF4, GATA4, α-fetoprotein (AFP), and HNF3β. Homozygous GATA6−− mice died between embryonic day (E) 6.5 and E7.5 and exhibited a specific defect in endoderm differentiation including severely down-regulated expression of GATA4 and absence of HNF4 gene expression. Moreover, widespread programmed cell death was observed within the embryonic ectoderm of GATA6-deficient embryos, a finding also observed in HNF4-deficient embryos. Consistent with these data, forced expression of GATA6 activated the HNF4 promoter in nonendodermal cells. Finally, to examine the function of GATA6 during later embryonic development, GATA6−−-C57BL/6 chimeric mice were generated. lacZ-tagged GATA6−− ES cells contributed to all embryonic tissues with the exception of the endodermally derived bronchial epithelium. Taken together, these data suggest a model in which GATA6 lies upstream of HNF4 in a transcriptional cascade that regulates differentiation of the visceral endoderm. In addition, these data demonstrate that GATA6 is required for establishment of the endodermally derived bronchial epithelium.

[Key Words: Endoderm; transcription factor; HNF4; GATA]

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Endodernally derived cell lineages have evolved to facilitate filtration and exchange of nutrients across tissues during embryonic and postnatal development. Each endodermal derived cell lineage subserves a unique function in developing vertebrates and these cell lineages can be distinguished morphologically and biochemically. Differentiation of endodermally derived cell lineages from the primitive endoderm occurs in a precise temporal and spatial pattern in vertebrate species. In the mouse, cells within the inner cell mass (ICM) proliferate and segregate to form an outer layer of extraembryonic primitive endoderm that can be recognized as early as embryonic day (E) 4.0 shortly before implantation (for review, see Gardner 1983; Hogan et al. 1983). Production of injection chimeras strongly suggests that primitive endoderm cells colonize the extraembryonic visceral and parietal endoderm and do not contribute to the endodermal tissues of the fetus (Gardner 1982,1983). At the late egg cylinder stage (E6.5), a population of cells within the epiblast migrates through the primitive streak and differentiates into the definitive embryonic endoderm. In addition, a small proportion of the definitive endoderm may be recruited directly from the overlying ectoderm (Tam and Beddington 1992). Immediately post-gastrulation (at E8.0), cells of the embryonic endoderm migrate through the embryo to line the primitive foregut, midgut, and hindgut cavities. The hepatic diverticulum arises from the junction of the primitive foregut and midgut at E8.5 and grows into the septum transversum. Finally, at E10.0, the tracheal diverticulum buds off the foregut and gives rise to the tracheal and bronchial epithelium, which can be recognized at E12.5.

A great deal has been learned about the developmental programs that control specification and differentiation of endoderm through the study of invertebrate species. Accumulating evidence suggests that a primordial function of transcription factors in the GATA family may be to specify endoderm and to activate the expression of endoderm-specific gene products (MacMorris et al. 1992; 3Corresponding author.
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In vertebrate species, members of the GATA family of type IV zinc finger transcription factors play key roles in transducing nuclear events that modulate cell lineage differentiation during vertebrate development (for review, see Orkin 1992; Simon 1995; Weiss and Orkin 1995). Six GATA factors have been identified in vertebrate species (Evans et al. 1988; Tsai et al. 1989; Wilson et al. 1991; Ho et al. 1991; Dorfman et al. 1992; Arceci et al. 1993; Kelley et al. 1993; Laverriere et al. 1994; Morrissey et al. 1996, 1997). However, only members of the GATA4/GATA5/GATA6 subfamily are expressed in the extraembryonic and embryonic endoderm during early development (Arceci et al. 1993; Laverriere et al. 1994; Morrissey et al. 1996, 1997). In the primitive streak mouse embryo, GATA4 and GATA6 are coexpressed in the visceral and parietal endoderm (Morrisey et al. 1996). Subsequently, GATA4, GATA5, and GATA6 are coexpressed in the embryonic heart and gut epithelium. However, coincident with formation of the embryonic lung bud, only GATA6 is expressed in the endodermally derived bronchial epithelium (Morrisey et al. 1997). Recently, we reported that GATA4-deficient embryos display profound defects in ventral morphogenesis and heart tube formation. However, embryos harboring a null mutation in the gene encoding GATA4 contain intact yolk sacs that express high levels of the endodermal markers GATA6, HNF4, and HNF3β (Kuo et al. 1997; Molkentin et al. 1997). Thus, it remains unclear what role, if any, GATA factors play in development of the endoderm in vertebrate species.

In the studies described in this report, gene targeting was used to examine the function of GATA6 in differentiating ES cells and in the developing mouse. Differentiated embryoid bodies derived from GATA6+/− ES cells lack a covering layer of visceral endoderm and the expression of both early and late markers of visceral endoderm is severely down-regulated or abolished. GATA6−/− embryos die at the early primitive streak stage (between E6.5 and E7.5) and exhibit a specific block in endoderm differentiation including severely attenuated expression of GATA4 and absence of HNF4 gene expression. In addition, cell death is observed within the embryonic ectoderm in GATA6−/− embryos beginning at E6.5. Consistent with these data, forced expression of GATA6 transactivated the HNF4 promoter. Finally, we observed that GATA6−/− ES cells fail to contribute to the embryonic bronchial epithelium. Taken together, these data demonstrate that GATA6 plays a critical role in differentiation of the visceral endoderm required for survival of the embryo past the early primitive streak stage. In addition, GATA6 plays an important role in establishment of the endodermally derived bronchial epithelium.

Results

Targeted disruption of GATA6 gene in ES cells

To produce a null mutation of the GATA6 gene, a targeting vector was constructed in which exons 4 and 5, encoding the amino- and carboxy-terminal zinc finger DNA-binding domain of the GATA6 protein, were replaced with a phosphoglycerokinase (PGK)-neomycin resistance (neo) cassette (Fig. 1A). The linearized targeting vector was electroporated into RW ES cells. Four of the 203 G418- and gancyclovir-resistant ES cell colonies screened were shown to be homologous recombinants by Southern blot analysis. Each of these GATA6−/− clones contained a single site of integration of the vector in the host genome as determined by Southern blot analyses performed with a neo probe (data not shown). To generate GATA6-deficient ES cells (GATA6−/−), independently derived GATA6−/− ES cell lines were subjected to growth selection in high concentrations of G418 (Mortenson et al. 1992). Southern blot analyses performed on high concentration G418-resistant colonies served to identify 24 independent GATA6−/− clones.

To confirm that we had generated a null mutation with this targeting strategy, Northern blot analyses were performed on RNA isolated from wild-type RW ES cells (GATA6+/+), heterozygous ES cells (GATA6+/−) and homozygous mutant ES cells (GATA6−/−) following their differentiation into embryoid bodies. High levels of GATA6 mRNA were detected in embryoid bodies derived from the wild-type (wt) and heterozygous (GATA6+/−) ES cells after 12 days of differentiation (Fig. 1C). In contrast, GATA6 mRNA was not observed in differentiated embryoid bodies derived from GATA6−/− ES cells (Fig. 1C). To confirm that GATA6 protein was not expressed in GATA6−/− ES cells, Western blot analyses were performed on whole cell extracts prepared from embryoid bodies derived from GATA6−/− and GATA6−/− ES cells with GATA6-specific polyclonal antiserum. As expected, the polyclonal antiserum recognized a 48-kD protein (Fig. 1D, arrow) in the wild-type ES cells following 12 days of differentiation. In contrast, GATA6 was not detected in whole cell extracts prepared from differentiated GATA6−/− ES cells (Fig. 1D). Moreover, in situ hybridization performed with a GATA6-specific riboprobe revealed that GATA6 mRNA was not expressed in GATA6-deficient embryos (see Fig. 5D below). Taken together, these data confirmed that the targeted ES cells contain a null mutation in the GATA6 gene.

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GATA6-deficient embryoid bodies exhibit a block in visceral endoderm differentiation

ES cells can be induced to aggregate and differentiate into cystic embryoid bodies (CEBs) that are covered by a layer of visceral endoderm (Doetschman et al. 1985; Soudais et al. 1995). Obvious differences were observed in the morphology of embryoid bodies derived from control wild-type (GATA6<sup>+/+</sup>) ES cells and homozygous mutant (GATA6<sup>−/−</sup>) ES cells after 12 days of differentiation (Fig. 2A,B). Of note, CEBs from wild-type and heterozygous (GATA6<sup>+/−</sup>) ES cells were indistinguishable over the 12-day course of differentiation (data not shown). Consistent with previous reports (Soudais et al. 1995), CEBs derived from wild-type (wt) and heterozygous (GATA6<sup>+/−</sup>) ES cells were cystic structures covered with a smooth layer of visceral endoderm (Fig. 2A,C). Cross-sections through embedded CEBs revealed that the visceral endoderm readily absorbed toluidine blue (Fig. 2C). Electron microscopic analysis confirmed that the covering layer of visceral endoderm contained a characteristic brush border with microvilli and numerous phagocytic vacuoles (Fig. 2E). In contrast, embryoid bodies derived from GATA6<sup>−/−</sup> ES cells were smaller and exhibited an irregular cobble-stoned surface (Fig. 2B). Cross-sections through embedded GATA6<sup>−/−</sup> mutant embryoid bodies revealed that they were relatively solid with well-orga-
nized interior structures (Fig. 2D). In addition, cells covering the surface of GATA6−/− embryoid bodies were relatively large, cuboidal, and lacked phagocytic vacuoles (Fig. 2D). The vast majority of these cells were not stained dark blue with toluidine blue (Fig. 2D). Ultrastructurally, the surface of GATA6-deficient embryoid bodies contained relatively undifferentiated cells that lacked microvilli and phagocytic vacuoles and did not phenotypically resemble visceral endoderm (Fig. 2F).

To determine whether embryoid bodies derived from GATA6−/− ES cells expressed genes encoding markers of the visceral endoderm, Northern blot analyses were performed with RNA prepared from differentiated embryoid bodies derived from GATA6+/+ and GATA6−/− ES cells. Abundant induction of genes encoding endodermal markers, including α-fetoprotein (AFP), HNF3β, HNF4, and GATA4, was observed in embryoid bodies beginning 4 days after induction of differentiation with levels of gene expression increasing over the 12-day course of differentiation (Fig. 3, lanes 1-6). In contrast, AFP, HNF4, and GATA4 gene expression was not induced in differentiated embryoid bodies derived from GATA6−/− ES cells, whereas a small, but detectable, induction of HNF3β mRNA was observed in these cells (Fig. 3, lanes 7-12). Of note, HNF3β is expressed in both endoderm and the notochordal floorplate of the developing neural tube (Lai et al. 1993; Sasaki and Hogan 1994; Weinstein et al. 1994). The failure of GATA6−/− embryoid bodies to express genes encoding markers of the endodermal lineage(s) was observed in multiple independent GATA6−/− ES cell clones and during multiple rounds of differentiation. Taken together, these data demonstrate that embryoid bodies derived from GATA6−/− ES cells exhibit a profound defect in endoderm differentiation.

**Generation of GATA6-deficient embryos**

Four independently derived GATA6+/− ES cell lines were used to generate chimeric mice, and these chimeric mice were crossed to C57BL/6 mice to transmit the targeted allele through the germ line as determined by in situ hybridization that does not distinguish between +/− and +/+ genotypes.

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**Table 1. Genotype of offspring from GATA-6+/− × GATA-6+/− matings**

| Age      | +/+ | +/− | −/− | Resorbed |
|----------|-----|-----|-----|----------|
| E6.5     | 26+ | 10  | 1   |          |
| E7.5     | 36+ | 0   | 11  |          |
| E8.5     | 38+ | 0   | 11  |          |
| Newborn  | 75  | 137 | 0   | none     |

*Genotype was determined by in situ hybridization that does not distinguish between +/− and +/+ genotypes.

Because GATA6-deficient embryos died between E6.5 and E7.5, it was of interest to determine the precise temporal and spatial pattern of GATA4 and GATA6 gene expression during early embryonic development. Therefore, in situ hybridization analyses were performed on post-implantation mouse embryos utilizing GATA4- and GATA6-specific riboprobes. Low level GATA4 gene expression was detected in E5.5 embryos exclusively within the visceral (VE) and parietal (PE) endoderm (Fig. 4B). In contrast, in E5.5 embryos, no differences were detected between the patterns of the hybridization observed with the antisense and control sense GATA6 riboprobes (Fig. 4C and data not shown). At E6.5, the genes encoding GATA4 and GATA6 were coexpressed within the yolk sac endoderm (Fig. 4E,F). High power views demonstrate hybridization of the GATA4 and GATA6 antisense riboprobes to both VE and PE (Fig. 4F). Hybridization of the control sense GATA4 and GATA6 riboprobes to adjacent sections was not observed. Thus, at the level of sensitivity afforded by these analyses, GATA-4 is expressed at least 12-24 hr prior to the expression of GATA6 in the extra-embryonic VE and PE. Moreover, the lethality observed in GATA6-deficient embryos occurs coincident with, or shortly after, the initiation of GATA6 gene expression in the advanced egg cylinder/early primitive streak mouse embryo.
ectoderm (EC). By E7.5, all GATA6−/− embryos were either resorbed, or undergoing resorption (Table 1).

To determine whether GATA6-deficient embryos express genes encoding early endodermal markers, in situ hybridization analyses were performed on staged E6.5 GATA6+/+ control embryos and GATA6−/− mutant embryos. As expected, GATA6 mRNA was detected exclusively within the VE and PE of control embryos (Fig. 5C) but was undetectable in the VE or PE of GATA6−/− embryos (Fig. 5D). Consistent with previous reports (Morrissey et al. 1996; Kuo et al. 1997), GATA4 was expressed in the VE and PE (arrowheads) of control embryos (Fig. 5E). In contrast, GATA4 gene expression (arrowheads) was detectable, but severely attenuated, in the VE and PE of GATA6-deficient embryos (Fig. 5F). HNF4 is a zinc finger transcription factor in the steroid hormone recep-

![Figure 4](image-url)  
**Figure 4.** Expression of the GATA4 and GATA6 genes in the postimplantation mouse embryo. Histological and in situ hybridization analyses performed on E5.5 (A–C) and E6.5 (D–F) murine embryos. (A) A hematoxylin and eosin-stained section through the E5.5 embryo. (B) Hybridization of the GATA4 antisense riboprobe to yolk sac visceral endoderm (VE) and parietal endoderm (PE) of E5.5 embryos. (C) Hybridization of the antisense GATA6 riboprobe to the E5.5 embryo. (D) A hematoxylin and eosin-stained section through the E6.5 embryo. (VE) Visceral endoderm, (PE) parietal endoderm, (EC) embryonic ectoderm. (E) Hybridization of the GATA4 (E) and GATA6 (F) antisense riboprobe to E6.5 embryos.

GATA6−/− embryos exhibit a specific defect in early endoderm differentiation

The phenotype of differentiated embryoid bodies derived from GATA6−/− ES cells suggested that GATA-6 might play an important role in the specification and/or differentiation of the extra-embryonic or embryonic endoderm. In E6.5 wild-type and heterozygous control embryos, a single layer of VE and PE endoderm was observed (Fig. 5A). Cells of the extra-embryonic region (X-VE) were cuboidal, or columnar, with large numbers of apical vacuoles (Fig. 5A). In contrast, the visceral endoderm at the embryonic pole (E-VE) was squamous in morphology (Fig. 5A). In addition, several layers of embryonic ectoderm (EC) were visualized within the epiblast (Fig. 5A). Mutant E6.5 GATA6−/− embryos were consistently smaller (10%–20%) than their heterozygous and wild-type littermates (Fig. 5, cf. A and B). These embryos possessed an intact layer of extra-embryonic (X-VE) and embryonic (E-VE) visceral endoderm, suggesting that endoderm is specified correctly in GATA6-deficient embryos (Fig. 5B). At E7.0, GATA6−/− embryos could be detected, but were severely growth retarded compared with their wild-type littermates (Fig. 5, cf. A and B). Whereas E7.0 null embryos contained VE and PE, there was a dramatic reduction in size of the epiblast with frequent pyknotic nuclei observed within the embryonic

![Figure 5](image-url)  
**Figure 5.** Expression of endodermal markers in wild-type and GATA6−/− mutant E6.5 embryos. Histological and in situ hybridization analyses performed on wild-type (wt) and GATA6−/− (−/−) mutant E6.5 embryos. (A,B) A hematoxylin and eosin-stained section of VE and PE endoderm. (X-VE) Visceral endoderm at the extra-embryonic pole, (E-VE) visceral endoderm at the embryonic pole of the embryo, (PE) parietal endoderm, embryonic ectoderm (EC). (C,D) In situ hybridization performed with a radiolabeled GATA6 antisense riboprobe on a wild-type (C) and GATA6−/− (D) embryo. (E,F) In situ hybridization performed with a radiolabeled GATA4 antisense riboprobe on a wild-type (E) and GATA6−/− (F) embryo. (G,H) In situ hybridization performed with a radiolabeled HNF4 antisense riboprobe on a wild-type (G) and GATA6−/− (H) embryo. (Arrowheads) Extraembryonic VE. (I,J) In situ hybridization performed with a radiolabeled Pem antisense riboprobe in wild-type (I) and GATA6−/− (J) embryos. (EPC) Ectoplacental cone, (VE) proximal and distal visceral endoderm.
tor superfamily that is expressed exclusively within the extra-embryonic endoderm at E6.5 (Sladek et al. 1990; Duncan et al. 1994). Although HNF4 mRNA (white arrowhead) was detected within the extra-embryonic VE of control embryos (Fig. 5G), at the level of sensitivity afforded by these analyses, the gene encoding HNF4 was not expressed in GATA6−/− embryos (Fig. 5H). In contrast, expression of Pem, a homeobox gene expressed in the proximal and distal VE, PE, and ectoplacental cone (EPC) at E6.5 (Lin et al. 1994), was expressed at comparable levels in control and GATA6−/− mutant embryos (Fig. 5I,J). Hybridization of control GATA6, GATA4, HNF4, and Pem sense riboprobes was not observed to adjacent sections of either wild-type or GATA6-deficient embryos (data not shown).

To confirm that the defect in expression of endodermal markers did not reflect a generalized down-regulation of gene expression throughout the embryo, the expression of genes that are expressed within the embryonic ectoderm was also examined. Otx2 is a homeobox gene that is one of the earliest markers of the ectoderm in vertebrate species (Simeone et al. 1992). An Otx2 antisense riboprobe hybridized to the embryonic ectoderm (arrowheads) of wild-type (GATA6+/+) and GATA6−/− embryos (Fig. 6C,D). Similarly, ENC1, which encodes a Kelch-related protein that is an early marker of the neuroectoderm within the epiblast (Hernandez et al. 1997), was expressed in both wild-type and GATA6−/− mutant embryos (Fig. 6E,F).

Cell death appears in the embryonic ectoderm of GATA6-deficient embryos

To determine whether the embryonic lethality observed in GATA6-deficient embryos was related to programmed cell death, the TUNEL (Tdt-mediated dUTP-biotin nick end labeling) reaction was performed on sections through E6.5 and E7.0 wild-type and GATA6−/− mutant embryos. In all GATA6 mutant embryos examined there was an increase in TUNEL-positive, brown-stained nuclei (black arrowheads) in the embryonic ectoderm compared with wild-type E6.5 or E7.0 embryos (Fig. 7, cf. C and D). In addition, rare TUNEL-positive cells were observed within the PE of GATA6−/− embryos (Fig. 7D, blue arrowhead). Of note, this pattern of cell death within the embryonic ectoderm closely resembled that demonstrated previously in embryos harboring a null mutation in HNF4 (Chen et al. 1994).

GATA6 activates transcription of the HNF4 promoter in nonendodermal cells

Analysis of the phenotype of differentiated GATA6−/− ES cells and GATA6-deficient embryos suggested that HNF4 might be a bona fide transcriptional target of GATA6 within the visceral endoderm. To determine whether forced expression of GATA6 can activate the HNF4 promoter, mesodermally derived NIH-3T3 cells were cotransfected with a luciferase reporter plasmid containing the murine HNF4 promoter (pHNF4Pr.luc) (Taraviras et al. 1994) and the pcDNAG6 expression vector encoding the murine GATA6 protein (Fig. 8). As anticipated, the pHNF4Pr.luc reporter plasmid was not activated by the control pcDNA3 eukaryotic expression plasmid (Fig. 8, lane 1). However, cotransfection of the pHNF4Pr.luc reporter plasmid with increasing amounts of the pcDNAG6 expression plasmid resulted in a stepwise increase in luciferase activity up to 60-fold above background levels (Fig. 8, lanes 2–4). Thus, GATA6 can transactivate the HNF4 promoter in nonendodermal cells.

GATA6−/− ES cells fail to contribute to the endodermally derived bronchial epithelium

The early lethality observed in GATA6 null embryos precluded assessment of the function of GATA6 within other tissues in which the gene is expressed. To deter-
mine the capacity of homozygous GATA6−/− ES cells to contribute to other tissues during later embryonic development, lacZ-tagged GATA6+/− ES cells were injected into C57BL/6 blastocysts and GATA6+/−-C57BL/6 chimeric embryos were produced (Fig. 9A). At E10.5, GATA6 is expressed within the primitive heart and the endodermally derived gut epithelium. In E10.5 chimeric embryos, blue-stained GATA6−/− cells contributed to most tissues including the neural tube (nt), somites (s), primitive atria (a) and ventricles (v), mesenchyme, body wall, and the primitive gut epithelium (Fig. 9B, arrow). In addition, an occasional TUNEL-positive cell (blue arrowhead) was observed within the parietal endoderm of GATA6+/− mutant embryos.

At E12.5, coincident with formation of the primitive lung bud, GATA6 is the only member of the GATA family expressed in the endodermally derived bronchial epithelium (Fig. 9C). Remarkably, in multiple independent E13.5 chimeric embryos, lacZ−/−-GATA6−/− cells were never observed within the bronchial epithelium (Fig. 9D, E, arrowheads). In contrast, lacZ−/−-GATA6−/− cells did contribute to the pulmonary mesenchyme in these embryos (Fig. 9D, E). Moreover, the lacZ+/−-GATA6+/− ES cells contributed to other endodermally derived cell lineages in these embryos (data not shown). Of note, in control GATA6+/−-C57BL/6 chimeric embryos, heterozygous lacZ-tagged GATA6+/− ES cells participated in formation of the embryonic bronchial epithelium (Fig. 9F). Thus, these data confirm that a cell intrinsic defect exists in the capacity of GATA6+/− ES cells to contribute to the bronchial epithelium. These data suggest that GATA6 plays an important role in establishing this endodermally derived cell lineage.

**Discussion**

The function of GATA6 in the late egg cylinder/early primitive streak embryo

We used gene targeting to examine the function of the GATA6 transcription factor during differentiation of ES cells and in the developing mouse. The data presented include the first conclusive evidence that a member of the GATA4/GATA5/GATA6 subfamily is required for differentiation of VE in vivo. We observed that differentiated GATA6−/− mutant embryoid bodies do not develop VE and fail to induce expression of genes encoding endodermal markers including GATA4, HNF4, HNF3β, and AFP. More importantly, GATA6 null embryos demonstrated a specific block in endoderm differentiation including attenuated expression of GATA4 and absence of HNF4 gene expression. The defect observed within GATA6-deficient embryos appears to represent a specific block in the transcriptional program controlling endo-

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**Figure 7.** Cell death is observed in E7.0 GATA6+/− embryos. (A,B) Histological sections of E7.0 wild-type (wt) (A) and GATA6+/− (−/−) (B) embryos. (VE) Visceral endoderm, (PE) parietal endoderm. (C,D) E7.0 wild-type (C) and GATA6+/− mutant (D) embryos were subjected to the TUNEL reaction. Brown-stained nuclei indicate end incorporation in DNA. Rare TUNEL-positive nuclei were observed in red blood cells within the yolk sac cavity in wild-type embryos (C). In contrast, multiple TUNEL-positive nuclei (black arrowheads) were observed within the embryonic endoderm of E7.0 GATA6+/− mutant embryos (D). In addition, an occasional TUNEL-positive cell (blue arrowhead) was observed within the parietal endoderm of GATA6+/− mutant embryos.

**Figure 8.** GATA6-modulated transactivation of the HNF4 promoter in NIH-3T3 cells. NIH-3T3 cells were transfected with 2.5 µg of the luciferase reporter plasmid (pCHNF4Pr.luc) containing the murine HNF4 promoter and varying amounts (µg) of an expression plasmid encoding GATA6 (pcDNAG6) or the control plasmid (pcDNA3). All transfection mixtures also contained 1 µg of the pMSVβgal reference plasmid. Forty-eight hours after transfection, luciferase and β-galactosidase activities were determined. Luciferase activities corrected for differences in transfection efficiencies were normalized to luciferase activity obtained following transfection of the pHNF4Pr.luc plasmid with the pcDNA3 control plasmid. The data are presented as relative luciferase activities ±S.E.M. The mean absolute light units obtained following transfection of the pHNF4Pr.luc reporter plasmid and the control plasmid pcDNA3 was 353.
ectodermal markers, and wild-type embryos. Moreover, the down-regulation of endoderm differentiation, as Pem, a homeobox gene expressed in all extra-embryonic tissues, including endoderm, was expressed at comparable levels in GATA6−/− and wild-type embryos. Moreover, the down-regulation in early endodermal markers does not represent general growth retardation, as equivalent expression of the ectodermal markers, Otx2 and ENC1, were observed within the epiblast of GATA6−/− and wild-type E6.5 embryos. These data also establish that GATA-6 is critical for survival of the early primitive streak embryo as lethality was observed within ~12 hr of the onset of GATA6 gene expression.

The defect represents a specific block in endoderm differentiation, as opposed to endoderm specification, because (1) the primitive endoderm is recognized as early as E4.0, whereas the gene encoding GATA6 is not expressed until ~E6.5; (2) histological analyses of GATA6-deficient embryos demonstrated cells phenotypically resembling VE and PE; and (3) the genes encoding Pem and GATA4 (albeit at a reduced level) are expressed in the visceral and parietal endoderm of GATA6-deficient embryos. Therefore, GATA6 and the primitive GATA factors end-1 and SERPENT/ABF share the capacity to activate genes encoding endoderm-specific proteins required for differentiation of this cell lineage (MacMorriss et al. 1992; Stroehrer et al. 1994; Rehorn et al. 1996). However, it remains to be determined whether GATA6 (possibly in concert with GATA4 and/or GATA5) regulates specification of the primitive endoderm and/or any endodermally derived cell lineage.

HNF4 is a bona fide transcriptional target of GATA6 in the visceral endoderm

Previous studies have revealed that HNF4 is required for the late stages of visceral endoderm formation and lies downstream, or in an alternate pathway, from GATA4 in a transcriptional cascade controlling differentiation of the visceral endoderm (Duncan et al. 1997). Embryoid bodies derived from HNF4−/− ES cells form a morphological endoderm and express early markers of the endoderm lineage, including GATA4, Apo-E, and vHnf-1, but do not express later markers of visceral endoderm including AFP, TTR, Apo-AI, Apo-AIV, Apo-B, RBP, and TFN (Duncan et al. 1997). Consistent with these findings, normal or upregulated expression of GATA-4 was observed in E8.5 HNF4-deficient embryos, whereas expression of the genes encoding AFP, TFN, Apo-AI, Apo-AIV, and Apo-B was almost undetectable in these mutant embryos (Duncan et al. 1997). We have shown through both loss-of-function (analysis of GATA6−/− ES cells and GATA6−/+ embryos) and gain-of-function (transactivation of the HNF4 promoter by forced expression of GATA6) experiments that HNF4 gene expression is regulated by GATA6. Of note, the absence of HNF4 and other markers of visceral endoderm in GATA6-deficient embryos was not observed in GATA4-deficient embryos (Kuo et al. 1997) demonstrating that HNF4 is differentially regulated by GATA6 and GATA4 in the visceral endoderm of early primitive streak embryos. Taken together, these data demonstrate that in the visceral endoderm of the advanced egg cylinder/early primitive streak embryo HNF4 is a bona fide transcriptional target of GATA6.

GATA6: a hierarchical model of visceral endoderm formation

For many years it has been suggested that formation and differentiation of visceral endoderm is a multistep process (Duncan et al. 1997; Gardner et al. 1983). The data presented in this work are consistent with a hierarchical model and serve to place GATA6 and HNF4 within the context of this model (Fig. 10). The absence of HNF4 gene expression in GATA6−/− ES cells and GATA6−/+ embryos coupled with the finding that forced expression of GATA6 activates the HNF4 promoter, strongly suggests that GATA6 lies upstream, and regulates (directly or indirectly) the expression of HNF4 in
the visceral endoderm at E6.5–E7.5. HNF4, in turn, is required to activate the expression of sets of genes encoding secreted proteins (and late markers of endoderm differentiation) including AFP, TTR, Apo-AI, Apo-AIV, Apo-B, RBP and TFN. Moreover, these data demonstrate that GATA6 is required to maintain basal levels of GATA4 gene expression in the late egg cylinder/early primitive streak embryo. Conversely, the finding that GATA6 is upregulated in GATA4-deficient embryos suggests that GATA4 may suppress expression of GATA6 in some embryonic tissues (Kuo et al. 1997). This model also aids in understanding the molecular basis of the lethality observed at E7.0 in GATA6-deficient embryos. Despite the fact that GATA4 is expressed exclusively in the VE and PE at this developmental stage, widespread programmed cell death was observed within the embryonic ectoderm beginning at E6.5, 12–24 hr after the onset of expression of GATA4 in wild-type mice (see Fig. 4). Cell death was also observed in the embryonic endoderm of HNF4-deficient embryos beginning at ~E6.5 (Chen et al. 1994). These data suggest that the lethality observed in GATA6-deficient embryos may result solely from abnormal differentiation of the extraembryonic visceral endoderm, which, in turn, results in abnormal expression of one, or more, factor(s) required for survival and/or proliferation of the embryonic ectoderm. The severity of the phenotype exhibited in GATA6 null embryos, which uniformly die by E7.5, whereas HNF4-deficient embryos survive to E10.5, may be due to the fact that GATA6 lies upstream of HNF4 and thereby controls alternative pathways regulating endoderm differentiation possibly including those activated directly or indirectly by GATA4.

GATA6 and development of the endodermally derived bronchial epithelium

As the lung bud arises from the primitive foregut, GATA6 is expressed at high levels within the embryonic bronchial epithelium serving to identify GATA6 as a candidate regulator of this endodermally derived cell lineage (Morrisey et al. 1996). Thus, it was noteworthy that GATA6−/− ES cells did not contribute to the embryonic bronchial epithelium, but did contribute to other endodermally derived cell lineages that coexpress GATA4, GATA5, and GATA6 including the stomach epithelium and the small and large intestinal epithelium. This cell-intrinsic defect was restricted to the bronchial epithelium, as lacZ-tagged GATA6−/− ES cells were observed throughout the pulmonary mesenchyme and other tissues. It is tempting to speculate that this cell-intrinsic defect may reflect the fact that GATA6 is the only GATA factor expressed within the embryonic bronchial epithelium, effectively abrogating functional redundancies that may exist between members of the GATA4, GATA5, and GATA6 subfamily elsewhere. In addition, these data demonstrate that GATA6 may be required for the specification, survival and/or differentiation of the bronchial epithelium. Thus, it is noteworthy that the human surfactant protein C, rat surfactant protein A, and mouse CC10 promoters each contain embedded GATA motifs (Korfhagen et al. 1992; Ray et al. 1993; Wert et al. 1993). Identification of the bona fide transcriptional targets of GATA6 in the embryonic lung should provide fundamental insights into the transcriptional programs that control lung development.

Materials and methods

Plasmids

A genomic clone including the 5’ end of the murine GATA6 gene was isolated from a SV129 mouse library by use of the full-length mouse GATA6 cDNA as a probe (Morrisey et al. 1996). The targeting vector was generated in the pPNT plasmid (Tybulewicz et al. 1991), which contains the PGK-neo-poly(A) and PGK-tk-poly(A) cassettes for positive and negative selection, respectively. As schematically represented in Figure 1A, the pPNT G6 targeting vector, contains the 4.5-kb NotI (5’ end)-Xhol (3’ end) GATA6 genomic subfragment, which includes sequences in exon 3 through intron 3 subcloned into NotI-Xhol-digested pPNT, and the 2.5-kb Ncol–Ncol GATA6 genomic subfragment subcloned into the BamHI site of pPNT. In pPNT G6, exons 4 and 5 encoding the amino- and carboxy-terminal zinc fingers, respectively, are replaced with the PGK-neo cassette. The pEFI/4F2.lacZ eukaryotic expression vector encoding the bacterial lacZ gene under the transcriptional control of the human EF1α promoter and the murine 4F2 heavy chain enhancer has been described previously (Kuo et al. 1997). The pGEX6 (amino acids 357–444) bacterial expression plasmid contains the mouse GATA6 cDNA encoding the carboxy-terminal region of the protein (amino acids 357–444) into SalI–Xhol-digested pGEX4T3 (Pharmacia). The pcDNA3 control plasmid and the pcDNA G6 eukaryotic expression plasmid encoding the murine GATA6 protein have been described previously (Morrisey et al. 1996). The pHNF4Pr.lacZ plasmid contains the 581-bp murine

![Figure 10](image-url). A transcriptional cascade controlling differentiation of the visceral endoderm. As described in Results and published previously (Duncan et al. 1997; Kuo et al. 1997), analysis of the phenotypes of GATA6−/−, GATA4−/−, and HNF4−/− mutant embryos has shown that GATA6 activates (directly or indirectly) the expression of the early endodermal marker HNF4, which in turn, activates the expression of late endodermal markers, including α1-fetoprotein (AFP), transferrin (TFN), Apo-AI, Apo-AIV, and ApoB. In addition, GATA4 gene expression is down-regulated in GATA6-deficient embryos, whereas GATA6 gene expression is up-regulated in GATA4-deficient embryos suggesting cross-regulation between GATA6 and GATA4.
HNF4 promoter (Tavariras et al. 1994) subcloned into the Xhol-KpnI sites of the pGL2-basic luciferase reporter plasmid.

Preparation of GATA6 polyclonal antiserum and Western blot analysis

The pGEXG6 (amino acids 357–444) plasmid was transformed into bacteria and glutathione S-transferase (GST)-GATA6 (amino acids 357–444) fusion protein was induced and purified from bacterial extracts as described previously (Morrisey et al. 1997). Rabbits were immunized with the purified GST-GATA6 (amino acids 357–444) fusion protein as per standard protocol of the Pocono Rabbit Farm and Laboratory (Canadensis, PA) for fusion proteins. Preimmune and immune IgG were isolated from the serum by protein A-Sepharose chromatography. Western blot analysis was performed with polyclonal antisera raised against the carboxyl terminus of the murine GATA6 protein and in vitro translated murine GATA4, GATA5, and GATA6 protein, as described previously (Morrisey et al. 1997). A 48-kD band, corresponding to the expected size of GATA6 protein, was identified with the GATA6 polyclonal antisera, but not with preimmune serum (data not shown). This antisera did not recognize GATA4 or GATA5 protein (data not shown).

Generation of GATA6+/− and GATA6+/+ ES cells and mice

The GATA6 targeting construct was linearized with NotI and electroporated into RW ES cells as described previously (Soudais et al. 1995). After 24 hr, neomycin-resistant transfectants were selected in 250 µg/ml G418 and 1 µg gancyclovir for 8–10 days. DNA from resistant ES cell clones was analyzed by Southern blot analysis after BamHI digestion with a radiolabeled probe derived from genomic sequences located 3' of the targeting vector (see Fig. 1A). GATA6+/− ES cells were obtained by subjecting independently derived GATA6+/− clones to selection in G418 at a concentration of 750 µg/ml as described previously (Soudais et al. 1995).

To generate GATA6-deficient mice, ES cells from four independently derived GATA6+/− clones were microinjected into C57BL/6 donor blastocysts that were implanted into pseudopregnant CD1 females (Bradley 1987). GATA6+/− ES cells were obtained by subjecting resistant ES cell clones to selection in G418 at a concentration of 750 µg/ml as described previously (Soudais et al. 1995). To generate GATA6+/− mice, ES cells from four independently derived GATA6+/− clones were microinjected into C57BL/6 donor blastocysts that were implanted into pseudopregnant CD1 females (Bradley 1987; Hogan et al. 1994). The resulting male chimeras were mated with C57BL/6 females and agouti offspring were genotyped by Southern blot analysis as described previously (Kuo et al. 1997). GATA6+/− mice were then outbred with C57BL/6 and CD1 mice to generate heterozygous GATA6+/− mice that were interbred for phenotypic analysis. All animal experimentation was performed according to National Institutes of Health (NIH) guidelines in the University of Chicago Animal Care Facility.

Northern blot analyses of embryoid bodies derived from wild-type and GATA6+/− ES cells

ES cells were differentiated into embryoid bodies in suspension culture as described (Doetschman et al. 1985). Embryoid bodies were collected after 0, 2, 4, 6, 8, and 12 days of liquid culture. RNA was isolated, and Northern blot analysis performed as described previously (Parmacek and Leiden 1989; Soudais et al. 1995). Probes included the mouse GATA6 cDNA (basepairs 1138–1450) (Morrisey et al. 1996) and the mouse GATA4 cDNA (basepairs 1678–1907). The mouse HNF4, mouse HNF3β, and mouse AFP cDNA probes have been described previously (Kuo et al. 1997).

Transfections and luciferase assays

NIH-3T3 cells (1 × 106) were transfected with 2.5 µg of luciferase reporter plasmid, between 0 and 25 µg of the pcDNA4- lacZ expression plasmid or the control pcDNA3 plasmid, and 1 µg of the pMSV β-gal reference plasmid with Lipofectin Reagent as described previously (Ip et al. 1994). Of note, in these experiments the total amount of plasmid DNA transfected was held constant. Forty-eight hours following transfection, cell lysates were prepared and normalized for protein content by a commercially available kit (Bio-Rad, Hercules, CA). Luciferase and β-galactosidase reporter plasmids were assayed for g418 and lacZ expression. Three independently derived GATA4−/− ES cells and GATA6−/−/C57/BL-6 chimeric mice.

The pEF1α2-lacZ expression vector was linearized with BsaI and electroporated into GATA6−/− and GATA6+/− ES cells as described previously (Kuo et al. 1997). At 8 day postselection in 175 µM hygromycin, resistant colonies were stained for β-galactosidase expression. Three independently derived GATA4−/− and GATA6−/− lacZ cell lines were microinjected into C57BL/6 blastocysts and implanted into pseudopregnant CD1 females as described (Bradley 1987). GATA6−/−/C57BL/6 and GATA6−/−/C57BL/6 chimeric embryos were micromanipulated from pregnant females and stained for β-galactosidase activity as described previously (Chang et al. 1995).

Histological and in situ hybridization analysis

Pregnant females from GATA6−/− × GATA6−/− matings were anesthetized and perfused through the descending aorta with 4% paraformaldehyde in PBS to fix embryos in utero as described previously (Kuo et al. 1997). For electron microscopy, differentiated embryoid bodies were fixed in 2.5% gluteraldehyde in PBS, postfixed with 1% osmium tetroxide, and stained with 1% uranyl acetate as described previously (Soudais et al. 1995). Electron micrographs were produced with a JEOL-CXII electron microscope. In situ hybridization with 35S-labeled cRNA probes was performed essentially as described by Eichele and coworkers (Kuratani et al. 1994). The cRNA probes used for in situ hybridizations were generated from the following cDNA templates subcloned into pGEM7Z or pGEM11Z; GATA6 (bp 1139–1450) (Morrisey et al. 1996), Ott2 (bp 212–509) (Simeone et al. 1992), ENC1 (bp 1412–1739) (Hernandez et al. 1997), Pem (bp 142–449) (Lin et al. 1994). The HNF4, HNF3β, and GATA4 cRNA probes were generated as described previously Kuo et al. (1997). Histological sections and dark-field micrographs were made with a Zeiss Axiophot microscope.

TUNEL analysis

Embryos for TUNEL assays were fixed, embedded, and sectioned in the same manner as for the in situ hybridization studies. TUNEL reaction to detect incorporation of biotinylated dUTP mediated by terminal deoxynucleotransferase was performed as described on sections of E6.5 and E7.0 mouse GATA6−/− and GATA6−/− embryos (Gavioli et al. 1992).

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