Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro

Claire Soudais[1], Malgorzata Bielinska[6], Markku Heikinheimo[5,6], Craig A. MacArthur[6], Naoko Narita[6], Jeffrey E. Saffitz[7], M. Celeste Simon[2,4], Jeffrey M. Leiden[1,3] and David B. Wilson[6,8,*]

Departments of [1]Medicine, [2]Molecular Genetics and Cell Biology, and [3]Pathology and [4]The Howard Hughes Medical Institute, University of Chicago School of Medicine, Chicago, IL, USA
[5]Children's Hospital, University of Helsinki, Helsinki, Finland
Departments of [6]Pediatrics, [7]Pathology, and [8]Molecular Biology and Pharmacology, Washington University School of Medicine, St Louis, MO 63110, USA

*Author for correspondence at Department of Pediatrics, Box 8116, Washington University School of Medicine, 1 Children's Place, St Louis, MO 63110, USA
The first two authors contributed equally to this work.

SUMMARY

Transcription factor GATA-4 belongs to a family of zinc finger proteins involved in lineage determination. GATA-4 is first expressed in yolk sac endoderm of the developing mouse and later in cardiac tissue, gut epithelium and gonads. To delineate the role of this transcription factor in differentiation and early development, we studied embryoid bodies derived from mouse embryonic stem (ES) cells in which both copies of the *Gata4* gene were disrupted. Light and electron microscopy demonstrated that embryoid bodies formed from wild-type and heterozygous deficient ES cells were covered with a layer of visceral yolk sac endoderm, whereas no yolk sac endoderm was evident on the surface of the homozygous deficient embryoid bodies. Independently selected homozygous deficient cell lines displayed this distinctive phenotype, suggesting that it was not an artifact of clonal variation. Biochemical markers of visceral endoderm formation, such as α-feto-

protein, hepatocyte nuclear factor-4 and binding sites for *Dolichos biflorus* agglutinin, were absent from the homozygous deficient embryoid bodies. Examination of other differentiation markers in the mutant embryoid bodies, studies of ES cell-derived teratocarcinomas and chimeric mouse analysis demonstrated that GATA-4-deficient ES cells have the capacity to differentiate along other lineages. We conclude that, under in vitro conditions, disruption of the *Gata4* gene results in a specific block in visceral endoderm formation. These homozygous deficient cells should yield insights into the regulation of yolk sac endoderm development and the factors expressed by visceral endoderm that influence differentiation of adjoining ectoderm/mesoderm.

Key words: knockout, yolk sac, primitive endoderm, mutagenesis, GATA-4, mouse, visceral endoderm

INTRODUCTION

The yolk sac of the rodent plays a vital role in nutrition prior to formation of the chorioallantoic placenta (Jollie, 1990; Cross et al., 1994). Two specialized types of endodermal cells within the yolk sac, parietal and visceral cells, facilitate nutrient, gas and waste exchange between the maternal circulation and the rapidly growing embryo (Gardner, 1983). Parietal and visceral endoderm cells are derived from a common precursor, primitive endoderm. The fate of primitive endoderm descendants is dependent on interactions with other cell types (Hogan and Tilly, 1981). The parietal endoderm layer develops in association with the trophectoderm and functions as a barrier between the maternal and embryonic systems. Parietal endoderm cells contain abundant rough endoplasmic reticulum, synthesize large amounts of type IV collagen and laminins, and assemble these extracellular matrix proteins into Reichert's membrane, a specialized basement membrane that

surrounds the embryo and filters nutrients (Gardner, 1983). Visceral endoderm cells develop in association with the inner cell mass and resemble gut enterocytes, both morphologically and functionally. Visceral endoderm cells have microvilli to enhance absorption, contain numerous phagocytic and pinocytic vesicles, and form a polarized epithelium with apical tight juctions. These cells synthesize and secrete proteins involved in nutrient transport including α-fetoprotein (AFP), transferrin and apolipoproteins (Rossant, 1986). Through unknown signals, visceral endoderm also participates in the induction of embryonic blood cells and vessels from adjacent extraembryonic mesoderm, thereby establishing a nutrient delivery system for the embryo (Coffin et al., 1991; Wilt, 1965; Milura and Wilt, 1969; Yoder et al., 1994; Palis et al., 1995).

Despite intensive investigation over the past decade, little is known about the factors that control yolk sac endoderm formation or the interactions between visceral endoderm cells and adjoining ectoderm/mesoderm. In the current study, we

present evidence suggesting that transcription factor GATA-4 plays an important role in yolk sac endoderm formation and function. This factor belongs to a family of zinc finger proteins that recognize a consensus DNA sequence known as the 'GATA' motif, which is an essential *cis*-regulatory element in the promoters and enhancers of numerous genes (Orkin, 1992). GATA-binding proteins have emerged as important regulators of lineage determination in various tissues. For example, gene targeting experiments in the mouse have established that GATA-1 (Pevny et al., 1991; Simon et al., 1992; Weiss et al., 1994; Pevny et al., 1995) and GATA-2 (Tsai et al., 1994), two transcription factors expressed by erythroid and megakaryocytic progenitors, are essential for hematopoiesis. The tissue distribution of GATA-4 differs from other GATA-binding proteins, suggesting that this factor has a distinctive role in development. During mouse embryogenesis, GATA-4 first appears in visceral and parietal endoderm; later in development, GATA-4 is expressed in other cell types including promyocardium, mature cardiomyocytes, gut epithelium, liver and gonads (Arceci et al., 1993; Heikinheimo et al., 1994a; Laverriere et al., 1994). Through promoter studies, GATA-4 has been implicated in the regulation of several genes expressed in cardiocytes, including *α-myosin heavy chain* (Molkentin et al., 1994), *cardiac troponin C* (Ip et al., 1994) and *brain type natriuetic peptide* (Grépin et al., 1994; Thuerauf et al., 1994). In gut, GATA-4 may control gastric parietal cell gene expression (Tamura et al., 1993). The function of transcription factor GATA-4 in yolk sac, liver and gonads is not well understood. To clarify the role of GATA-4 in mouse development, we have disrupted both copies of the *Gata4* gene in ES cells and examined the consequences of GATA-4 deficiency on in vitro differentiation. Herein we show that targeted mutagenesis of the *Gata4* gene disrupts in vitro differentiation of visceral endoderm, suggesting that this transcription factor plays a role in yolk sac development.

MATERIALS AND METHODS

Construction of a GATA-4 targeting vector

A genomic clone encompassing the 5′-end of the *Gata4* gene was isolated from a 129/Sv♂ mouse library by screening with GATA-4 cDNA termed λG14A (Arceci et al., 1993). The targeting construct was generated in the pPNT vector (Tybulewicz et al., 1991), which contains *PGK-neo*-poly(A) and *PGK-tk*-poly(A) cassettes for positive and negative selection, respectively. A 5.6 kb *Kpn*I-*Nhe*I fragment from the *Gata4* gene was cloned downstream from the *PGK-neo*-poly(A) cassette via a 5′-*Kpn*I site and 3′-*Xba*I site, as shown in Fig. 1. A 2 kb fragment of the *Gata*4 gene, generated via PCR using the forward primer 5′-TTCTCGAGCTAGCCAAACATA-CATCTCAGGTACACTGAT-3′, the reverse primer 5′-TTGCGGC-CGCAATGCTTCTAACAATTGCTTCAGGGTCCT-3′ and the genomic clone as template, was cloned upstream of the *PGK-neo*-poly(A) cassette via 5′-*Xho*I and 3′-*Not*I sites. In the resultant construct, *Gata4* sequences were oriented opposite to the *PGK-neo*-poly(A) cassette.

ES cell selection and screening

Early passage CCE ES cells (Robertson, 1987) were routinely cultured on mitomycin C-treated STO feeder cells in culture medium consisting of high glucose DMEM supplemented with 15% fetal calf serum, 0.1 mM 2-mercaptoethanol, non-essential amino acids, nucleosides, L-glutamine and penicillin/streptomycin (Doetschman et al., 1985). ES cells (2×10^7) were electroporated with 25 μg of NotI-lin-

earized targeting plasmid (250V, 500 μF, electrode distance 0.4 cm) and the cells were then seeded into 100 mm plates (Wurst and Joyner, 1993). After 24 hours, 250 μg/ml G418 and 1 μM ganciclovir were added. DNA isolated from resistant colonies was analyzed by Southern blotting (Sambrook et al., 1989) using a 1.2 kb *Eco*RI/*Xho*I probe as described in Fig. 1. Targeted clones were also checked for single integration by hybridization with a *neo* probe after digestion of the DNA with *Eco*RI. Homozygous deficient ES cells were obtained by step selection with G418 (0.5, 1 and 2 mg/ml) as originally described by Mortensen et al. (1992). ES cells were differentiated in suspension culture using the method of Doetschman et al. (1985).

RNase protection analysis

RNA was isolated by the method of Chomczynski and Sacchi (1987). RNase protection assays were performed with a commercially available kit (Ambion) and 20 μg of total RNA. ^{32}P-labelled antisense riboprobes recognizing various transcripts were prepared by in vitro transcription in the presence of [^{32}P]αCTP (600-800 Ci/mmol) using the following plasmid templates, linearizing restriction endonucleases and polymerases: GATA-4, pBluescript clone λG14A (Arceci et al., 1983) *Not*I, T7; FGF-8a, pBluescript subclone FGF-8a (Heikinheimo et al., 1994b), *Hin*dIII, T7; hepatocyte nuclear factor-4 (HNF-4), pBluescript subclone p4-is (Chen et al., 1994), *Cla*I, T7; TGFβ1, pBluescript subclone (Millan et al., 1991), *Eco*RI, T3; TGFβ2, pBluescript subclone (Millan et al., 1991), *Hin*dIII, T7; connexin-45 (Cx45), pGEM subclone of the cDNA isolated by Hennemann et al. (1992), *Eco*RI, Sp6; Flk-1, pBluescript subclone (Millauer et al., 1993), *Hin*dIII, T3; FGF-3, pBluescript subclone int-2 (Wilkinson et al., 1989), *Eco*RI, T3. Antisense β-actin probe was prepared using a template supplied by the manufacturer (Ambion). The sizes of the full length and protected RNA probes (in nucleotides) were as follows: GATA-4, 491 and 430 ; FGF-8a, 286 and 189; HNF-4, 290 and 259; Cx45, 530 and 500; TGFβ1, 630 and 600; TGFβ2, 534 and 501; Flk-1, 560 and 530; FGF-3, 478 and 430; β-actin, 300 and 250.

In situ hybridization

Embryoid bodies were washed in PBS, fixed 2 hours in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned (4 μm) and subjected to in situ hybridization using ^{33}P-labelled GATA-4 antisense and sense riboprobes (Heikinheimo et al., 1994a).

Light/electron microscopy of embryoid bodies

Embryoid bodies were washed with serum-free medium, fixed >2 hours in Karnovsky's solution, postfixed with OsO₄ and embedded in Spurr epoxy resin. 1 μm sections were stained with toluidine blue and viewed under light microscopy. For electron microscopy, ultrathin sections (70 nm) stained with lead citrate and uranyl acetate and viewed on a JEOL 1200 EX electron microscope.

Western blotting of embryoid bodies

Embryoid bodies from wild-type and homozygous deficient ES cells were washed twice with PBS and then lysed by sonication NET buffer (Sambrook et al., 1989) supplemented with 80 μg/ml PMSF, 0.4% aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin. The samples were centrifuged at 11,000 *g* and the resultant supernatants collected. Protein concentrations were determined using Bradford reagent (Biorad) and 75 μg samples of soluble protein were subjected to SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes. Western blotting was performed as described (Harlow and Lane, 1988), using a 1:500 dilution of rabbit anti-mouse AFP serum (ICN) followed by ^{125}I-protein A (1×10^6 cts/minute/ml, Amersham). Signals were quantified by phosphorimage analysis.

Staining of embryoid bodies with lectins or antibodies

Wild-type and homozygous deficient embryoid bodies were fixed for 10 minutes at room temperature in 2% glutaraldehyde, washed and then stained 30 minutes at room temperature with 0.5 mg/ml FITC-

labelled *Dolichos biflorus* agglutinin (Sigma) in the presence or absence of 0.1 mM GalNAc, as described elsewhere (Sato and Muramatsu, 1985; Wu et al., 1983). The cells were then washed twice in PBS and photographed using an inverted fluorescence microscope. Frozen sections of embryoid bodies were fixed with acetone and subjected to immunostaining with anti-SSEA-1 IgM (Solter and Knowles, 1978), followed by a peroxidase-conjugated goat anti-mouse IgM secondary IgG (Cappel) (Harlow and Lane, 1988).

ES cell derived teratocarcinomas

The flanks of BALB/cBy-*nu* nude mice (Jackson Labs) were injected subcutaneously with 1.5×10^6 wild-type or GATA-4 homozygous deficient ES cells that had been passaged twice through LIF media to remove feeder cells. Teratocarcinomas were harvested from the animals after 2-3 weeks, when the tumors were 0.5-1.5 cm in diameter. The teratocarcinomas were fixed in buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin/eosin. Tumor histology was reviewed by a mouse pathologist who was unaware of the source of the tissue. Portions of the unfixed tumors were also freeze-thawed and subjected to glucose phosphate isomerase (Gpi-1) isozyme analysis (Papaioannou and Johnson, 1993) to determine the percentage of tumor cells that were ES-derived.

Generation of chimeric mice

Established methods were used to produce chimeric mice for analysis (Papaioannou and Johnson, 1993). GATA-4 homozygous deficient ES cells were injected into 3.5 day p.c. C57BL/6J blastocysts and the injected blastocysts were then reimplanted into the uterii of pseudopregnant CD1 females. Chimeric offspring were identified by coat color. DNA was isolated from the organs of 6 week old chimeric animals (Sambrook et al., 1989), digested with *NcoI+ClaI* and then subjected to Southern analysis using as a probe the 1.2 kb *EcoRI/XhoI* fragment described in Fig. 1.

RESULTS

Generation of ES cells deficient in GATA-4

To target the *Gata4* gene in ES cells, we employed a positive-negative selection strategy (Mansour et al., 1988). The targeting vector contained a total of 7.6 kb of *Gata4* DNA flanking a *neomycin resistance* (*neo*) cassette that was transcribed in the opposite direction (Fig. 1A). An HSV *thymidine kinase* (*tk*) cassette was included at the 5'-end of the construct to permit selection against random integration events. Homologous recombination between this vector and the *Gata4* gene resulted in disruption of most of exon II, including sequences encoding the translation start site and N-terminal *trans*-activation domain of the GATA-4 molecule (Arceci et al., 1993). The linearized targeting vector was introduced into ES cells and the cells were selected with G418 plus ganciclovir. To screen for homologous recombination events, DNA from resistant colonies was subjected to

Southern analysis with a probe 3' to the recombination site. The wild-type *Gata4* allele displayed a 4.6 kb band on Southern blotting of *XhoI+NotI*-digested DNA, whereas the disrupted locus showed a 3.5 kb band (Fig. 1D). Of 174 colonies of G418- and ganciclovir-resistant cells examined, 8 had undergone a single homologous recombination event. Correct targeting in these lines was verified by Southern analysis with additional probes, including the *neo* gene (data not shown).

To prepare cells homozygous deficient in GATA-4, independent ES cell lines containing a single targeted *Gata4* allele were subjected to stepwise selection with increasing concentrations of G418. Southern analysis showed that 3 of 24 ES lines resistant to high concentrations of G418 had undergone gene conversion, resulting in disruption of both alleles (two such lines are shown in Fig. 1E). These independently selected homozygous deficient lines, derived from different heterozygous lines, displayed the same distinctive phenotype (see below), suggesting that the phenotype was not an artifact of clonal variation.

GATA-4 expression in wild-type ES cells and the targeted cell lines

When cultured in suspension, ES cells differentiate into embryoid bodies, a process that recapitulates aspects of early embryonic development. A variety of tissue types appear in differentiating embryoid bodies, including yolk sac endoderm,
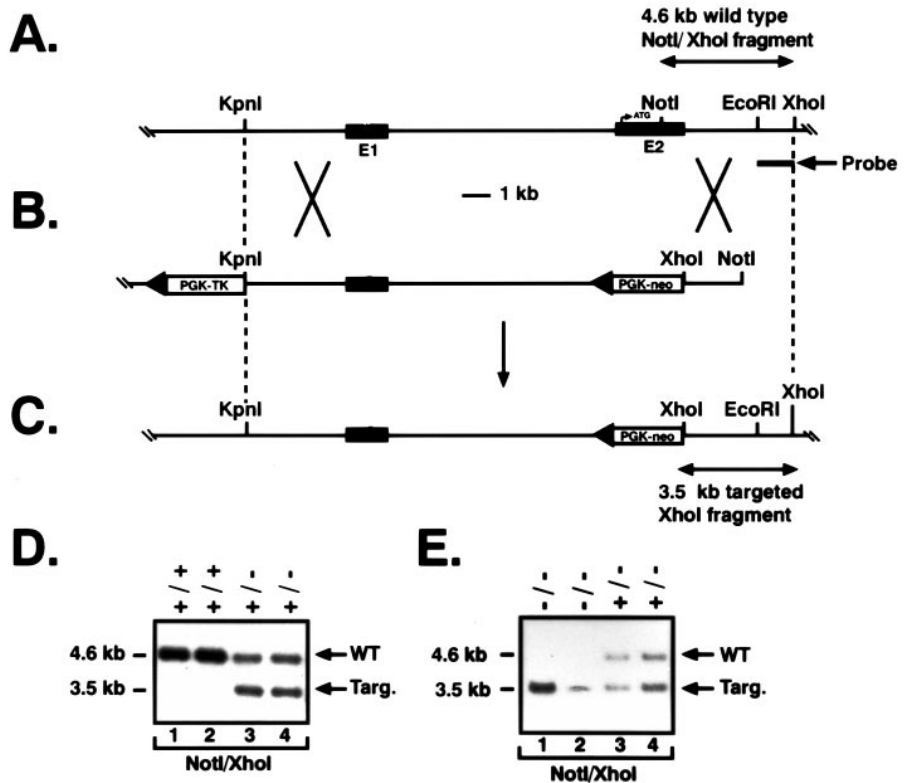

Fig. 1. Gene targeting strategy. (A) Schematic representation of a portion of the *Gata4* gene. The *EcoRI/XhoI* fragment used as a probe for Southern analysis is indicated. (B) Linearized targeting vector. (C) Targeted *Gata4* allele. Exon II (E2), which contains the translation start site, has been replaced by the *neo* cassette. (D) Southern analysis of *XhoI/NotI*-digested wild-type (lanes 1,2) and heterozygous deficient (lanes 3,4) ES cell DNA. (E) Southern analysis of XhoI/NotI digested DNA from homozgyous (lanes 1,2) and heterozygous (lanes 3,4) ES cell lines.

cardiac muscle and hematopoietic elements (Doetschman et al., 1985). The patterns of tissue-specific gene expression observed in differentiating embryoid bodies parallel the patterns seen during early mouse embryo development, making in vitro ES cell differentiation an invaluable system for the study of developmental programs such as skeletal myogenesis (Rohwedel et al., 1994), cardiac myogenesis (Sánchez et al., 1991; Miller-Hance et al., 1993), vasculogenesis (Wang et al., 1992; Risau et al., 1988) and hematopoiesis (Weiss et al., 1994; Keller et al., 1993).

Previously, we reported that GATA-4 RNA is one of the transcripts induced during differentiation of embryoid bodies (Arceci et al., 1993). To determine whether GATA-4 RNA expression was altered in the targeted cell lines, we used RNase protection assays and in situ hybridization to measure GATA-4 transcripts in embryoid bodies derived from wild-type and GATA-4 homozygous deficient ES. No evidence of GATA-4 transcripts was detected in the homozygous deficient embryoid bodies using RNase protection assays (data not shown), whereas GATA-4 message was evident in differentiated wild-type embryoid bodies (see below, Fig. 9A). In situ hybridization of GATA-4 antisense probe to day 8 wild-type embryoid bodies revealed that expression of GATA-4 message was limited to yolk sac endoderm on the surface of the bodies; no GATA-4 expression was detected in undifferentiated cells in the core of these embryoid bodies (Fig. 2A-D). In agreement with the RNase protection findings, antisense probe did not hybridize to day 8 homozygous deficient embryoid bodies (Fig. 2G-I). Thus, disruption of exon II of the *Gata4* gene with the *neo* cassette not only obliterated sequences encoding the translation start site and N-terminal portion of the GATA-4 molecule, it also dramatically reduced steady state levels of GATA-4 transcripts. These findings suggest that the targeting event produced a true null allele.

Visceral yolk sac differentiation is blocked in homozygous deficient embryoid bodies

Using inverted light microscopy, we found that embryoid bodies derived from either wild-type or GATA-4 heterozygous deficient ES cells had a different morphology than homozygous deficient embryoid bodies (Fig. 3). These differences in morphology were most apparent after 10 days in culture. Many of the older wild-type and heterozygous deficient embryoid bodies were large and cystic; the outer surfaces of these embryoid bodies were smooth (Fig. 3A,B,D,E). In contrast, the homozygous deficient embryoid bodies were generally smaller and non-cystic; the surfaces of these embryoid bodies had a cobblestone appearance (Fig. 3C,F).

Sections through embryoid bodies revealed

that wild-type and heterozygous embryoid bodies were covered with a well-developed epithelium of visceral endoderm (Fig. 4). Cells in the visceral endoderm layer of wild-type embryoid bodies stained intensely with toluidine
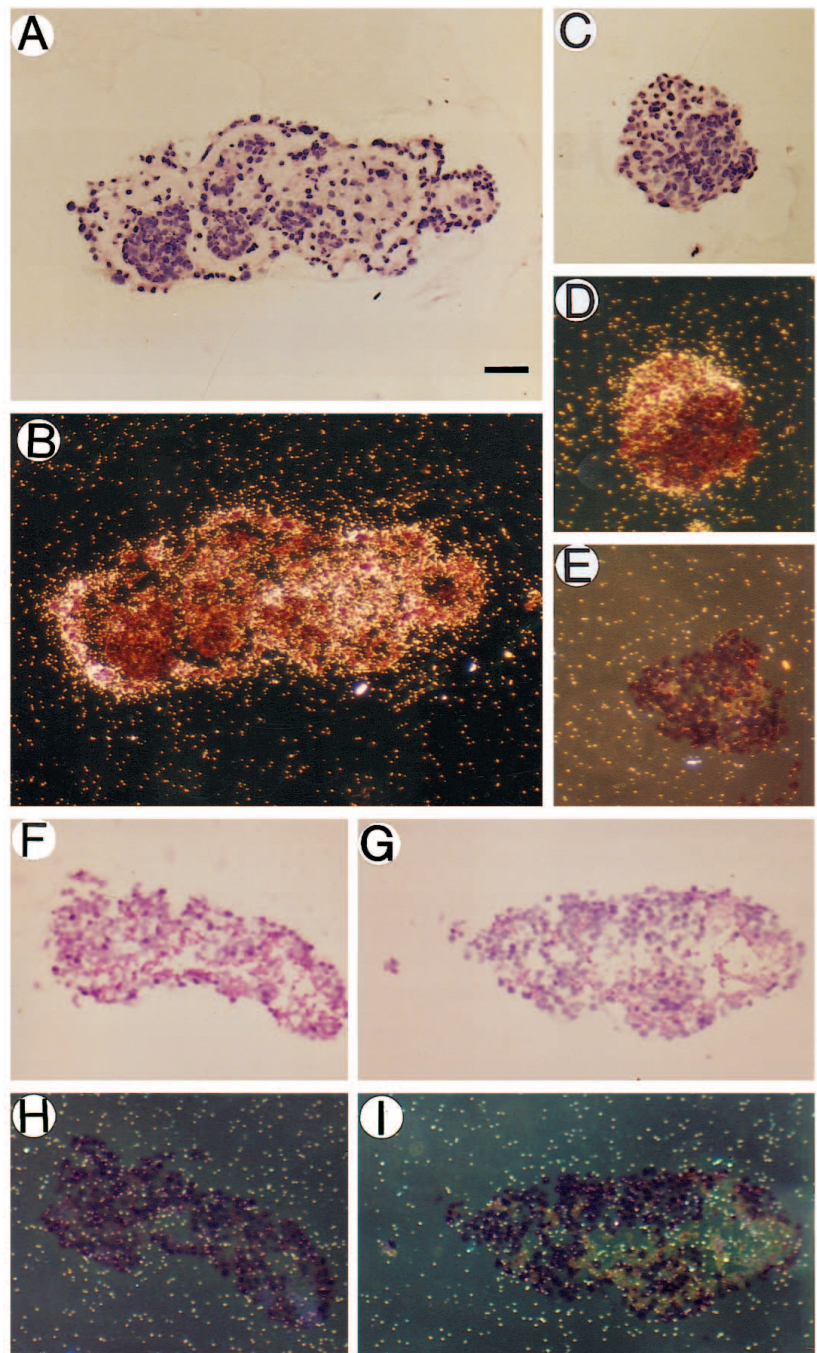

Fig. 2. In situ hybridization of day 8 embryoid bodies derived from wild-type and GATA-4 homozygous deficient ES cells. (A,B and C,D) Corresponding light- and dark-field views of wild-type embryoid bodies hybridized to GATA-4 antisense riboprobe. GATA-4 expression is restricted to yolk sac endoderm cells on the surface of the embryoid bodies; less differentiated cells on the interior of the embryoid body do not express GATA-4 message. (E) Negative control of a wild-type embryoid body hybridized to GATA-4 sense riboprobe. (F,G and H,I) Corresponding light- and dark-field views of homozygous deficient embryoid bodies hybridized to antisense probe. No GATA-4 expression is seen in the homozygous deficient cells. Bar, 100 μm.

blue, contained numerous vacuoles and had a brush border of microvilli (Fig. 4A-D). Large cysts were evident in sections of older (>10 days) wild-type and heterozygous deficient embryoid bodies (Fig. 4B,C). In agreement with the work of others (Doetschman et al., 1985; Wang et al., 1992), we found that the walls of these cysts were composed of visceral endoderm cells, mesothelial cells, endothelial cells and blood cell islands (Fig. 4B,C). Within the cores of older wild-type embryoid bodies, we observed pockets of undifferentiated cells separated by foci of mesenchymal and epithelial cells (Fig. 4C,D).

In contrast, there was no visceral endoderm on the surface of the homozygous deficient embryoid bodies (Fig. 4E-H). Between 4 and 10 days of differentiation, the surface of the homozygous deficient embryoid bodies consisted of loosely associated, relatively undifferentiated cells (Fig. 4E). At later stages of differentiation (>10 days), some of the embryoid bodies developed a flat, simple epithelium of cells that lacked vacuoles, a brush border, or a thick basement membrane (Fig. 4F,G). At no point during in vitro differentiation were visceral endoderm-covered cystic structures evident in the homozygous deficient embryoid bodies. Furthermore, the mutant embryoid bodies did not contain recognizable blood vessels or blood islands, possibly due to a lack of induction of mesodermal precursors by visceral endoderm. While yolk sac endoderm was not seen in the mutant embryoid bodies, patches of differentiated mesenchymal cells were evident within these bodies (Fig. 4F-H), suggesting that some aspects of differentiation are not blocked in GATA-4-deficient cells. This was confirmed by examination of the expression of stage-specific embryonic antigen-1 (SSEA-1), an surface oligosaccharide epitope present on stem cells, the ICM and primitive ectoderm that is down regulated as these cells differentiate (Solter and Knowles, 1978). Wild-type and GATA-4-deficient embryoid bodies exhibited similar patterns of SSEA-1 expression (Fig. 5). The cores of the embryoid bodies contained undifferentiated cells that expressed the antigen, while the surrounding, more differentiated cells did not express SSEA-1.

The ultrastructural features of wild-type, heterozygous deficient and homozygous deficient embryoid bodies were also compared by electron microscopy. On the surface of wild-type embryoid bodies we observed cells with the classic features of visceral endoderm, including phagocytic vacuoles, lipid droplets, glycogen deposits, apical tight juctions and numerous microvilli (Fig. 6A,D). Beneath the visceral endoderm layer were relatively undifferentiated cells reminiscent of stem cells. Embryoid bodies derived from the heterozygous deficient cells also had cells with the hallmark features of visceral endoderm (Fig. 6B,E). In striking contrast, the surface of the homozygous deficient embryoid bodies lacked all characteristics of visceral endoderm (Fig. 6C,F). Instead, the surface of these embryoid bodies was covered with undifferentiated cells that lacked microvilli, phagocytic vacuoles, apical tight junctions, or a well-developed basement membrane. These marked differences in surface ultrastructural features between the wild-type and homozygous deficient day 9 embryoid bodies persisted at a later stages of development (i.e., day 16), suggesting that the block in visceral endoderm formation in the mutant cells was not merely temporal in nature.
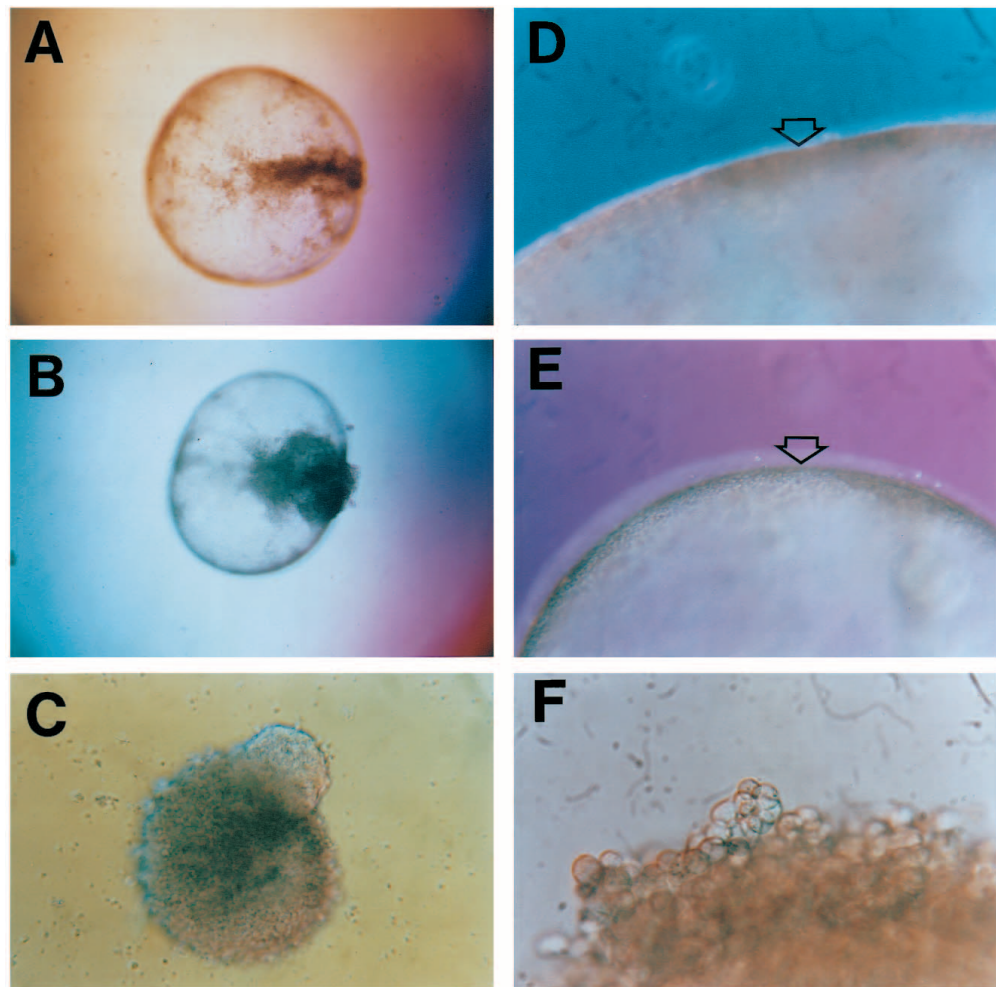

Fig. 3. Light microscopic appearance of day 10 wild-type (A,D), GATA-4 heterozygous deficient (B,E) and GATA-4 homozygous deficient (C,F) embryoid bodies. Arrows denote the smooth, endoderm-covered surfaces of wild-type and heterozygous embryoid bodies. Magnifications: (A,B) ×40; (C-E) ×100; (F) ×200.

GATA-4-deficient embryoid bodies lack biochemical markers of visceral endoderm

To confirm and extend these morphologic findings, we compared expression of several visceral endoderm markers in the wild-type and GATA-4-deficient embryoid bodies. First, AFP in the wild-type and mutant embryoid bodies was quantified by western blotting. As shown in Fig. 7, abundant AFP was detected within the wild-type embryoid bodies, but only trace AFP was present in the GATA-4-deficient embryoid bodies. Using phosphorimage analysis, the amount of AFP in lysates of the homozygous deficient cells was determined to be 25-100 times less than the wild-type cells in multiple experiments with independently selected clones of GATA-4-deficient cells. Similar results were obtained when radiolabelled AFP was immunoprecipitated from culture supernatants of [35S]methionine-labelled embryoid bodies (not shown).

Another class of markers useful for documenting changes in epithelia during embryogenesis are developmentally regulated surface oligosaccharides. The lectin *Dolichos biflorus* agglutinin (DBA) recognizes certain GalNAc-containing oligosaccharides present on visceral endoderm but not on undifferentiated cell types (Sato and Muramatsu, 1985). We examined the ability of this lectin to bind to the surface of wild-type and GATA-4 homozygous deficient embryoid bodies. As shown in Fig. 8A, abundant FITC-labelled DBA bound to the surface of day 10 wild-type embryoid bodies. Addition of excess GalNAc blocked this binding (Fig. 8B). There was minimal FITC-DBA binding to the surface of the homozygous deficient embryoid bodies either in the absence or presence of excess GalNAc (Fig. 8C,D), indicating that surface oligosaccharides characteristic of visceral endoderm are not expressed on the homozygous deficient cells.

We next used RNase protection assays to quantify expression of another yolk sac marker, HNF-4, a developmentally regulated transcription factor first expressed in visceral endoderm and later in liver, intestine, and nephrogenic tissue (Duncan et al., 1994; Taravirus et al., 1994). In wild-type embryoid bodies, HNF-4 RNA first appeared at 5-6 days of differentiation and then increased over the ensuing days of culture (Fig. 9A). GATA-4 expression in the wild-type embryoid bodies preceded HNF-4 expression by approximately 1 day. In embryoid bodies derived from two independently selected GATA-4-deficient ES lines (clones 9c and 10b) expression of HNF-4 message was markedly reduced, reflecting the defect in visceral endoderm development (Fig. 9A).

Non-yolk sac differentiation markers are expressed in the mutant embryoid bodies

To show that other differentiation programs were not disrupted by GATA-4 deficiency, we compared expression of other inducible transcripts in wild-type and mutant embryoid bodies. The transcripts examined included the following: connexin-45 (Cx45), a gap junction protein expressed in a variety of early embryonic tissues including lung, brain, skin, heart, intestine
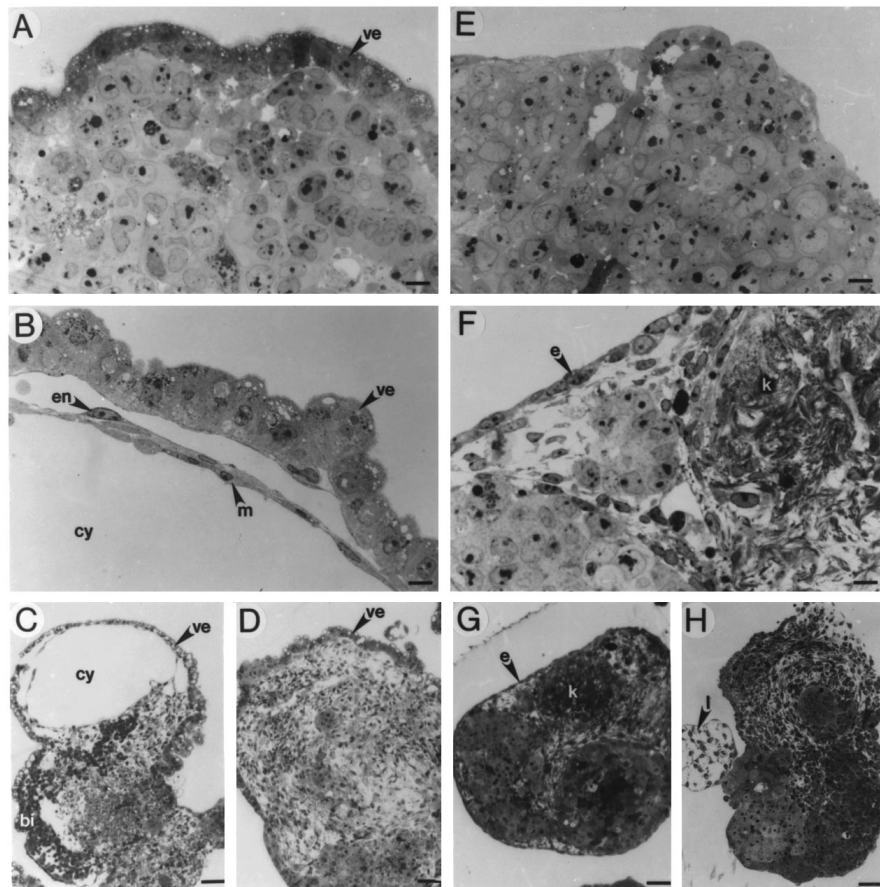

Fig. 4. Toluidene-blue-stained 1 μm sections of embryoid bodies derived from wild-type (A-D) or homozygous deficient (E-H) cells. (A) The surface of this day 9 wild-type embryoid body is covered with a layer of visceral endoderm, which stains darkly, has apical vacuoles and a brush border. Bar, 20 μm. (B) Lining of a cystic cavity in a day 16 wild-type embryoid body demonstrating the outer visceral endoderm layer, endothelial cells in a vascular channel and mesothelial cells. Bar, 20 μm. (C,D) Lower magnification views of day 16 wild-type embryoid bodies, illustrating visceral endoderm, a blood island and pockets of other differentiated cells within the core of the embryoid bodies. Bars, 80 μm. (E) Surface of a day 9 homozygous deficient cell. No visceral endoderm layer is seen. Bar, 20 μm. (F) Surface of a day 16 homozygous deficient cell revealing a thin epithelial layer that lacks features of visceral endoderm. Areas of keratinization reflecting cell differentiation are present in the core of this mutant embryoid body. Bar, 20 μm. (G,H) Lower magnification views of homozygous deficient embryoid bodies, showing patches of differentiated cells within the embryoid bodies. A surface lobule, or bud, composed of loosely organized mesenchymal cells without endoderm is also seen. Bars, 80 μm. Abbreviations: bi, blood island; cy, cystic cavity; e, epithelium (simple squamous); en, endothelial cell; k, keratin swirl; l, lobule of mesechymal cells; m, mesothelial cell; ve, visceral endoderm.
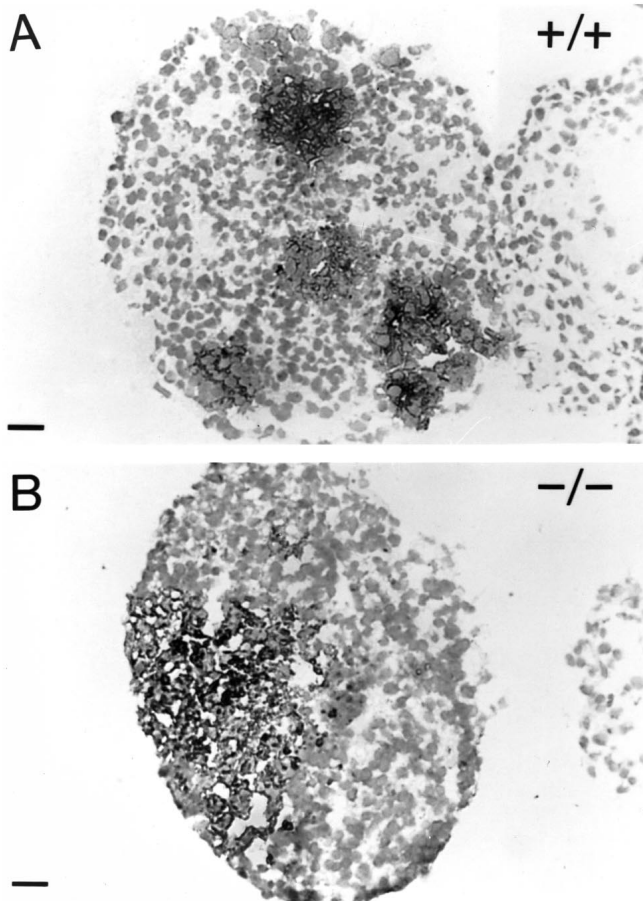
Fig. 5. Expression of SSEA-1 in wild-type and homozygous deficient embryoid bodies. Frozen sections of day 12 wild-type (A) or GATA-4 homozygous deficient (B) embryoid bodies were subjected to immunohistochemistry with anti-SSEA-1 antibody followed by a peroxidase conjugated secondary antibody. Bars, 100 μm.

and kidney (Hennemann et al., 1992); Flk-1, a receptor tyrosine kinase restricted to angioblasts and endothelial cells (Millauer et al., 1993); transforming growth factors β1 and β2 (TGFβ1 and TGFβ2), which are expressed by in a variety of embryonic epithelia (Millan et al., 1991); fibroblast growth factor-3 (FGF-3), a transcript found in parietal endoderm, extraembryonic mesoderm and the primitive streak (Wilkinson et al., 1989); and FGF-8, a recently identified member of the fibroblast growth factor family expressed in limb bud ectoderm, branchial arches, nasal pits and regions of the brain (Heikinheimo et al., 1994b; Crossley and Martin, 1995). Each of these transcripts exhibited a similar pattern of induction during in vitro differentiation of wild-type and GATA-4-deficient embryoid bodies (Fig. 9B). In separate RT-PCR experiments, we documented normal expression of two other differentiation markers, Brachyury and GATA-2, in the mutant embryoid bodies (data not shown). These findings imply that differentiation of many embryonic lineages are not disrupted in the GATA-4-deficient embryoid bodies.

ES-cell derived teratocarcinomas

As independent proof that gene targeting of GATA-4 does not produce a generalized block in lineage commitment, we compared the capacity of wild-type and homozygous deficient ES cells to differentiate into various tissues by injecting cells into the flanks of nude mice and analyzing the resultant teratocarcinomas. The incidence and gross appearance of the teratocarcinomas was similar for the two groups; after 2 weeks tumors were seen in 10/12 mice injected with wild-type cells versus 12/13 mice injected with the GATA-4-deficient cells. Gpi-1 isoenzyme analysis confirmed that >95% of cells in the tumors were derived from ES cells rather than the host animal (data not shown). Histological analysis of the teratocarcinomas revealed that both the wild-type and mutant ES cells can differentiate into a wide range of tissue types, including skeletal muscle, squamous epithelium, cartilage, bone, smooth muscle, respiratory epithelium and neuronal tissue (Fig. 10). These various tissue types appeared with roughly equal frequencies in the wild-type and homozygous deficient teratocarcinomas (data not shown). Thus, both the normal and mutant ES cells give rise to ectodermal, mesodermal and definitive endodermal derivatives. We were not able to use teratocarcinoma formation to assess whether GATA-4 is essential for in vivo yolk sac differentiation, since neither the wild-type nor mutant ES teratocarcinomas contained morphologically recognizable yolk sac elements. On the basis of these teratocarcinoma studies, we conclude that GATA-4 deficiency does not produce a generalized effect on lineage commitment.

Characterization of chimeric mice derived from GATA-4-deficient ES cells

The ability of GATA-4-deficient cells to contribute to various tissue types was also assessed by preparation of chimeric mice. Wild-type or GATA-4 homozygous deficient ES cells were injected into C57BL/6J host blastocysts and chimeric offspring identified by coat color. DNA was isolated from the organs of 6 week old chimeric animals (15-20% coat chimerism) and subjected to Southern analysis. The results of one such analysis are shown in Fig. 11. In these experiments, we employed a modification of the Southern analysis scheme outlined in Fig. 1. Rather than digesting DNA with *Xho*I+*Not*I, the combination of *Nco*I+*Cla*I was used because digestion with these endonucleases resulted in stronger hybridization signals. The targeted allele gave a 5.75 kb band on Southern analysis, while the wild-type allele gave a 5.3 kb band. DNA from the homozygous deficient cells was detected in a diverse array of organs, including heart, skeletal muscle, stomach and kidney. Similar results were obtained with additional chimeric animals. These findings confirm that GATA-4-deficient stem cells can contribute to a variety of tissue types and are therefore not generally defective in differentiation.

DISCUSSION

Through gene targeting, we have established that transcription factor GATA-4 is essential for in vitro differentiation of yolk sac endoderm. Unlike wild-type cells, GATA-4 homozygous deficient cells do not form morphologically recognizable visceral endoderm when differentiated into embryoid bodies. The mutant embryoid bodies are also deficient in expression of several markers of visceral endoderm, such as AFP, HNF-4

Fig. 6. Electron microscopy of the surface of embryoid bodies derived from wild-type, heterozygous deficient and homozygous deficient cells. Two magnifications are shown: (A-C) bars, 2.5 µm; (D-F) bars, 1 µm. (A,D) The surface of wild-type embryoid bodies is covered with electron-dense visceral endoderm cells that have abundant microvilli, phagocytic vacuoles and secretory vesicles. (B,E) The heterozygous deficient cells also have visceral endoderm cells on their surface, though in some areas the visceral endoderm is not fully developed (left side of B). (C,F) No visceral endoderm is seen on the homozygous deficient cells. Only an incidental short microvillus is evident on these surface cells.
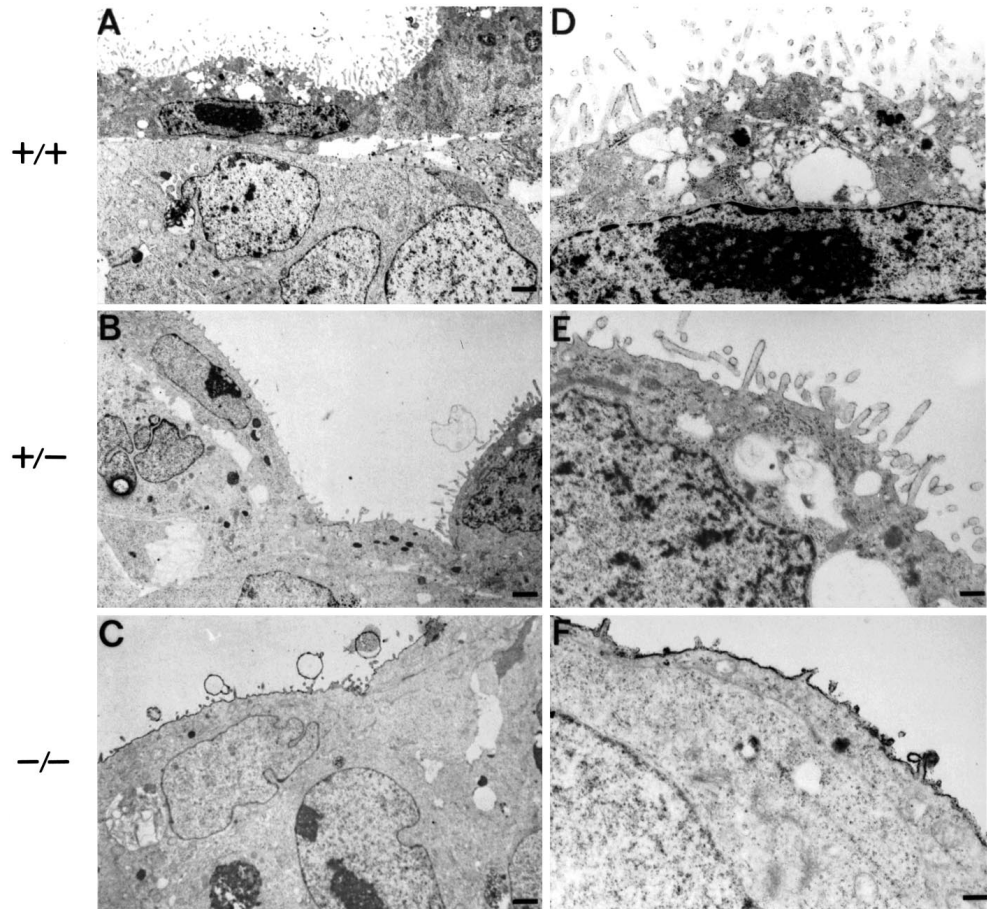
and *Dolichos biflorus* agglutinin binding sites. GATA-4 deficiency does not produce a generalized block in lineage commitment, as shown by non-yolk sac marker analysis, blastocyst injection experiments and teratocarcinoma analysis.

Although our results demonstrate that GATA-4 deficiency blocks the in vitro differentiation of stem cells into visceral endoderm, the precise stage of this developmental block is currently unknown. Both visceral and parietal endoderm cells are derived from primitive endoderm cells that appear along the blastocoelic surface of the inner cell mass of the day 5 mouse embryo (Gardner, 1993; Rossant, 1986). In theory, the phenotype of the GATA-4-deficient embryoid bodies could be due to either a lack of formation of primitive endoderm or a block in differentiation of primitive endoderm cells into visceral endoderm cells. Because there are no known morphologic or biochemical markers that unequivocally distinguish primitive endoderm cells (Kahan and Adamson, 1983), the presence of primitive endoderm cells in the GATA-4 homozygous deficient embryoid bodies could not be directly verified. It is possible that some of the cells on the surface of the mutant embryoid bodies are primitive endoderm cells. Cells with the classic features of parietal endoderm (expanded rough endoplasmic reticulum, thick basement membrane) were not evident in the GATA-4-deficient embryoid bodies; however, the culture conditions employed (Doestchman et al., 1985) do not promote parietal endoderm differentiation in vitro from wild-type ES cells. Thus, we do not know whether the homozygous deficient ES cells can differentiate into parietal endoderm.

To address this issue, we are currently trying different conditions for culture of the embryoid bodies.

Homozygous deficient embryoid body analysis is a powerful tool for defining the role of genes in development, but the limitations of this approach should be kept in mind. The potential exists for ES cells to nonspecifically acquire mutations during propagation, resulting in clonal variation among ES lines that could include a loss of differentiation capacity. It seems unlikely that the phenotype of the GATA-4-deficient embryoid bodies is due to clonal variation. Independently selected GATA-4-deficient ES cell lines displayed the same phenotype, while none
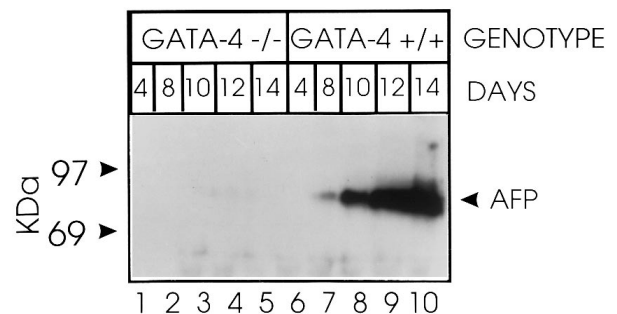

Fig. 7. AFP synthesis by wild-type and GATA-4 homozygous deficient embryoid bodies. Embryoid bodies were differentiated for the indicated lengths of time, solubilized and then equivalent amounts of soluble protein were subjected to Western blotting with anti-AFP serum followed by ^{125}I-protein A.
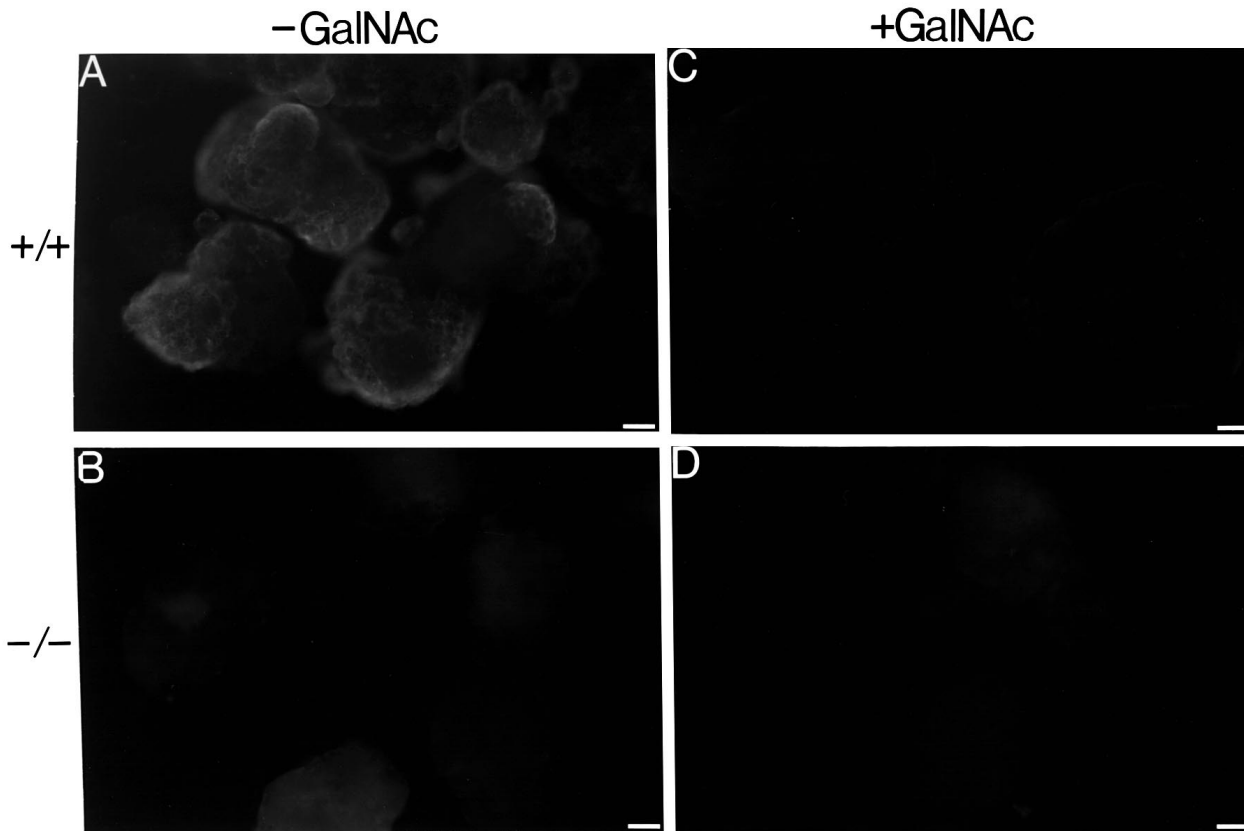
Fig. 8. *Dolichos biflorus* agglutinin binding to wild-type and homozygous deficient embryoid bodies. Day 10 wild-type (A,B) or GATA-4 homozygous deficient (C,D) were fixed and then incubated with FITC-DBA in the absence (A,C) or presence (B,D) of 0.1 mM GalNAc. Bars, 100 μm.

of the wild-type or heterozygous deficient ES lines (including those propagated in G418 for extended periods of time) showed evidence of a block in visceral endoderm differentiation. Formal proof that the in vitro phenotype is secondary to GATA-4 deficiency per se awaits gene reintroduction experiments.

While our results argue that GATA-4 is essential for in vitro visceral endoderm formation, the in vivo consequences of GATA-4 deficiency on yolk sac development are currently unknown. To address this issue, we are attempting to generate and characterize GATA-4 homozygous deficient mouse embryos. In light of the embryoid body results, we anticipate that yolk sac function will be impaired in the GATA-4-deficient embryos, possibly resulting in an early lethal phenotype.

Despite intensive study of embryonic stem cell differentiation over the past decade, few other factors have been shown to be essential for yolk sac endoderm differentiation or development. Some genes involved in the differentiation process have been identified through studies of retinoic-acid-treated embryonal carcinoma cells (Gudas, 1994). For example, targeted

disruption of the retinoid receptors RARα and RARγ in mouse F9 embryonal carcinoma cells is associated with decreased expression of some, but not all, yolk sac differentiation markers (Boylan et al., 1993, 1995). Targeted deletion of β1-integrins in F9 cells affects in vitro morphologic differentiation of yolk sac endoderm but not tissue-specific gene expression
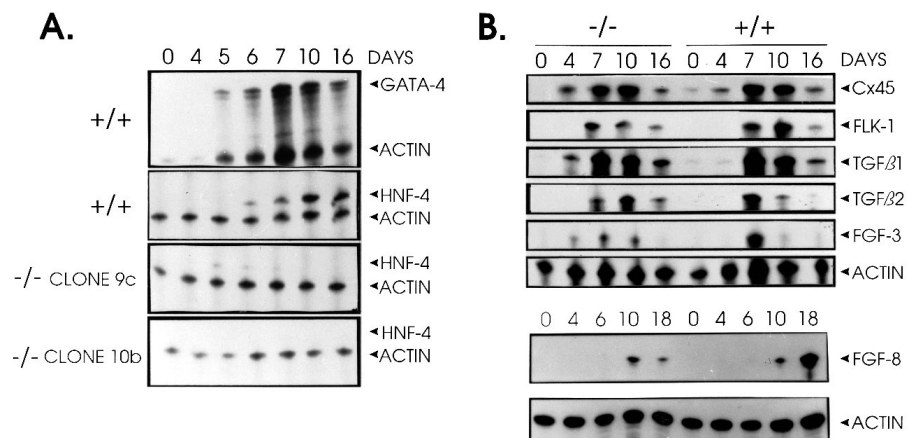

Fig. 9. (A) Expression of HNF-4 transcripts in wild-type (+/+) and GATA-4-deficient (−/−) embryoid bodies using RNase protection assays. Two independently selected GATA-4-deficient lines are shown. (B) Expression of other differentiation markers in wild-type and GATA-4-deficient (clone 9c) embryoid bodies. RNase protection assays for Cx45, Flk-1, TGFβ1, TGFβ2, FGF-3, actin and FGF-8 are shown.

of markers such as AFP, laminin, or SSEA-1 (Stephens et al., 1993). These findings contrast with the GATA-4-deficient embryoid bodies, which show defects in both endoderm formation and tissue-specific gene expression.

Few genes have been described that are abundantly expressed in murine yolk sac endoderm and whose disruption results in an early lethal phenotype. Among these only *evx1* homozygote embryos fail to differentiate yolk sac tissue or form an egg cylinder (Spyropoulos and Capecchi, 1994). Targeting of *HNF-4* results in no obvious changes in morphology or endocytic fuction of visceral endoderm. Rather, the lethal phenotype appears to be related to cell death in embryonic ectoderm and impaired embryonic/extraembryonic mesoderm formation (Chen et al., 1994). The murine lethal transgene insertion *Hβ58* disrupts another gene expressed preferentially by visceral endoderm that is essential for embryonic ectoderm differentiation (Lee et al., 1992). Embryos and embryoid bodies homozygous deficient in the gene *msd*, whose biochemical nature is unknown, display a block in mesoderm development both in yolk sac and embryonic tissues (Holdener et al., 1994). Visceral and parietal endoderm formation appears normal in *msd*-deficient embryos. The developmental disturbances manifested in homozygotes deficient for the *HNF-4*, *Hβ58* and *msd* may reflect a lack of production by visceral endoderm of regulatory molecules critical for differentiation of adjacent tissue. Analysis of these and other mutations affecting mouse yolk sac development promises to yield insights into interactions between endoderm and mesoderm.

The phenotype of the GATA-4-deficient embryoid bodies resembles the appearance of wild-type embryoid bodies that have been cultured in the presence of certain agents. Tunicamycin, an inhibitor of N-linked glycosylation, has been shown to reversibly block formation of yolk sac endoderm in embryoid bodies derived from PSA-1 stem cells (Grabel et al., 1983). Similarities between the GATA-4 homozygous deficient and the tunicamycin-treated embryoid bodies suggest that GATA-4 may be essential for expression of a glycoprotein gene or genes involved in visceral endoderm differentiation. The target genes for GATA-4 in yolk sac endoderm are currently unknown, though our GATA-4-deficient ES lines should be useful in establishing these target genes. F9 cell embryoid bodies cultured in high concentrations of laminin also develop a reversible block in yolk sac endoderm differentiation (Grover et al., 1983). The mechanisms whereby laminin suppresses endoderm differentiation are not understood.

Besides yolk sac endoderm, GATA-4 is expressed in certain other tissues of the fetal and adult mouse, notably cardiac
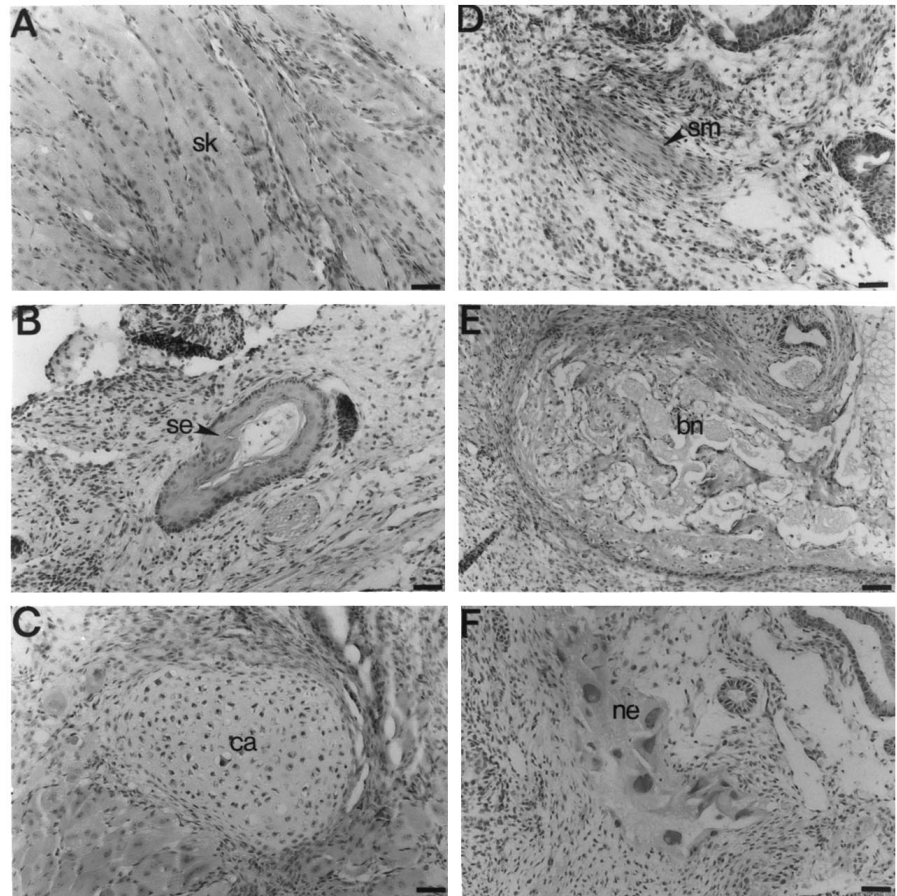

Fig. 10. Tissue types present in teratocarcinomas derived from GATA-4 homozygous deficient ES cells. ES cells were injected into the flanks of nude mice, harvested at 2-3 weeks, sectioned and stained with H&E. (A) Skeletal muscle, (B) squamous epithelium, (C) cartilage, (D) smooth muscle, (E) bone and (F) neuronal cells. Bars, 100 μm.
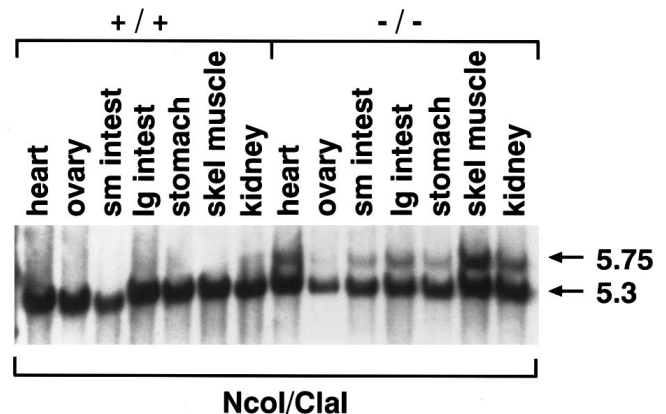

Fig. 11. Analysis of chimeric mice generated by injection of wild-type or GATA-4 homozygous deficient ES cells into blastocysts. DNA was isolated from the organs of 6 week old chimeric animals, digested with *Nco*I+*Cla*I and then subjected to Southern analysis using as a probe the 1.2 kb *Eco*RI/*Xho*I fragment described in Fig. 1. Gene targeting results in loss of an *Nco*I site normally found the second exon of the *Gata4* gene. As a consequence, the targeted allele gives a 5.75 kb band on Southern analysis, while the wild-type allele gives a 5.3 kb band.

muscle, gut epithelium and gonads (Arceci et al., 1993; Heikinheimo et al., 1994a; Laverriere et al., 1994; Grépin et al., 1994; Tamura et al., 1993). The role of GATA-4 in these other cell types is under study. In the heart, GATA-4 has been implicated in the regulation of several genes encoding myocardial proteins (Ip et al., 1994; Molkentin et al., 1994; Grépin et al., 1994; Thuerauf et al., 1994), while in stomach GATA-4 is thought to regulate expression of gastric parietal cell genes (Tamura et al., 1993). It is possible that GATA-4 also controls a battery of genes involved in more general aspects of epithelial function, such as extracellular matrix biosynthesis, which accounts for the presence of GATA-4 in a variety of epithelial tissues, including yolk sac endoderm, heart, gut lining and gonads (discussed in Kelley et al., 1993). Our GATA-4-deficient ES lines should facilitate an investigation of the role of GATA-4 in each of these tissues.

REFERENCES

Arceci, R. J., King, A. A. J., Simon, M. C., Orkin, S. H. and Wilson, D. B. (1993). Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermal derivatives and heart. *Mol. Cell. Biol.* **13**, 2235-2246.

Boylan, J. F., Lohnes, D, Taneja, R., Chambon, P. and Gudas, L. J. (1993). Loss of RARγ function by gene disruption results in aberrant hoxa-1 expression and differentiation upon retinoic acid treatment. *Proc. Natl. Acad. Sci. USA* **90**, 9601-9605.

Boylan, J. F., Lufkin, T., Achkar, C. C., Taneja, R., Chambon, P. and Gudas, L. J. (1995). Targeted disruption of retinoic acid receptor α (RARα) and RARγ results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. *Mol. Cell. Biol.* **15**, 843-851.

Chen, W. S., Manova, K., Weinstein, D. C., Duncan, S. A., Plump, A. S., Prezioso, V. R., Bachvarova, R. F. and Darnell, J. E. Jr. (1994). Disruption of the *HNF-4* gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev.* **8**, 2466-2477.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Coffin, J. D., Harrison, J., Schwartz, S. and Heimark, R. (1991). Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. *Dev. Biol.* **148**, 51-62.

Cross, J. C., Webb, Z. and Fisher, S. J. (1994). Implantation and the placenta: key pieces of the development puzzle. *Science* **266**, 1508-1518.

Crossley, P. H. and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. and Kelmer, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands, and myocardium. *J. Embryol. Exp. Morph.* **87**, 27-45.

Duncan, S. A., Manova, K., Chen, W. S., Hoodless, P., Weinstein, D. C., Bachvarova, R. F. and Darnell, J. E. Jr. (1994). Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. *Proc. Natl. Acad. Sci. USA* **91**, 7598-9602.

Gardner, R. L. (1983). Origin and differentiation of extraembryonic tissues in the mouse. *Intl. Rev. Exp. Path.* **24**, 63-133.

Grabel, L. B., Singer, M. S., Rosen, S. D. and Martin, G. R. (1983). The role of carbohydrates in the intercellular adhesion and differentiation of teratocarcinoma stem cells. In *Teratocarcinoma Stem Cells.* (ed. L. M. Silver, G. R. Martin and S. Strickland), pp. 145-161. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Grépin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T. and Nemer, M. (1994). A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol. Cell. Biol.* **14**, 3115-3129.

Grover, A., Andrews, G. and Adamson, E. D. (1983). Role of laminin in epithelium formation by F9 aggregates. *J. Cell Biol.* **97**, 137-144.

Gudas, L. J. (1994). Retinoids and vertebrate development. *J. Biol. Chem.* **269**, 15399-15402.

Harlow, E. and Lane, D. (1988) *Antibodies: a Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Heikinheimo, M., Scandrett, J. M. and Wilson, D. B. (1994a). Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev. Biol.* **164**, 361-373.

Heikinheimo, M., Lawshé, A., Shackleford, G. M., Wilson, D. B. and MacArthur, C. A. (1994b) *Fgf-8* expression in the post-gastrulation mouse suggest roles in the development of the face, limbs, and central nervous system. *Mech. Devel.* **48**, 129-138.

Hennemann, H., Schwarz, H. J. and Willecke, K. (1992). Characterization of gap junction genes expressed in F9 embryonic carcinoma cells: molecular cloning of mouse connexin31 and -45 cDNAs. *Eur. J. Cell Biol.* **57**, 51-58.

Hogan, B. L. M. and Tilly, R. (1981). Cell interactions and endoderm differentiation in cultured mouse embryos. *J. Embryol. Exp. Morph.* **62**, 379-394.

Holdener, B. C., Faust, C., Rosenthal, N. S. and Magnuson, T. (1994). *msd* is required for mesoderm induction in mice. *Development* **120**, 1335-1346.

Ip, H. S., Wilson, D. B., Heikinheimo, M., Tang, Z., Ting, C.-N., Simon, M. C., Leiden, J. M. and Parmacek, M. S. (1994). The GATA-4 transcription factor transactivates the cardiac-specific troponin C promoter-enhancer in non-muscle cells. *Mol. Cell. Biol.* **14**, 7517-7526.

Jollie, W. P. (1990). Development, morphology, and function of the yolk-sac placenta of laboratory rodents. *Teratol.* **41**, 361-381.

Kahan, B. and Adamson, E. D. (1983). A teratocarcinoma-derived bipotential cell line with primitive endoderm properties. In *Teratocarcinoma Stem Cells.* (eds. L. M. Silver, G. R. Martin and S. Strickland), pp. 131-144. Cold Spring Harbor, N. Y. : Cold Spring Harbor Laboratory.

Keller, G., Kennedy, M., Papyannopoulou, T. and Wiles, M. V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell. Biol.* **13**, 473-487.

Kelley, C., Blumberg, H., Zon, L. I. and Evans, T. (1993). GATA-4 is a novel transcription factor expressed in the endocardium of the developing heart. *Development* **118**, 817-827.

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. E. and Evans, T. (1994). GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J. Biol. Chem.* **269**, 23177-23184.

Lee, J. J., Radice, G., Perkins, C. P. and Costantini, F. (1992). Identification and characterization of a novel, evolutionarily conserved gene disrupted by the murine *Hβ58* embryonic lethal transgene insertion. *Development* **115**, 277-288.

Mansour, S. L., Thomas, K. R. and Capecchi, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336**, 348-352.

Millan, F. A., Denhez, F., Kondaiah, P. and Akhurst, R. J. (1991) Embryonic gene expression patterns of TGFβ 1, 2, and 3 suggest different developmental functions in vivo. *Development* **111**, 131-141.

Millauer, B. Wizigmann-Voos, S., Schnurch, H.,, Martinez, R., Moller, N. P. H., Risau, W. and Ullrich, A. (1993) High affinity VEGF binding and developmental expression suggest flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* **72**, 835-846.

Miller-Hance, W. C., LaCorbiere, M., Fuller, S. S., Evans, S. M., Lyons, G.,

Schmidt, C., Robbins, J. and Chien, K. R. (1993). In vitro chamber specificiation during ES cell embryogenesis. Expression of the *ventricular myosin light chain-2* gene is independent of heart tube formation. *J. Biol. Chem.* **268**, 25244-25252.

Milura, Y. and Wilt, F. (1969). Tissue interactions and the formation of the first erythroblasts of the chick embryo. *Dev. Biol.* **19**, 201-210.

Molkentin, J. D., Kalvakolanu, D. V. and Markham, B. E. (1994). Transcription factor GATA-4 regulates cardiac muscle-specific expression of the *α-myosin heavy-chain* gene. *Mol. Cell. Biol.* **14**, 4947-4957.

Mortensen, R. M., Conner, D. A., Chao, S., Geisterfer-Lowrance, A. A. T. and Seidman, J. G. (1992) Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* **12**, 2391-2395.

Orkin, S. H. (1992). GATA-Binding transcription factors in hematopoietic cells. *Blood* **80**, 575-581.

Palis, J., McGrath, K. E. and Kingsley, P. D. (1995). Initiation of hematopoiesis and vasculogenesis in murine yolk sac explants. *Blood* **86**, 156-163.

Papaioannou, V. and Johnson, R. (1993). Production of chimeras and genetically defined offspring from targeted ES cells. In *Gene Targeting: a Practical Approach*. (ed. A. L. Joyner), pp. 107-146, Oxford: IRL Press.

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S., D'Agati, V., Orkin, S. H. and Costantini, F. (1991). Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**, 257-260.

Pevny, L., Lin, C.-S., D'Agati, V., Simon, M. C., Orkin, S. H. and Costantini, F. (1995). Development of hematopoietic cells lacking transcription factor GATA-1. *Development* **121**, 163-172.

Risau, W., Sariola, H., Zerwes, H.-G., Sasse, J., Ekblom, P., Kemler, R. and Doetschman, T. (1988). Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development* **102**, 471-478.

Robertson, E. J. (1987). In *Teratocarcinomas and Embryonic Stem Cells: a Practical Approach*. (ed. E. J. Robertson). Oxford: IRL Press.

Rohwedel, J., Maltsev, V., Bober, E., Arnold, H.-H., Hescheler, J. and Wobus, A. M. (1994). Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expresssion of myogenic determination genes and functional expression of ionic currents. *Dev. Biol.* **164**, 87-101.

Rossant, J. (1986) Development of extraembryonic lineages in the mouse embryo. In *Experimental Approaches to Mammalian Embryonic Development.* (eds. J. Rossant and R. A. Pedersen), pp. 97-119, Cambridge, UK: Cambridge University Press.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sánchez, A., Jones, W. K., Gulick, J., Doetschman, T. and Robbins, J. (1991). *Myosin heavy chain* gene expression in mouse embryoid bodies: an in vitro developmental study. *J. Biol. Chem.* **266**, 22419-22426.

Sato, M. and Muramatsu, T. (1985). Reactivity of five N-acetylgalactosamine-recognizing lectins with preimplantation embryos, early postimplantation embryos, and teratocarcinoma cells of the mouse. *Differentiation* **29**, 29-38.

Simon, M. C., Pevny, L., Wiles, M. V., Keller, G., Costantini, F. and Orkin, S. H. (1992). Rescue of erythroid development in gene targeted GATA-1⁻ mouse embryonic stem cells. *Nature Gen.* **1**, 92-98.

Solter, D. and Knowles, B. B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA* **75**, 5565-5569.

Spyropoulos, D. D. and Capecchi, M. R. (1994). Targeted disruption of the *even-skipped* gene, *evx1*, causes early postimplantation lethality of the mouse conceptus. *Genes Dev.* **8**, 1949-1961.

Stephens, L. E., Sonne, J. E., Fitzgerald, M. L. and Damsky, C. H. (1993) Targeted deletion of β1-integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. *J. Cell Biol.* **123**, 1607-1620.

Tamura, S., Wang, X.-H., Maeda, M. and Futai, M. (1993). Gastric DNA-binding proteins recognize upstream sequence motifs of parietal cell-specific genes. *Proc. Natl. Acad. Sci. USA* **90**, 10876-10880.

Taraviras, S., Monaghan, A. P., Schutz, G. and Kelsey, G. (1994). Characterization of the mouse *HNF-4* gene and its expression during mouse embryogenesis. *Mech. Devel.* **48**, 67-79.

Thuerauf, D. J., Hanford, D. S. and Glembotski, C. C. (1994). Regulation of rat brain natriuetic peptide transcription: a potential role for GATA-related transcription factors in myocardial gene expression. *J. Biol. Chem.* **269**, 17772-17775.

Tsai, F.-Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-226.

Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T. and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the *c-abl* proto-oncogene. *Cell* **65**, 1153-1163.

Wang, R., Clark, R. and Bautch, V. L. (1992). Embryonic stem cell-derived cystic embryoid bodies form vascular channels: an in vitro model of blood vessel development. *Development* **114**, 303-316.

Weiss, M., Keller, G. and Orkin, S. H. (1994). Novel insights into erythroid development revealed through in vitro differentiation of GATA-1⁻ embryonic stem cells. *Genes Dev.* **8**, 1184-1197.

Wilkinson, D. G., Bhatt, S. and McMahon, A. P. (1989). Expression pattern of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development. *Development* **105**, 131-136.

Wilt, F. (1965). Erythropoiesis in the chick embryo: the role of endoderm. *Science* **147**, 1588-1590.

Wu, T.-C., Wan,Y.-J. and Damjanov, I. (1983). Fluoroscein-conjugated *Bandeiraea simplicifolica* lectin as a marker of endodermal, yolk sac, and trophoblastic differentiation in the mouse embryo. *Differentiation* **24**, 55-59.

Wurst, W. and Joyner, A. L. (1993). Production of targeted embryonic stem cell clones. In *Gene Targeting: a Practical Approach*. (ed. A. L. Joyner), pp. 33-61, Oxford: IRL Press.

Yoder, M. C., Papaioannou, V. E., Breitfeld, P. P. and Williams, D. A. (1994) Murine yolk sac endoderm- and mesoderm-derived cell lines support in vitro growth and differentiation of hematopoietic cells. *Blood* **83**, 2436-2443.