Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 286 (2005) 574 – 586

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Genomes & Developmental Control

Perception of differentiation cues by GATA factors in primitive endoderm lineage determination of mouse embryonic stem cells

Callinice D. Capo-chichi[a], Malgorzata E. Rula[a], Jennifer L. Smedberg[a], Lisa Vanderveer[a], Michael S. Parmacek[b], Edward E. Morrisey[b], Andrew K. Godwin[a], Xiang-Xi Xu[a,*]

[a]*Ovarian Cancer and Tumor Cell Biology Programs, Department of Medical Oncology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111-2497, USA*

[b]*Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

Received for publication 1 July 2005, revised 9 July 2005, accepted 11 July 2005
Available online 12 September 2005

Abstract

The formation of the primitive endoderm covering the inner cell mass of early mouse embryos can be simulated in vitro by the differentiation of mouse embryonic stem (ES) cells in culture following either aggregation of suspended cells or stimulation of cell monolayers with retinoic acid. The developmentally regulated transcription factors GATA-4 and GATA-6 have determining role in mouse extraembryonic endoderm development. We analyzed the in vitro differentiation of mouse embryonic stem cells deficient of GATA factors and conclude that GATA-4 is required for ES cells to perceive a cell positioning (cell aggregation) signal and GATA-6 is required to sense morphogenic (retinoic acid) signal. The collaboration between GATA-6 and GATA-4, or GATA-6 and GATA-5 which can substitute for GATA-4, is involved in the perception of differentiation cues by embryonic stem cells in their determination of endoderm lineage. This study indicates that the lineage differentiation of ES cells can be manipulated by the expression of GATA factors.

Keywords: Disabled-2; GATA factors; Primitive endoderm; Retinoic acid; Cell growth suppression; Differentiation

Introduction

In implanting blastocysts, three cell lineages derived from the fertilized ova are the trophectoderm cells lining the outer surface of the blastocysts, endoderm cells lining the blastocoel cavity, and the pluripotent cells of the inner cell mass (Lu et al., 2001). The trophectoderm and endoderm cells contribute exclusively to extraembryonic tissues and will form the placenta and yolk sac, respectively. The inner cell mass is pluripotent and gives rise to all cell lineages in the future definite embryos. Pluripotent embryonic stem (ES) cells can be derived from the inner cell mass of implanting blastocysts and maintained in culture (Hogan and Tilly, 1977, 1978; Martin, 1981; Evans and Kaufman, 1981). Upon aggregation in vitro, ES cells undergo spontaneous differentiation into extraembryonic endoderm-like cells (Coucouvanis and Martin, 1995, 1999). The differentiation of ES or EC (embryonic carcinoma) cells into extraembryonic endoderm cells in cultures mimics one of the earliest steps in embryonic development (Martin and Evans, 1975; Sherman and Miller, 1978). Upon differentiation, extraembryonic endoderm markers such as GATA-4, GATA-6, laminin, collagen IV, SPARC, and Dab2 are induced (Bielinska et al., 1999), and the stem cell marker Oct-3/4 is lost (Niwa et al., 2000). GATA genes/proteins are the determining factors in formation of extraembryonic endoderm in vivo and endoderm lineage commitment in vitro (Soudais et al., 1995; Bielinska and Wilson, 1997; Morrisey et al., 1998; Koutsourakis et al., 1999).

GATA factors belong to a family of transcription factors that bind DNA fragments with an “A/T GATA A/G” core sequence (Weiss and Orkin, 1995; Molkentin, 2000; Patient and McGhee, 2002). The GATA factors are conserved among species and involved in embryonic development and maintenance of cell differentiation in adults (Bodmer and

* Corresponding author. Fax: +1 215 728 2741.
E-mail address: X_XU@fccc.edu (X.-X. Xu).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.07.037

Venkatesh, 1998). Six GATA genes are present in mammals (Patient and McGhee, 2002). GATA-1, 2, and 3 are involved in hematopoiesis (Weiss and Orkin, 1995; Orkin, 1992), and GATA-4, 5, and 6 first identified in chicken are implicated in heart and organ development (Laverriere et al., 1994; Charron and Nemer, 1999; Molkentin, 2000). In early mouse embryonic development, GATA-4 and GATA-6 are two of the few genes induced in the first cell lineage (the primitive endoderm) that differentiates from the pluripotent inner cell mass of the early blastocyst (Arceci et al., 1993; Morrisey et al., 1996). The expression of GATA-4 and GATA-6 is restricted to the primitive endoderm and the derived visceral and parietal endoderm of the extraembryonic tissues (Arceci et al., 1993; Morrisey et al., 1998; Koutsourakis et al., 1999). Targeted deletion of GATA-4 results in embryonic lethality at ~E9.5 due to defects in heart morphogenesis (Kuo et al., 1997; Molkentin et al., 1997). However, ES cells homozygous deficient of GATA-4 fail to undergo spontaneous endoderm differentiation in vitro (Soudais et al., 1995), indicating a role in extraembryonic endoderm differentiation that can be rescued by a redundant pathway in vivo, possibly a retinoic acid-regulated pathway (Bielinska and Wilson, 1997). Nevertheless, the extraembryonic endoderm formed in GATA-4 (−/−) embryos is defective and is the cause of cardia bifida, and a wild type extraembryonic endoderm supplied to GATA-4 null embryos is sufficient to rescue the cardiac developmental defect (Narita et al., 1997; Watt et al., 2004). GATA-6 (−/−) ES cells fail to undergo endoderm differentiation both in vitro and in vivo (Morrisey et al., 1998; Koutsourakis et al., 1999). Deletion of GATA-6 results in a decrease in GATA-4 expression in extraembryonic endoderm (Morrisey et al., 1998; Koutsourakis et al., 1999) but not in the heart (Zhao et al., 2005). In contrast, GATA-4-deficient embryos exhibit an increase in GATA-6 expression (Kuo et al., 1997; Molkentin et al., 1997). Consideration of these observations led to the suggestion of a linear pathway in the regulation of endoderm differentiation that places GATA-4 downstream of GATA-6 (Morrisey et al., 1998). Nevertheless, feedback regulatory loops are likely present.

One of the GATA-6-induced genes is Disabled-2 (Dab2) (Morrisey et al., 2000). Dab2 expression is detectable in the primitive endoderm of implanting blastocysts at E4.5, and is expressed exclusively in the visceral and parietal endoderm, but not in ectoderm or mesoderm up to E7.5 mouse embryos (Yang et al., 2002). Dab2 is essential for the formation of primitive endoderm (Yang et al., 2002), as shown by gene knockout experiments. In Dab2 knockout embryos, although primitive endoderm differentiation occurs, the cells are unable to form an epithelial structure. Dab2 is not expressed in embryo proper until much later (E9.0), and thus is a specific marker for extraembryonic endoderm differentiation of ES and embryonic carcinoma (EC) cells. The expression of collagen IV and laminin, markers for the extraembryonic endoderm lineage (Gudas et al., 1990), is also suppressed in GATA-6 (−/−) ES cells (Morrisey et al., 2000). Laminin also has critical role in the formation of the extraembryonic endoderm, since mouse embryos deficient of laminin gamma also die at E5.5–6.5 due to failure in the formation of extraembryonic endoderm (Smyth et al., 1998, 1999).

The mechanism underlying the induction of endoderm differentiation of ES cells is of interest, since this is one of the earliest steps in embryonic development. In this study, we explore the role of GATA-4 and GATA-6 in ES cell differentiation in response to environmental and chemical cues. We show that GATA-4 and GATA-6 mediate cell aggregation and the retinoic acid-induced Dab2 expression and endoderm differentiation in ES cells. Deficiency of GATA-4 or GATA-6 differentially alters the response of the ES cells to differentiation cues, cell aggregation and retinoic acid, and results in deficiency of endoderm lineage commitment.

Materials and methods

Reagents

All-*trans*-retinoic acid (RA) was purchased from Sigma. Tissue culture supplies were obtained from Fisher Scientific, Inc. (Springfield, NJ). DMEM medium was purchased from Mediatech (Herndon, VA); fetal bovine serum (FBS) was obtained from Gibco; TRIzol reagent, 100× antibiotic-antimycotic solution, Lipofectamine, and serum-free Opti-MEM I medium were purchased from Gibco BRL (Grand Island, NY); the ECL Super-Signal West Dura Extended Duration Substrate immunodetection reagents were purchased from PIERCE (Rockfort, IL); Hybrisol I hybridization solution came from Intergen (Purchase, NY); positively-charged nylon membranes were from Boehringer Mannheim; [α-^{32}P]dCTP was from NEN (Boston, MA). All other general chemicals and supplies, including DMSO, ethanol, isopropanol, and agarose, were from Sigma or Fisher and were reagent grade or higher. Mouse GATA-6 cDNA (2.6 kb) was cloned in the *Bam*HI/*Xho*I site of pcDNA3 vector (Ed Morrisey, University of Pennsylvania School of Medicine, Philadelphia, PA) (Morrisey et al., 1996). Mouse GATA-5 cloned in the pcDNA3 vector was isolated by Ed Morrisey (Morrisey et al., 1997). Mouse GATA-4 cDNA (1.7 Kb) was cloned in the *Eco*RI site of pMT2 vector, and was generously given by Dr. David B. Wilson (Washington University School of Medicine, St. Louis, MO). All the GATA cDNAs were flag-tagged at the N-terminal of the proteins.

Cell culture

Mouse embryonic carcinoma cells were cultured on swine skin gelatin-coated tissue culture plates in ES cell media containing $NaHCO_3$, HEPES, NEAA, pyr, L-glut, L-Asp, L-Arg, nucleoside, 2-mercaptoethanol, antibiotic-antimytotic, LIF (1000 u/ml), and 15% FBS (Robertson, 1997). The plates were coated overnight at 4°C with sterile gelatin solution (0.1%), then washed three times with PBS prior to

use. Cell differentiation was induced by treating with 1 μM RA for four days.

Parental RW-4 mouse embryonic stem cells of wild type and of GATA-4 (−/−) (Soudais et al., 1995) and GATA-6 (−/−) (Morrisey et al., 1998) genotypes were maintained by the Tissue Culture Facility of Fox Chase Cancer Center according to standard protocol. Prior to experiments, the embryonic stem cells were plated on gelatin-coated plates or slide chambers without feeder cells and LIF. In experiments to analyze the formation of embryoid bodies, cells were also maintained in petri dishes for 4 days to allow aggregation. Briefly, confluent ES cells were resuspended with 0.04% trypsin in PBS, and were seeded on petri dishes in ES medium supplemented with 15% fetal bovine serum without LIF. Medium was changed every other day for 4 days to 1 week. The spheroids were then collected with a transfer pipet and transferred to a 15 ml tube, washed two times with PBS, and then resuspended in 1 ml of PBS. The cell aggregations were transferred to an Eppendorf tube and centrifuged at 2000×*g* for 1 min. The cell aggregations were then collected for Western or Northern blot analysis. For histochemical analysis, the cell aggregations were fixed in 1 ml of formalin for 2 h, washed two times with PBS, and resuspended in 1% agarose to be processed for paraffin embedding.

Cell transfection

Embryonic stem cells were seeded at 2×10^5/dish in 35 mm diameter gelatin-coated plates in ES cell media. Plasmid DNA was purified using Qiagen Maxiprep columns. Transfections of ES cells with plasmid DNA were performed using Mirus Trans1T-LT1 reagent (Mirus Co., Madison, WI) according to the manufacturer's protocol. Briefly, transfection was performed in ES cell monolayers of about 60% confluence using 8 μl of Mirus Trans1T-LT1 reagent for 2 μg of plasmid-cDNA. Media were changed 24 h after transfection. Cells were used for immunofluorescence assay 72 h post-transfection. ES cells were transfected with GATA-4, GATA-5, or GATA-6 and the effects on cell differentiation were assayed by immunofluorescence using Dab2 and laminin expression as marker of cell differentiation. Each transfection experiment shown as a figure in the manuscript was repeated at least three times and a representative experiment is shown.

Immunofluorescence microscopy

Immunofluorescence was performed as previously described (Capo-chichi et al., 2003). Embryonic stem cells were treated with RA or transfected with GATA-4, GATA-5, or GATA-6. Cells were plated on 22 × 40 mm cover slides in 6 well dishes and fixed with 4% paraformaldehyde (15 min). Cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed with PBS, and blocked with 3% BSA in PBS containing 0.1% Tween-20 (room temperature for 30 min). Anti-GATA-4, GATA-5, GATA-6, Dab2, Oct-3/4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-Flag M2-Cy3, and anti-Laminin (Sigma) were used at 1/200 dilution in 1% BSA in PBS containing 0.1% Tween-20 and incubated at 37°C for 2 h. The cellular localization of the antigens was revealed by fluorescein or Texas Red conjugated secondary antibodies (Jackson Immuno-Research lab., West Grove, PA) at 1/200 dilution. The secondary antibodies were: donkey anti-mouse IgG conjugated with Texas Red and donkey anti-rabbit IgG conjugated with Fluorescein. Rabbit anti-GATA-4 or rabbit anti-GATA-5 or goat anti-GATA-6 IgG was used with mouse anti-Dab2 antibodies for double labeling. Nuclei were marked by Dapi staining. The Nikon Eclipse E 800 epifluorescence microscope with 60× oil immersion objective linked to a Roper Quantix CCD (charged coupled device) camera was used for observation and image acquisition. Images were merged by overlaying using the Adobe-Photoshop program.

Northern blot analysis

Total RNA was isolated from monolayer or aggregate cell culture as previously described (Capo-chichi et al., 2003). RNA was separated on 1% agarose gel containing 7% formaldehyde and 20 mM MOPS buffer, blotted to positively-charged nylon membranes using 20× SSC buffer, and fixed by baking. The DNA probes were labeled with [α-^{32}P]dCTP using a random prime labeling kit (Amersham). The hybridization and Northern blotting followed standard procedures as described previously (Capo-chichi et al., 2003).

Western blot analysis

The cells were washed twice with 2 ml PBS, collected by scraping with a cell rubber scraper in 1 ml PBS, and centrifuged at 5000 rpm for 5 min. SDS buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5% SDS, 5% 2-mercaptoethanol, 40% glycerol) was added to the pellet and mixed before heating for 5 min. The samples were then centrifuged for 5 min at 4°C and the supernatants were kept frozen at −20°C until use. GATA-4, GATA-5, GATA-6, Dab2, and β-actin were analyzed in the cell extract using SDS-polyacrylamide gels (10%). Anti-Dab2 (p96) monoclonal antibodies were purchased from BD/Transduction Laboratories (Lexington, KY). Anti-GATA-4, anti-GATA-5, and anti-GATA-6 came from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin came from Sigma.

Results

Endoderm lineage differentiation of embryonic stem cells induced by aggregation and/or retinoic acid

The mechanism underlying the induction of endoderm differentiation of ES cells is of special interest, since this is one of the earliest steps in embryonic development. We used the loss of Oct-3/4 as an indication of ES cell differentiation,

and the induction of Dab2, collagen IV, and laminin as the markers for commitment to the extraembryonic endoderm lineage to investigate the endoderm differentiation of murine ES cells. Retinoic acid induced the differentiation of ES cells grown as monolayer, as indicated by the dramatic reduction of Oct-3/4 level, and the induction of Dab2 expression, mainly the p67 isoform of the protein, as an indication of endoderm lineage (Fig. 1A). Upon aggregation of ES cells

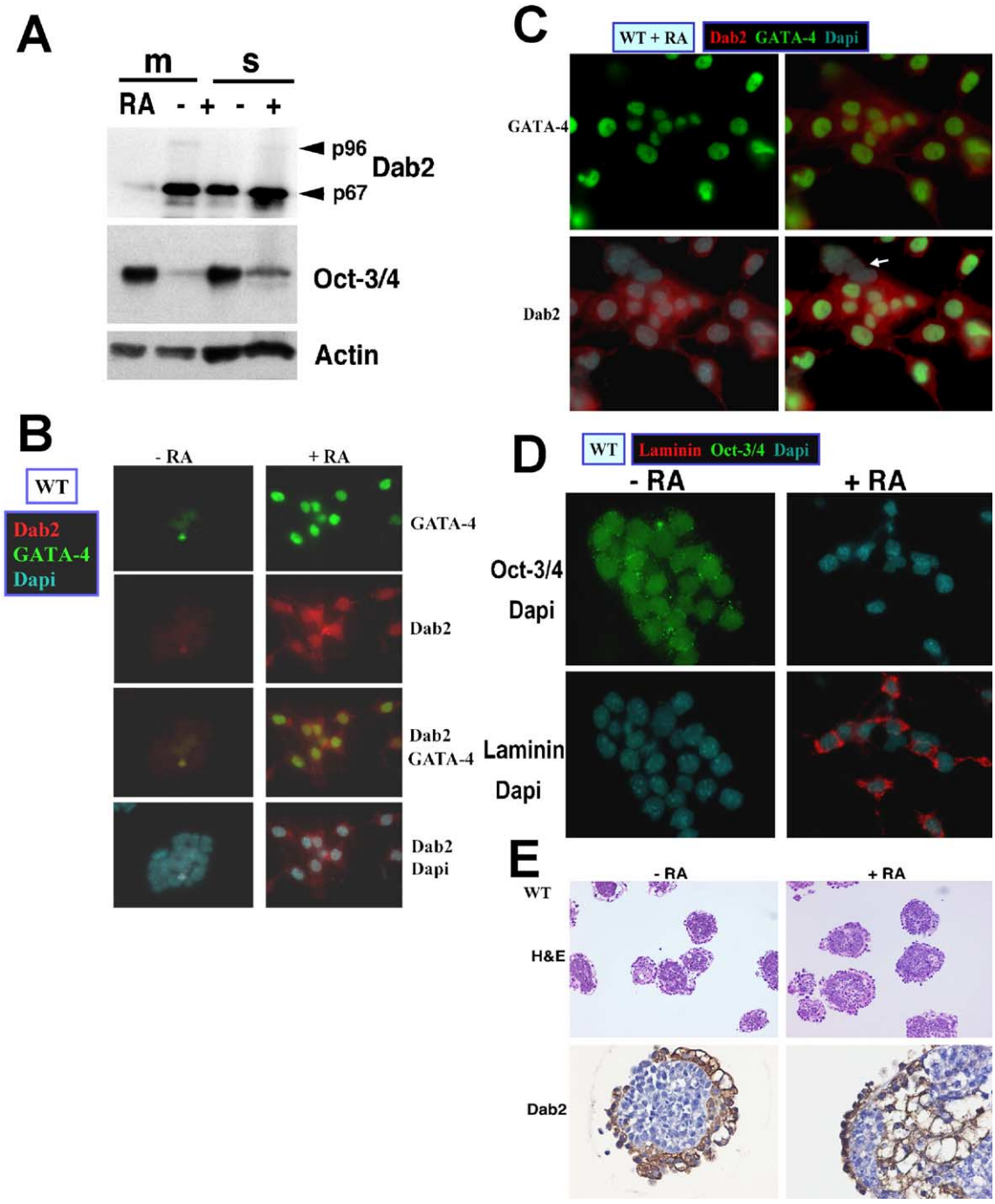

Fig. 1. Endoderm lineage differentiation of ES cells in vitro. (A) Approximately 1×10^6 ES cells were plated on 100-mm plates as a monolayer culture ("m") or cultured in suspension to allow cell aggregation to form spheroids ("s"). Cells were treated with 1 μM retinoic acid (RA) or DMSO control for 4 days and the cell lysate was prepared and subjected to Western blotting to detect expression of Oct-3/4 and Dab2 (both p96 and p67 proteins). Beta-actin was determined as a loading control. (B) ES cells in monolayers were treated with or without retinoic acid (1 μM) for 4 days. Immunofluorescence staining of Dab2 (red) and GATA-4 (green) was performed. DAPI (blue) was used for nuclear counterstaining. (C) Another example of retinoic acid-treated ES cells stained with Dab2 (red), GATA-4 (green), and DAPI (blue). Arrow indicates Dab2- and GATA-4-negative cells that are not primitive endoderm cell type. (D) ES cells in monolayers were treated with or without retinoic acid (1 μM) for 4 days. Immunofluorescence staining of laminin (red) and Oct-3/4 (green) was performed. DAPI (blue) was used for nuclear counterstaining. (E) Morphology and Dab2 immunostaining of embryoid bodies from a 4-day suspension culture of ES cells incubated with or without 1 μM retinoic acid. Representative images of H&E staining (100×) and Dab2 immunostaining (400×) are shown.

for 4 days to form spheroids (or embryoid bodies), Dab2 expression was induced to a similar level. Oct-3/4 was only slightly reduced in cell aggregates compared to undifferentiated ES cells cultured as monolayers, and was further reduced in cell aggregates upon addition of retinoic acid (Fig. 1A). In this 4-day time course, ES cells differentiate mainly toward extraembryonic endoderm lineage (Rohwedel et al., 1999; Mummery et al., 1990). Thus, it appears that endoderm lineage differentiation of ES cells, as indicated by Dab2 expression, is achieved by either cell aggregation or retinoic acid treatment of cell monolayer. However, a fraction of cells with stem cell characteristics, as indicated by the expression of Oct-3/4, are preserved in embryoid bodies, but are mostly lost when treated with retinoic acid.

The endoderm differentiation of monolayer ES cells can be also monitored by immunofluorescence microscopy to detect the expression of Oct-3/4, Dab2, laminin, and GATA factors (Figs. 1B, C). In untreated ES cell cultures, cells positive for Dab2 or GATA-4 were rarely observed (Fig. 1B), indicating the undifferentiated state of the cells. Following treatment with retinoic acid for 4 days, about 90% of the cells became GATA-4- and Dab2-positive (Fig. 1B). An example (Fig. 1C) shows that three cells are negative for Dab2 and GATA-4 (indicated by an arrow), which are either undifferentiated ES cells or differentiated cells of non-primitive endoderm lineage. Another example is shown in Fig. 1D, in which undifferentiated ES cells are positive for Oct-3/4 in the nucleus, and upon retinoic acid treatment, Oct-3/4 is lost and laminin is induced. Thus, when ES cells in monolayer were treated with retinoic acid, the majority of cells committed to the primitive endoderm lineage. Following suspension of the ES cells for 4 days, spheroid/embryoid bodies were formed with a well-differentiated endoderm outer layer that is Dab2-positive (Fig. 1E). Retinoic acid treatment further induced differentiation and a portion of the interior cells are Dab2-positive, suggesting that these cells have undergone endoderm lineage differentiation. Thus, in the majority of cell aggregates, only the outer layer of ES cells in the embryoid bodies undergoes endoderm differentiation; however, retinoic acid treatment can also induce the endoderm differentiation in the interior of the spheroids.

Requirement of GATA-6 in retinoic acid-induced endoderm lineage differentiation of embryonic stem cells in monolayer culture

We investigated the endoderm differentiation of ES cells deficient for GATA-4 or GATA-6 when induced by retinoic acid in culture. As indicated by the immunoblots (Fig. 2A), monolayers of wild type, GATA-4 (−/−), or GATA-6 (−/−) ES cells underwent differentiation as indicated by the loss of Oct-3/4 when stimulated with retinoic acid for 4 days. GATA-6 (−/−) ES cells, however, retained a fraction of Oct-3/4 and did not differentiate to endoderm, as indicated by the lack of Dab2 expression. GATA-4 (−/−) ES cells did express Dab2 when induced by retinoic acid, but the Dab2 expression level was less than wild type. GATA-6 was induced by retinoic acid in both wild type and GATA-4 (−/−) ES cells. GATA-4 was induced by retinoic acid in wild type but not in GATA-6 (−/−) cells. Thus, it appears that GATA-6, but not GATA-4, is necessary for retinoic acid-induced endoderm commitment in the differentiation of ES cells, and GATA-4 is not necessary but it enhances GATA-6-mediated endoderm differentiation. The differentiation of individual ES cells in monolayer culture was investigated by immunofluorescence microscopy. GATA-4 (−/−) ES cells differentiated following treatment with retinoic acid for 4 days; however, consistently, we observed that the differentiation was partial: around 40% of GATA-4 (−/−) ES cells underwent endoderm differentiation as indicated by the expression of laminin, and Dab2 and the loss of Oct-3/4 (Figs. 2B, C). In GATA-6 (−/−) ES cells, retinoic acid also induced cell differentiation as indicated by the loss of Oct-3/4, but failed to induce endoderm lineage commitment, noted by the lack of GATA-4-, Dab2-, and laminin-positive cells (Figs. 2D, E). Thus, in wild type ES cells treated with retinoic acid, the majority of the cells select endoderm lineage, and loss of GATA-4 reduces the fraction of cells that undergo endoderm lineage differentiation. GATA-6 is absolutely necessary for retinoic acid-induced endoderm commitment of ES cells.

Requirement of GATA-4 in aggregation-induced endoderm lineage differentiation of embryonic stem cells

We next tested the aggregation-induced differentiation of the wild type and GATA-deficient ES cells by both Western blot (Fig. 3A) and Northern blot (Fig. 3B). The wild type ES cells, when aggregated, underwent endoderm differentiation as indicated by the expression of Dab2, GATA-4, GATA-6, laminin, and collagen IV, and the differentiation was enhanced further by the addition of retinoic acid.

For the GATA-4 (−/−) ES cells, aggregation-induced endoderm lineage differentiation was impaired, but was restored by retinoic acid, consistent with previous reports (Soudais et al., 1995; Bielinska and Wilson, 1997). In aggregated GATA-4 (−/−) ES cells without retinoic acid, GATA-6 expression was absent (Fig. 3B), indicating that, in the absence of GATA-4, aggregation does not induce GATA-6 expression and primitive endoderm differentiation of the cells. When retinoic acid was added, GATA-6 expression level was induced to a higher level in GATA-4 (−/−) than that in wild type cells, and Dab2, collagen IV, and laminin were induced (Fig. 3B). In several experiments, it was noted that Dab2 protein (Fig. 3A) and mRNA (Fig. 3B) levels did not correlate well, even though the same preparation of cells was used for both Western and Northern blot analysis. Consistent with these biochemical data, the morphological features of cell aggregation from GATA-4 (−/−) ES cells demonstrated that GATA-4 is required for the aggregation signal but is dispensable for retinoic acid induction of the endoderm lineage. In spheroids from

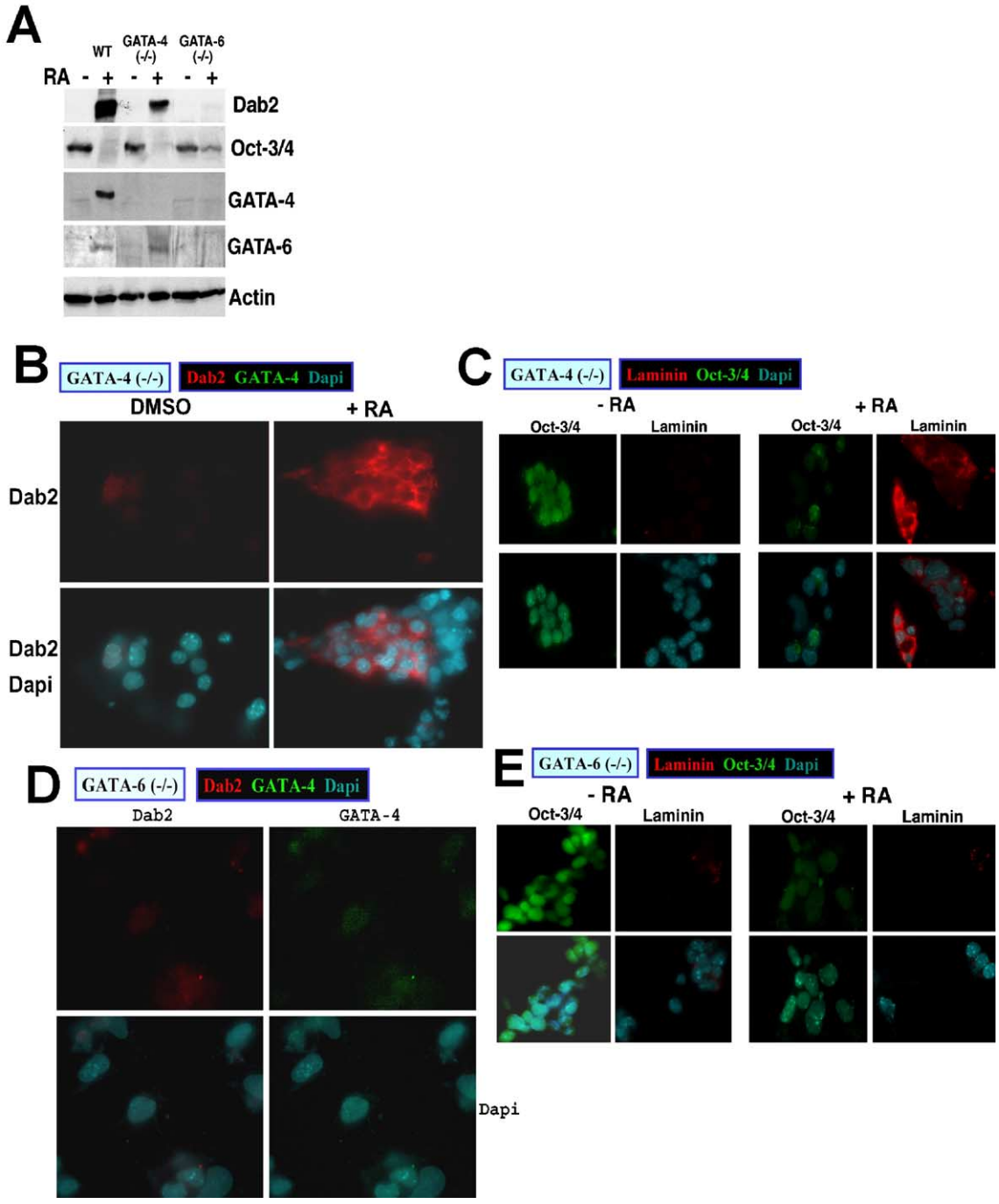

Fig. 2. Retinoic acid-induced endoderm differentiation of GATA-deficient ES cells in monolayer culture. (A) Western blot analysis: wild type embryonic stem cells and those homozygous deficient in GATA-4 or GATA-6 were cultured as monolayers and treated with DMSO carrier control or 1 μM retinoic acid (RA) for 4 days. Cell lysates were prepared for Western blot analysis. GATA-4 (−/−) (B, C) and GATA-6 (−/−) (D, E) ES cells in monolayer cultures were treated with or without retinoic acid (1 μM) for 4 days. Indirect immunofluorescence staining of Dab2, GATA-4, laminin, and Oct-3/4 was performed. Dapi was used for nuclear counterstaining. In some cases, two individual signals were combined by overlaying the images using Adobe Photoshop. Representative images are shown: (B) GATA-4 (−/−) ES cells were stained with Dab2 (red) and Dapi (blue, lower panels). (C) GATA-4 (−/−) ES cells were stained with Oct-3/4 (green), laminin (red), and Dapi (blue, lower panels). (D) GATA-6 (−/−) ES cells were stained with Dab2 (red), GATA-4 (green), and Dapi (blue, lower panels). (E) GATA-6 (−/−) ES cells were stained with laminin (red), Oct-3/4 (green), and Dapi (blue, lower panels).

GATA-4 (−/−) ES cells, no surface epithelial structure (Fig. 3C) or Dab2 expression (Fig. 3D) was observed. With retinoic acid treatment, the GATA-4 (−/−) embryoid bodies exhibited surface endoderm structure (Fig. 3C) and both surface and internal Dab2 expression (Fig. 3D), indistinguishable from wild type control.

For GATA-6 (−/−) ES cells, endoderm commitment is defective also in spheroids and cannot be rescued by the

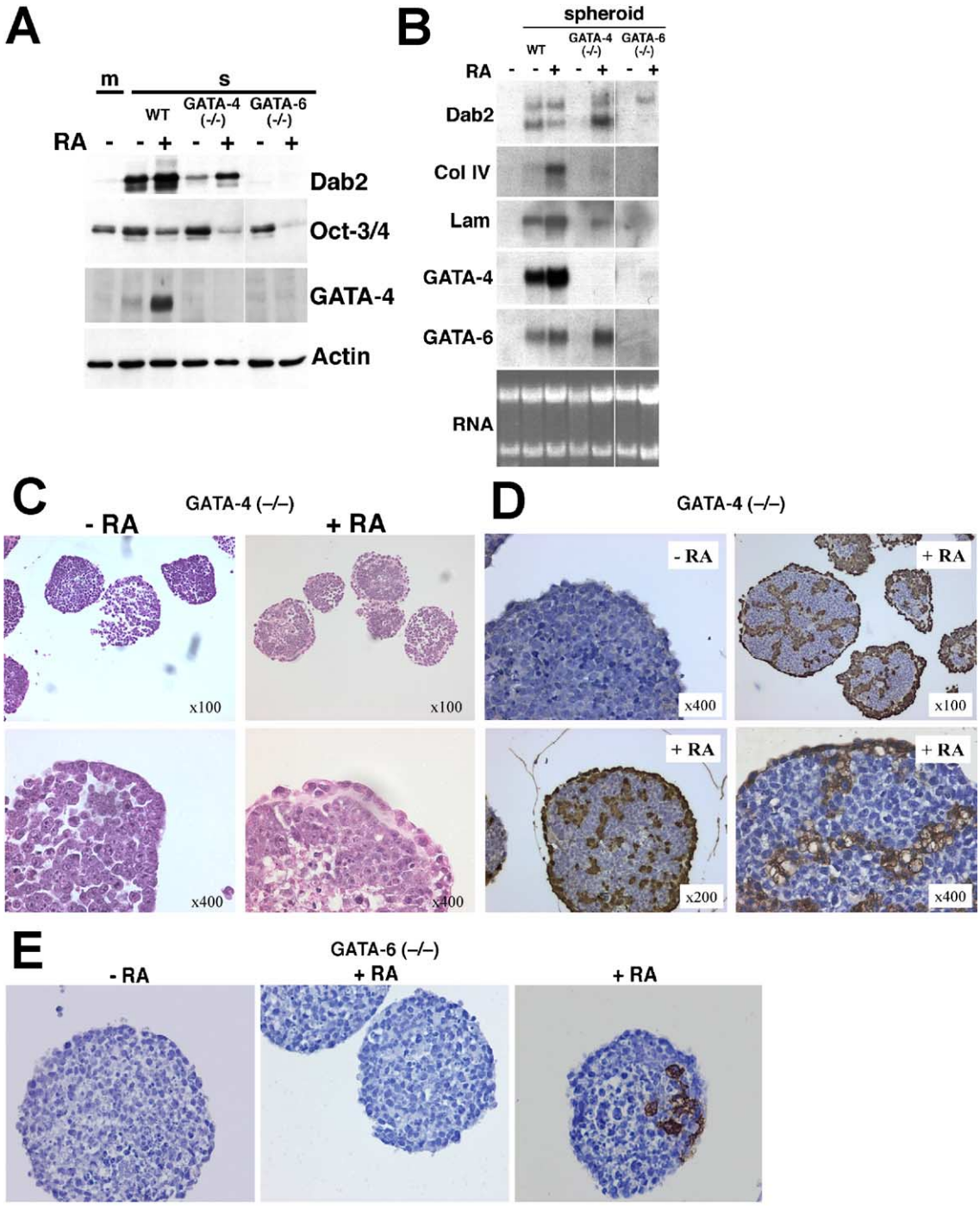

Fig. 3. Retinoic acid and aggregation-induced endoderm differentiation of GATA-deficient ES cells. Wild type embryonic stem cells and those homozygous deficient for GATA-4 or GATA-6 were cultured in medium lacking LIF in suspension to allow the formation of cell aggregates ("s") on gelatin-coated Petri dishes, with or without 1 μM retinoic acid (RA) for 4 days. ES cells in monolayer culture ("m") were used as control. The total cell lysate was prepared from the embryoid bodies/cell aggregates in 2% SDS gel loading buffer for (A) Western blot and (B) Northern blot analysis for Dab2, collagen IV α1 (Col IV), laminin γ (Lam), GATA-4, GATA-5, and GATA-6. (C) H&E staining of embryoid bodies from a 4-day suspension culture of GATA-4 (−/−) ES cells treated with or without 1 μM retinoic acid. (D) Dab2 immunostaining of embryoid bodies from a 4-day suspension culture of GATA-4 (−/−) ES cells with or without 1 μM retinoic acid. Images of 100×, 200×, and 400× are shown. (E) Dab2 immunostaining of embryoid bodies from a 4-day suspension culture of GATA-6 (−/−) ES cells with or without 1 μM retinoic acid. Most of the embryoid bodies are Dab2-negative regardless of retinoic acid treatment. The image at the far right represents a rare (around 1%) embryoid body containing Dab2-positive cells.

addition of retinoic acid. As shown in Fig. 3E, aggregates of GATA-6 (−/−) ES cells lack an outer endoderm layer and are Dab2-negative either with or without retinoic acid. In retinoic acid-treated GATA-6 (−/−) ES cell aggregates, a few spheroids (less than 1%) were observed to contain Dab2-positive cells (Fig. 3E, far right panel). However, no

morphological normal endoderm layer was observed. Thus, deficiency of GATA-4 results in defects in the formation of embryoid bodies, and the defects can be overcome by supplementation with retinoic acid. In contrast, GATA-6 is required for in vitro differentiation and endoderm formation of ES cells induced either by aggregation or retinoic acid.

Induction of endoderm lineage differentiation of embryonic stem cells following ectopic expression of GATA factors

Ectopic expression of GATA-4 or GATA-6 has been reported to be sufficient to induce endoderm differentiation of ES cells (Fujikura et al., 2002). Using immunofluorescence to detect Dab2 or laminin in individual cells as an indication of endoderm differentiation, we confirmed that ectopic expression of GATA-4, GATA-5, or GATA-6 is sufficient to induce expression of Dab2, GATA-4, laminin, and thus endoderm differentiation of ES cells. First, using flag-tag to detect cDNA expression, as an example shown for the transfection of flag-tagged GATA-4 into ES cells (Fig. 4A), we observed that the cells expressing GATA-4 (green) also showed flag staining (red). The intensities of GATA-4 and flag staining are corresponsive, suggesting that GATA-4 expression was the result of the transfection. The transfection efficiency of this particular transfection was estimated to be 36%. In many transfection over time, we observed that the transfection efficiency ranged from 10% to 50%. Transfection of GATA-4 into wild type ES cells resulted in endoderm differentiation: co-expression of Dab2 (red) as a dotted pattern and GATA-4 (green) in the nucleus occurred in 10–20% of the presumably transfected cells (Fig. 4A). In vector-transfected controls, there were few (less than 0.1%) GATA-4- and Dab2-positive cells, which were likely the result of spontaneous differentiation. Examples of transfection of GATA-6-induced Dab2 (Fig. 4C) are also presented. We conclude that ectopic transfection/expression of either GATA-4 or GATA-6 alone is sufficient to induce endoderm differentiation of ES cells in monolayer culture.

Requirement of collaborating GATA factors in endoderm lineage differentiation of embryonic stem cells

We next tested the ability of individual GATA factors to induce endoderm differentiation when transfected into ES cells that are deficient for one of the other GATA factors. As anticipated, when GATA-4 was transfected into GATA-6 (−/−) ES cells, expression of GATA-4 (green) was not able to induce Dab2 (red) in GATA-6 (−/−) ES cells (Fig. 4D), confirming the critical role of GATA-6 in endoderm lineage differentiation.

A somewhat surprising finding was that transfection of GATA-6 was not able to induce Dab2 in GATA-4 (−/−) cells (Fig. 4E), although these GATA-4 (−/−) ES cells did express Dab2 when transfected with GATA-4 (not shown). Additionally, the transfected cells as identified by GATA-6 staining were also still positive for Oct-3/4 (red, Fig. 4F). Thus, expression of GATA-6 alone without GATA-4 is not sufficient to induce Dab2 expression and endoderm lineage commitment. Furthermore, expression of GATA-6 alone is not sufficient to down-regulate Oct-3/4 that is associated with the differentiation and the loss of pluripotency of ES cells, indicating that a collaborating GATA factor (in this case, GATA-4) with GATA-6 is required for the differentiation of ES cells.

From the above experiments, we conclude that GATA-4 may have a collaborating role for GATA-6-mediated Dab2 induction and endoderm lineage commitment, since transfection of GATA-6 in wild type but not GATA-4 (−/−) ES cells induces Dab2 expression. However, retinoic acid was able to induce GATA-6 and Dab2 in the absence of GATA-4 (Fig. 2), suggesting that the collaborating role of GATA-4 with GATA-6 is dispensable in the presence of retinoic acid. We considered the possibility of a redundant factor induced by retinoic acid.

Redundant role of GATA-5 in endoderm lineage differentiation of embryonic stem cells

Indeed, GATA-5, which normally is not significantly expressed during E4.5 to E6.5 stages of the mouse embryos and is not considered to have a role in extraembryonic endoderm development (Morrisey et al., 1997; Molkentin et al., 2000), was induced in ES cells with either GATA-4 or GATA-6 deletion (Fig. 5). In wild type ES cells, however, GATA-5 was not significantly induced by retinoic acid, suggesting that the expression of GATA-5 is suppressed by the presence of both GATA-4 and GATA-6.

When GATA-4 (−/−) ES cells were transfected with GATA-5, however, GATA-6 was induced and Dab2 was expressed in the presumably GATA-5-transfected cells (Fig. 6). Nevertheless, GATA-5 transfection into GATA-6 (−/−) ES cells failed to induce Dab2 (not shown), suggesting that even in the presence of GATA-5 expression, GATA-6 is still necessary for endoderm differentiation of ES cells.

Thus, we propose that GATA-5 can be induced by retinoic acid in the absence of either GATA-4 or GATA-6. In GATA-4 (−/−) ES cells, retinoic acid induces GATA-5 and GATA-6, and GATA-5 can substitute for GATA-4 in its collaborating role with GATA-6 to induce Dab2 expression and endoderm differentiation. It is also suggested from the above experiments that, in the absence of GATA-4, GATA-5 is able to induce GATA-6, but GATA-6 is not able to induce GATA-5.

Discussion

Differentiation of stem cells into various lineages underlies the principle of development and is a fascinating question in biology. The differentiation of embryonic stem

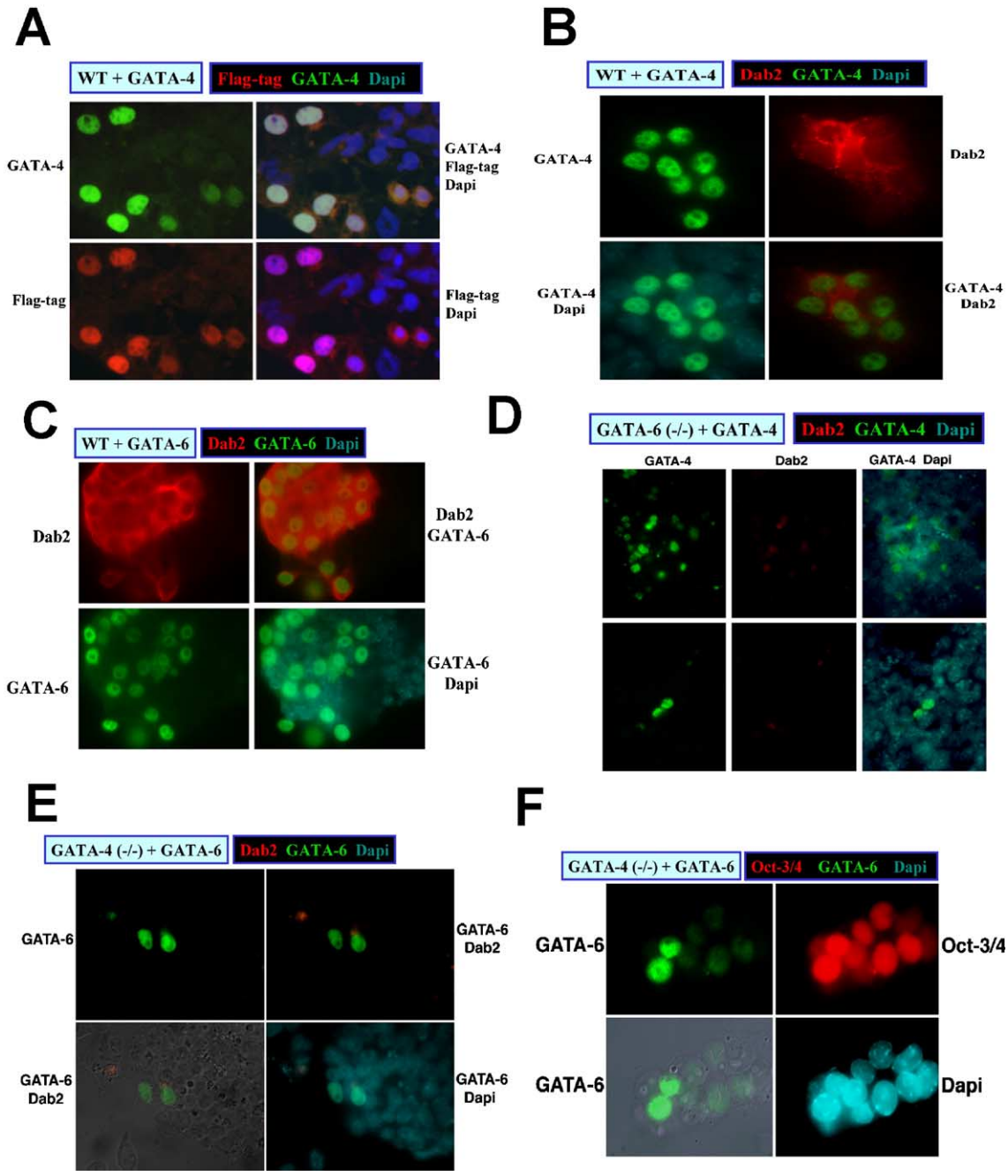

Fig. 4. Ectopic GATA factor expression induces endoderm differentiation of ES cells: immunofluorescence microscopy analysis. Wild type ES cells in monolayers were transfected with vector control or expression constructs of GATA-4 or GATA-6. Expression of Dab2, Oct-3/4, laminin, GATA-4, and GATA-6 was analyzed by immunofluorescence microscopy. Dapi was used for nuclear counter staining. Representative images from more than three transfections of each cDNA are shown. (A) ES cells were transfected with flag-tagged GATA-4, and the expression of GATA-4 (green), flag-tag (red), and Dapi (blue) was determined by immunofluorescence microscopy. The images were also over-layered. (B) ES cells were transfected with GATA-4, and the expression of GATA-4 (green), Dab2 (red), and Dapi (blue) was determined by immunofluorescence microscopy. (C) ES cells were transfected with GATA-6, and the slides were stained for Dab2 (red), GATA-4 (green), and Dapi (blue). (D) GATA-6 (−/−) cells were transfected with GATA-4. GATA-4 (green) and Dab2 (red) were double labeled. Two images are shown. (E) GATA-4 (−/−) cells were transfected with GATA-6. GATA-6 (green), Dab2 (red), and Dapi (blue) were stained. Note that the light red nuclear staining in transfected cells is background leaked from the strong Dapi signal. (F) GATA-4 (−/−) cells were transfected with GATA-6. GATA-6 (green) and Oct-3/4 (red) were labeled, and Dapi (blue) was used for counterstaining.

cells into primitive endoderm lineage provides a unique model to investigate genes and the mechanism for the response of ES cells to differentiation cues (Abe et al., 1996; Coucouvanis and Martin, 1995). The current study has systematically investigated the requirement of GATA factors in ES cell differentiation in response to retinoic acid, a chemical cue, and cell aggregation, an environmental cue.

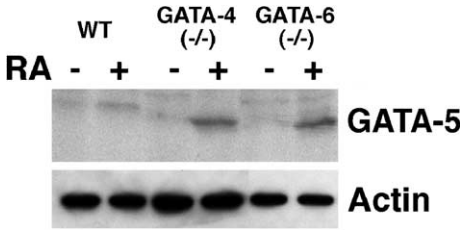

Fig. 5. Retinoic acid induces GATA-5 expression in GATA-4- or GATA-6-deficient ES cells. ES cells of wild type (WT), GATA-4 (−/−), or GATA-6 (−/−) were plated on 100-mm plates as a monolayer culture and were treated with 1 μM retinoic acid (RA) or DMSO control for 4 days. The cell lysate was prepared and subjected to Western blotting to detect expression of GATA-5. Beta-actin was determined as a loading control.

Two inductive routes for extraembryonic endoderm differentiation of embryonic stem cells in vitro

The differentiation of embryonic stem cells can be studied in vitro in monolayer culture or in aggregates (Abe et al., 1996; Coucouvanis and Martin, 1995). In additional to morphological criteria, we used collagen IV and laminin, as well as the expression of Dab2, as molecular markers for the differentiation of ES cells into the primitive endoderm lineage. Collagen IV and laminin are the main components of the basement membrane specifically expressed by the extraembryonic endoderm in the early embryo, and have been used in previous studies as an indication of primitive endoderm differentiation of ES and EC cells (Gudas et al., 1990; Fujikura et al., 2002). Dab2 is first expressed and is restricted to the primitive endoderm of the E4.5 blastocysts (Yang et al., 2002), and is restricted in the extraembryonic endoderm until E9.0 to E9.5, when Dab2 expression is also detectable in the developing liver and heart (DH Yang and XX Xu, unpublished observations). Dab2 can be used as a marker for the primitive endoderm lineage differentiation of ES cells (Morrisey et al., 2000; Fujikura et al., 2002).

Consistent with earlier reports (Soudais et al., 1995; Bielinska and Wilson, 1997; Morrisey et al., 1998; Koutsourakis et al., 1999), we found that GATA-4-deficient ES cells fail to differentiate into primitive endoderm following aggregation but the ability can be restored by retinoic acid. However, GATA-6-deficient ES cells are not responsive to cell aggregation cue and also fail to undergo primitive endoderm differentiation upon retinoic acid induction. Thus, in primitive endoderm lineage differentiation, the aggregation signal requires both GATA-4 and GATA-6 to induce differentiation, and retinoic acid inductive signal is GATA-6 dependent, as illustrated in a working model in Fig. 7.

Order of GATA factors in determination of extraembryonic endoderm fate of embryonic stem cells

Previously, from analysis of knockout mice and embryonic stem cells, it was proposed that GATA-6 is an upstream regulator of GATA-4 (Morrisey et al., 1998). With information from the current study, we propose a model for the order of induction between GATA-4 and GATA-6 (Fig. 7), for their roles in endoderm differentiation of ES cells in vitro. The endoderm differentiation induced by retinoic acid is dependent on GATA-6, suggesting that GATA-6 is upstream of GATA-4 in responding to retinoic acid signal, and either GATA-4 or GATA-5 is dispensable for this signal. Expression of GATA-6, either induced by retinoic acid or by ectopic expression, appears to be sufficient to induce endoderm lineage differentiation of ES cells. GATA-4 is necessary for the ES cells to sense an aggregation signal, but GATA-6 is also required for induction of differentiation. This can be explained by proposing that GATA-4 is required to sense an aggregation signal and is upstream of GATA-6. Thus, in the case of retinoic acid signal, GATA-4 is downstream of GATA-6,

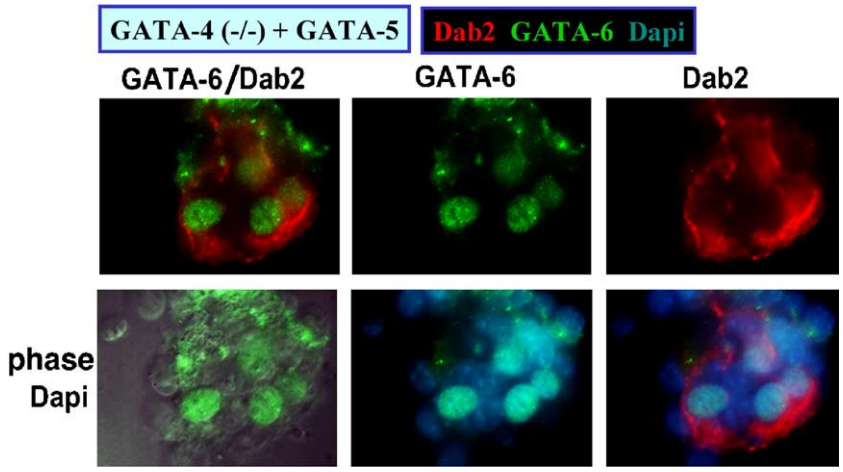

Fig. 6. Ectopic GATA-5 expression induces endoderm differentiation of GATA-4 (−/−) ES cells: immunofluorescence microscopy analysis. GATA-4 (−/−) ES cells in monolayers were transfected with vector control or expression constructs of GATA-5 for 2 days. Expression of Dab2 (red) and GATA-6 (green) was analyzed by immunofluorescence microscopy. Dapi (blue) was used for nuclear counter staining.

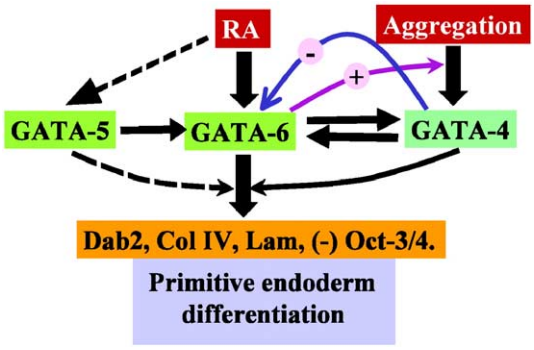

Fig. 7. Schematic model illustrates the role of GATA transcription factors for extraembryonic endoderm differentiation in embryonic stem cells. Physical (aggregation) or chemical (retinoic acid) cues induce extraembryonic endoderm differentiation of ES cells. We propose that, in the regulatory cascade, GATA-4 is required to perceive a cell aggregation signal, and GATA-6 is required to sense the retinoic acid signal. GATA-5 can be induced by retinoic acid in the absence of either GATA-4 or GATA-6 (dashed black arrow), and may be able to substitute for some function of GATA-4 (collaboration with GATA-6). Retinoic acid induces GATA-6; aggregation induces GATA-4; and GATA-4 and GATA-6 have mutual inductive activities. GATA-6 collaborates with GATA-4 or GATA-5 (when GATA-4 is deleted) to induce primitive endoderm differentiation as indicated by the expression of Dab2, collagen IV, and laminin. From the observation of GATA factor expression in ES cells deficient of a particular GATA factor, we propose that GATA-4 suppresses GATA-6 expression; and GATA-6 enhances GATA-4 expression.

and in the case of aggregation signal, GATA-4 is upstream of GATA-6.

Collaboration, redundancy, and inter-regulation of gene expression among GATA factors

The GATA factors exhibit inter-regulation for the expression of individual factor. Besides, inductive expression of a GATA factor can also modulate the expression level of another.

In GATA-4 knockout mice and differentiated GATA-4 (−/−) ES cells, it was observed that GATA-6 level is elevated (Soudais et al., 1995; Bielinska and Wilson, 1997). In contrast, in both GATA-6 knockout mouse embryos and differentiated GATA-6 (−/−) ES cells, GATA-4 expression is suppressed (Morrisey et al., 1998; Koutsourakis et al., 1999). Thus, it is thought that GATA-4 suppresses GATA-6 expression, and GATA-6 is required for, or is able to stimulate, GATA-4 expression. Similar inter-regulation between the expression of GATA-4 and GATA-6 was observed in our in vitro experiments with GATA-deficient ES cells. It should be cautioned that the requirement of GATA-6 for expression of GATA-4 may only be applied to ES cells and extraembryonic endoderm, since loss of GATA-6 in heart does not affect GATA-4 expression (Zhao et al., 2005).

The expression of GATA-4 and GATA-6 has not been reported in GATA-5 knockout mice (Molkentin et al., 2000). GATA-5 is expressed strongly only in GATA-4 or GATA-6-deficient ES cells during differentiation, suggesting that GATA-5 expression is suppressed by either GATA-4 or GATA-6.

A previous study (Fujikura et al., 2002) on the differentiation of ES cells by GATA-4 and GATA-6 concluded that GATA factors are expressed at two levels: strong and weak. Perhaps, weak expression of a particular GATA factor is able to induce other regulatory genes, such as another GATA family member. We postulate that, in wild type ES cells, aggregation first induces a weak level of GATA-4 expression, which induces GATA-6 expression. GATA-6 then enhances GATA-4 to a stronger expression. The high level of GATA-4 is able to suppress GATA-6 expression. Such a circuitous regulatory loop of interaction may balance both GATA-4 and GATA-6 expression levels (Fig. 7). In GATA-6 (−/−) ES cells, aggregation stimulates a weak GATA-4 expression. Hence, in the absence of enhancement by GATA-6, GATA-4 expression remains weak (Fig. 7).

Transfection experiments also revealed the necessary collaborating role of GATA-6 with GATA-4 in the induction of Dab2 expression and endoderm lineage. This conclusion is based on the inability of GATA-6 transfection to induce Dab2 in GATA-4 (−/−) ES cells. However, the requirement of GATA-4 to collaborate with GATA-6 can be substituted by retinoic acid-induced GATA-5 (Fig. 7). We propose that GATA-5 induced by retinoic acid can substitute GATA-4 for a collaborating role in endoderm differentiation.

Comparing embryonic stem cell differentiation in vitro and the development of primitive endoderm of the mouse embryos

The in vitro differentiation of ES cells into primitive endoderm-like cells is one of the best studied developmental cell lineage selection mimicking the development of the primitive endoderm (Martin and Evans, 1975; Strickland et al., 1980; Coucouvanis and Martin, 1995; Abe et al., 1996; Fujikura et al., 2002). However, differentiation inducing factors in the derivation of primitive endoderm of the blastocysts are not yet fully understood (Bielinska et al., 1999). We suggest that the formation of primitive endoderm in the early embryos probably involves both an aggregation cue to induce GATA-4 expression and a retinoic acid-like cue to induce GATA-6 expression (Fig. 7). However, both GATA-4 and GATA-5 are dispensable for the formation of primitive endoderm in mouse embryos during E4.5 to E8.5 stages in terms of gross morphology of the extraembryonic endoderm structure (Kuo et al., 1997; Molkentin et al., 2000). However, the GATA-4-deficient extraembryonic endoderm is defective as reflected in the heart development, which can be rescued by wild type extraembryonic endoderm (Narita et al., 1997; Watt et al., 2004_Hlt112052179]) and the gastrulation failure in 50% of the GATA-4 (−/−) embryos (Molkentin et al., 1997).

Nevertheless, the GATA-4 (−/−) extraembryonic endoderm is defective in regulating cardiac morphogenesis. In the absence of GATA-4 that is required to perceive

aggregation cue, a retinoic acid or a similar inducing cue, either embryonic or maternal origin, likely activates GATA-6 expression and enables the formation the primitive endoderm (albeit defective) during these embryonic stages. The involvement of retinoic acid as a morphogenic agent in embryonic development has been well considered (De Luca, 1991; Ross et al., 2000), but whether retinoic acid has a role at such an early stage, the induction of primitive endoderm, is not certain (Bielinska et al., 1999). Our current study suggested that the formation of primitive endoderm in the early embryos probably involves both an aggregation cue to induce GATA-4 expression and a retinoic acid-like cue to induce GATA-6 expression (Fig. 7). In the absence of GATA-4, we speculate that GATA-5 may be expressed and have redundant function in extraembryonic endoderm formation during E4.5 to E9.5 stages.

This study implies that by manipulating GATA genes, we may be able to modulate lineage determination of ES cells in vitro by changing their perceptions of differentiation cues. The finding may have implication for the application of embryonic stem cells for tissue engineering.

Acknowledgments

We appreciate the generous gifts of mouse GATA-4 cDNA expression constructs and GATA-4 (−/−) ES cells from Dr. David Wilson. We thank Isabelle H. Roland for her technical advice and assistance, and appreciate the assistance and contribution of Jonathan Boyd of the Fox Chase Cancer Center (FCCC) Imaging Facility, Sharon Howard of the FCCC Tissue Culture Facility, Cass Renner of the FCCC Pathology and Histochemistry Facility, and Tony Yeung of the FCCC Biotechnology Facility. We appreciate Drs. Elizabeth R. Smith, Myung Shin, and Corrado Caslini for helpful suggestions and comments during the preparation of the manuscript, and Ms. Patricia Bateman for her excellent secretarial support. The studies were supported by grants R01 CA099471, R01 CA79716, and R01 CA75389 to X.X. Xu from NCI, NIH. The work was also supported by an appropriation from the Commonwealth of Pennsylvania.

References

Abe, K., Niwa, H., Iwase, K., Takiguchi, M., Mori, M., Abe, S.I., Abe, K., Yamamura, K.I., 1996. Endoderm-specific gene expression in embryonic stem cells differentiated to embryoid bodies. Exp. Cell Res. 229, 27–34.

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

Bielinska, M., Wilson, D.B., 1997. Induction of yolk sac endoderm in GATA-4-deficient embryoid bodies by retinoic acid. Mech. Dev. 65, 43–54.

Bielinska, M., Narita, N., Wilson, D.B., 1999. Distinct roles for visceral endoderm during embryonic mouse development. Int. J. Dev. Biol. 43, 183–205.

Bodmer, R., Venkatesh, T.V., 1998. Heart development in *Drosophila* and vertebrates: conservation of molecular mechanisms. Dev. Genet. 22, 181–186.

Capo-chichi, C.D., Roland, I.H., Vanderveer, L., Bao, R., Yamagata, T., Hirai, H., Cohen, C., Hamilton, T.C., Godwin, A.K., Xu, X.X., 2003. Anomalous expression of epithelial differentiation-determining GATA factors in ovarian tumorigenesis. Cancer Res. 63, 4967–4977.

Charron, F., Nemer, M., 1999. GATA transcription factors and cardiac development. Semin. Cell Dev. Biol. 10, 85–91.

Coucouvanis, E., Martin, G.R., 1995. Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. Cell 83, 279–287.

Coucouvanis, E., Martin, G.R., 1999. BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. Development 126, 535–546.

De Luca, L.M., 1991. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J. 5, 2924–2933.

Evans, M.J., Kaufman, M.H., 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156.

Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki Ji, J., Niwa, H., 2002. Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev. 16, 784–789.

Gudas, L.J., Grippo, J.F., Kim, K.W., Larosa, G.J., Stoner, C.M., 1990. The regulation of the expression of genes encoding basement membrane proteins during the retinoic acid-associated differentiation of murine teratocarcinoma cells. Ann. N. Y. Acad. Sci. 580, 245–251.

Hogan, B., Tilly, R., 1977. In vitro culture and differentiation on normal mouse blastocysts. Nature 265, 626–629.

Hogan, B., Tilly, R., 1978. In vitro development of inner cell masses isolated immunosurgically from mouse blastocysts: II. Inner cell masses from 3.5- to 4.0-day p.c. blastocysts. J. Embryol. Exp. Morphol. 45, 107–121.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R., Grosveld, F., 1999. The transcription factor GATA6 is essential for early extraembryonic development. Development 126, 723–732 (Corrected and republished in: Development 126:723–732).

Kuo, C.T., Morrisey, E.E., Anandappa, R., Sigrist, K., Lu, M.M., Parmacek, M.S., Soudais, C., Leiden, J.M., 1997. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 11, 1048–1060.

Laverriere, A.C., MacNeill, C., Mueller, C., Poelmann, R.E., Burch, J.B., 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.

Lu, C.C., Brennan, J., Robertson, E.J., 2001. From fertilization to gastrulation: axis formation in the mouse embryo. Curr. Opin. Genet. Dev. 11, 384–392.

Martin, G.R., 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U. S. A. 78, 7634–7638.

Martin, G.R., Evans, M.J., 1975. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc. Natl. Acad. Sci. U. S. A. 72, 1441–1445.

Molkentin, J.D., 2000. The zinc finger-containing transcription factors GATA-4, -5, and-6 ubiquitously expressed regulators of tissue-specific gene expression. J. Biol. Chem. 275, 38949–38952.

Molkentin, J.D., Lin, Q., Duncan, S.A., Olson, E.N., 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev. 11, 1061–1072.

Molkentin, J.D., Tymitz, K.M., Richardson, J.A., Olson, E.N., 2000. Abnormalities of the genitourinary tract in female mice lacking GATA5. Mol. Cell. Biol. 20, 5256–5260.

Morrisey, E.E., Ip, H.S., Lu, M.M., Parmacek, M.S., 1996. GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. Dev. Biol. 177, 309–322.

Morrisey, E.E., Ip, H.S., Tang, Z., Lu, M.M., Parmacek, M.S., 1997. GATA-5: a transcriptional activator expressed in a novel temporally and

spatially-restricted pattern during embryonic development. Dev. Biol. 183, 21–36.
Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S., Parmacek, M.S., 1998. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev. 12, 3579–3590.
Morrisey, E.E., Musco, S., Chen, M.Y., Lu, M.M., Leiden, J.M., Parmacek, M.S., 2000. The gene encoding the mitogen-responsive phosphoprotein Dab2 is differentially regulated by GATA-6 and GATA-4 in the visceral endoderm. J. Biol. Chem. 275, 19949–19954.
Mummery, C.L., Feyen, A., Freund, E., Shen, S., 1990. Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. Cell Differ. Dev. 30, 195–206.
Narita, N., Bielinska, M., Wilson, D.B., 1997. Wild-type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. Dev. Biol. 189, 270–274.
Niwa, H., Miyazaki, J., Smith, A.G., 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. 24, 372–376.
Orkin, S.H., 1992. GATA-binding transcription factors in hematopoietic cells. Blood 80, 575–581.
Patient, R.K., McGhee, J.D., 2002. The GATA family (vertebrates and invertebrates). Curr. Opin. Genet. Dev. 12, 416–422.
Robertson, E.J., 1997. Derivation and maintenance of embryonic stem cell cultures. Methods Mol. Biol. 75, 173–184.
Rohwedel, J., Guan, K., Wobus, A.M., 1999. Induction of cellular differentiation by retinoic acid in vitro. Cells Tissues Organs 165, 190–202.
Ross, S.A., McCaffery, P.J., Drager, U.C., De Luca, L.M., 2000. Retinoids in embryonal development. Physiol. Rev. 80, 1021–1054.
Sherman, M.I., Miller, R.A., 1978. F9 embryonal carcinoma cells can differentiate into endoderm-like cells. Dev. Biol. 63, 27–34.
Smyth, N., Vatansever, H.S., Meyer, M., Frie, C., Paulsson, M., Edgar, D., 1998. The targeted deletion of the LAMC1 gene. Ann. N. Y. Acad. Sci. 857, 283–286.
Smyth, N., Vatansever, H.S., Murray, P., Meyer, M., Frie, C., Paulsson, M., Edgar, D., 1999. Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J. Cell Biol. 144, 151–160.
Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C.A., Narita, N., Saffitz, J.E., Simon, M.C., Leiden, J.M., Wilson, D.B., 1995. Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. Development 121, 3877–3888.
Strickland, S., Smith, K.K., Marotti, K.R., 1980. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. Cell 21, 347–355.
Watt, A.J., Battle, M.A., Li, J., Duncan, S.A., 2004. GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. Proc. Natl. Acad. Sci. U. S. A. 101, 12573–12578.
Weiss, M.J., Orkin, S.H., 1995. GATA transcription factors: key regulators of hematopoiesis. Exp. Hematol. 23, 99–107.
Yang, D.H., Smith, E.R., Roland, I.H., Sheng, Z., He, J., Martin, W.D., Hamilton, T.C., Lambeth, J.D., Xu, X.X., 2002. Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. Dev. Biol. 251, 27–44.
Zhao, R., Watt, A.J., Li, J., Luebke-Wheeler, J., Morrisey, E.E., Duncan, S.A., 2005. GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. Mol. Cell. Biol. 25, 2622–2631.