GATA transcription factors are important regulators of tissue-specific gene expression during development. GATA2 and GATA3 have been implicated in the regulation of trophoblast-specific genes. However, the regulatory mechanisms of GATA2 expression in trophoblast cells are poorly understood. In this study, we demonstrate that Gata2 is transcriptionally induced during trophoblast giant cell-specific differentiation. Transcriptional induction is associated with displacement of GATA3-dependent nucleoprotein complexes by GATA2-dependent nucleoprotein complexes at two regulatory regions, the −3.9- and +9.5-kb regions, of the mouse Gata2 locus. Analyses with reporter genes showed that, in trophoblast cells, −3.9- and +9.5-kb regions function as transcriptional enhancers in GATA motif-independent and dependent fashions, respectively. We also found that knockdown of GATA3 by RNA interference induces GATA2 in undifferentiated trophoblast cells. Interestingly, three other known GATA motif-dependent Gata2 regulatory elements, the −1.8-, −2.8-, and −77-kb regions, which are important to regulate Gata2 in hematopoietic cells are not occupied by GATA factors in trophoblast cells. These elements do not show any enhancer activity and also possess inaccessible chromatin structure in trophoblast cells indicating a context-dependent function. Our results indicate that GATA3 directly represses Gata2 in undifferentiated trophoblast cells, and a switch in chromatin occupancy between GATA3 and GATA2 (GATA3/GATA2 switch) induces transcription during trophoblast differentiation. We predict that this GATA3/GATA2 switch is an important mechanism for the transcriptional regulation of other trophoblast-specific genes.

In the early mouse embryo, trophoectoderm overlaying the inner cell mass contains trophoblast stem (TS) cells (1). During development, TS cells give rise to distinct highly differentiated trophoblast subtypes, which build the functional units of the organ, the placenta (2). Trophoblast cells are important for the anchorage of the embryo to the mother, for establishing a vascular connection for nutrient and gas transport to the embryo, and expression of hormones that are required for the successful progression of pregnancy (3). In rodents, multiple differentiated cell types can be derived from TS cells: trophoblast giant cells, spongiotrophoblast, syncytiotrophoblast, glycogen trophoblast cells, and invasive trophoblasts (2, 4). Trophoblast giant cells are characterized by endoreduplication and expression of members of the prolactin gene family. During pregnancy, these cells invade into the uterus and promote local and systemic adaptations in the mother that are necessary for embryonic growth and survival (2, 3). Differentiation of trophoblast giant cells occurs in a spatially and temporally highly organized manner and multiple transcription factors, including GATA2 and GATA3, have been implicated in the transcriptional regulation of trophoblast giant cell-specific gene expression (5–8).

The GATA family of transcription factors, GATA1–GATA6, controls multiple developmental processes by regulating tissue-specific gene expression by binding to W(A/T)GATA motifs (GATA motifs) of regulatory elements (9, 10). GATA family members have been subdivided into two subfamilies based on their expression and functional analysis. GATA1, GATA2, and GATA3 regulate the development of different hematopoietic lineages: erythroid, hematopoietic progenitor, and T-lymphoid, respectively (11–13). Similarly, GATA4, GATA5, and GATA6 have been shown to be involved in cardiac, genitourinary, and multiple endodermal developmental events (14–16).

GATA2 was initially cloned from chicken reticulocyte as a GATA motif-binding factor and was shown to be present in all developmental stages of erythroid cells (17). Targeted deletion of Gata2 resulted in embryonic lethality at embryonic day 10.5–11.5 due to ablation of blood cell development (12). However, GATA2 is also expressed in other hematopoietic cells, neurons, and cells of developing heart, liver, pituitary, and in trophoblasts (7, 18–22).

GATA3 was first cloned as a T cell-specific transcript (23, 24). Germ line deletion of Gata3 results in embryonic lethality...
due to a multitude of phenotypic abnormalities, including growth retardation, severe deformities of the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis (13). Interestingly, expression analysis during early mouse development showed that GATA3 is most abundantly expressed in trophoblast cells prior to embryonic day 10.5 (25).

Although GATA2 and GATA3 are expressed in trophoblast cells, very little is known about GATA factor function and their regulation in this context. Studies in a choriocarcinoma-derived rat trophoblast stem cell line (Rcho-1 trophoblast cells) showed that both GATA2 and GATA3 regulate trophoblast-specific expression of placent lactogen 1 (PL-1; also known as Prl3d1) gene (7). Studies with knock-out mice showed that placentas develop in Gata2 and Gata3-null embryos (8). However, placentas lacking Gata2 or Gata3 exhibited reduced PL-I and proliferin (also known as Prl2c2) gene expression, with Gata2-null placentas having greater reductions in proliferin (8). Besides, placenta sites lacking GATA2 have significantly less neovascularization compared with wild-type placentas in the same uterus (8). Important mechanistic information regarding Gata2 transcriptional regulation has come from analysis of the native nucleoprotein structure of the endogenous Gata2 locus in hematopoietic precursor cells (26–29). These studies indicated that during erythroid differentiation GATA1 and GATA2 directly regulate Gata2 transcription in a reciprocal fashion (26). Analysis of the mouse Gata2 locus in erythroid progenitors showed that in the transcriptionally active state, GATA2 occupies four conserved upstream elements (−77, −3.9, −2.8, and −1.8 kb) along with an intronic (+9.5 kb) conserved element (26, 28, 29). GATA1-mediated repression of Gata2 transcription was tightly coupled with displacement of GATA2 by GATA1 (GATA2/GATA1 switch) from those regulatory elements. Studies with GATA factor cofactor friend of GATA1 (FOG1)-null cells showed that FOG1 plays an unique role in this regulatory mechanism, in which it facilitates the chromatin occupancy of GATA1 displacing GATA2 from the Gata2 locus (27). These findings support a model in which GATA2 positively autoregulates transcription by binding to its own locus. In erythroid progenitors, this autoregulation is abrogated by a FOG1-dependent GATA2/GATA1 switch that triggers formation of regulatory complexes leading to repression of transcription.

To begin to understand the role of GATA factors in trophoblast function, we studied Gata2 transcriptional regulation in Rcho-1 trophoblast stem cells (30, 31) and mouse TS cells (32), and during their differentiation to the trophoblast giant cell lineage. Herein, we demonstrate that in trophoblast stem cells GATA3 directly represses Gata2 by occupying the −3.9- and +9.5-kb regulatory elements at the Gata2 locus. During trophoblast differentiation, GATA2 displaces GATA3 thereby forming a transcriptionally favorable nucleoprotein complex at the Gata2 locus. This GATA2-mediated displacement of GATA3 (GATA3/GATA2 switch) is associated with displacement of cofactor FOG1 and recruitment of cofactor Mediator1/TRAP220 (MED1/TRAP220) at the Gata2 locus. These studies define an important mechanism of Gata2 regulation in trophoblast cells and implicate a GATA3/GATA2 switch as an important molecular determinant for gene regulation during trophoblast differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents.—**Rcho-1 trophoblast cells were cultured as mentioned earlier (31). Cells were maintained in a proliferative state by culturing under subconfluent conditions with RPMI 1640 medium (Invitrogen) supplemented with 20% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 50 μM 2-mercaptoethanol (2-ME) (Sigma), 1 mM sodium pyruvate, and 1% penicillin/streptomycin (Invitrogen). Differentiation was induced by replacing the culture medium with NCTC 135 culture medium (Sigma) supplemented with 1% horse serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2.3 μg/ml HEPES, 2.2 μg/ml sodium bicarbonate, and 1% penicillin/streptomycin. Differentiation was continued for a period of 8 days at which point most of the cells appeared to be giant cells. Mouse TS cells were initially cultured on a feeder layer of primary mouse embryonic fibroblasts (MEF) in the presence of 25 ng/ml fibroblast growth factor 4 (FGF4; Sigma) and heparin (1 μg/ml) in TS cell medium (RPMI 1640 supplemented with 20% fetal bovine serum, 2-mercaptoethanol (100 μM), sodium pyruvate (1 mM), l-glutamine (2 mM), and 1% penicillin/streptomycin). For experiments, mouse TS cells were expanded in a proliferative state without MEF feeders by culturing in the presence of 70% MEF-conditioned medium, 30% TS cell medium containing, 20% fetal bovine serum, 25 ng/ml FGF4 (Sigma), and 1 μg/ml heparin (Sigma). MEF-conditioned medium was produced by addition of 10.5 ml of TS cell medium to 100-mm culture plates containing 2 × 10^6 mitomycin-C (10 μg/ml; Sigma)-treated MEFs. Differentiation of TS cells was induced by culturing them in medium devoid of FGF4, heparin, and MEF-conditioned medium. Human embryonic kidney-293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum.

**Quantitative RT-PCR.—**RNA was extracted from different cell samples with TRIzol reagent (Invitrogen). cDNA was prepared by annealing RNA (1 μg) with 250 ng of a 5:1 mixture of random and oligo(dT) primers heated at 68 °C for 10 min. This was followed by incubation with Moloney murine leukemia virus reverse transcriptase (50 units) (Invitrogen) combined with 10 mM dithiothreitol, RNasin (Promega, Madison, WI), and 0.5 mM dNTPs at 42 °C for 1 h. Reactions were diluted to a final volume of 100 μl and heat inactivated at 97 °C for 5 min. A 20-μl PCR contained 2 μl of cDNA, 10 μl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and corresponding primer sets. Control reactions lacking reverse transcriptase (RT) yielded very low signals. Relative expression levels were determined from a standard curve of serial dilutions of the proliferative Rcho-1 trophoblast cell and undifferentiated TS cell cDNA samples and were normalized to the expression of 18 S ribosomal RNA (18S rRNA) and glyceraldehyde-3-phosphate dehydrogenase, respectively.

Forward and reverse primers for quantitative RT-PCR were (5′-3′): mouse Gata2, GCAGAGAAGCAAGGCTCGC and CAGTTGACACACCTCCGGG; mouse Gata3, CGGTTCG-GATGTAAGTCGA and GTAGAGGTTGCCCCGAGT; rat
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Gata2, TAAGCAGCGCAAGCAAGGCTC and AGCTTCA- GATTCTATTCCCAGA; rat Gata3, CCGGGTGGAGATG- TAACTGGA and CCGCGATGTCACCACCTCC; rat Gata1, TCTCTAGGCGCCAGAAGAG and AGTATGCTGATT- GGGTCGCTG; rat Gata4, GCCAACCTTGCGAAGAC and CTCCTGCGTCTGGAAGATCTCAA; rat Gata5, CCTC- CCTGGCCCGAG and CAGGGAACTCTTCCAGAAGT; rat Gata6, CTCCCCGTGACCAGG and TCTCCGAGAG- GCCCTCCAAC; rat Fogl, ATAGAGGAGCCCAATGTC and GTGCCAGACTGATTGGACC; rat Fogg, GACCAT- GCTTCTATTTGCT and AATGCCCCAGACTTCGA- CAAG; rat caudal type homeobox 2 (Cdx2), AGCTCGAC- GTCCCTAGGA and CCGGTATTTGTCTTTCGCTG; rat transcription factor EB (Tfeh), GATGCCCTAACAAGTCAC- CCTGT and TCTAGAGATCCTGTGCT; and rat trophoblast-specific protein α (Tphpa), GCAAAGCACAGA- GGGTAAAGAAGG and TTTCTATGTCAGTTGACAG; rat Cdx2, GCTTCTATTTTGCCT and AAATGCCACAGGACTTG- GAGTG; and rat caudal type homeobox 2 (Cdx2), ATAGAGGAGCCCCCAAGTCC and GCTTCTATTTTGCCT and AAATGCCACAGGACTTG- GAGTG; and rat caudal type homeobox 2 (Cdx2), ATAGAGGAGCCCCCAAGTCC and GCTTCTATTTTGCCT and AAATGCCACAGGACTTG- GAGTG.

Northern Blot Analysis—For Northern blot analysis total RNA was extracted from undifferentiated and day 8 differentiated Rcho-1 trophoblast cells using TRIzol reagent. Total RNA (20 μg/lane) was resolved in 1% formaldehyde-agarose gels, transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Keene, NH), and cross-linked. Blots were probed with 32P-labeled cDNAs (PerkinElmer Life Sciences) for Gata2, NM_033442 (GenBank); and PL-II, NM_012535 (GenBank). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) cDNA was used to evaluate the integrity and equal loading of RNA samples. Probes were generated using Prime-it II random primer labeling kits (Stratagene). Probes were incubated with the blots at 42 °C overnight and washed twice with 2 SSPE, 0.1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). Protein concentrations were determined by the Bradford assay (Bio-Rad) and resolved by 8 or 10% PAGE. Monoclonal anti-GATA3 (Hg3–31) antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-β-actin was obtained from Calbiochem, San Diego, CA. Polyclonal rabbit anti-GATA2 and anti-FOG1 antibodies used for our study were described earlier (27). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse from Santa Cruz Biotechnology were used as secondary antibodies. To detect FOG1, Rcho-1 trophoblast cells lysates were prepared in RIPA buffer. Lysates were cleared by centrifugation at 13,000 × g for 30 min at 4 °C, and immunoprecipitated with preimmune rabbit serum or rabbit anti-FOG1 polyclonal antibody. Immune complexes were adsorbed to protein A-Sepharose, and resolved on 8% PAGE and analyzed by Western blotting using ECL Plus reagent (GE Healthcare).

RNA Interference—Lentiviral vectors containing short hairpin RNAs (shRNAs) targeting rat Gata3 mRNA were cloned in pLK01 (Open Biosystems, Huntsville, AL). Lentiviral supernatants were produced in human embryonic kidney-293T cells by transfection with calcium phosphate as described earlier (33). Lentiviral supernatants were collected after 24 and 48 h of incubation. Undifferentiated Rcho-1 trophoblast cells grown to 70% confluence were incubated with 8 μg/ml Polybrene (Sigma) containing medium for 30 min followed by infection with lentiviral supernatants. Infected Rcho-1 trophoblast cells were selected by addition of 3 μg/ml of puromycin (Sigma) after 48 h of infection. After 3 days samples were prepared for mRNA and protein analysis. The Gata3 target sequence 5´-GCCCTGCGACCTACATATAAA-3´ successfully knocked down expression of the target gene. For control experiments cells were infected either with empty viral vector or vectors expressing shRNAs against the Gata3 target sequence 5´-CGGATGTAGTA- GTCAGGAGGCACG-3´, which did not knock-down GATA3 expression.

Quantitative ChIP Assay—Real-time PCR-based quantitative ChIP analysis was performed according to an earlier described protocol (34). Undifferentiated and differentiated Rcho-1 trophoblast and mouse TS cells were trypsinized, washed, and resuspended in phosphate-buffered saline, and protein-DNA cross-linking was conducted by treating cells with formaldehyde at a final concentration of 1% for 10 min at room temperature with gentle agitation. Glycine (0.125 m) was added to quench the reaction. Antibodies against GATA2, GATA3, FOG1, MED1/TRAP220 (M-255; Santa Cruz), CBP/ P300 (A-22; Santa Cruz), diacetylated histone 3 (acH3; Millipore, Billerica, MA), tetra-acetylated histone 4 (acH4; Millipore), and RNA polymerase II (Pol II; N20, Santa Cruz) were used to immunoprecipitate protein-DNA cross-linked fragments. Immunoprecipitated DNA was analyzed by real-time PCR (ABI 7500, Applied Biosystem, Foster City, CA). Primers were designed to amplify 60- to 100-bp amplicons and were based on sequences in the Ensembl data base for mouse and rat Gata2 loci. Samples from three or more immunoprecipitations were analyzed. Products were measured by SYBR Green fluorescence in 25-μl reactions. The amount of product was determined relative to a standard curve of input chromatin. Dissociation curves showed that PCRs yielded single products. Primer sequences are available on request.

Transient Transfection Assay—Plasmid constructs (in pGL3 basic vector; Promega) containing a luciferase reporter gene fused to hematopoietic cell-specific (1S) promoter (35) of mouse Gata2 gene alone or in combination with Gata2 regulatory elements have been described earlier (28, 29) and were kind gifts from Dr. Emery H. Bresnick (University of Wisconsin Madison, Madison, WI). For transient transfection analysis, undifferentiated or day 4 differentiated Rcho-1 trophoblast
cells were transfected with an equal amount of each plasmid (3 ωg). Plasmids were added to 150 ωl of Opti-MEM (Invitrogen) reduced serum medium, incubated with Lipofectamine reagent (Invitrogen) for 20 min at room temperature, and then added to the cells. After 3 h of incubation the transfection mixture was replaced with culture medium. Cell lysates were harvested 48 h post-transfection and luciferase activity was measured in a Veritas Microplate Luminometer using the luciferase assay buffer (Promega). The luciferase activity for each sample was normalized to the protein concentration of the lysate. At least three independent preparations of each plasmid were analyzed.

**DNase I Hypersensitive Site Mapping.**—DNase I hypersensitive sites (DHSs) were mapped according to the procedure described by Follows et al. (36) with a few modifications. Briefly, to generate whole genome DHS libraries from undifferentiated and day 8 differentiated Rcho-1 trophoblast cells, nuclei were generated by lysing cells (3 \times 10^6 cells for each condition) in cell lysis buffer (300 mM sucrose, 10 mM Tris, pH 7.4, 15 mM NaCl, 5 mM MgCl_2, 0.1 mM EDTA, 60 mM KCl, 0.2% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 20 εg/ml leupeptin, 5 mM dithiothreitol). Nuclei were gently resuspended in reaction buffer containing different units of DNase I (New England Biolabs, Beverly, MA) and left to incubate at 4 °C. After 1 h, 700 ωl of nuclear lysis buffer (100 mM Tris-HCl, pH 8, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) and 50 εg of proteinase K were added to each set and incubated at 55 °C for 1 h followed by a 30-min incubation at 37 °C with 10 εg of RNase A. Digested DNA fragments were extracted with phenol/chloroform, blunted ended with T4 polymerase (New England Biolabs), and ligated with an asymmetric double-stranded linker (LP21; GAAATTCAGATCTCCGGGTCA-LP25; GCGGTGACCCGAGATCTGAGATCC linker). The precipitated ligated DNA was amplified using Vent exo-polymerase (New England Biolab) and a biotinylated LP25 primer. Following amplification, products were extracted using Dynal-streptavidin beads (Dynabeads M-270, Dynal Biotech) and suspended in TE buffer. From the library, DHSs at the Gata2 locus were determined by measuring relative enrichment of DNase I-treated samples versus DNase I-untreated samples by real-time PCR using region-specific primers. We used the same primers that were used for ChIP analysis. Quantification of samples was done using SYBR Green (Applied Biosystems) where standard curves were generated with known amounts of genomic DNA.

**RESULTS**

**Induction of Gata2 Expression during Trophoblast Giant Cell-specific Differentiation.**—To determine whether GATA2 expression is dynamically regulated during trophoblast differentiation, we used Rcho-1 trophoblast cells as a model system. Rcho-1 trophoblast cells represent a faithful model for studying rat trophoblast cells in undifferentiated and differentiated states (31, 37, 38). These cells can be maintained in a proliferative stem cell state and can be induced to undergo endoreduplication and differentiation along the trophoblast giant cell lineage.

As mentioned earlier under “Experimental Procedures,” over a period of 8 days, Rcho-1 trophoblast cells can be differentiated to trophoblast giant cells by replacing the culture condition. We performed a time course analysis with undifferentiated and differentiating Rcho-1 trophoblast cells to determine Gata2 mRNA expression. Quantitative RT-PCR analysis showed that the Gata2 mRNA level was significantly induced after 4 days of differentiation (Fig. 1A), and a 8-fold induction in the Gata2 mRNA level was observed after day 6 of differentiation. The maximum Gata2 mRNA level was maintained during the later period of differentiation. To validate this transcriptional induction we also performed Northern blot analysis with undifferentiated and day 8 differentiated cells. Northern blot analysis validated the RT-PCR analysis (Fig. 1B). The transcriptional induction PL-II, a prolactin family member that is only expressed in trophoblast giant cells (39) confirmed differentiation toward trophoblast giant cell lineage. We also performed Western blot analysis to determine GATA2 protein levels. Western blot analysis validated that the induced Gata2 mRNAs were translated to produce GATA2 proteins in differentiated Rcho-1 trophoblast cells (Fig. 1C).
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To further validate GATA2 induction during trophoblast differentiation we used mouse TS cells. In the absence of a FGF4 and within a course of 6 days, mouse TS cells differentiate to generate polyploid trophoblast giant cells (32). Therefore, we determined Gata2 mRNA and protein expression in undifferentiated TS cells and after 6 days of differentiation. Similar to Rcho-1 trophoblast cells, differentiation of mouse TS cells significantly induced Gata2 mRNA and protein expression (Fig. 1, D and E). Low level GATA2 expression was also detected in mouse TS cells grown in the presence of FGF4. This is consistent with the observation that a small percentage of TS cells undergo differentiation to generate giant cells even in the presence of FGF4 (32).

GATA3 Directly Represses Gata2 Transcription in Trophoblast Stem Cells—Studies in mouse hematopoietic precursors showed that, at the transcriptionally active Gata2 locus, GATA2 occupies five conserved cis-elements (Fig. 2A), designated −77-, −3.9-, −2.8-, −1.8-, and +9.5-kb regions (26, 28, 29) corresponding to their position with respect to the hematopoietic cell-specific 1S (35) promoter. These regions contain multiple conserved WGATAR motifs (Fig. 2B). Occupancy of GATA2 at the transcriptionally active Gata2 locus indicated that GATA2 might positively autoregulate its own transcription. However, this autoregulation mechanism has not been tested in other GATA2 expressing cell types.

As Gata2 is induced during trophoblast differentiation (Fig. 1), we wanted to test whether transcriptional activation is associated with GATA2 binding to the regulatory cis elements of the Gata2 locus. So, we performed quantitative ChIP analysis across a ~100-kb region of the Gata2 locus (Fig. 2A) in undifferentiated and differentiated mouse TS cells to determine GATA2 chromatin occupancy. Quantitative ChIP analysis showed that, in differentiated mouse TS cells, GATA2 occupies only the −3.9- and +9.5-kb regions (Fig. 2C). As a small population of mouse TS cells spontaneously differentiate to GATA2 expressing trophoblast giant cells, we also found very low amounts of GATA2 binding at those regions in ChIP assays with undifferentiated TS cells. GATA2 occupancy was highly induced with differentiation. Interestingly, we were not able to detect GATA2 binding at other Gata2-regulatory regions (Fig. 2C) as well as at other conserved WGATAR motifs within the ~100-kb Gata2 locus (data not shown).

We also tested GATA2 occupancy in Rcho-1 trophoblast cells. According to the Ensembl data base (Ensembl protein coding gene: ENSRNOG000000012347), in the rat Gata2 locus, the −80.4-, −8.4-, −7.3-, −6.3-, and +5.0-kb regions relative to rat Gata2 promoter correspond to mouse −77-, −3.9-, −2.8-, −1.8-, and +9.5-kb regions, respectively. However, for simplification, in this article we will describe regulatory regions of both mouse and rat Gata2 loci according to their positions at the mouse locus. Similar to mouse TS cells, ChIP analysis in Rcho-1 trophoblast cells detected GATA2 occupancy only at the −3.9- and +9.5-kb regions of differentiated Rcho-1 trophoblast cells (Fig. 2D).

Genetic complementation studies in GATA1-null erythroid progenitor cells showed that during erythroid differentiation repression of Gata2 transcription is associated with recruitment of GATA1 and displacement of GATA2 from the Gata2 locus (26–29). These results indicate that GATA1 directly represses Gata2 transcription in erythroid precursors. However, the mechanism of Gata2 repression in other cell types that do not express GATA1 is poorly understood. As Gata2 is repressed in the undifferentiated trophoblast stem cells, we hypothesized that Gata2 repression in those cells is directly mediated by other GATA factor(s). Earlier studies indicated that at least another GATA factor, GATA3, is expressed in the Rcho-1 trophoblast cells and in the trophoblast giant cells of the mouse placenta (7, 8, 25). However, we took an unbiased
approach and determined the expression of all other GATA factors in undifferentiated Rcho-1 trophoblast cells. As shown in Fig. 3, mRNA analysis revealed that only Gata3 mRNA is highly expressed in undifferentiated Rcho-1 trophoblast cells (Fig. 3A). Analysis in mouse TS cells also showed the similar GATA factor expression pattern (data not shown).

The Gata3 mRNA levels do not change significantly in the Rcho-1 trophoblast cells or mouse TS cells with differentiation (Fig. 3B). In mouse TS cells, Western blot analysis validated the Gata3 mRNA expression patterns (Fig. 3C, right panel). However, Western blot analysis showed that GATA3 protein levels are reduced in the differentiated Rcho-1 trophoblast cells (Fig. 3C, left panel) indicating the involvement of post-transcriptional mechanisms in the regulation of GATA3 in Rcho-1 trophoblast cells.

To test the hypothesis that GATA3 directly represses Gata2 transcription in undifferentiated trophoblast stem cells, we tested GATA3 chromatin occupancy at the Gata2 locus in Rcho-1 trophoblast cells. As shown in Fig. 3D (top panel), we found that GATA3 occupies the −3.9- and +9.5-kb regions of the repressed Gata2 locus in undifferentiated Rcho-1 trophoblast cells. Furthermore, analysis of mouse TS cells also determined GATA3 occupancy at the −3.9- and +9.5-kb regions in undifferentiated cells (Fig. 3D, bottom panel). Interestingly, although GATA3 protein is present in differentiated Rcho-1 and TS cells, GATA3 occupancy was not detected at the transcriptionally active Gata2 locus.

To further validate the functional role of GATA3 in Gata2 repression, we utilized an RNA interference approach to knockdown GATA3 in trophoblast stem cells. As a small population of TS cells undergoes spontaneous differentiation in our culture conditions, we used the undifferentiated Rcho-1 trophoblast cells for the knockdown study. We found that when GATA3 was knocked down by ~65% in undifferentiated Rcho-1 trophoblast cells, Gata2 mRNA was induced by ~2.5-fold (Fig. 4A). Western blot analysis also validated the knockdown of GATA3 and induction of GATA2 protein (Fig. 4B).

These results, in combination with ChIP analyses, indicate that, in trophoblast stem cells, GATA3 directly represses Gata2 transcription, and Gata2 transcriptional induction during tro-
These results indicate that altered expression levels of GATA3 and GATA2 might be an important determinant of trophoblast giant cell-specific differentiation.

Context-dependent Function of Gata2 Regulatory Elements in Trophoblast Cells—Interestingly, GATA factor occupancy was not detected at the −77-, −2.8-, and −1.8-kb regions in mouse TS cells and their corresponding regions in Rcho-1 trophoblast cells. In different cell types individual regulatory elements of a locus can function distinctly if the regulatory complexes are assembled by distinct cellular signals. In that context, distinct cell type-specific regulatory complexes containing unique transcription factors and cofactors might assemble at individual elements to regulate tissue-specific expression of the locus. Thus, in trophoblast cells, the lack of GATA factor occupancy to these elements indicates two possibilities: (i) functions of these elements are not dependent on GATA factor binding, or (ii) these elements do not regulate Gata2. To further compare functional properties of distinct Gata2 regulatory elements in trophoblast cells, we measured their activities in transient transfection assays in undifferentiated and differentiated Rcho-1 trophoblast cells expressing high levels of GATA3 and GATA2, respectively.

Previously, it was demonstrated that each of the five regulatory elements activated the mouse Gata2 1S promoter when fused to a luciferase reporter gene in transient transfection assays in hematopoietic cells (28, 29). By contrast, we found that constructs containing the −77-, −2.8-, and −1.8-kb regions linked to the Gata2 1S promoter had no significant enhancer activity in Rcho-1 trophoblast cells.

In accordance with the results of transient transfection analysis, DHSs were detected at −3.9- and −9.5-kb regions in both undifferentiated and differentiated Rcho-1 trophoblast cells (Fig. 6, A and B). However, in contrast to the findings in hematopoietic cells (28, 29), DHSs were not detected at the −1.8-, −2.8-, and −77-kb regions of the active Gata2 locus in differentiated Rcho-1 trophoblast cells.

We also found that, in Rcho-1 trophoblast cells, the aCH3 and aCH4 levels are enriched at the +9.5- and −3.9-kb regions but
not at the −1.8, −2.8-, and −77-kb regions of both repressed and active Gata2 locus (Fig. 6C). As increased histone acetylation facilitates factor access to nucleosomes, these results along with lack of DHSs indicate that although −1.8-, −2.8-, and −77-kb regions are important functional Gata2 regulatory elements in hematopoietic cells, in trophoblast cells these elements are probably inaccessible to trans-acting factors.

Dynamic Recruitment of Cofactors at the Gata2 Locus during Trophoblast Differentiation—Cofactor FOG1 has been implicated in transcriptional regulation of Gata2 (27, 40). FOG1 has been shown to occupy the regulatory regions of the Gata2 locus in both transcriptionally active and inactive states, and plays a crucial role in GATA2 repression by facilitating GATA1 chromatin occupancy to displace GATA2 during erythroid differentiation. FOG1 also interacts with GATA3, and GATA3-FOG1 complexes repress transcription of several genes in T lymphocytes (41, 42). As GATA3 represses Gata2 expression in trophoblast stem cells, we wanted to determine whether this repression is mediated via a GATA3-FOG complex. So, we tested FOG1 and FOG2 expression in Rcho-1 trophoblast cells. As shown in Fig. 7A, quantitative RT-PCR analysis showed that
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Fog1 is expressed in Rcho-1 trophoblast cells and Fog1 mRNA expression was induced by ~2-fold in differentiated Rcho-1 trophoblast cells. Protein analysis also validated FOG1 expression in Rcho-1 trophoblast cells (Fig. 7B). However, we found that Fog2 is not expressed in Rcho-1 trophoblast cells (data not shown).

As FOG1 is expressed in both undifferentiated and differentiated trophoblast cells and has both coactivator and corepressor activity, it could function in different ways in Gata2 regulation during trophoblast differentiation: (i) as a corepressor of GATA3, (ii) as a coactivator of GATA2, or (iii) as a chromatin occupancy facilitator to facilitate GATA2 binding and to displace GATA3 from the Gata2 locus. So, we performed ChIP analysis to ask whether FOG1 co-occupies the Gata2 locus with GATA factors in undifferentiated and differentiated Rcho-1 trophoblast cells. We found that FOG1 occupies the −3.9- and +9.5-kb regions in the undifferentiated Rcho-1 trophoblast cells (Fig. 7C, top panel). However, despite the fact that GATA2-FOG1 complexes form at the transcriptionally active Gata2 locus in hematopoietic precursors, FOG1 occupancy at the active Gata2 locus was not detected in the differentiated Rcho-1 trophoblast cells.

Studies in hematopoietic cells showed that cofactor CBP/P300 can function as a coactivator of GATA factors (43) and co-localizes with GATA2 at the regulatory regions of the activated Gata2 locus (28, 29). However, in our analysis we were able to detect CBP recruitment at the +9.5-kb regions of both the repressed and activated Gata2 locus in Rcho-1 trophoblast cells (Fig. 7C, middle panel). This is in line with the fact that we did not observe any significant differences in histone H3 and H4 acetylation (Fig. 6C) between the repressed and activated Gata2 locus in trophoblast cells. Thus, CBP recruitment and changes in histone modifications do not correlate with Gata2 activation in trophoblast cells.

During transcriptional activation the Mediator complex serves as an interface for regulatory factors and Pol II (44). Targeted knock-out of the mediator subunit MED1/TRAP220 revealed its critical role in placental development (45, 46). Previous studies have suggested that MED1/TRAP220 functions as a coactivator of GATA factors (47). Furthermore, it has been shown that MED1/TRAP220 physically interacts with GATA2 and functions as a coactivator of GATA2 to regulate certain genes (48, 49). Thus, we wanted to determine whether MED1/TRAP220 functions as a coactivator to regulate GATA2 transcription in trophoblast cells. We performed quantitative ChIP analysis to determine MED1/TRAP220 binding at the Gata2 locus in undifferentiated and differentiated Rcho-1 trophoblast cells and found that MED1/TRAP220 is recruited only to the −3.9- and +9.5-kb regions of the activated Gata2 locus in differentiated Rcho-1 trophoblast cells (Fig. 7C, bottom panel).

These results indicate the possibility that during trophoblast giant cell-specific differentiation a GATA2-Mediator complex forms at the Gata2 locus that positively regulates Gata2 transcription.

Our analysis showed that along with a switch in chromatin occupancy between GATA3 and GATA2, dynamic recruitment of FOG1 and MED1/TRAP220 at the Gata2 locus are associated with transcriptional activation of Gata2 during trophoblast giant cell-specific differentiation. Interestingly, we found significant Pol II occupancy at the −3.9- and +9.5-kb regions of the repressed Gata2 locus, and transcriptional activation of Gata2 is associated with Pol II recruitment at the promoter regions (Fig. 7D).

As we detected Pol II binding at the repressed Gata2 locus, we further tested whether Pol II at the repressed Gata2 locus is transcriptionally competent. To that end, we measured whether occupied Pol II at the −3.9- and +9.5-kb regions are phosphorylated at serine 5 (Ser(P)-5) at the carboxyl-terminal domain of the Pol II large subunit. Ser(P)-5 at the Pol II COOH-terminal domain is a key modification for the transition from preinitiation to transcriptional initiation and elongation. ChIP analysis utilizing a monoclonal antibody (H14: Covance), specific for Ser(P)-5-Pol II detected a strong Ser(P)-5 signal at the −3.9-kb region (Fig. 7E) but not at the +9.5-kb region, indicating the presence of a functional Pol II at the −3.9-kb region of repressed Gata2 locus. However, quantitative RT-PCR analysis revealed that very low levels of transcripts arise from both −3.9- and +9.5-kb regions in the undifferentiated cells (data not shown).

The presence of Pol II, enriched histone acetylation, and presence of DHSs at the −3.9- and +9.5-kb regions of repressed Gata2 locus indicates that Pol II containing “complexes” pre-assemble at those regions in trophoblast stem cells (Fig. 8) and a GATA3/GATA2 switch relocates Pol II to the promoter region. Thus, based on our findings, it is attractive to propose a mechanism in which GATA3-FOG1 complexes, formed at the −3.9- and +9.5-kb regions, repress Gata2 transcription in trophoblast stem cells and displacement of GATA3-FOG1 complexes by GATA2-MED1/TRAP220 activator complexes recruits Pol II at the promoter region leading to transcriptional activation during trophoblast giant cell-specific differentiation.

DISCUSSION

Although evidence is emerging regarding the important functional roles of GATA factors in trophoblast cells (7, 8, 50–52), molecular mechanisms of their regulation in trophoblast cells are poorly understood. The results described in this study establish a molecular mechanism that regulates Gata2 transcription during trophoblast giant cell-specific differentiation. We have provided evidence for three different aspects of Gata2 regulation in trophoblast cells: (i) we have delineated the regulatory regions of the Gata2 locus that confers enhancer activity in the trophoblast cells, (ii) we have shown that GATA3 directly represses GATA2 expression in trophoblast stem cells, and (iii) we have shown that the transcriptional regulation is associated with dynamic recruitment of cofactors FOG1 and MED1/TRAP220 at the Gata2 locus.

Multiple experimental approaches like analyses of reporter gene expression, identification of regulatory factors by chromatin immunoprecipitation, and functional analysis in vivo can be used to define regulatory regions of tissue-specific gene expression. Based on the findings that −3.9- and +9.5-kb regions showed enhancer activity in trophoblast cells, it is suggestive that these regions regulate endogenous Gata2 expression in trophoblast cells during placental development. However, dele-
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Intriguingly, transient transfection analysis (Fig. 5) and DHS mapping (Fig. 6) indicated that −1.8-, −2.8-, and −77-kb regions lack enhancer activity and do not contain accessible chromatin structures in trophoblast cells (Fig. 5). Furthermore, the enhancer activity of the −3.9-kb region is not dependent on conserved GATA motifs. Rather GATA motifs in that region are probably required for GATA3-mediated transcriptional repression. Analysis in transgenic mice indicated that a 3.1-kb fragment of the Gata2 locus that contains the −1.8- and −2.8-kb regions can drive expression of a Gata2 promoter-green fluorescence protein transgene in multipotent hematopoietic progenitors (53). Other studies showed that the +9.5-kb region but not −3.9- and −77-kb regions can function as an autonomous enhancer to drive Gata2 expression in endothelial cells in vitro and during embryonic development (54–56). These studies along with our findings strongly indicate intrinsic differences among the Gata2 regulatory regions. These intrinsic differences, at least in part, are contributed by other tissue-specific transcription factors, which function in combination with GATA factors from the regulatory regions (54, 55). Therefore, determining those factors in trophoblast cells is an area of further research.

Our results and studies in erythroid progenitors and TS cells revealed that both GATA1 and GATA3 directly repress Gata2 transcription in erythroid progenitors, and trophoblast stem cells, respectively. Furthermore, GATA2 might positively auto-regulate transcription. These findings indicate a general mechanism of GATA2 regulation in which multiple GATA factors, depending on their expression pattern and cellular signaling could regulate GATA2 expression in diverse tissues by directly modulating the nucleoprotein structure of the locus. So, the question arises how do different GATA factors function in a different fashion from the same GATA motifs? One possible explanation is the differential interaction with cofactors. We have shown here that in the repressed locus FOG1 co-localizes with GATA3, whereas in the activated locus MED1/TRAP220 co-localizes with GATA2. Although, we have not been able to correlate CBP/P300 binding with the Gata2 activation in trophoblast cells, it has been demonstrated in Gata2 regulation in hematopoietic cells. In erythroid progenitors CBP/P300 binding correlates with the DNase I hypersensitivity at Gata2 regulatory regions (28). All these observations provide evidence that formation of different GATA factor-cofactor complexes in response to diverse cellular signaling contribute to the functional outcome of the regulatory GATA motifs at the Gata2 locus.

Although the expression of several placental genes is reduced in Gata2−/− and Gata3−/− mice, the lack of an overt placental phenotype led to the prediction that these two factors might function redundantly during placental development. However, experiments have not been done in a context where both GATA2 and GATA3 are limiting. Many placenta-expressed genes are expressed in a spatial and temporal pattern during the course of gestation. So, placental functions beyond embryonic day 11.5 (Gata2-null mice die at E10.5 and Gata3-null mice die at E11.5), specifically differential expression of placental hormones during late gestation (39, 57) might be regulated by specific GATA factors. In that context, we predict the GATA3/GATA2-switch is an important mechanism for regulating expression levels of multiple genes in trophoblast cells. The presence of conserved GATA motifs in the regulatory regions of multiple prolactin family genes (58) further supports this prediction. However, as both GATA3 and GATA2 proteins are present in the differentiated trophoblast giant cells, they might have both unique and shared target genes in those cells. Thus, two important experimental approaches would greatly expand our understanding of GATA factor function in the placenta: (i) determining trophoblast function in the absence of both GATA2 and GATA3 and (ii) identifying GATA target genes in trophoblast cells.
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