GATA-2 and GATA-3 regulate trophoblast-specific gene expression in vivo

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SUMMARY

We previously demonstrated that the zinc finger transcription factors GATA-2 and GATA-3 are expressed in trophoblast giant cells and that they regulate transcription from the mouse placental lactogen I gene promoter in a transfected trophoblast cell line. We present evidence here that both of these factors regulate transcription of the placental lactogen I gene, as well as the related proliferin gene, in trophoblast giant cells in vivo. Placentas lacking GATA-3 accumulate placental lactogen I and proliferin mRNAs to a level 50% below that reached in the wild-type placenta. Mutation of the GATA-2 gene had a similar effect on placental lactogen I expression, but led to a markedly greater reduction (5- to 6-fold) in proliferin gene expression. Placentas lacking GATA-2 secrete significantly less angiogenic activity than wild-type placentas as measured in an endothelial cell migration assay, consistent with a reduction in expression of the angiogenic hormone proliferin. Furthermore, within the same uterus the decidual tissue adjacent to mutant placentas displays markedly reduced neovascularization compared to the decidual tissue next to wild-type placentas. These results indicate that GATA-2 and GATA-3 are important in vivo regulators of trophoblast-specific gene expression and placental function, and reveal a difference in the effect of these two factors in regulating the synthesis of related placental hormones.

Key words: angiogenesis, GATA factor, placenta, placental lactogen, proliferin, transcription factor, trophoblast

INTRODUCTION

The trophectoderm, the outer layer of the blastocyst, is the first cell lineage to differentiate in the mammalian embryo (Ilgren, 1983). Upon migration away from the inner cell mass, some trophoctoderm cells further differentiate into trophoblast giant cells (Müntener and Hsu, 1977; Ilgren, 1983), which have amplified, polytene genomes (Varmuza et al., 1988). The giant cells produce a number of placental hormones, including four members of the prolactin (PRL)/growth hormone (GH) family in the mouse: placental lactogen I (PL-I), placental lactogen II (PL-II), proliferin (PLF), and proliferin-related protein (PRP) (Ogren and Talamantes, 1988). PL-I and PL-II bind to the PRL receptor (Harigaya et al., 1988; MacLeod et al., 1989) and enhance the maintenance of the corpus luteum, regulate maternal carbohydrate metabolism in the liver, and stimulate the development of the mammary gland for postpartum lactation (Ogren and Talamantes, 1988). PLF and PRP, which bind neither the PRL receptor nor the GH receptor, are major positive and negative regulators, respectively, of angiogenesis (Jackson et al., 1994); PLF has also been found to stimulate uterine cell proliferation (Nelson et al., 1995).

Analysis of the expression of two of these hormones, PL-I and PLF, may be especially useful in characterizing early molecular events underlying trophoblast differentiation, since transcription of the PL-I and PLF genes occurs exclusively in giant cells and begins at the time of implantation (Lee et al., 1988; Nieder and Jennes, 1990; Faria et al., 1991; Carney et al., 1993). The temporal patterns of PL-I and PLF expression are very similar, with peak mRNA and protein levels for both of these hormones occurring on day 10 of gestation (Linzer et al, 1985; Colosi et al., 1987; Lee et al., 1988; Ogren et al., 1989). Thus, the PL-I and PLF genes may have critical transcriptional regulatory elements in common and may be under the coordinate control of one or a small number of regulatory factors.

Our previous studies on the PL-I gene have identified a region of only 274 bp immediately upstream of the transcriptional start site that is sufficient for trophoblast-specific expression in transfected cell cultures (Shida et al., 1993). Within this region are regulatory elements recognized by the AP-1 transcription factor (Shida et al., 1993) and by two members of the GATA family of transcription factors, GATA-2 and GATA-3 (Ng et al., 1994). Both the AP-1 sites and the GATA sites are critical for promoter activity in transfected Rcho-1 cells (Shida et al., 1993; Ng et al., 1994), a rat choriocarcinoma cell line that differentiates in culture into hormone-producing giant cells (Faria and Soares, 1991). Furthermore, we have demonstrated that GATA-2 and GATA-3 are expressed in trophoblast giant cells; that increased levels of GATA-2 and GATA-3 result in greater transcriptional activity of the PL-I promoter in Rcho-1 cells, indicating that these factors are present in limiting amounts; and that the

expression of either GATA-2 or GATA-3 in mouse fibroblasts converts those cells into a permissive environment for PL-I promoter activity, suggesting that these factors may positively and directly contribute to trophoblast giant cell differentiation (Ng et al., 1994).

The analysis of the PLF gene promoter has also revealed a critical AP-1 element (Mordacq and Linzer, 1989; Groskopf and Linzer, 1994) and the presence of GATA-2/3 consensus binding sites (Ng et al., 1994). The locations of the AP-1 and GATA sites with respect to the transcription start site are similar in the PLF and PL-I gene promoters. Furthermore, in both promoters one of the GATA elements is in close proximity to an AP-1 site, suggesting that these factors might work together in a complex regulating the expression of both of these genes.

GATA-2 and GATA-3 are expressed in distinct but overlapping patterns during embryonic development (Yamamoto et al., 1990; Dorfman et al., 1992; Oosterwegel et al., 1992; Leonard et al., 1993; George et al., 1994; Ng et al., 1994). Gene disruption experiments revealed that both GATA-2 and GATA-3 are required for fetal development, with GATA-2 essential for hematopoiesis (Tsai et al., 1994) and GATA-3 for fetal nervous system development and fetal liver hematopoiesis (Pandolfi et al., 1995). However, since these factors are also expressed in the placenta and have been implicated in placental-specific gene expression (Ng et al., 1994; Steger et al., 1994), it is possible that disruption of the GATA-2 or GATA-3 genes also has significant consequences on placental development and function. To explore this possibility, PL-I and PLF gene expression have been examined in mice bearing targeted mutations in the GATA-2 or GATA-3 gene. These studies indicate that both GATA-2 and GATA-3 are required for maximal PL-I and PLF expression in vivo.

MATERIALS AND METHODS

Animals

Mice heterozygous for a disrupted GATA-2 gene (Tsai et al., 1994) were mated, and pregnant female mice were killed on day 9.5 of gestation. Similarly, mice heterozygous for a disrupted GATA-3 gene (Pandolfi et al., 1995) were mated, and pregnant females were killed at day 10.5 or 11.5. The day of appearance of a vaginal plug was designated as day 0.5. Mice were maintained on a cycle of 14 hours light/10 hours dark, with food and water freely available. All procedures were approved by the Animal Care and Use Committee.

Genotype determination

Embryos and placentas were separated, rapidly frozen on dry ice, and stored at $-80°C$. Genomic DNA was extracted from embryos or yolk sacs as described by Hogan et al. (1994). Samples were digested in 100 μl of proteinase K (2 mg/ml in 50 mM Tris-HCl, pH 9.0, 50 mM EDTA, 400 mM NaCl, 5 mM DTT, 2.5 mM spermidine, and 1% SDS) at 55°C overnight. The lysate was extracted with phenol/chloroform and the DNA was precipitated with ethanol. GATA-2 and GATA-3 genotypes were analyzed by PCR (Tsai et al., 1994; Pandolfi et al., 1995). Briefly, 0.2 μg of genomic DNA was combined with 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 μM of each primer, 1× PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.1 mg/ml gelatin), and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA) in a 20 μl volume. After 32-35 cycles, PCR products from the wild-type and mutant alleles were detected by agarose gel electrophoresis.

RNA isolation and filter hybridization

Total RNA was isolated from selected placentas based on the genotyping results. For RNA isolation, placental tissue was homogenized in guanidinium thiocyanate, and the lysate was purified by centrifugation through a CsCl cushion (Ausubel et al., 1995). 5 μg of total RNA were denatured and subjected to formaldehyde-agarose gel electrophoresis. RNA was then transferred onto nitrocellulose membranes, immobilized by UV cross-linking, and hybridized to radiolabeled PL-I (Colosi et al., 1987), PLF (Linzer and Nathans, 1984), or ribosomal protein S2 (Harpold et al., 1979) cDNAs. Hybridization intensities were quantified by phosphorimaging, and the amount of the ribosomal protein S2 mRNA was used for normalization.

In situ hybridization

In situ hybridization was performed on frozen placental tissue as previously described (Ng et al., 1994). Tissue sections (10-20 μm) were cut, fixed in 5% paraformaldehyde, and acetylated. Antisense and sense riboprobes for PL-I and PLF were synthesized by in vitro transcription using SP6 or T7 RNA polymerase to incorporate [α-^{35}S]UTP and purified by Sephadex G-50 column chromatography. Sections were hybridized with 10^7 cpm/ml of riboprobe at 47°C in heavy mineral oil chambers. After a 12- to 16-hour hybridization, slides were treated with RNase A (20 μg/ml) at 37°C for 30 minutes. Slides were washed progressively in 1× SSC, 0.5× SSC, and 0.25× SSC at 47°C, then coated with NTB-2 emulsion and exposed for two weeks at 4°C. After development, slides were stained with hematoxylin.

To determine the number of giant cells, tissue sections were stained in 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, from Sigma Chemical Company, St. Louis, MO) solution for 10 minutes at room temperature followed by washing in phosphate-buffered saline. Stained, giant nuclei were counted by fluorescence microscopy.

Placental cultures and endothelial cell migration

Conceptuses arising from matings of mice heterozygous at the GATA-2 locus were isolated on day 9.5 of gestation. Fetal tissue was used for genotyping, and the corresponding placentas were minced and placed into culture in 0.5 ml serum-free Dulbecco's Modified Eagle's Medium (DMEM, GIBCO-BRL, Grand Island, NY). Culture media were collected after 24 hours, clarified by centrifugation, and protein concentrations were determined by a dye binding assay (BioRad, Hercules, CA).

Primary cultures of bovine adrenal capillary endothelial cells were propagated in DMEM supplemented with 10% fetal calf serum and endothelial cell mitogen (100 μg/ml) (Biomedical Technologies, Stoughton, MA). Cells to be used for migration assays were incubated overnight in DMEM without serum but with 0.1% bovine serum albumin (BSA), and then transferred to gelatinized 0.5 mm filters (Nucleopore Corporation, Pleasanton, CA) in a Boyden chamber at $1.3×10^4$ cells per well. The upper chambers contained DMEM with 0.1% BSA, and the lower chambers DMEM with each sample (5 μg/ml placental conditioned medium or 10 ng/ml basic fibroblast growth factor, bFGF, as a positive control). After 6 hours at 37°C, the filters were removed from the chambers, fixed and stained, and the number of cells that had migrated to the lower surface of each filter was determined.

Immunostaining of blood vessels

Conceptuses arising from matings of mice heterozygous at the GATA-2 locus were isolated on day 9.5 of gestation. Fetal tissue was excised and used for genotyping. The remaining tissue, including the placenta and the associated decidual tissue from the uterus, was immediately frozen, sectioned and fixed in paraformaldehyde. Tissue sections were treated with an antibody against the endothelial cell surface marker PECAM-1 (Vecchi et al., 1994), and antibody binding was visualized using the Vectastain kit (Vector Laboratories, Burlingame, CA). Sections were then counterstained with hematoxylin.
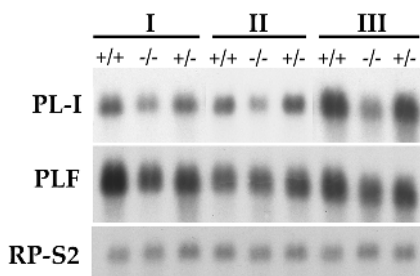
RESULTS

Placental gene expression in mice lacking GATA-3

To investigate the in vivo role of GATA factors in the expression of trophoblast-specific genes, mice heterozyygous for a disrupted GATA-3 gene were mated, and placental and fetal tissue were harvested at day 10.5 of gestation, the time of peak synthesis of both PL-I and PLF. Since the trophoblast lineage derives from the embryo and not from the mother, these matings were expected to yield, within each litter, placentas and embryos that are wild type, heterozygous, or homozygous mutant at the GATA-3 locus. RNA was purified from each placenta to analyze hormone mRNA levels; DNA was extracted from the corresponding fetus in each case and analyzed by PCR to determine the genotype.

Placentas that develop without GATA-3 express the PL-I and PLF mRNAs (Fig. 1), suggesting that GATA-3 is not absolutely required for trophoblast giant cell differentiation. However, the levels of both of these mRNAs are reduced in these placentas, indicating that GATA-3 is required for maximal PL-I and PLF gene expression. The analysis of five litters (three are shown in Fig. 1) revealed that PL-I mRNA levels are higher in wild type (2.3 ± 0.2-fold after normalization to the ribosomal protein S2 mRNA, $n=5$) and heterozygotes (2.7 ± 0.3-fold, $n=5$) than in the homozygous mutants. A similar effect was observed on PLF expression, with wild type (1.8 ± 0.3-fold, $n=5$) and heterozygous (2.0 ± 0.2-fold, $n=5$) placentas containing higher concentrations of this mRNA than the homozygous mutant placentas. No significant differences in PL-I and PLF mRNA levels were detected between heterozygous mutant and wild-type placentas, and no change was seen in the amount of the control ribosomal protein S2 mRNA among the three genotypes. Some variation in the level of hormone gene expression from litter to litter was observed, though, probably in part because of the narrow window of maximal PL-I and PLF gene expression and because the litters were of slightly different gestational ages; comparisons were therefore always made within individual litters.
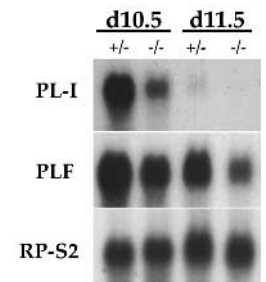
Temporal pattern of placental hormone gene expression

One explanation for the decreased level of PL-I and PLF mRNAs in GATA-3 homozygous mutant placentas might be
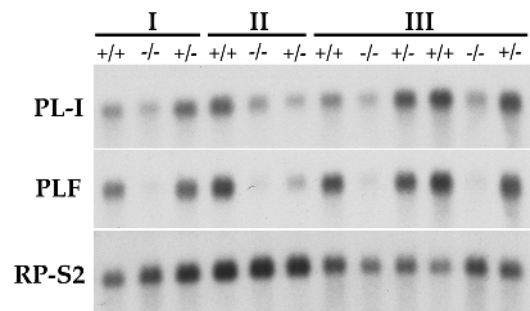

Fig. 2. Temporal pattern of hormone gene expression in GATA-3 mutant placentas. Total placental RNA at day 10.5 and day 11.5 of gestation was analyzed by filter hybridization to the PL-I (top panel) or PLF (middle panel) cDNA, or to the control ribosomal protein S2 cDNA (bottom panel). The heterozygous (+/−) or homozygous (−/−) GATA-3 mutant genotype is indicated for each of the placental samples.

that the absence of GATA-3 resulted in a shift in the temporal pattern of hormone gene expression such that the peak levels of PL-I and PLF mRNA, which normally occur on day 10, are delayed. However, the amount of PL-I mRNA rapidly declines between day 10.5 and day 11.5 in wild type (not shown), heterozygous, and homozygous mutant placentas, and the level at day 11.5 is still greater in the heterozygous placenta compared to the homozygous mutant placenta (Fig. 2). Although the concentration of PLF mRNA does not decline as rapidly as PL-I mRNA, decreased levels are detected from day 10.5 to day 11.5 in all three genotypes, as well, (shown for heterozygous and homozygous mutant samples in Fig. 2) and again, the heterozygous placenta has a higher concentration of PLF mRNA compared to the homozygous mutant placenta. Thus, the absence of GATA-3 results in decreased expression of the PL-I and PLF genes, rather than a delay in their expression.

Placental gene expression in mice lacking GATA-2

The 2-fold decrease in PL-I mRNA levels seen in the GATA-3 mutant background was also observed for placentas in which the GATA-2 gene had been disrupted (Fig. 3); the ratio of PL-I mRNA in wild-type compared to homozygous mutant placentas was found to be 2.7 ± 0.6 ($n=5$), and 1.9 ± 0.2 ($n=5$) for heterozygous compared to homozygous mutant placentas. In contrast, mutation of the GATA-2 gene had a more pronounced effect on PLF gene expression than did the disruption of the GATA-3 gene (Fig. 3); the ratio of PLF mRNA in wild type compared to homozygous mutant placentas was found to be 6.2 ± 1.2 ($n=5$), and 4.7 ± 0.6 ($n=5$) for heterozygous compared to homozygous mutant placentas. For this analysis, RNA levels

Fig. 1. Hormone mRNA levels in GATA-3 mutant placentas. Total placental RNA from day 10.5 of gestation was analyzed by sequential filter hybridization to PL-I (top panel), PLF (middle panel), and control ribosomal protein S2 (bottom panel) cDNAs. Wild-type (+/+), heterozygous GATA-3 mutant (+/−), or homozygous GATA-3 mutant (-/-) placental RNA samples from three litters (Roman numerals) were assayed.

Fig. 3. Hormone mRNA levels in GATA-2 mutant placentas. Total placental RNA from day 9.5 of gestation was analyzed by sequential filter hybridization to the PL-I (top panel) or PLF (middle panel) cDNA, or to the control ribosomal protein S2 cDNA (bottom panel). Roman numerals indicate three distinct litters, and the wild-type (+/+), heterozygous mutant (+/-), or homozygous mutant (−/−) GATA-2 genotype is indicated for each of the placental samples.
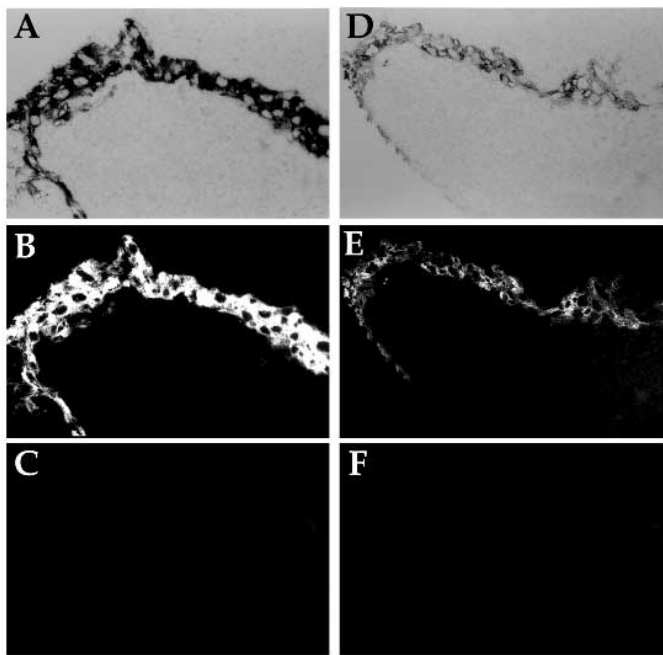
Fig. 4. PL-I gene expression in GATA-3 mutant trophoblast giant cells. Wild-type (A-C) or homozygous GATA-3 mutant (D-F) placentas were sectioned and hybridized to the PL-I antisense (A-B and D-E) or control sense (C,F) riboprobes. Hybridization, detected as dark silver grains in the bright-field photomicrographs (A,D) or as bright regions in the dark-field images (B,E), is seen specifically in the trophoblast giant cells. No hybridization was detected in the surrounding uterine cells or in other cell types within the placenta, and no hybridization was observed with the sense probes (C,F). The height of each panel in the figure corresponds to a distance of 0.6 mm.

were measured at day 9.5 of gestation since GATA-2 homozygous mutants die on day 10 (Tsai et al., 1994).

PL-I and PLF gene expression in individual trophoblast giant cells

The reduction in PL-I and PLF gene expression in GATA factor-deficient placentas could be due to a decrease in hormone synthesis within each trophoblast giant cell, to a decrease in the number of giant cells, or to a combination of these two effects. To address this question, placental tissue from wild type and mutant littermates was analyzed by in situ hybridization. Hybridization of placental tissue with a PL-I probe revealed that expression of the PL-I mRNA is still restricted to the giant cells in the homozygous GATA-3 mutant placenta, but that the level of PL-I mRNA in each giant cell is reduced relative to the wild-type placenta (Fig. 4). Similarly, less PLF mRNA appears to be present per giant cell in the homozygous mutant GATA-3 placenta compared to wild type (Fig. 5). An apparently greater reduction in PL-I (Fig. 6) and PLF (Fig. 7) mRNA levels in each trophoblast giant cell is seen in the GATA-2 homozygous mutant placenta.

Although the size of the giant cell layer in the GATA-3 and GATA-2 homozygous mutants often appears to be reduced, no significant difference was detected between the number of giant cell nuclei in wild-type and

homozygous mutant placentas as determined by counting of DAPI-stained sections. The number of giant cell nuclei per section from three pairs (i.e., in the same pregnant female) of wild-type and homozygous mutant placentas were calculated as the mean ± standard deviation from up to eight sections with the following results: 130±22 (wild type) and 136±13 (mutant); 114±9 (wild type) and 125±18 (mutant); and 238±29 (wild type) and 213±31 (mutant); the variation in the number of nuclei seen between pairs was probably due to slight differences in gestational ages. In addition to the finding that the number of giant cells is indistinguishable in the wild-type and mutant placentas, inspection of the morphology of the giant cell layer did not reveal and gross structural defects. These results indicate that the reduced level of PL-I and PLF mRNA in placentas lacking GATA-3 or GATA-2 can be attributed primarily to decreased gene expression within each giant cell.

Reduced angiogenic activity of GATA-2 deficient placentas

PLF stimulates endothelial cell migration and neovascularization, and is therefore thought to stimulate the growth of maternal uterine blood vessels towards the conceptus (Jackson et al., 1994). Since PLF represents the majority of the angiogenic activity secreted from the mid-gestation placenta in an endothelial cell migration assay (Jackson et al., 1994), it seemed likely that the total angiogenic activity secreted by GATA-2 mutant placentas, which have very low PLF mRNA levels, would be greatly reduced.

To examine this hypothesis, tissue from three homozygous mutant placentas and from three wild-type or heterozygous mutant placentas was cultured in serum-free medium. The conditioned media from the three homozygous mutant placenta cultures had significantly less stimulatory activity in the endothelial cell migration assay than the conditioned media from
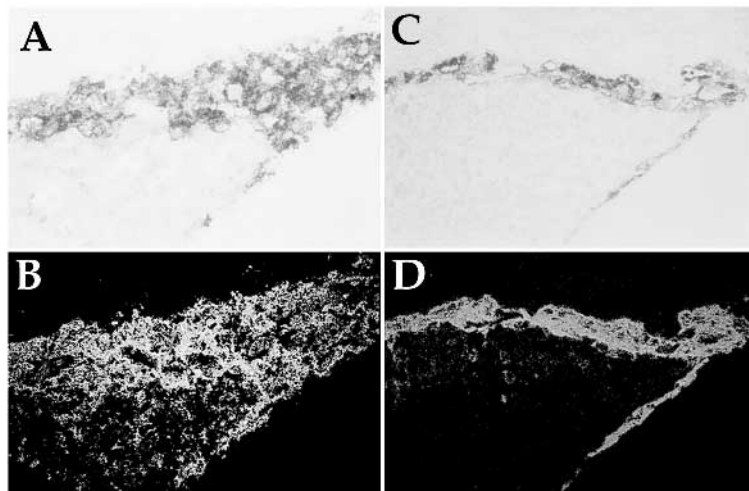
Fig. 5. PLF gene expression in GATA-3 mutant trophoblast giant cells. Wild-type (A-B) or homozygous GATA-3 mutant (C-D) placentas were sectioned and hybridized to a PLF antisense riboprobe. Hybridization is detected in the trophoblast giant cells as dark silver grains in the bright-field photomicrographs (A,C) or as bright regions in the dark-field images (B,D); no hybridization was detected in other cell types, and no hybridization was observed with a control sense probe (not shown). The height of each panel in the figure corresponds to a distance of 0.6 mm.
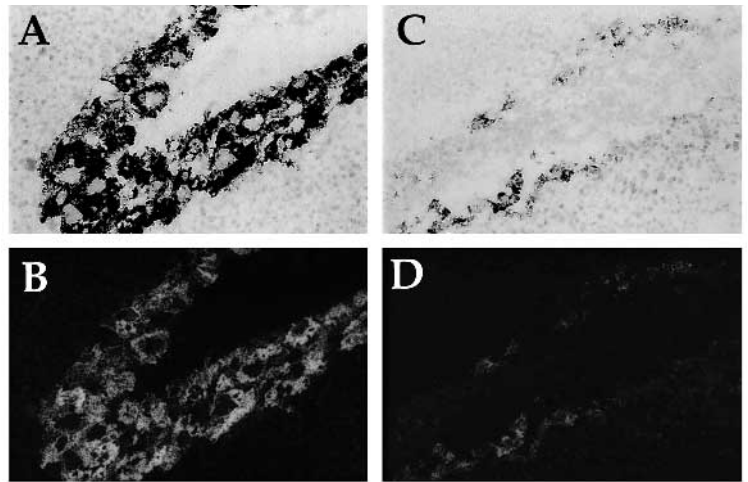
Fig. 6. PL-I gene expression in GATA-2 mutant trophoblast giant cells. Wild-type (A,B) or homozygous GATA-2 mutant (C,D) placentas were sectioned and hybridized to a PL-I antisense riboprobe. Hybridization to the trophoblast giant cells is detected as dark silver grains in the bright-field photomicrographs (A,C) or as bright regions in the dark-field images (B,D); no hybridization was detected in other cell types, and no hybridization was observed with a control sense probe (not shown). The height of each panel in the figure corresponds to a distance of 0.6 mm.

the wild-type or heterozygous placenta cultures (Fig. 8). Some residual activity was detected in the homozygous mutant samples, consistent with the presence of other angiogenic factors (Jackson et al., 1994). Thus, one consequence of the absence of GATA-2 in the developing placenta is a deficiency in the secretion of angiogenic factors, possibly leading to abnormal placental and decidual vascularization.

The in vivo consequences of reduced placental angiogenic activity was analyzed by comparing neovascularization in decidual tissue adjacent to wild-type and homozygous GATA-2 mutant conceptuses within individual pregnant females. Fetal tissue from the conceptuses was excised to determine the genotype of the placenta. Neovascularization in the decidual tissue associated with each conceptus was visualized by immunostaining with an antibody against an endothelial cell surface protein, PECAM-1. A significant reduction in neovascularization in the decidual tissue adjacent to the mutant conceptuses is seen compared to the growth of blood vessels in the uterus near wild-type conceptuses (Fig. 9).

DISCUSSION

The present analysis of mice lacking the GATA-2 or GATA-3 transcription factors has demonstrated that both of these factors are required for maximal expression of both the PL-I and PLF genes in vivo. We had previously shown that both factors regulate PL-I promoter activity in co-transfected Rcho-1 cells (Ng et al., 1994), so the current results support the use of this rat trophoblast cell line as a model system for studying trophoblast gene expression. Since the PL-I and PLF genes are co-expressed in trophoblast giant cells at the same stage of gestation, these results further suggest that GATA-2 and GATA-3 may coordinately control the transcription of a set of genes in giant cells, and therefore may be important regulators of trophoblast giant cell differentiation. However, disruption of neither the GATA-2 nor the GATA-3 gene alone was sufficient to prevent giant cell differentiation, suggesting that these factors may have complementary activities and that mutation of both genes

may be necessary to observe a requirement for GATA factors in placental development. Attempts to produce mice that are heterozygous mutant at both the GATA-2 and GATA-3 loci have yielded the surprising result that the double heterozygous embryos die during gestation, and therefore it is not currently possible to generate mice null for both GATA-2 and GATA-3 to test this hypothesis; the basis for this fetal lethality in the double heterozygotes is under investigation.

Even though GATA-2 and GATA-3 may have overlapping activities, disruption of the GATA-2 gene was found to have a more pronounced effect on PLF gene expression than did mutation of the GATA-3 gene. One possible explanation is that GATA-2 and GATA-3 have identical activities on this gene promoter, but that in the wild-type placenta GATA-2 protein is simply more abundant than GATA-3. In this case, the effects of these factors could be additive and total GATA factor activity
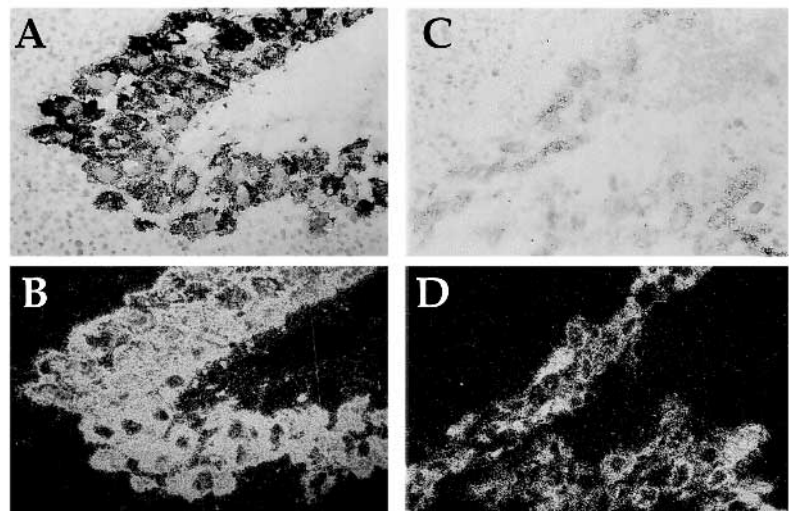
Fig. 7. PLF gene expression in GATA-2 mutant trophoblast giant cells. Wild-type (A,B) or homozygous GATA-2 mutant (C,D) placentas were sectioned and hybridized to a PLF antisense riboprobe. Hybridization is detected in the trophoblast giant cells as dark silver grains in the bright-field photomicrographs (A,C) or as bright regions in the dark-field images (B,D); no hybridization was detected in other cell types, and no hybridization was observed with a control sense probe (not shown). The height of each panel in the figure corresponds to a distance of 0.6 mm.

Fig. 8. Angiogenic activity secreted by GATA-2 mutant placentas. Cultures of placental tissue from day 9.5 of gestation were prepared from two separate litters with (A) one wild-type (+/+), one heterozygous mutant (+/−), and one homozygous mutant (−/−) GATA-2 placenta, or (B) one wild-type (+/+) and two homozygous mutant (−/−) placentas. Conditioned media from the cultures were collected and tested (at 5 µg/ml) for the ability to stimulate endothelial cell migration; the numbers of migrating cells in 10 high-magnification microscope fields were counted. Angiogenic activity (mean ± s.e.m. of three measurements) secreted from wild-type and heterozygous mutant placentas was similar to the positive control, 10 ng/ml bFGF. The activity secreted from the homozygous mutant placentas was significantly reduced relative to the wild-type and heterozygous mutant placentas ($P<0.001$ in a Student's t-test). Endothelial cells treated with bovine serum albumin (BSA) served to determine the background level of migration.
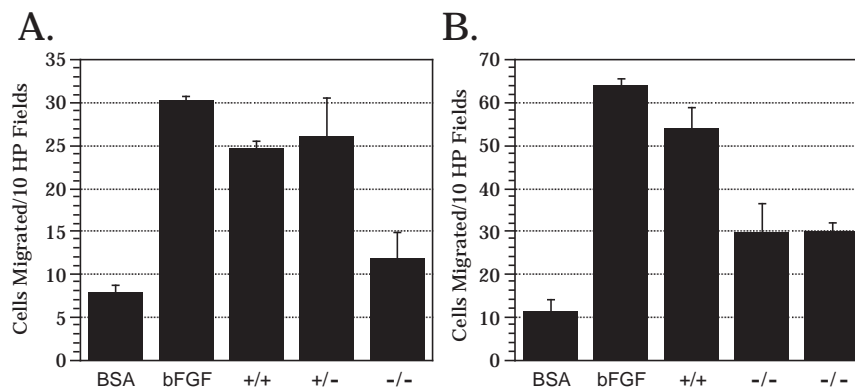
would be limiting for hormone gene expression in giant cells. Consistent with this explanation are the observations that both these factors have an indistinguishable ability to induce transcription from the PL-I promoter in co-transfected trophoblast cell cultures (Ng et al., 1994), and are equally active in stimulating transcription from the human glycoprotein α-subunit gene in a transfected pituitary cell line (Steger et al., 1994). However, unlike the effect on the PLF gene, disruption of the GATA-2 gene compared to the GATA-3 gene had a similar impact on PL-I gene expression in vivo. Furthermore, GATA-2 and GATA-3 mRNA levels in giant cells are very similar (Ng et al., 1994), suggesting that the concentrations of these two transcription factors may not be much different in the placenta.

Another possible explanation for the greater effect of the GATA-2 mutation on PLF mRNA levels is that GATA-2 may interact more strongly than GATA-3 with cis-regulatory elements in the PLF gene promoter in vivo, or GATA-2 may cooperate more effectively with the other transcription factors that regulate the PLF gene. Such differences in biochemical activities may mean that GATA-2 and GATA-3 would only partially complement each other, and therefore these factors may have distinct roles in placental development. In support of this possibility is the observation that GATA-2, but not GATA-3, is uniquely able to stimulate proliferation and block differentiation of erythroid precursors (Briegel et al., 1993).

The fetal lethality that results from a homozygous null mutation of either the GATA-2 or GATA-3 locus has been attributed to a direct requirement for these factors in fetal development (Tsai et al., 1994; Pandolfi et al., 1995). However, since these factors are also important regulators of placental hormone synthesis, and quite likely the synthesis of other placental proteins, a placental defect may also contribute to fetal death. We have previously demonstrated that the mouse placenta secretes peak levels of an angiogenic activity at midgestation (day 10). The angiogenic activity is principally due to PLF protein (Jackson et al., 1994). In the GATA-2 null mutant, PLF mRNA levels were significantly reduced, suggesting that neovascularization in the conceptus may be compromised. Indeed, the total amount of angiogenic secretions from these mutant placentas, as measured in an endothelial cell mgration assay, was significantly reduced compared to wild-type or heterozygous mutant placentas. Furthermore, neovascularization in the uterine decidual tissue next to GATA-2

homozygous mutant placentas is significantly reduced compared to blood vessel growth in the decidual tissue next to wild-type placentas. Since the decidual tissue is maternal, and therefore the genotype of the vascular endothelial cells throughout this tissue is identical, local differences in decidual neovascularization are not cell autonomous, but instead are most likely to arise from differences in the levels of angiogenic factors secreted from the adjacent embryo-derived placenta. The reduced growth of blood vessels is consistent with the lower levels of proliferin expression in the mutant placentas, and therefore these data support a model in which proliferin diffuses locally from trophoblast giant cells to stimulate uterine neovascularization. However, angiogenic activity caused by tissue secretions is typically multi-factorial, with combinations of angiogenic and anti-angiogenic factors being produced, so
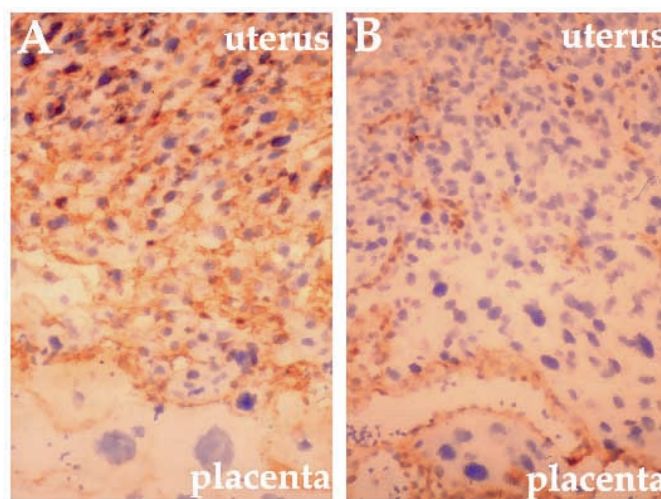
Fig. 9. Defects in decidual neovascularization. Maternal anti-mesometrial uterine tissue adjacent to a GATA-2 wild-type placenta (A) and next to a homozygous mutant placenta (B) within the same pregnant GATA-2 heterozygous female at day 9.5 were analyzed for neovascularization by immunostaining (yellow-brown) with an antibody against the endothelial cell surface marker, PECAM-1. The decreased decidual neovascularization associated with GATA-2 mutant placentas was detected in all three pregnancies that were examined. The width of each panel in the figure corresponds to a distance of 0.3 mm.

the lack of GATA-2 in trophoblasts may have effects on the expression of multiple genes encoding angiogenesis regulatory factors in addition to the proliferin gene.

The reduced expression of PLF by the GATA-2 mutant placenta might have consequences on processes other than angiogenesis, as well. PLF stimulates uterine cell proliferation (Nelson et al., 1995), and this hormone enters the fetus where it binds to specific structures and may participate in fetal development (Lee et al., 1988; Jackson and Linzer, 1996). Transport of this hormone into the fetus may occur preferentially within a conceptus rather than through the maternal bloodstream, so the decreased level of PLF protein in one conceptus may not be compensated for by wild-type levels of hormone secretion from a neighboring conceptus. In contrast, the known effects of PL-I are on maternal organs including the ovary and liver (Ogren and Talamantes, 1988), and thus a lower level of hormone production from one placenta may be offset by the secretion of higher levels from other placentas. Some paracrine effects of PL-I are likely, though, since this hormone can bind to PRL receptors expressed in the decidual tissue (Gu et al., 1996), and thus the reduction in PL-I levels in homozygous GATA-2 mutant conceptuses may also have developmental consequences even in heterozygous crosses.

Additional effects of GATA-2 and GATA-3 on trophoblast function may also be revealed as other target genes for these factors are identified. How these factors may cooperate with other transcriptional regulators in controlling placental development, including the basic helix-loop-helix proteins Hxt, which has also been shown to stimulate transcription from the PL-I promoter (Cross et al., 1995), and Mash-2, which is required for the formation of the spongiotrophoblast cell layer that underlies and gives rise to the giant cells (Guillemot et al., 1994), remains to be determined.

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