Identification of Integrin-α4, Rb1, and Syncytin A as Murine Placental Target Genes of the Transcription Factor GCMa/Gcm1

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Members of the GCM (glial cells missing) transcription factor family have been shown to act as master regulators in different cells during mammalian and fly development being responsible for processes including gliogenesis, hematopoiesis, placental formation, and development of the parathyroidea. In the central nervous system of flies, several target genes for GCM have been reported, namely repo, pointed, and tramtrack. In mammals, two GCM genes are known (GCMa and GCMb), but the knowledge of their target genes is very limited. Here, we present for the first time a global approach aimed to identify GCMa target genes. We found 66 genes up-regulated and 11 genes down-regulated in GCMa-deficient chorionic tissue of mice at embryonic day 9.5. Moreover, we verified by quantitative reverse transcription-PCR all 11 down-regulated genes. The two most strongly down-regulated genes, integrin-α4 and retinoblastoma (Rb1), were further analyzed by promoter studies. Additionally, we identified down-regulation of the murine syncytin A gene, which is fundamental for syncytotrophoblast formation. Finally, we proved strong down-regulation of integrin-α4 and Rb1 transcript levels by in situ hybridization in murine GCMa-deficient placenta at embryonic day 9.5. Our data demonstrate for the first time that genes encoding key regulators of placental tissue formation and architecture are regulated by GCMa.

All GCM family members are involved as master regulators in key steps of differentiation processes. The prototypical GCM protein of Drosophila melanogaster turned out to be mandatory for gliogenesis (7–9). Later, GCM in flies was also shown to be responsible for the specification of the plasmatocyte/macrophage lineage during hematopoiesis (10). Apart from these main functions, fly GCM proteins are additionally important for neurogenesis in the developing visual system and the postembryonic brain and for terminal tendon cell differentiation (11–13).

Only two GCM proteins have been reported in mammals, GCMa/Gcm1 and GCMb/Gcm2. GCMa is necessary for the differentiation of trophoblasts to syncytiotrophoblasts during placental labyrinth formation (14, 15). Consequently, GCMa knock-out mice die at mid-embryogenesis due to the failure of placental labyrinth formation and subsequent lack of nutrient and oxygen supply to the embryo. Accordingly, GCMa is the first transcription factor capable of initiating syncytiotrophoblast formation. The placental phenotype of the GCMa knock-out mice resembles human placental pathologies such as pre-eclampsia or intrauterine growth restrictions associated with fetal to neonatal mortality and morbidity (14). Pre-eclampsia, clinically defined by hypertension and proteinuria (14), is still one of the main causes of maternal and perinatal morbidity. In fact, both decreased and increased placental GCMa transcript amounts have been reported in mothers with pre-eclampsia (16, 17). In adult mice, additional GCMa expression sites have been reported in kidney, thymus, and brain (18, 19). GCMb is required for the generation of parathyroid glands (20). Consequently, GCMb knock-out mice suffer from hypoparathyroidism characterized by affected calcium and phosphate homeostasis.

To date, several target genes of GCM have been identified in flies and in mammals. In flies, three transcription factors, encoded by the loci repo, pointed, and tramtrack, are expressed in GCM-dependent fashion and are all required for terminal differentiation of lateral glia (21–23). In addition, fly GCM is able to regulate its own expression (24, 25). In flies, a number of further GCM target genes were identified by a genome-wide analysis (26). In mammals, only aromatase and syncytin are reported as placental targets of human GCMa (27, 28). These targets have been of limited help for understanding placental abnormalities.

To further understand how GCMa is involved in the process of placental labyrinth formation, we conducted a comparative...
analysis of placental genes in the absence of GCMa in mouse placenta tissue by microarray technology. This is the first genome-wide analysis for mammalian GCMa targets. A number of genes are up- or down-regulated in GCMa-ablated placenta. We further focused on those genes that were down-regulated in the absence of GCMa in the placenta. We proved their transcriptional down-regulation by quantitative RT-PCR. The two most down-regulated genes in placenta lacking GCMa were verified by promoter and in situ hybridization studies. Our data suggest that GCMa acts at a crucial step of placental development, and its further characterization might help to understand placental pathologies in humans.

**EXPERIMENTAL PROCEDURES**

**Primers Used for Quantitative RT-PCR and Plasmid Constructs**—GCMa targets derived by microarray studies were verified by quantitative RT-PCR (LightCycler, Roche Applied Science) using the 5′- and 3′-primers listed in supplemental Table 1.

For integrin-α4 reporter studies, an ~6-kb EcoRV/HindIII integrin-α4 promoter fragment derived from a genomic clone (4H6-1; gift of Joy Yang, The Johns Hopkins University (JHU) School of Medicine, Baltimore, MD) was subcloned in Smal/HindIII-digested pGL2-Basic (Promega). An Rb1 luciferase reporter construct was generated using a Bluescript plasmid containing an ~6.4-kb Rb1 promoter fragment (pmRbP; (129).lacZ; gift of Eldad Zacksenhaus, University of Toronto, Canada). The Rb1 promoter was amplified by PCR primers 5′-ATA GGT ACC GAT CCC GTT CTT CAC AGC AG-3′ and 5′-TAT GGA TCC GAG CAC GCC GGG CCG GC-3′ and subcloned in KpnI/BglII-digested pGL2-Basic (Promega). For promoter studies, different 5′-truncations of integrin-α4 and Rb1 promoters were created. For the integrin-α4 promoter region, a 3′-primer (5′-GAT CAA GCT TGC GCT CTT TCT GGT GGA GAA CAT T-3′) was combined with the following 5′-primers: 5′-GAA GTA TGA TTC AGG ATA TC-3′ (~2009 to +235), 5′-GAT CGG ATC CAC TTT ATG GAG TAG GAG ACT GAA TC-3′ (~1493 to +235), 5′-GAT CGG ATC CGC CTC CTG CTC CAG GAT GAA GAA-3′ (~1022 to +235), 5′-GAT CGG ATC CGC AGG AGG TTT GGC TTC TGC-3′ (~513 to +235) to amplify 5′-truncated promoter fragments by PCR and subclone them in BglII/HindIII-digested pGL2-Basic (Promega). For the Rb1 promoter region, a 3′-primer (5′-TAT GGA TCC GAC GCC GAG CCG GC-3′) was used with the following 5′-primers: 5′-ATA GGT ACC GCA GTA CGT GAG ACT GAA TC-3′ (~2814 to +150), 5′-ATA GGT ACC TTG GTG GCA GTG ATG AGT TC-3′ (~827 to +150), 5′-ATA GGT ACC TCT CCA GAA GGC CAC CAA TG-3′ (~399 to +150). The PCR fragments were subsequently inserted into KpnI/BglII-digested pGL2-Basic (Promega). For the generation of an in situ probe, full-length GCMa subcloned in pBluescript (Stratagene) was linearized by EcoRI to generate an antisense and by SacI to make a sense riboprobe. For an Rb1 riboprobe, the 3′-part of the open reading frame was subcloned as a 969-bp PCR fragment into pGEM-Teasy (Promega) using primers 5′-GGA TGG AGA AGG ACC TGA TA-3′ and 5′-AGT TTC TGT TGG AAT TTT GA-3′ as described (29). A plasmid for the generation of an in situ probe for integrin-α4 was kindly provided by Joy Yang (JHU, School of Medicine, Baltimore, MD).

**Tissue Culture, Transfection, and Luciferase Assay**—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum (Invitrogen). The cells were transfected in 60-mm dishes with 1 μg of luciferase reporter and 0.1 μg of cytomegalovirus-driven GCMa expression vector using DNA calcium phosphate precipitates. At 48 h after transfection, cells were harvested for luciferase assays as described (30). All experiments were repeated independently at least three times.

**Dissection of Placental Tissue, Genotyping, RNA Extraction, Reverse Transcription, and LightCycler PCR**—Previously, constitutive GCMa knock-out mice were generated by replacing exons 3–6 of GCMa by a complete lacZ open reading frame (15). GCMa<sup>lacZ/lacZ</sup> mice are embryonically lethal, representing GCMa-null mutants and trophoblast cells in pregnant mothers expressing β-galactosidase in place of GCMa. Here, we intercrossed GCMa<sup>+/lacZ</sup> mice and obtained GCMa<sup>++/+</sup>, GCMa<sup>+</sup>/lacZ, and GCMa<sup>lacZ/lacZ</sup> genotypes according to Mendelian inheritance. Embryos were lysed and genotyped as described at E9.5, the time of dissection (15). GCMa<sup>++/+</sup> (wild-type) or GCMa<sup>lacZ/lacZ</sup> (mutant) chimeras were used for further experiments.

A minimum of 40 either GCMa<sup>++/+</sup> or GCMa<sup>lacZ/lacZ</sup> chimeras were pooled, and total RNA was extracted using TRizol reagent (Invitrogen) (30). The total RNA was further purified by DNaseI digestion and column purification (Qiagen DNaseI-, Qiagen RNasey kit) according to the manufacturer’s instructions. After reverse transcription, first strand cDNA was used in PCR experiments. Amplification products were separated on 2% (w/v) agarose gels after 30 cycles (for β-actin) or 40 cycles for integrin-α4 and Rb1. PCR products were subcloned and verified by DNA sequencing. Quantitative PCR reactions were performed using the LightCycler FastStart DNA Master SYBR Green kit and the Light-Cycler thermal cycle system (Roche Applied Science) according to the manufacturer’s instructions. Densitometrical analysis of PCR fragment band intensities using conventional PCRs was performed using the Scion Image program (Scion Corp.).

**Microarray Analysis**—RNA extraction and purification from a minimum of 40 placental tissues at stage E9.5 were performed as described (see above). Synthesis of double-stranded cDNA, generation of biotinylated cRNA, hybridization to Mouse Genome430_2.0 arrays (Affymetrix, Santa Clara, CA), washing, staining, and scanning in a GeneChip Scanner3000 of the arrays were done as recommended in the Affymetrix Expression s2_manual (41). Signal intensities and expression changes were determined using the GCOS1.4 software (Affymetrix). A scaling across all probe sets of a given array to a target intensity of 1000 was included to compensate for variations in the amount and quality of the cRNA samples and other experimental variables.

**In Situ Hybridization Experiments**—Extra-embryonic placental tissues from mice at E9.5 were dissected and fixed in 4% paraformaldehyde, deposited in 12-well plates, and hybridized with sense or antisense RNA specific for integrin-α4, Rb1, Dlx3, or GCMa according to protocols previously described (32).
RESULTS

Overview of Genes Differentially Expressed in the Absence of GCMa in the Placenta—To identify placental GCMa target genes, we decided to analyze the pattern of genes down-regulated in the absence of GCMa. To this end, we dissected chorions of murine GCMa+/+ or GCMalacZ/lacZ placentae and extracted RNA for further use in microarray studies. Previous reports demonstrated normal development of GCMa-ablated chorions until embryonic stage E9.5. Thereafter the labyrinth layer failed to form (14, 15). We collected RNA from wild-type and mutant (GCMalacZ/lacZ) chorions at E9.5. At this embryonic stage, syncytiotrophoblast differentiation and tissue morphogenesis were not impaired in mutant chorions (14, 15). We dissected >200 chorions, used the associated embryos for DNA isolation and genotyping (data not shown), and grouped the different chorions according to their genotype as GCMa+/+, GCMa+/lacZ, GCMa+/lacZ, or GCMa−/−. As shown before, the genotype of the chorions followed strictly Mendelian rules of inheritance (14, 15). Although no haploinsufficiency was ever observed for GCMa heterozygotes, we decided to compare for further studies only RNAs derived from GCMa+/lacZ chorions with GCMa+/+ chorions. We used >40 chorionic tissues of each genotype, extracted RNA, and performed Affymetrix microarray studies. Using stringent analysis parameters for data mining analysis (signal log ratio >1.1 or <−1.1; p value <0.001 or >0.999), 66 genes were up-regulated, and 11 genes were down-regulated (Table 1; for up-regulated genes, see supplemental Table 2). Transcript amount of GCMa itself was significantly down-regulated in GCMa-deficient chorion tissue (Table 1). The down-regulation of GCMa transcript amount is explainable by loss of GCMa mRNA parts complementary to array probes as the array probes are directed against exon 6 of GCMa, whereas exons 3–6 are deleted in GCMa+/lacZ tissue (15). Although integrin-α4 mRNA level was down-regulated by only 0.76-fold on the array, we decided to further examine its level because integrin-α4 is known to play a fundamental role during choriogenesis at E9.5 and might therefore be a target of GCMa (33). Until now, all reported GCMa targets were shown to be induced by GCMs (21–23, 27, 28, 34). Therefore, we decided to focus only on the genes down-regulated in the absence of GCMa in the placenta as they might be induced as primary targets by GCMa.

Analysis of Differentially Expressed GCMa Targets by Quantitative RT-PCR—To verify the GCMa target genes identified by microarray technology, we performed quantitative RT-PCR of all 11 down-regulated candidates. As template, we generated first strand cDNA from RNAs extracted from dissected chorions of GCMa+/+ or GCMa-deficient placentae. Most strongly down-regulated were transcript levels of integrin-α4 and retinoblastoma (Rb1) gene (0.01- and 0.32-fold, respectively; Table 1). Previously, it was reported that human GCMa regulates the transcription of the fusogenic syncytin gene important for syncytiotrophoblast formation. By quantitative RT-PCR studies, we also observed for the first time a significant down-regulation of murine syncytin A in GCMa-deficient chorions (0.52-fold; Table 1, Fig. 1A).

Using conventional RT-PCR, we even failed to observe a PCR-amplified fragment for integrin-α4 after gel electrophoresis (Fig. 1B). In the case of Rb1, the amount of PCR-amplified fragment was significantly reduced (Fig. 1B).

Further, we quantified the degree of down-regulation of integrin-α4 and Rb1 transcript levels by densitometrical analysis (Fig. 1C). Note that the degree of down-regulation of integrin-α4 and Rb1 is similar independent of using either microarray or quantitative RT-PCR experiments (compare Table 1 with Fig. 1C).
upstream of the ATGs of integrin-α4 and Rb1 genes. In fact, several putative GCM binding sites are present within both promoters (Fig. 2, A and B).

To find out which of these GCM binding sites are of importance for transcriptional activation, we created a series of consecutive 5′-deletions for each promoter, transiently transfected these reporter plasmids in cultured cells, and examined their inducibility by GCMa (Fig. 3, A–C). We detected for none of the four 5′-deletions of the integrin-α4 promoter reporters the same high transcriptional activation as for the whole 6-kb fragment observed (Figs. 2A and 3, A and B). Additionally, if we used only a 513-bp fragment upstream of the ATG of integrin-α4 for reporter studies, we measured an 8.3-fold induction. Accordingly, our data demonstrate that the relevant GCMa-dependent transactivation sites are located within the integrin-α4 promoter between −5022 and −2009 and downstream of −513. For the Rb1 promoter, we generated three consecutive 5′-deletions of the used −6-kb promoter (Fig. 3A). GCMa induced all Rb1 reporter constructs except −399 to +150, indicating that GCMa-dependent transactivation of Rb1 gene requires the sites at −675 and −449 (Fig. 3C).

Analysis of Spatial Expression of GCMa Targets Integrin-α4 and Rb1 by in Situ Hybridization of Murine Placentae—So far, our data proved the impact of integrin-α4 and Rb1 as potential placental GCMa targets by in vitro methodology. To verify both genes as GCMa targets in vivo, we decided to analyze their spatial transcription pattern by whole mount in situ hybridization of placenta. We used placenta of mice at E9.5. At this time point, no signs of impaired tissue development was observed in GCMa-deficient placentae (14, 15). First, we analyzed the spatial transcription profile of GCMa and mammalian Distal-less homolog Dlx3, which is expressed in the labyrinthine layer in a subset of trophoblast cells (15). As reported previously, in GCMa-deficient placentae, the transcription of Dlx3 was not affected (data not shown) (15). In placental tissue from GCMa+/+ mice, we detected GCMa in the layer of the placenta containing the labyrinthine trophoblasts as shown before (14, 15). In GCMa-ablated placentae, we failed to detect any signal for GCMa transcripts (Fig. 4, A and B). In agreement with the in vivo data, we also failed to detect transcripts of integrin-α4 and only a weak signal intensity of Rb1, arguing for rare transcription of the latter in the labyrinthine layer of placenta (Fig. 4B). We evaluated by densitometrical analysis the observed pattern and obtained less than 1% of the signal intensity found in GCMa+/+ placentae for integrin-α4 and 31.4% for Rb1 in GCMa-deficient placentae (Fig. 4C).

DISCUSSION

The transcription factor GCMa has been shown to play a fundamental role as master regulator conducting trophoblast
Placental GCMa Targets

A

FIGURE 4. Spatial expression profiles of integrin-α4 and Rb1 in wild-type or GCMa-deficient placentae at E9.5. A, a scheme of a placenta tissue at E9.5 days postcoital. The boxed area was used for whole mount in situ hybridization experiments. B, images of placentae cut in half after whole mount in situ hybridization and staining using probes as indicated on the left. C, densitometrical analysis of staining intensities of images shown in B. GCMa+/+ = wild-type, GCMam2/m2 = mutant.

differentiation and syncytiotrophoblast formation (14, 15). Accordingly, GCMa is expected to be important in different human placental pathologies. In fact, several reports correlated changed GCMa expression levels with pre-eclampsia (16, 17). Additionally, GCMa was shown to induce gene expression of the human fusogenic syncytin gene, which plays a crucial role during differentiation of trophoblast to syncytiotrophoblast (27). In this respect, it fits that syncytin was also shown to be down-regulated in pre-eclampsia (36).

For better understanding of how GCMa is involved in placental pathologies, we decided to search for the first time by genome-wide analysis for mammalian placental GCMa target genes. We compared by array studies GCMa+/+ and GCMa-deficient murine chorionic tissue and identified 11 down-regulated genes in the absence of GCMa (Table 1). Human syncytin, a protein encoded by the envelope gene of a recently identified endogenous defective human retrovirus, human endogenous hetrovirus-W (HERV-W), is highly expressed in placental tissue, and as mentioned above, regulated by GCMa. Previously, a mouse syncytin homologue was not believed to exist, but recently, two murine genes of substantial homology were identified, and one of them (syncytin A) was shown to play a role in mouse placental development (37, 38). In fact, we confirmed by this study that murine syncytin A is also a GCMa target (Table 1). Aromatase as another described target of GCMa in humans could be neglected in this study as, curiously, aromatase is not expressed in mouse placenta (39). Finally, we focused on the characterization of the two most strongly down-regulated genes in the absence of GCMa in murine placenta, namely integrin-α4 and Rb1.

As a matter of fact, the promoters of both integrin-α4 and Rb1 contain a number of potential GCM binding sites. Our effort to map both promoters revealed that for integrin-α4, regions containing GCM binding sites at positions −3252, −2458, −2355, −317, and −225 are crucial for strong induction of integrin-α4 promoter activity (Fig. 3B). In the case of Rb1, two GCM binding sites at positions −675 and −449 are effectively regulating GCMa-dependent Rb1 gene expression as side effects such as abortions due to impaired placental integrin-α4 expression (33). It might be worth considering whether other known drugs leading to abortions are causally associated with yet unknown impaired placental GCMa expression. Data from another study indicated that a VCAM1-integrin-α4 interaction is necessary for the induction of GCMa expression (31). In agreement with those previous data, our data suggest that GCMa and integrin-α4 regulate their expression in an interdependent manner.

Recently, excessive proliferation of trophoblast cells and a severe disruption of the normal labyrinthine architecture in the placenta were identified as the actual cause of the embryonic lethality of Rb1-deficient mice (35). Surprisingly, our studies identified Rb1 and integrin-α4 as targets of GCMa, placing GCMa in a very key position in development of trophoblast cells, reminiscent of its prototypical role as master regulator of gliogenesis in D. melanogaster. It appears that it is a common theme that GCM proteins regulate very important steps of developmental processes. Further studies are necessary to understand the role of GCMa in human placental pathologies.

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