Stimulation of GCMa and syncytin via cAMP mediated PKA signaling in human trophoblastic cells under normoxic and hypoxic conditions

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Abstract Glial cells missing a (GCMa) belongs to a new transcription factor family. Synctin was shown to be a target gene of GCMa. Here, we demonstrate that the protein kinase A (PKA) pathway acts upstream of GCMa. After transient transfection of BeWo cells with PKA, GCMa transcriptional activity and both GCMa and syncytin transcripts were upregulated. This increase was accompanied by further cellular differentiation. Using normoxic or hypoxic conditions to mimic pathophysiological settings known to diminish trophoblast differentiation, we found that gene repressive effects of oxygen deficiency were compensated by the induction of the PKA pathway. We propose that GCMa-driven syncytin expression is the key mechanism for syncytiotrophoblast formation.
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1. Introduction

Gial cells missing a (GCMa) is a mammalian member of a recently identified transcription factor family, characterized by a zinc-coordinating DNA binding domain at the amino-terminus. This DNA binding domain is composed of two subdomains each largely consisting of β-sheets. The interaction of GCM family members with their cognate octameric GCM binding motif 5'-ATGCCGGT-3' is mediated by contacts between amino acid residues of the β-sheets and the major groove of the DNA [1]. All members of the GCM family so far identified are key regulators of differentiation processes [2]. In mammals, GCMa gene expression is restricted to placentas, kidney and thymus [3–5]. Ablation of GCMa in mice resulted in midembryonal death due to failure to develop a functional placental syncytiotrophoblast layer [6,7]. During embryonic development, the syncytiotrophoblast provides the embryo with nutrients and oxygen. The human syncytiotrophoblast originates from adjacent cytotrophoblast cells. The basic mechanisms of syncytiotrophoblast formation and the regulatory influence of oxygen still have to be elucidated. A known key molecule for placental syncytium formation is syncytin, a fusogenic glycoprotein originally derived from an endogenous retrovirus [8,9]. Syncytin was reported as one of the first placental target genes stimulated by GCMa [10]. Its production in the placenta is followed by placental differentiation processes, such as the formation of intercellular gap junctions, cell syncytialization, and an increase in β-human chorionic gonadotropin (β-HCG) secretion [11]. Accordingly, the expression pattern of the gap junction forming connexins, such as connexin-43 (Cx43) is related specifically to the stage of placental differentiation [12,13].

Pre-eclampsia, clinically defined by hypertension and proteinuria [14], is still one of the main causes of maternal and perinatal morbidity. Although the pathogenesis is unexplained, an abnormal placental development is an underlying cause along with alterations in local oxygen availability [15,16]. It was shown that syncytin is downregulated under hypoxic conditions at the transcriptional level [17,18]. Furthermore, a diminished syncytin gene expression and deregulated protein distribution are found in placentas of pre-eclamptic women [19,20]. It seems likely that in pre-eclampsia placental oxygen deficiency is responsible for the reduction of syncytin transcription. Additionally, decreased placental GCMa mRNA and protein levels have been found in placentas of pre-eclamptic women [21]. For some time, molecular mechanisms of the embryonic lethality of GCMa-deficient mice were considered syncytin independent, since no syncytin was identified in rodents. But recently, two murine syncytin genes of retroviral origin have been identified, giving rise to the assumption that GCMa-deficient mice die due to missing syncytin gene expression in murine syncytiotrophoblast progenitor cells [22]. Here, we elucidate upstream GCMa/syncytin signaling mechanisms by demonstrating that the dibutyryl-cAMP or 8-Br-cAMP (cAMP)-driven protein kinase A (PKA) signaling pathway stimulates syncytin and GCMa expression in both primary trophoblasts and BeWo choriocarcinoma cells. The

Abbreviations: cAMP, either dibutyryl-cAMP or 8-Br-cAMP; Cx43, connexin-43; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; β-HCG, β-human chorionic gonadotropin; PKA, protein kinase A; FAM, 6-carboxy-fluorescein; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; GCMa, glial cells missing a; LTR, long terminal repeat; TAMRA, 6-carboxy-tetramethylrhodamine; GFP, green fluorescent protein; RFP, red fluorescent protein.

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PKA-dependent signaling pathway also stimulates GCMa transcriptional activity in BeWo choriocarcinoma cells. Subsequently, we found that hypoxia-mediated downregulation of GCMa and syncytin expression is compensated by concurrent induction of the PKA signaling pathway.

2. Materials and methods

2.1. Cell culture

BeWo cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and cultivated as described [17]. After approval by the local ethics committee, term trophoblast cells were isolated using a trypsin-DNA-cell percoll gradient centrifugation method [23,24] and cultured in DMEM with 10% fetal calf serum (FCS). Cells were grown at 37 °C under normoxia with 95% air, 5% CO₂, or low oxygen conditions with either 3% or 1% O₂ (Forma Scientific). Oxygen tension was 142 mmHg for the normoxic setting compared to 21 and 7 mmHg in the hypoxic setting. For further experimental studies, cells were seeded into triplicate wells of 6-well-plates (Life Technologies) at a density of 1 × 10⁵ cells/cm² and, after adherence, maintained under normoxic or hypoxic conditions for 24–48 h in the presence or absence of different stimuli and chemicals as indicated. Stimulation of the cAMP pathways was performed using 100 µM forskolin or 150 µM cAMP. For the inhibition of de novo protein synthesis 30 min before cAMP treatment 100 µg of cycloheximide (Sigma Chemicals) per ml medium was added. All experiments were repeated at least three times.

2.2. Cell vitality and detection of apoptosis

Loss of viability was ruled out by using trypan blue staining. Apoptosis was detected by fluorescence-activated cell sorting (FACS) with Annexin-V and propidium iodine double staining (Alexis Biochemicals), using the FACS Calibur flow cytometer and CellQuest software (Becton–Dickinson) as published earlier [25].

2.3. Fluorescence immunocytochemistry

Configuration of gap junctions was shown by immunolabeling for Cx43 and cell fusion by a reduction of the amount of desmoplakin. Cells were fixed with 4% paraformaldehyde and incubated with a rabbit polyclonal antibody reactive against Cx43 (dilution 1:200), followed by an Alexa 488-conjugated goat anti-rabbit IgG (dilution 1:250) treatment [26]. For desmoplakin analysis, cells were fixed with ice-cold monochloroacetone. A monoclonal antibody against desmoplakin was used (dilution 1:200) followed by an Alexa 488-conjugated goat-anti-mouse IgG (dilution 1:250) treatment. All antibodies were obtained either from Chemicon or MoBiTEC. Western Blot was performed using a BX60 microscope (Olympus) and the software and camera system F-View II-Kamera (Soft Imaging System).

2.4. Molecular analysis and real-time PCR

Reverse transcription (RT) of 1.5 µg to 1 mg total RNA was performed after RNA extraction with the phenol/guanidine isothiocyanate method followed by DNase-I digestion [17]. GCMa expression studies were carried out using the LightCycler-FastStart DNA Master SYBR Green kit and the LightCycler Thermal Cycle system (Roche Applied Science) [27]. Gene expression of syncytin was quantified using real-time PCR. For this purpose, we applied in BeWo cells and in primary term human trophoblasts or BeWo cells incubated with lower oxygen doses. As reporter dye (6-carboxy-fluorescein, FAM) at the transfection efficiency. For TaqMan PCR, fluorogenic probes have been used, containing a reporter dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3’-end [17]. The following RT-PCR primers have been used: Syncytin 5’GTGACCTATACCTGCCGTGTAA3’, 5’GGTTTTTGGGCCGAGACCT3’, 5’FAM-CCTATTTAATACCCTCAC-TGGGCTCCA-(TAMRA)3’, 5’AGCACGGCATGCTGAACCAAC3’, 5’GCCGTGTTGAGCTGTA3’. The CMV expression plasmids encoding the DNA-binding domain of GCMa (GCMa-220) have been described [27]. As marker for GCMa-220 co-transfected cells a CMV expression plasmid encoding red fluorescent protein (RFP) (Clontech) was used. Statistical analysis was performed using unpaired two-tailed t-test or Mann–Whitney test.

2.5. Transfection, luciferase assays, extract preparation and Western blot

A luciferase reporter plasmid containing six tandemly arranged modules of GCM-binding sites and a TATA box was transiently transfected into BeWo cells to measure the activity of endogenous GCMa protein [28]. For luciferase assays 60-mm dishes were used and transfected with 2 µg of total DNA. Transfected cells were either incubated with 100 µM forskolin or 150 µM cAMP for up to 48 h. At 48 h post-transfection, cells were harvested for luciferase assays or extract preparation as described [27]. Luciferase activities were compared with mock-treated cells. Experiments were performed in duplicate three independent times. A polyclonal rabbit serum against the carboxyl-terminal half of GCMa served as primary antibody (1:3000 dilution), horseradish-peroxidase-coupled-protein A as secondary detection agent in Western blots using the ECL detection system (Amersham Biosciences).

2.6. Hormone measurements

Active free β-hCG in culture media was measured using an enzyme-linked immunosorbent assay kit (DRG International) with a sensitivity of 1.0 mIU/mL and inter- and intra-assay variation coefficients of less than 10%.

3. Results

3.1. GCMa and syncytin gene expression is stimulated by cAMP and preserved during concomitant hypoxia

Hypoxia was triggered by incubation of BeWo cells with forskolin or cAMP for 24–48 h enhanced syncytin expression by 3–4-fold (Fig. 1A). Primary human trophoblasts exhibited an even higher response than 10%.

Stimulation of BeWo cells with forskolin or cAMP for 24–48 h enhanced syncytin expression by 3–4-fold (Fig. 1A). Primary human trophoblasts exhibited an even higher response after incubation with either of the two compounds by up to 8-fold compared to un-stimulated control cells (Fig. 1B). Subsequently, we studied the influence of lower oxygen levels, because a decrease of oxygen concentration in the placenta results in disturbed placentogenesis such as pre-eclampsia. Considering the low level of oxygen in the placental tissue we incubated the trophoblastic cells either with 3% or with 1% O₂.
simultaneously treated with forskolin or cAMP (Fig. 1A,B).
Therefore, the hypoxia-related decline of syncytin expression in BeWo cells and trophoblasts was, to a great extent, preventable by the co-stimulation with cAMP agonists (Fig. 1A,B).

Subsequently, we studied the effect of cAMP agonists on the transcription of GCMa. A previous report demonstrated syncytin as target of GCMa, suggesting that PKA dependent upregulation of GCMa might be responsible for the increase of syncytin transcripts via two GCMa-binding sites in the up-stream region of the 5'-LTR of the syncytin gene [10]. As observed for syncytin, the treatment with either forskolin or cAMP for 24–48 h under normoxic conditions increased the expression of endogenous GCMa up to 5-fold in BeWo cells compared to un-stimulated control cells (Fig. 1C). Under hypoxic conditions, GCMa transcript amounts were significantly downregulated in both untreated BeWo cells and primary trophoblasts (Fig. 1C,D; \( P = 0.003, P = 0.012 \), respectively). When BeWo cells cultured under low oxygen levels were treated with cAMP agonists, a comparable increase in GCMa transcript levels was observed as for BeWo cells grown under normoxic conditions (Fig. 1C). Interestingly, cAMP agonists counteract a loss of GCMa transcripts in primary trophoblasts grown under hypoxia to a smaller extent compared to cells cultured under normoxia (Fig. 1D). To test whether the stimulation of the PKA pathway also increased endogenous GCMa protein activity, we carried out transient transfection on BeWo cells with a reporter built from six tandemly arranged modules of synthetic GCMa binding sites and a TATA box 5' to a luciferase gene. When cells were incubated post-transfection with either forskolin or cAMP agonists for 48 h, we observed an up to 3-fold increase in luciferase activity compared to mock-treated controls (Fig. 1E).
3.2. Cell viability, morphological and functional differentiation

To study functional differentiation of trophoblastic cells we measured β-HCG production and secretion in the cell supernatant. As expected, the increase of GCMa mRNA and protein activity along with syncytin gene transcription in trophoblastic cells was accompanied by a time-dependent β-HCG accumulation in the cell medium (Fig. 2A). Furthermore, with additional forskolin stimulation over 48 h, we observed 35-fold higher concentrations for β-HCG. After 48 h of cell growth under hypoxic conditions, cAMP-stimulated β-HCG concentrations in the cell culture medium were 25-fold higher than under control conditions. For hypoxic experiments, it was of interest to control for cell viability and apoptotic response. When investigating the apoptotic rate, we found a mean apoptotic rate of 4–10% in normoxic and 3–21% in hypoxic trophoblastic cells and a mean cell viability of 79–93% for both BeWo cells and primary trophoblasts.

We also analyzed the presence of Cx43, a major gap junctional protein in trophoblast. Cx43 is localized, for example, between cytotrophoblastic cells and the syncytiotrophoblast, allows intracellular communication, and is associated with the fusion process. Trophoblastic cells exhibited an intensive punctuate Cx43 immunostaining during cell differentiation predominantly at the cell boundary and around nuclei (labeled with 4,6-diamidine-2-phenylindole dihydrochloride, DAPI) as early as 24 h after the onset of incubation with cAMP agonists or forskolin compared to unstimulated control cells (Fig. 2B–E). This phenomenon is indicative for an increase of intercellular gap junction formation. Adenylate cyclase activator forskolin preserved Cx43 immunofluorescence at the cell boundaries and around nuclei under hypoxia with 3% or 1% O2 for 24 h compared to hypoxic cells without cAMP stimulation (Fig. 2D,E). As expected, activation of the cAMP/PKA pathway led to a diminished immunofluorescent staining for desmosomal protein desmoplakin and an increased number of DAPI-stained nuclei within syncyta compared to standard culture conditions, whereas the co-application of hypoxic stimuli and forskolin led to a weaker diminution of desmoplakin immunoreactivity, in accordance with a reduced but continuing cell fusion (data not shown).

![Fig. 2. Adenylate cyclase activator forskolin stimulates functional and morphological differentiation under normoxia and is largely protective under hypoxia. Treatment of BeWo cells with cAMP agonists induces functional differentiation as depicted on increased β-HCG secretion in the cell culture supernatant (A). Measurements were performed by ELISA from triplicates. P-values <0.01. Immunocytochemical detection of gap junctional protein Cx43 in BeWo cells incubated under normoxic conditions without (B) and with 100 μM forskolin (C) for 24 h and under 1% O2 in the presence (D) or absence of forskolin (E) for the same period. Cx43 is visible as green punctuate immunofluorescence at the cell boundaries and around nuclei. Cell nuclei were labeled with DAPI (blue color). In forskolin stimulated cells Cx43 punctuate immunofluorescence is increased. Hypoxia diminishes Cx43 immunostaining compared to normoxia. Hypoxia-related arrest of gap junctional Cx43 immunoreactivity is, to a great extent, compensated for by the induction of cAMP/PKA pathways.](image)

3.3. Endogenous GCMa and syncytin expression increased in BeWo cells transfected by a constitutively active catalytic PKA subunit

Up to now, our data indicate that the stimulation of PKA signaling leads to an increase of GCMa transcript levels, which most likely is responsible for a concomitant increase of the amount of syncytin transcript. To investigate the involvement of the PKA pathway directly, we analyzed if constitutively active PKA expression in trophoblastic cells is able to increase the transcription rate of GCMa and syncytin. We therefore transfected the catalytic subunit of PKA into BeWo cells, and performed RNA extraction after 48 h followed by cDNA synthesis and real-time PCR to test for a potential increase of either GCMa or syncytin transcript levels. Although only ≈10% of the BeWo cells were transfected as evidenced by co-transfected GFP (data not shown), we still observed a 2-fold increase of the amount of GCMa transcripts (Fig. 3A). Next, we determined if the increase of GCMa mRNA correlates with an increase of GCMa protein activity. Indeed, we observed an almost 8-fold increase in luciferase activity if we co-transfected plasmids containing the catalytical subunit of PKA together with a GCM-dependent luciferase reporter (Fig. 3B). At the same time, we observed a 3-fold increase of the amount of syncytin transcripts (Fig. 3C). Indeed, by transiently transfecting BeWo cells with the catalytical subunit of PKA and immunostaining them with the marker Cx43, we observed a far stronger signal compared to BeWo cells transfected with the empty vector (Fig. 3D,E). This demonstrates that PKA activity is sufficient to increase cell differentiation in trophoblastic cells.
3.4. PKA-mediated stimulation of GCMa requires de novo protein synthesis and stabilizes GCMa protein

We tested if de novo protein synthesis is required for the stimulation of GCMa and syncytin expression by PKA. For this purpose, we pretreated BeWo cells with cycloheximide and incubated them with cAMP over different time periods, followed by the determination of GCMa and syncytin transcripts using real-time RT-PCR. In BeWo cells not treated with cycloheximide, the amount of GCMa mRNA increased after treatment of cells with cAMP (Fig. 4A). Interestingly, a significantly higher increase of the mRNA level of GCMa was observed after simultaneous treatment with cycloheximide for 6–8 h (Fig. 4A). This increase of GCMa transcripts is comparable to an increase of transcript amounts of several proteins in response to cycloheximide treatment, like c-fos [29], presumably due to mRNA stabilization. Syncytin transcript levels increased slightly after cAMP treatment for up to 8 h (Fig. 4B). Surprisingly, in the presence of cycloheximide we observed higher levels of syncytin transcripts (Fig. 4B).

The half-life of GCMa protein is approximately 2.5 h under standard cell culture conditions [30]. To analyze whether GCMa protein is stabilized in the presence of PKA, we transiently transfected GCMa alone or together with the catalytical subunit of PKA into BeWo cells. We observed a significantly higher amount of GCMa protein in GCMa plus PKA co-transfected BeWo cells than in cells only transfected with GCMa (Fig. 4C). Since endogenous GCMa protein is not detected at the short exposure time used for detection of the Western blot in control cells or after transfection with PKA alone, we conclude that transfected GCMa protein is stabilized in the presence of PKA activity, thereby stimulating the transcription of the syncytin gene in the absence of de novo GCMa protein synthesis.

3.5. A dominant-negative GCMa protein inhibits cell fusion events in choriocarcinoma cells

Previously, it has been demonstrated that syncytin is a direct target of GCMa, since the syncytin promoter contains two GCM-binding motifs [10,31]. We asked if under PKA-activated conditions there is still a link between GCMa/syncytin expression and cellular differentiation. First, we proved that a protein fragment of GCMa, the isolated GCMa DNA-binding domain, is able to act as a dominant-negative mutant. Transient transfection of a GCMa DNA-binding domain (GCMa-220) containing an expression plasmid together with full length GCMa almost completely abolished GCMa-dependent reporter gene expression (Fig. 5A). We then transiently transfected BeWo cells with GCMa-220 together with RFP. After incubation of these cells with cAMP for 24 h we immunostained them with Cx43 as marker for cell differentiation and gap junction formation. A considerable lower amount of Cx43 staining indicated that RFP-labeled cells do not differentiate further, most likely due to blocked GCMa and syncytin expression (Fig. 5B,C).

4. Discussion

So far, upstream pathways regulating GCMa/syncytin signaling were entirely unknown. Our data illustrate that the GCMa/syncytin signaling is upregulated by the cAMP-driven PKA pathway. The in vivo impact of our results is supported by the fact that PKA signaling stimulates GCMa and syncytin expression in primary trophoblasts. Therefore, GCMa controlled syncytin expression by PKA might be a key mechanism for human syncytiotrophoblast formation.

It has been demonstrated previously that PKA regulates transcription through phosphorylation [32,33]. Indeed, using
bioinformatics we succeeded in identifying two such PKA phosphorylation sites within the primary sequence of mouse GCMa (data not shown). Thus, GCMa might be directly phosphorylated by PKA activity and as a consequence be stimulated in its transcriptional activity. Another possibility would be that phosphorylated GCMa has a longer half life thus indirectly increasing GCMa amounts and activity. Indeed, we observed that GCMa protein is stabilized in response to PKA activity. In addition to activation and stabilization of GCMa through phosphorylation by PKA it might also be possible that PKA activates cofactors through phosphorylation such as CREM binding protein, CBP. Previously, CBP has been shown to act jointly with proteins such as CREM/CREB/ATF1 or NFkB [34,35] and might also be able to associate and thereby modulate the DNA binding capacity of GCMa. Furthermore, stimulation of GCMa activity is also arranged through increased expression of the GCMa gene, as shown in this study. Transcription factors such as CREM or CREB may be activated by PKA and directly interact with the GCMa promoter. Interestingly, binding motifs for CREM and CREB are localized in the GCMa promoter (data not shown).

Concomitantly in this study, we demonstrate that hypoxia-related downregulation of syncytin transcription in trophoblasts can be, to a great extent, compensated by stimulating the cAMP-driven PKA pathway, facilitating further cellular differentiation of syncytiotrophoblasts, as evidenced by an increase of gap junction formation. Interestingly, under hypoxic conditions the transcription of GCMa was compensated to a lower degree compared with the transcription of syncytin by stimulation of the PKA pathway. This may indicate that, at

Fig. 4. Increase of syncytin transcript levels in BeWo cells after inhibition of de novo protein synthesis is due to GCMa protein stabilization. BeWo cells were incubated with cAMP alone or together with cycloheximide (CHX) as indicated. Endogenous mRNA levels of GCMa (A) or syncytin (B) were then measured by real-time RT-PCR, normalized to β-actin. P-values <0.05. BeWo cells were transiently transfected with the empty vector, GCMa, PKA, or GCMa plus PKA. GCMa protein was detected from cell extracts by western blot (C). Note that in the presence of PKA the amount of GCMa protein is considerably higher.

Fig. 5. A dominant-negative GCMa protein blocks PKA-GCMa-synctin mediated increase of Cx43. Transient transfection of BeWo cells with six tandemly arranged GCM-binding motifs containing Luciferase as reporter together with either GCMa alone or GCMa and a dominant-negative GCMa fragment (A; GCMa-220). P-values <0.05. BeWo cells were transiently co-transfected with RFP (as marker for the identification of transfected cells) and a dominant-negative GCMa fragment. After stimulation with cAMP for 24 h, cells were immunostained for Cx43 as a marker for functional cellular differentiation. A representative example of two RFP labelled cell (B), exhibiting less intense Cx43 immunostaining is depicted (C, circled area).
least under hypoxia, syncytin gene expression is modulated not only by increased GCMa expression.

During syncytialization the cell-to-cell communication requires Cx43-dependent formation of gap junctions for the flux of signal molecules such as cAMP and Ca2+. Previous findings show that Cx43 can be regulated by cAMP, β-HCG, estrogens, and possibly by syncytin in human trophoblasts and may be directly involved in the formation of syncytiotrophoblast [26]. Cx43 may, therefore, be a putative effector molecule for the cAMP/PKA mediated GCMa-syncytin pathway. In support of this assumption, it was demonstrated that in granulosa cells Cx43-type gap junctions are regulated by PKA [36]. It was reported that syncytin gene expression is downregulated under hypoxia and that diminished syncytin transcription and deregulated protein distribution can be found in placentas from pre-eclamptic women [19, 20]. We therefore propose that an altered syncytin activation downstream the cAMP-driven PKA-GCMa signaling cascade is a major risk factor for diminished trophoblast differentiation and impaired syncytiotrophoblast formation, further increasing the risk of placental dysfunction in the course of placental hypoxia. Elucidation of the underlying mechanisms of oxygen variation has shown that hemoproteins and mitochondria may play a pivotal role in the detection of local oxygen tension. Additionally, adaptive response to oxygen deprivation is mediated through the increase of HIF-1α protein, a mechanisms that may be affected in pre-eclampsia [37].

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