Involvement of Epac1/Rap1/CaMKI/HDAC5 signaling cascade in the regulation of placental cell fusion

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ABSTRACT: The placental transcription factor glial cell missing 1 (GCM1) and its target gene syncytin-1 are involved in cAMP-stimulated trophoblastic fusion for syncytiotrophoblast formation. GCM1 DNA-binding activity is inhibited by sumoylation, whereas GCM1 stability is decreased by deacetylation. CAMP enhances GCM1 desumoylation through the Epac1/Rap1/CaMKI signaling cascade and CaMKI is known to down-regulate class IIa HDAC activity. In this paper, we study whether the Epac1/Rap1/CaMKI signaling cascade regulates GCM1 activity and placental cell fusion through class IIa HDACs. Interaction and co-localization of GCM1 and HDAC5 were characterized by co-immunoprecipitation analysis and immunofluorescence microscopy (IFM). Regulation of GCM1 transcription activity and syncytin-1 expression by HDAC5 was studied by transient expression. Phospho-specific antibodies against HDAC5, RNA interference and IFM were used to examine the de-repression of GCM1 activity, syncytin-1 expression and cell–cell fusion by Epac1/Rap1/CaMKI signaling cascade in placental BeWo cells expressing constitutively active Epac1 and CaMKI. We demonstrate that both GCM1 and HDAC5 are expressed in the syncytiotrophoblast layer of full-term placenta and the nuclei of BeWo cells. The interaction between HDAC5 and GCM1 facilitates GCM1 deacetylation and suppresses its transcriptional activity. In contrast, Epac1 stimulates HDAC5 phosphorylation on Ser259 and Ser498 in a Rap1- and CaMKI-dependent manner leading to nuclear export of HDAC5 and thereby de-repression of GCM1 transcriptional activity. Importantly, HDAC5 suppresses syncytin-1 expression and cell–cell fusion in BeWo cells, which is counteracted by Epac1 and CaMKI. Our results reveal a new layer of regulation of GCM1 activity and placental cell fusion through the Epac1/Rap1/CaMKI signaling cascade by restraining HDAC5 from interacting with and mediating GCM1 deacetylation.

Key words: HDAC5 / GCM1 / Epac1 / placenta / cell–cell fusion

Introduction

Human placenta is composed of villous tissues the outer surface of which is covered by a multinucleated cell layer termed syncytiotrophoblast. Cell–cell fusion of subjacent mononucleated cytotrophoblasts is required for formation of the syncytiotrophoblast layer. Such cell fusion events involve the fusogenic membrane protein syncytin, which is regulated by the placental transcription factor glial cell missing 1 (GCM1; Mi et al., 2000; Yu et al., 2002). GCM1 is a labile protein that has been subjected to a variety of post-translational modifications. Specifically, the FBW2 F-box protein in the SCFFBW2 E3 ligase complex recognizes Ser322-phosphorylated GCM1 to facilitate GCM1 ubiquitination (Yang et al., 2005; Chiang et al., 2009). In contrast, GCM1 activity is positively stimulated by cAMP signaling. In this scenario, protein kinase A (PKA) activated by cAMP facilitates the association of GCM1 and dual-specificity phosphatase 23, which dephosphorylates Ser322 and promotes interaction between the CERB-binding protein (CBP) and GCM1 (Lin et al., 2011). As a result, CBP acetylates and stabilizes GCM1 and functions as a co-activator of GCM1-mediated transcriptional activation (Chang et al., 2005).

Protein acetylation is a reversible modification in which histone deacetylases (HDACs) are crucial for removing acetyl groups from modified lysine residues. In addition, recruitment of HDACs to co-repressor complexes such as N-CoR/SMRT and NuRD facilitates histone deacetylation resulting in transcriptional silencing. Interestingly, the functions of class IIa HDACs (HDAC4, -5, -7 and -9) are regulated by phosphorylation in response to environmental cues (Haberland et al., 2009; Parra and Verdin, 2010). For instance, Ser259 and Ser498 phosphorylation in HDAC5 is mediated by calmodulin-dependent protein kinase I/IV (CaMKI/IV) in C2C12 myoblasts undergoing myogenesis or by protein kinase D in the cardiomyocytes under hypertrophic stress (McKinsey et al., 2000; Vega et al., 2004). Phosphorylated HDAC5 recruits 14-3-3 resulting in...
nuclear export of HDAC5 into cytoplasm (Grozinger and Schreiber, 2000). Consequently, myocyte enhancer factor 2 in stimulated cells dissociates from HDAC5 and thereafter associates with transcriptional co-activator p300 to activate expression of skeletal muscle or hypertrophic genes (Slepak et al., 2001; Haberland et al., 2009).

Exchange protein directly activated by CAMP I (EpacI) is a CAMP-binding protein with guanine nucleotide exchange factor (GEF) activity for small GTPases, Ras-related protein 1 (Rap1) and Rap2. Structural analysis indicates that binding of cAMP induces a conformation change in EpacI and directly relieves autoinhibition of GEF activity to activate Rap GTPases (Glericher and Bos, 2010). Our previous study indicates that sumoylation of GCM1 suppresses its DNA-binding activity (Chou et al., 2007). In this context, we have further demonstrated that cAMP can activate the EpacI/Rap1/CaMKI signaling cascade to enhance interaction between GCM1 and SUMO1/sentrin-specific peptidase 1 (SENP1), which desumoylates GCM1 and thereby elevates GCM1 activity in placental cells (Chang et al., 2011).

Although HDAC4 and HDAC5 are involved in the regulation of skeletal muscle differentiation, it remains unclear whether they participate in placental cell differentiation. Furthermore, we were curious about whether the cAMP/EpacI/Rap1/CaMKI signaling pathway modulates HDAC4 and -5 to regulate GCM1 activity and placental cell fusion. Here, we demonstrated that HDAC5 exhibits higher GCM1-binding efficiency than HDAC4 and both GCM1 and HDAC5 are co-localized in the nuclei. Moreover, HDAC5 mediates GCM1 deacetylation and suppresses its transcriptional activity. Interestingly, constitutively active EpacI stimulates Ser259 and Ser498 phosphorylation in HDAC5 depending on both Rap1 and CaMKI. Both EpacI and CaMKI facilitate nuclear export of HDAC5 and enhance GCM1 acetylation and synctin-1 expression in placental BeWo cells. Importantly, fusogenic activity of BeWo cells is suppressed by HDAC5, which can be reversed by EpacI and CaMKI. Therefore, cAMP may activate the EpacI/Rap1/CaMKI signaling cascade to counteract the suppressive effect of HDAC5 on GCM1 activity leading to increased synctin-1 expression and placental cell fusion.

Materials and Methods

Plasmid constructs

pHDAC4-FLAG, pHDAC5-FLAG, pHDAC3-Myc and pHA-GCM1 expression plasmids and GCM1-specific reporter plasmid, p(GBS)4E1BLuc, have been described previously (Chuang et al., 2006). The pHDAC52A-FLAG expression plasmid is similar to pHDAC-FLAG except that the encoded HDAC5 harbors a serine-to-alanine mutation in CaMKI phosphorylation sites, Ser259 (S259) and Ser498 (S498). A lentivirus strain harboring an HDAC5-FLAG expression cassette has been established using a modified pCDH expression plasmid (SBI, Mountain View, CA). The pSyn1(27 950/28 314)LUC and different combinations of pHDAC5-FLAG, pHA-GCM1 and pCACaMKI expression plasmids using TransIT LT1 reagent (Mirus, Madison, WI). To study the effect of HDAC5 and CaMKI on GCM1 transcriptional activity, 293T or BeWo cells were transfected with p(GBS)4E1BLuc reporter plasmid and different combinations of pHDAC5-FLAG and pCACaMKI expression plasmids. At 48 h post-transfection, cells were harvested for luciferase assays as previously described (Chen et al., 2000). Specific luciferase activities were normalized by protein concentration. Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL).

Co-immunoprecipitation analysis

To study interaction between GCM1 and HDAC4 or HDAC5, 293T cells were transfected with pHA-GCM1 and pHDAC4-FLAG or pHDAC5-FLAG for 48 h. Cells were then harvested in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM DTT, 5 mM NaF, 1 mM Na3VO4 and a protease inhibitor cocktail (Sigma, St Louis, MO) for consecutive immunoprecipitation and immunoblotting with HA and FLAG Abs (Sigma). Densitometry analysis was performed using the ImageJ software to quantify the relative binding efficiency between GCM1 and HDAC4 or HDAC5. The interaction of endogenous GCM1 and HDAC5 was studied by immunoprecipitation of BeWo cells and primary cytotrophoblast cells with normal rabbit IgG or GCM1 Ab and immunoblotting with a rabbit anti-HDAC5 Ab (Abcam, Cambridge, UK). To study regulation of HDAC5 phosphorylation by CaMKI and EpacI, 293T cells were transfected with pHDAC5-FLAG or pHDAC52A-FLAG and primary cytotrophoblast cells with normal rabbit IgG or GCM1 Ab and immunoblotting with a rabbit anti-HDAC5 Ab (Abcam, Cambridge, UK). To study interaction between GCM1 and HDAC4 or HDAC5, 293T cells were transfected with pHA-GCM1 and pHDAC4-FLAG or pHDAC5-FLAG for 48 h. Cells were then harvested in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM DTT, 5 mM NaF, 1 mM Na3VO4 and a protease inhibitor cocktail (Sigma, St Louis, MO) for consecutive immunoprecipitation and immunoblotting with HA and FLAG Abs (Sigma). Densitometry analysis was performed using the ImageJ software to quantify the relative binding efficiency between GCM1 and HDAC4 or HDAC5. The interaction of endogenous GCM1 and HDAC5 was studied by immunoprecipitation of BeWo cells and primary cytotrophoblast cells with normal rabbit IgG or GCM1 Ab and immunoblotting with a rabbit anti-HDAC5 Ab (Abcam, Cambridge, UK). To study regulation of HDAC5 phosphorylation by CaMKI and EpacI, 293T cells were transfected with pHDAC5-FLAG or pHDAC52A-FLAG and different combinations of expression plasmids for CAEpacI, CACAcaMKI, shRap1 and shCaMKI. At 48 h post-transfection, cells were harvested for immunoprecipitation with FLAG Ab and immunoblotting with anti-Ser259- and anti-Ser498-HDAC5 Abs (Abcam), respectively. In a separate experiment, BeWo cells were transduced with lentivirus strain harboring empty vector (CDH), CAEpacI and CACAcaMKI expression cassettes to generate mock (CDH) BeWo cells, CAEpacI- and CACAcaMKI-expressing BeWo cells (named as CDH-, CAEpacI- and CACAcaMKI-BeWo cells in this study), respectively. The CAEpacI-BeWo cells were split into three groups and further transduced with a lentivirus strain for scramble, Rap1 or CaMKI#1 shRNA for 72 h, followed by antibiotic selection with 5 μg/ml puromycin for an additional 48 h. The above-described groups of BeWo cells were harvested for immunoprecipitation with HDAC5 Ab and immunoblotting with anti-Ser498-HDAC5 Ab.

Immunohistochemistry and immunofluorescence microscopy

Full-term human placental tissues were fixed with formalin and embedded in paraffin wax. After deparaffinization, the sections were incubated with normal rabbit serum (NRS), normal mouse serum, HDAC5, cytokeratin 7 and placental cell fusion. BeWo cells as previously described (Chen et al., 2000). Specific luciferase activities were normalized by protein concentration. Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL).

Co-immunoprecipitation analysis

To study interaction between GCM1 and HDAC4 or HDAC5, 293T cells were transfected with pHA-GCM1 and pHDAC4-FLAG or pHDAC5-FLAG for 48 h. Cells were then harvested in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM DTT, 5 mM NaF, 1 mM Na3VO4 and a protease inhibitor cocktail (Sigma, St Louis, MO) for consecutive immunoprecipitation and immunoblotting with HA and FLAG Abs (Sigma). Densitometry analysis was performed using the ImageJ software to quantify the relative binding efficiency between GCM1 and HDAC4 or HDAC5. The interaction of endogenous GCM1 and HDAC5 was studied by immunoprecipitation of BeWo cells and primary cytotrophoblast cells with normal rabbit IgG or GCM1 Ab and immunoblotting with a rabbit anti-HDAC5 Ab (Abcam, Cambridge, UK). To study regulation of HDAC5 phosphorylation by CaMKI and EpacI, 293T cells were transfected with pHDAC5-FLAG or pHDAC52A-FLAG and different combinations of expression plasmids for CAEpacI, CACAcaMKI, shRap1 and shCaMKI. At 48 h post-transfection, cells were harvested for immunoprecipitation with FLAG Ab and immunoblotting with anti-Ser259- and anti-Ser498-HDAC5 Abs (Abcam), respectively. In a separate experiment, BeWo cells were transduced with lentivirus strain harboring empty vector (CDH), CAEpacI and CACAcaMKI expression cassettes to generate mock (CDH) BeWo cells, CAEpacI- and CACAcaMKI-expressing BeWo cells (named as CDH-, CAEpacI- and CACAcaMKI-BeWo cells in this study), respectively. The CAEpacI-BeWo cells were split into three groups and further transduced with a lentivirus strain for scramble, Rap1 or CaMKI#1 shRNA for 72 h, followed by antibiotic selection with 5 μg/ml puromycin for an additional 48 h. The above-described groups of BeWo cells were harvested for immunoprecipitation with HDAC5 Ab and immunoblotting with anti-Ser498-HDAC5 Ab.
(CK7) or GCM1 Ab and sequentially incubated with biotin-conjugated secondary Ab and horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Antigenic detection was performed using the chromogenic substrate 3,3′-diaminobenzidine tetrahydrochloride, and the sections were further counterstained with hematoxylin.

For cellular localization of GCM1 and HDACS, 293T cells were transfected with pH-A-GCM1 and pHADC5-FLAG. At 48 h post-transfection, cells were fixed with paraformaldehyde, permeated and incubated with HA or FLAG Ab. Subsequently, cells were incubated with FITC-conjugated FLAG and TRICT-conjugated HA Abs (Sigma). On the other hand, co-localization of GCM1 and HDACS in BeWo cells was performed using a guinea pig anti-GCM1 Ab and rhodamine-conjugated anti-rabbit IgG Abs (Jackson ImmunoResearch).

For regulation of HDACS nuclear export by Epac1 and CaMKI, 293T cells were transfected with pHADC5-FLAG plus the empty pCDH, which expresses GFP via an independent promoter, or the pCDH plasmid harboring CAEpac1 or CACaMKI. At 48 h post-transfection, cells were fixed and stained with FITC-conjugated FLAG Ab. In a separate experiment, the CDH-, CAEpac1- and CACaMKI-BeWo cells described in the ‘co-immunoprecipitation analysis’ subsection were fixed and stained with FITC-conjugated FLAG Ab. Immunofluorescence was examined under a Zeiss laser scanning confocal microscope (LSM510) (Carl Zeiss Microscopy, Jena, Germany). Images were prepared for presentation using Adobe Photoshop v7.0.

**GCM1 acetylation analysis**

Regulation of GCM1 acetylation by HDACS and CaMKI was investigated by transfection of 293T cells with different combinations of pH-A-GCM1, pHADC5-FLAG, pHADC5A-FLAG and pCACaMKI. At 48 h post-transfection, cells were harvested for immunoprecipitation with anti-acetylated-lysine (Ac-K) Ab (Cell Signaling, Beverly, MA) and immunoblotting with HA Ab. To study the roles of HDACS, CaMKI and Rap1 in regulation of GCM1 acetylation by Epac1, the CDH-, CAEpac1- and CACaMKI-BeWo cells were transfected with a lentivirus strain harboring an HDACS-FLAG expression cassette. Subsequently, the CAEpac1-BeWo cells co-expressing HDACS-FLAG were further transduced with a lentivirus strain for scramble, Rap1 or CaMKI#1 shRNA for 72 h, followed by antibiotic selection with 5 μg/ml puromycin for an additional 48 h. Cells were then harvested for immunoprecipitation with GCM1 Ab and immunoblotting with Ac-K Ab.

**Chromatin immunoprecipitation analysis**

The effect of HDACS on the acetylation of histone 3 lysine 9 (Ac-H3K9) in the syncytin-1 promoter was studied in the CDH-, CACaMKI- and CAEpac1-BeWo cells co-expressing HDACS-FLAG by chromatin immunoprecipitation (ChIP) analysis using Ac-H3K9 (Abcam) and RNA polymerase II (RNA Pol II, EMD Millipore, Billerica, MA) Abs. Primer sequences for PCR amplification of syncytin-1 promoter region harboring the proximal GCM1-binding site are 5′-GCCCATTTGCGATTGTAACATCTGCCAC-3′ and 5′-GCAAGATAATTGTGTATCCTCAGGC-3′.

**Cell fusion analysis**

A previously described co-culture system was applied to study the effects of HDACS, Epac1 and CaMKI on the fusogenic activity of BeWo cells (Yu et al., 2002). In brief, CDH-, CACaMKI- and CAEpac1-BeWo cells co-expressing with or without HDACS-FLAG described in the ‘GCM1 acetylation analysis’ subsection were co-cultured with 293T cells expressing mCherry fluorescent protein in 6-well tissue culture dishes for 24 h. Cells were then fixed and examined for syncytium formation under an Olympus fluorescence microscope equipped with a cooled charge-coupled device camera. Microscope images of the entire well for each sample were captured for quantification of fusion events and comparison of syncytium size in three independent experiments.

**Statistical analysis**

Data are presented as mean ± SD of three independent experiments. Differences were assessed by the Student’s t-test. A P-value of <0.05 was considered statistically significant (*P < 0.05; **P < 0.01).

**Results**

**Characterization of GCM1–HDACS interaction**

We examined the interaction between GCM1 and HDACS or HDAC5 by transfecting 293T cells with pH-A-GCM1, pHADC4-FLAG and pHADC5-FLAG expression plasmids, followed by co-immunoprecipitation analysis with HA and FLAG Abs. As shown in Fig. 1A, specific interaction between HDACS and GCM1 and between HDACS and HDAC5 was detected. Moreover, quantification by densitometry analysis indicated that HDACS exhibits higher GCM1-binding efficiency compared with HDAC4 (Fig. 1A, lower panel). Immunofluorescence microscopy (IFM) was conducted to further demonstrate nuclear co-localization of HA-GCM1 and HDACS-FLAG in 293T cells (Fig. 1B). Of note, HDAC4-FLAG signals were barely detected in the nuclei, which might underscore its lower binding efficiency to HA-GCM1 in the aforementioned co-immunoprecipitation analysis. We further demonstrated specific interaction between endogenous GCM1 and HDACS in placental BeWo cells using GCM1 Ab for immunoprecipitation and HDAC5 Ab for immunoblotting (Fig. 1C, left upper panel). A reciprocal co-immunoprecipitation experiment also demonstrated a specific interaction between GCM1 and HDACS (data not shown). Correspondingly, GCM1 and HDACS were co-localized in the nuclei of BeWo cells (Fig. 1C, right panel). The interaction between GCM1 and HDACS in placenta was also confirmed by co-immunoprecipitation analysis in primary cytotrophoblast cells purified from human full-term placenta (Fig. 1C, left lower panel). Finally, we examined the expression of GCM1 and HDACS in full-term human placenta by immunohistochemistry. As shown in Fig. 1D, both GCM1 and HDACS were detected in the nuclei of the syncytiotrophoblast layer, which expresses CK7 protein marker.

**Suppression of GCM1 acetylation and transcriptional activity by HDACS**

We now tested the effect of HDACS on GCM1 transcriptional activity. To this end, 293T cells were transfected with pH-A-GCM1 and p(GBS)E1BLuc, a GCM1-specific reporter construct, and increasing amounts of pHADC5-FLAG. As expected, the luciferase reporter gene is transactivated by HA-GCM1 in terms of luciferase activity (Fig. 2A). However, the observed transcriptional activation was suppressed by HDACS-FLAG in a dose-dependent manner (Fig. 2A). Likewise, the transcriptional activity of endogenous GCM1 was suppressed by HDACS-FLAG in BeWo cells transfected with p(GBS)E1BLuc and pHADC5-FLAG (Fig. 2B). We further studied the effects of trichostatin A (TSA), an HDACS inhibitor, on the regulation GCM1 transcriptional activity and acetylation by HDACS in placenta. To this end, BeWo cells were transduced with a lentivirus strain harboring empty vector (CDH) or HDACS-FLAG expression cassette. Cells were then transfected with...
p(GBS)ELuc in the presence or absence of TSA. As shown in Fig. 2C, TSA counteracted the suppressive effect of HDAC5-FLAG on GCM1 transcriptional activity in a dose-dependent manner (Fig. 2C). In a parallel experiment, the mock CDH-BeWo cells and HDAC5-FLAG-expressing BeWo cells treated with or without TSA were subjected to GCM1 acetylation analysis. As shown in Fig. 2D, the level of acetylated GCM1 decreased in the presence of HDAC5-FLAG, which was attenuated by TSA. Correspondingly, expression of the GCM1 target gene, syncytin-1, also decreased in the presence of HDAC5-FLAG, which was attenuated by TSA as well (Fig. 2D). Taken together, these results suggest that

Figure 1 Characterization of interaction between GCM1 and HDAC5. (A) GCM1 interacts with HDAC4 and HDAC5. 293T cells were transfected with different combinations of pHA-GCM1, pHDAC4-FLAG and pHDAC5-FLAG for reciprocal co-immunoprecipitation using HA and FLAG Abs. IP, immunoprecipitation; IB, immunoblotting. Densitometry analysis was performed for calculation of the relative band intensity ratios of GCM1 to HDAC (left) or HDAC to GCM1 (right). Each bar represents the means ± SD of three independent experiments. (B) Co-localization analysis of GCM1 and HDAC5. 293T cells were transfected with the expression plasmids described in (A) and subjected to IFM analysis as described in Materials and Methods. Nuclei were stained by DAPI (blue). (C) Interaction of GCM1 and HDAC5 in placental cells. BeWo cells (left upper) or primary cytotrophoblast cells (left lower) were subjected to co-immunoprecipitation with normal rabbit IgG, HDAC5 Ab and GCM1 Ab. In a separate experiment, BeWo cells were subjected to IFM analysis (right). (D) Immunohistochemistry of GCM1 and HDAC5 in placenta. Full-term human placenta sections were subjected to immunostaining for detection of HDAC5, GCM1 and CK7 expression. Note that zoom-in images are provided at the bottom of the panel. The inset shows a section immunostained by normal mouse serum. NRS, normal rabbit serum. Scale bar, 100 μm.
HDAC5 deacetylates GCM1 and suppresses its transcriptional activity to down-regulate syncytin-1 expression.

**Regulation of HDAC5 phosphorylation and nuclear export by Epac1 and CaMKI**

It is known that nuclear export of HDAC5 can be regulated by CaMKI (McKinsey et al., 2000; Vega et al., 2004). We have recently demonstrated that cAMP may activate the Epac1/Rap1/CaMKI signaling cascade to regulate GCM1 activity (Chang et al., 2011). This prompted us to investigate whether the aforementioned Epac1 signaling cascade may up-regulate GCM1 activity by facilitating HDAC5 nuclear export. Indeed, IFM revealed nuclear localization of HDAC5-FLAG in 293T cells and both CAEpac1 and CACaMKI facilitate HDAC5-FLAG nuclear export (Fig. 3A). We also tested whether Ser259 and Ser498 phosphorylation in HDAC5-FLAG is regulated by the Epac1 signaling cascade. As shown in Fig. 3B, Ser259 and Ser498 phosphorylation was stimulated by CAEpac1 and CACaMKI. However, the stimulatory effect by CAEpac1 was attenuated by CaMKI knockdown using two different shRNA expression constructs, shCaMKI#1 and #2 (lanes 4 and 5 in Fig. 3B). As a control, no Ser259 and Ser498 phosphorylation was detected in the HDAC52A-FLAG mutant in the presence of CACaMKI and CAEpac1 (lane 11 in Fig. 3B).

We now studied whether CAEpac1 and CACaMKI also regulate nuclear export of endogenous HDAC5 in BeWo cells. BeWo cells
were transduced with lentivirus strains harboring CAEpac1 and CACaMKI expression cassettes to generate CAEpac1- and CACaMKI-BeWo cells, respectively. By IFM, we demonstrated that HDAC5 nuclear export significantly increased in both CAEpac1- and CACaMKI-BeWo cells in comparison with mock CDH-BeWo cells (Fig. 3C). In addition, both CAEpac1 and CACaMKI stimulated Ser498 phosphorylation in HDAC5, which was abolished in the CAEpac1-BeWo cells further transduced with a lentivirus strain for shRap1 or shCaMKI (Fig. 3D). Therefore, both Rap1 and CaMKI are essential downstream effectors for Epac1-mediated HDAC5 phosphorylation and nuclear export in placental cells.

**Regulation of GCM1 activity by CaMKI and HDAC5**

Subsequently, we tested whether CaMKI can reverse the suppression of GCM1 transcriptional activity by HDAC5. To this end, BeWo cells were transduced with lentivirus strains harboring CAEpac1 and CACaMKI expression cassettes to generate CAEpac1- and CACaMKI-BeWo cells, respectively. By IFM, we demonstrated that HDAC5 nuclear export significantly increased in both CAEpac1- and CACaMKI-BeWo cells in comparison with mock CDH-BeWo cells (Fig. 3C). In addition, both CAEpac1 and CACaMKI stimulated Ser498 phosphorylation in HDAC5, which was abolished in the CAEpac1-BeWo cells further transduced with a lentivirus strain for shRap1 or shCaMKI (Fig. 3D). Therefore, both Rap1 and CaMKI are essential downstream effectors for Epac1-mediated HDAC5 phosphorylation and nuclear export in placental cells.

Figure 3  Regulation of HDAC5 nuclear export by Epac1 and CaMKI. (A) HDAC5 nuclear export is promoted by constitutively active Epac1 and CaMKI. 293T cells were transfected with different combinations of pHDAC5-FLAG, empty pCDH and the pCDH constructs for CAEpac1 and CACaMKI for IFM analysis of HDAC5-FLAG. (B) Regulation of S259 and S498 phosphorylation in HDAC5 by Epac1, Rap1 and CaMKI. 293T cells were transfected with different combinations of pHDAC5-FLAG, pHDAC52A-FLAG, pCAEpac1, pCACaMKI, pshCaMKI#1, pshCaMKI#2, pshsc (sc) and pshRap1, followed by co-immunoprecipitation with FLAG, anti-Ser259-HDAC5 and anti-Ser498-HDAC5 Abs. Note that Rap1 and CaMKI are required for HDAC5 phosphorylation regulated by CAEpac1. (C) Epac1 and CaMKI promote HDAC5 nuclear export in placental cells. BeWo cells were transduced with a lentivirus strain harboring empty vector (CDH), CAEpac1 or CACaMKI expression cassette for IFM analysis of HDAC5. (D) Stimulation of HDAC5 phosphorylation by Epac1 and CaMKI in placental cells. The CAEpac1-BeWo cells described in (C) were further transduced with lentivirus strains expressing scramble, Rap1 and CaMKI#1 shRNA, respectively. Cells were subjected to co-immunoprecipitation using HDAC5 and anti-Ser498-HDAC5 Abs.
Regulation of GCM1 acetylation by CaMKI and HDAC5

To study the effect of CaMKI on HDAC5-mediated GCM1 deacetylation, 293T cells were transfected with pHA-GCM1, pHDAC5-FLAG and pCACaMKI, followed by co-immunoprecipitation analysis with Ac-K and HA Abs. While the level of acetylated HA-GCM1 decreased under HDAC5-FLAG (compare lanes 2 and 3, left panel, Fig. 5A), this was counteracted and acetylated HA-GCM1 increased under CACaMKI (compare lanes 3 and 6, left panel, Fig. 5A). Recent studies have shown that class IIa HDACs, which lack intrinsic deacetylase activity, recruit HDAC3 and/or SIRT1 to facilitate deacetylation of target proteins (Fischle et al., 2002; Zhao et al., 2005). We tested whether HDAC5 interacts with HDAC3; and if yes, whether CaMKI affects the interaction between HDAC5 and HDAC3. To this end, 293T cells were transfected with different combinations of pHDAC5-FLAG, pHDAC52A-FLAG, pHDAC3-Myc and pCACaMKI, followed by co-immunoprecipitation analysis with FLAG and Myc Abs. As shown in the right panel of Fig. 5A, CACaMKI significantly impaired the interaction between HDAC3-Myc and HDAC5-FLAG, but not HDAC52A-FLAG (Fig. 5A, right panel). Therefore, while HDAC5 recruits HDAC3 to deacetylate GCM1, CaMKI regulates HDAC5 nuclear export and prevents GCM1 deacetylation by abolishing the HDAC5–HDAC3 interaction.
We further examined the effect of CaMKI and EpacI on HDAC5-mediated GCM1 deacetylation in CDH-, CACaMKI- and CAEpac1-BeWo cells transduced with HDAC5-FLAG-expressing lentivirus. We observed that HDAC5-FLAG mediates deacetylation of endogenous GCM1 (compare lanes 1 and 4, left panel, Fig. S5B). Both CACaMKI and CAEpac1 increased the level of acetylated GCM1 in the presence or absence of HDAC5-FLAG (lanes 2, 3, 5 and 6, left panel, Fig. S5B). Of note, the counteraction of HDAC5-FLAG-mediated GCM1 deacetylation by CAEpac1 was abolished in the CAEpac1-BeWo cells co-expressing HDAC5-FLAG when either CaMKI or Rap1 was knocked down (lanes 8 and 9, left panel, Fig. S5B). Along this line, CAEpacI counts on Rap1 and CaMKI to counteract HDAC5-FLAG and stimulate syncytin-1 expression (lanes 8 and 9, left panel, Fig. S5B). These results suggest that activation of Epac1/Rap1/CaMKI signaling cascade relieves the suppression of GCM1 acetylation and activity by HDAC5 to up-regulate syncytin-1 expression.

It has recently been reported that histone 3 lysine 9 (H3K9) in the syncytin-1 promoter is highly acetylated in BeWo cells (Trejbalova et al., 2011). Accordingly, we studied whether HDAC5, CaMKI and Epac1 affect H3K9 acetylation in the syncytin-1 promoter in the CDH-, CACaMKI- and CAEpac1-BeWo cells co-expressing HDAC5-FLAG by ChIP analysis. As shown in the right panel of Fig. 5B, none of the three factors significantly affected H3K9 acetylation in the syncytin-1 promoter.

**Regulation of placental cell fusion by HDAC5**

Because syncytin-1 mediates placental cell fusion, we investigated the interplay between HDAC5, CaMKI and Epac1 in regulation of BeWo cell fusion. To this end, CDH-, CACaMKI- and CAEpac1-BeWo cells co-expressing with or without HDAC5-FLAG were co-cultured with 293T cells expressing mCherry fluorescent protein. The fusogenic activity of BeWo cells was examined by the number of fusion events and the size of syncytium. Compared with CDH-BeWo cells, the number of fusion events increased in CAEpac1- and CAEpac1-BeWo cells by 1.93- and 1.55-fold, respectively (left panel, Fig. 5C). Interestingly, the size of syncytium was much larger in the CAEpac1- and CAEpac1-BeWo cells than CDH-BeWo cells (a–c, right panel, Fig. 5C). In contrast, both fusion events and syncytium size dramatically decreased in CDH-BeWo cells co-expressing HDAC5-FLAG (compare a and d, right panel, Fig. 5C), suggesting that HDAC5 suppresses the fusogenic activity of BeWo cells. Importantly, the number of fusion events in CAEpac1- and CACaMKI-BeWo cells increased by 6.75- and 5.91-fold, respectively, compared with CDH-BeWo cells in the presence of HDAC5-FLAG (left panel, Fig. 5C). Correspondingly, the size of syncytium in CAEpac1- and CACaMKI-BeWo cells co-expressing HDAC5-FLAG increased (d–f, right panel, Fig. 5C). These results suggested that HDAC5 suppresses BeWo cell fusion, which can be reversed by Epac1 and CaMKI.

**Discussion**

cAMP signal transduction is crucial for regulation of placental cell fusion and differentiation (Wice et al., 1990; Keryer et al., 1998). Given that cell surface antigen CD98, connexin 43 and phosphatidylserine exposure have been implicated in the regulation of placental cell fusion, the underlying mechanisms remain elusive (Lyden et al., 1993; Kudo et al., 2003; Dunk et al., 2012). Instead, syncytin fusogenic proteins, which are derived from endogenous retroviral envelope proteins, have been characterized as functional effectors for placental cell fusion. Importantly, expression of both syncytin-1 and its upstream regulator GCM1 are stimulated by cAMP and silencing of either syncytin-1 or GCM1 by anti-sense oligonucleotides or siRNA impedes cell—cell fusion of BeWo cells (Mi et al., 2000; Frendo et al., 2003; Baczynski et al., 2009). Indeed, we have demonstrated that GCM1 activity can be regulated by cAMP at the post-translational level in placental cells. Specifically, the cAMP/PKA/DUSP23/CBP signaling pathway protects GCM1 from ubiquitination and enhances GCM1 acetylation and stability (Lin et al., 2011). Moreover, the cAMP/Epac1/Rap1/CaMKI/SENP1 signaling pathway facilitates GCM1 desumoylation and maintains DNA-binding activity of GCM1 (Chang et al., 2011).

In the present study, we first demonstrated that HDAC5 co-localizes with GCM1 in the nucleus and facilitates GCM1 deacetylation as well as represses its transcriptional activity. Correspondingly, HDAC5 suppresses the expression of GCM1 target gene, syncytin-1 and placental cell fusion. We have previously shown that activation of Epac1 by the
cAMP analog, 8-(4-chlorophenylthio)-2′-O-Me-cAMP-AM, results in Rap1 and CaMKI activation in placental cells (Chang et al., 2011). Here, we used constitutively active Epac1, CAEpac1, to mimic Epac1 activation by cAMP. We showed that CAEpac1 may activate Rap1 and CaMKI to stimulate Ser259 and Ser498 phosphorylation in HDAC5 and nuclear export of HDAC5. Concomitantly, the Epac1/Rap1/CaMKI signaling cascade increases GCM1 acetylation and activity, which facilitates syncytin-1 expression and cell–cell fusion (Fig. 5D). Therefore, the present study reveals a suppressor role for HDAC5 in regulation of GCM1 activity and placental cell differentiation and a new cAMP signaling pathway through Epac1, Rap1 and CaMKI to counteract the suppressive effects of HDAC5 in placental cell differentiation.

We demonstrated that treatment with 25 ng/ml of TSA reverses the suppressive effect of exogenous HDAC5 on GCM1 transcriptional activity in BeWo cells (Fig. 2C). Interestingly, TSA at a higher concentration (50 ng/ml) not only counteracts the HDAC5 suppressive effect but also enhances GCM1 transcriptional activity. This raises the possibility that additional HDACs may be involved in suppression of GCM1 transcriptional activity in BeWo cells. Indeed, our previous study has shown that class I HDAC3 also mediates GCM1 deacetylation and down-regulates its transcriptional activity (Chuang et al., 2006). It has been demonstrated that class Ila HDACs may recruit HDAC3 and/or SIRT1 to deacetylate target proteins (Fischle et al., 2002; Zhao et al., 2005). Here we also observed that HDAC5 interacts with HDAC3, which is impaired by CaMKI most likely due to enhanced nuclear export of HDAC5 (Fig. 5A). Therefore, GCM1 activity may be regulated by both class I and class Ila HDACs in placenta (Fig. 5D).

Being a regulator of skeletal muscle differentiation, HDAC5 also plays an important role in osteoclast and adipocyte differentiation. HDAC5 deacetylates and represses the nuclear factor of activated T-cells 1 (NFATc1) in osteoclast precursor cells to impair RANKL-induced osteoclast differentiation (Kim et al., 2011). The expression of glucose transporter 4 (GLUT4) is repressed by HDAC5 in 3T3-L1 preadipocytes. Nevertheless, HDAC5 relocates to the cytosolic compartment and MEF2 is de-repressed and transactivates GLUT4 expression in differentiated 3T3-L1 adipocytes (Weems and Olson, 2011). In the present study, we further demonstrated that HDAC5 participates in placental cell differentiation through regulation of GCM1 activity and cell fusion. Together with our previous findings, the cAMP signal transduced by the Epac1/Rap1/CaMKI pathway bifurcates to relieve the suppression of GCM1 transcriptional activity by HDAC5 and to reverse the inhibition of GCM1 DNA-binding activity by sumoylation. Importantly, both events converge to elevate GCM1 activity in placental cells.

**Authors’ roles**


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**References**


Grozinger CM, Schreiber SL. Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. *Proc Natl Acad Sci USA* 2000; 97:7835–7840.


Lyden TW, Ng AK, Rote NS. Modulation of phosphatidylserine epitope expression by BeWo cells during forskolin treatment. Placenta 1993; 14:177–186.


