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Hautala, Laura C.

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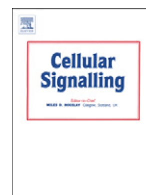

Repressed PKCδ activation in glycodelin-expressing cells mediates resistance to phorbol ester and TGFβ

Laura C. Hautala, Riitta Koistinen, Hannu Koistinen *

Department of Clinical Chemistry, Medicum, University of Helsinki and Helsinki University Central Hospital, Haartmaninkatu 8, 00290 Helsinki, Finland

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ABSTRACT

Glycodelin is a glycoprotein mainly expressed in well-differentiated epithelial cells in reproductive tissues. In normal secretory endometrium, the expression of glycodelin is abundant and regulated by progesterone. In hormone-related cancers glycodelin expression is associated with well-differentiated tumors. We have previously found that glycodelin drives epithelial differentiation of HEC-1B endometrial adenocarcinoma cells, resulting in reduced tumor growth in a preclinical mouse model. Here we show that glycodelin-transfected HEC-1B cells have repressed protein kinase C delta (PKCδ) activation, likely due to downregulation of PDK1, and are resistant to phenotypic change and enhanced migration induced by phorbol 12-myristate 13-acetate (PMA). In control cells, which do not express glycodelin, the effects of PMA were abolished by using PKCδ and PDK1 inhibitors, and knockdown of PKCδ, MEK1 and 2, or ERK1 and 2 by siRNAs. Similarly, transforming growth factor β (TGFβ)-induced phenotypic change was only seen in control cells, not in glycodelin-producing cells, and it was mediated by PKCδ. Taken together, these results strongly suggest that PKCδ, via MAPK pathway, is involved in the glycodelin-driven cell differentiation rendering the cells resistant to stimulation by PMA and TGFβ.

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1. Introduction

Glycodelin is a secreted glycoprotein with several well-characterized glycoforms [1]. It is expressed in differentiated epithelial cells, especially those of reproductive tissues, and hematopoietic cells of bone marrow, and is involved in cell recognition both in reproductive and immune systems [1]. Glycodelin is highly expressed in normal secretory endometrium, where its expression is regulated by progesterone [1]. In proliferative and postmenopausal endometrium the expression levels of glycodelin are very low, if present at all. In hormone-related cancers, glycodelin expression is reduced and more frequently observed in well-differentiated than in less differentiated tumors [2–4]. In malignant endometrium the expression is hardly detected [1]. Previously, coculturing Ishikawa endometrial adenocarcinoma cells with normal endometrial stromal cells in Matrigel basement membrane preparation has been shown to induce reversion of the cancer cells to resemble normal endometrial epithelium [5]. The differentiation was accompanied by induction of glycodelin synthesis. Similar effect was found when Ishikawa cells were exposed to histone deacetylase inhibitors (HDACIs) [6]. Furthermore, knockdown of glycodelin expression by RNA-interference blocked the reversion of the HDACI treated cells, indicating that glycodelin is crucial in this process. We have found that in HEC-1B endometrial adenocarcinoma cells glycodelin transfection changes the morphology of the cells when grown in 3D environment with Matrigel [7,8]. Glycodelin-producing cells were less cohesive and formed more acinar structures resembling those of normal endometrium [7]. In addition, the glycodelin-producing cells formed significantly smaller tumors in preclinical xenograft mice. Thus, it appears that glycodelin drives epithelial differentiation and changes cancer cells towards less malignant direction.

In HEC-1B cells gene expression changes induced by glycodelin were particularly enriched in mitogen-activated kinase (MAPK) pathways [7], which are involved in many crucial cellular processes such as cell growth, proliferation, differentiation, migration and apoptosis [9]. In these pathways, MAPKs are in central role mediating the effects of numerous extracellular and intracellular stimuli. Of MAPK pathways the extracellular signal-regulated kinase (ERK) pathway is commonly dysregulated in different cancers [9]. Several protein kinase C (PKC) isozymes have been reported to be involved in the activation of ERK pathway [10]. PKCs are serine/threonine protein kinases classified into three subgroups according to their activators. Classical or conventional PKCs (cPKCs; PKCα, PKCβI, PKCβII and PKCγ) are calcium dependent and activated by diacylglycerol (DAG), while novel PKCs (nPKC; PKCδ, PKCε, PKCη and PKCθ) are calcium independent, but activated by DAG, and atypical PKCs (aPKCs; PKCζ and PKCλ) are unresponsive both to calcium and DAG [10]. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), are able to act as analogues of DAG, thus effecting ERK pathway by activating classical and novel PKCs [11]. Partially because

* Corresponding author at: Department of Clinical Chemistry, Biomedicum Helsinki, Haartmaninkatu 8, P.O. Box 63, 00290 Helsinki, Finland.
E-mail address: hannu.k.koistinen@helsinki.fi (H. Koistinen).

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of this, phorbol esters are considered as tumor promoters [12]. In many cancers also different PKC isozymes are dysregulated [13]. However, the functions and effects of PKCs are very cancer and cell type specific, leading to opposing roles of PKC isozymes in different cancers where the same PKC isozyme can act both as a tumor promoter or suppressor [13]. Recently, it was reported that the majority of the cancer associated PKC mutations lead to inactivation of PKCs supporting their role as tumor suppressors [14].

ERK pathway can also be activated by transforming growth factor β (TGFβ) [15]. The activation of this noncanonical TGFβ pathway, through Ras, is dependent on the cell type [16]. Canonical, TGFβ pathway involves the activation of Smad proteins trough TGFβ type I and type II receptors [17]. Among several other functions, TGFβ drives epithelial-mesenchymal transition (EMT) of cancer cells, which leads epithelial cells to acquire mesenchymal characteristics with increased migratory and invasive capabilities [18]. As with PKCs, the effects of TGFβ are cell type specific and depend on cellular context [17].

Despite the evident role of glycodelin in epithelial differentiation the underlying mechanisms of this has not yet been delineated. Our previous results show that the MAPK pathway is a strong candidate for a mediator of the glycodelin-induced differentiation. One of the regulators of the classical MAPK pathway is PKCδ, the molecule which have been implicated in pathogenesis of different cancers, including endometrial cancer [19]. In the present study, we examined the effects of glycodelin on HEC-1B endometrial cancer cells and found that glycodelin expression reduces the activation of PKCδ, making the cells unresponsive to PMA- and TGFβ-induced morphological changes and migration trough the ERK pathway.

2. Materials and methods

2.1. Cell line and cell culture

Stable glycodelin-producing cell clones with full-length protein-encoding region (including the signal sequence) of glycodelin and control cell clones (containing glycodelin cDNA in antisense orientation) were created using HEC-1B human endometrial adenocarcinoma cells (HTB-113, American Type Culture Collection) as previously described [7]. HEC-1B cell clones were authenticated using microsatellite markers (Promega GenePrint 10 System) by the Institute for Molecular Medicine Finland FIMM Technology Centre, University of Helsinki. The concentration of glycodelin from the culture media was detected by an immunofluorometric assay. The glycodelin-transfected cells produced 34–235 ng glycodelin/10^6 cells/24 h while the control cells did not produce detectable amounts of glycodelin [7]. The cells were cultured at +37 °C in RPMI-1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidified atmosphere with 5% CO_2.

2.2. Western immunoblotting

Four different glycodelin-transfected and four control cell clones were cultured in 6-well plates (1×10^5 cells per well) in MEM (Invitrogen) with the same supplements as above. The cells were grown for 4 days, after which 0–2 nM PMA (Sigma-Aldrich) was added to the wells and incubated for an additional 4 h. To detect the levels of phosphorylated PDK1, or the effect of treatment of the cells with 10 nM PMA for 15 min on ERK1/2 phosphorylation or PKCδ (Thr505) phosphorylation when the cells were pretreated with 30 μM GSK2334470 (Sigma-Aldrich) for 30 min before addition of PMA, the cells were seeded on 6-well plates and incubated overnight before treatments.

The cells were lysed with sample buffer containing 2.5% β-mercaptoethanol, denatured by boiling and ~20 μg of protein was loaded onto SDS-polyacrylamide gel (NuPAGE® Novex® 4–12% Bis-Tris Gel, Life Technologies™). After electrophoresis the proteins were transferred to membranes, which were then blocked with 5% milk in TBS for 1 h at room temperature and incubated with the primary antibody overnight at +4 °C. After washing with TBS containing 0.1% Tween, membranes were first incubated with biotinylated antimouse or antirabbit IgG for 50 min at room temperature and then with streptavidin conjugated horseradish peroxidase (both from GE Healthcare). Detection was performed with ECL solution (Amersham). Quantitation was performed using Gene Tools (Syngene) or Image Lab 5.2.1 (Bio-Rad) softwares and normalized with β-actin (#A2066 from Sigma-Aldrich). The antibody against PKCδ (#610397) was obtained from BD Transduction Laboratories, and phospho-PKCδ (Thr505) (#9374), phospho-PKCs (Antibody Sampler kit, #9921), phospho-PDK1 (Ser241) (#3061), p44/42 MAPK (ERK1/2, #9102), phospho-p44/42 MAPK (pERK1/2, #4376), Smad2 (#5339) and phospho-Smad2 (#3108) were all from Cell Signaling Technology. Molecular masses were estimated using molecular weight markers (Precision Plus Protein™ Dual Color Standards, #1610374, Bio-Rad).

2.3. Determination of PKCδ translocation

The cells were grown overnight on glass coverslips and when ~80% confluent transfected with 0.5 μg pEGFP-C3 vector containing PKCδ cDNA and encoding a PKCδ-GFP fusion protein [20] (provided by Professor Yusuf A. Hannun) using JetPRIME transfection reagent (Polyplus-transfection Inc.) according to manufacturer's instructions. Generally 20–50% of the cells were found to be positive for GFP. After 24 h incubation the cells were exposed to 10 nM PMA for 10 min, washed with PBS, fixed with 4% PFA and mounted with VECTASHIELD mounting media containing DAPI (Vector Laboratories). Zeiss LSM 510 Meta confocal microscope and LSM software were used for imaging the cells.

2.4. Morphological changes

To monitor the morphological changes associated with PMA and TGFβ (R&D Systems) stimulation the cells were plated on Matrigel basement membrane preparation (Becton Dickinson) in a 96-well plate (4000 or 5000 cells per well) and incubated for 1 h before addition of 0–10 nM PMA or 5 ng/ml TGFβ. Some of the cells were preincubated 30 min with 30 μM GSK2334470, 10 μM Rottlerin (Sigma-Aldrich) or Bryostatin 1 (Calbiochem). Morphological changes were monitored under a light microscope.

2.5. RNA interference (RNAi)

siRNAs (ON-TARGETplus SMARTpool siRNA) for PKCδ (PRKCD), ERK1 (MAPK3), ERK2 (MAPK1), MEK1 (MAP2K1) and MEK2 (MAP2K2) were all purchased from Dharmacon Research, Inc. (sequences shown in Supplementary Table 1). For PKCδ four individual siRNAs of the SMARTpool were also obtained. The cells were seeded on 6-well plates and incubated overnight. When the cells were 50–80% confluent the transfections were performed using JetPRIME transfection reagent or Mirus TransIT-X2 System (Mirus Bio LLC) according to manufacturer's instructions (the final siRNA concentration 45 nM). Experiments were performed 48 h after transfection. ON-TARGETplus Non-targeting siRNA #1 (Dharmacon Research, Inc.) was used as a control.

2.6. Pathway reporter assay

The cells were plated on 96-well plates and incubated 24 h. The MAPK/ERK pathway activity was quantified by transfecting a mixture including inducible Elk-1/SRF transcription factor responsive construct with firefly luciferase reporter gene and a construct constitutively expressing Renilla luciferase for controlling transfection efficiency (Cignal Finder 10-Pathway Reporter Array, Qiagen). The transfection was performed using JetPRIME transfection reagent according to

manufacturer's instructions. After 24 h incubation the wells were washed and 0 or 10 nM PMA was added for 5 h 30 min. Luciferase activity was measured using Dual-Glo Luciferase Assay System according to manufacturer's instructions (Promega).

2.7. Cell migration

For wound healing test the cells were grown on 4-well chamber slides until they formed a monolayer after which scratches were made using a pipet tip. Chambers were washed with PBS and 0–10 nM PMA was added. Cells were monitored under microscope (Zeiss 3i Stallion HSI). Quantitation was performed by measuring the area of the cell monolayer after 0, 9 and 19 h using ImageJ software [21].

2.8. Statistics

Two-tailed student's *t*-test, assuming unequal variances, was used for the comparison of differences between the glycodelin-producing and control cells.

3. Results

3.1. Reduced activation of PKCδ in glycodelin-producing cells

Our previous results suggested that MAPK pathway is involved in the cell differentiating effect of glycodelin [7]. Therefore, in further analyses we concentrated on PKC isoforms which regulate the classical MAPK pathway. The level of phosphorylated PKCδ (Thr505) in glycodelin-producing cells was found to be significantly lower ($p = 0.04$) than in the control cells, while the total PKCδ level was similar in both cell types (Fig. 1A). The levels of phosphorylated PKC-α/βII, -ζ/λ and –μ were similar or undetectable in glycodelin-producing and control cells (data not shown). An exposure of four hours to PKC-activator PMA increased the phosphorylated PKCδ and downregulated total PKCδ levels in both cell types (Fig. 1A). However, even after exposure to PMA, the content of phosphorylated PKCδ in glycodelin-producing cells was considerably lower than in the control cells without PMA treatment, showing that the glycodelin-producing cells were less responsive to PMA. After 10 min exposure of the cells to 10 nM PMA, transfected PKCδ-reporter translocated to plasma membrane in both cell types (Fig. 1B), indicating that the activation of PKCδ can take

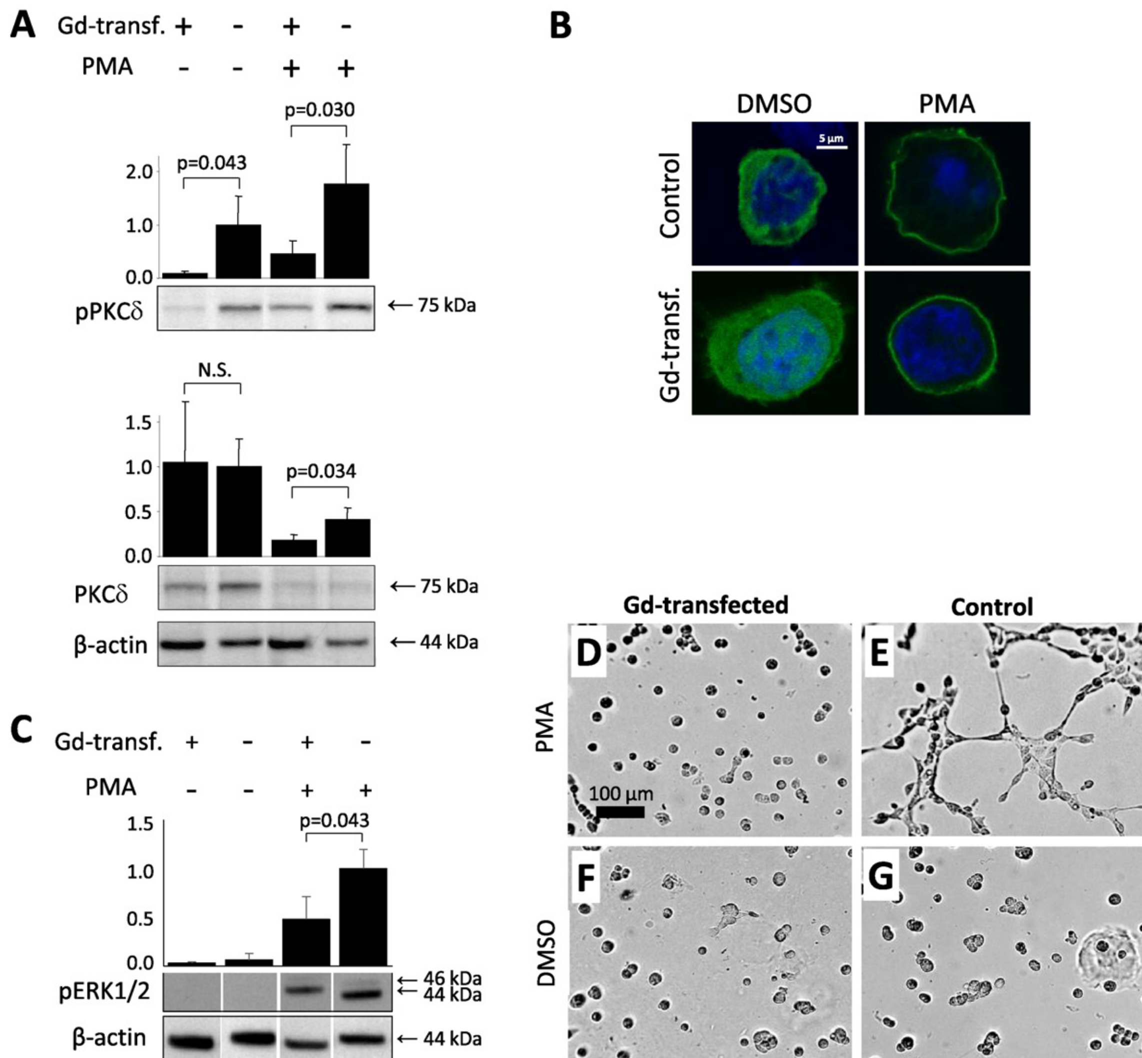

Fig. 1. Glycodelin-producing cells have reduced levels of phosphorylated PKCδ (Thr505) and reduced response to the PMA-induced phosphorylation of PKCδ and ERK1/2 (A, C). Cells were grown on plastic with and without 2 nM PMA. The relative levels of the proteins were detected by Western blotting and normalized against β-actin (mean + SD, quantitation is based on 3–4 cell clones in each group). Exposure of the cells to 10 nM PMA for 10 min induces translocalization of PKCδ-GFP fusion protein to plasma membrane both in glycodelin-producing and control cells (B). Glycodelin prevents PMA-induced morphological changes in HEC-1B cells. Live cell images of glycodelin-producing (D, F) and control cells (E, G) cultured on Matrigel and treated with 2 nM PMA (D, E) or with DMSO as a control (F, G) for 24 h. B and D–G are representative examples of 2–3 individual experiments with 2–4 different glycodelin-producing and control cell clones.

place in both cell types [22]. PMA treatment also induced ERK1/2 phosphorylation (Fig. 1C). Interestingly, the levels of phosphorylated PDK1, which participates in PKC maturation, were somewhat reduced in glycodelin-producing cells (0.58-fold as compared to control cells, $p > 0.05$, $n = 3$ for both). Furthermore, PDK1 inhibitor GSK2334470-treated control cells showed reduced PKCδ phosphorylation of Thr505 when exposed to PMA (0.47-fold, $p < 0.01$, $n = 3$).

3.2. Glycodelin-producing cells are resistant to phenotypic change induced by PMA and TGFβ

Previously we have found that glycodelin induces differentiation in the cells grown with Matrigel for ~2 weeks [7]. Since we found that the amount of phosphorylated PKCδ was reduced in glycodelin-producing cells, we studied whether stimulation of PKCδ by PMA affects the cell phenotype. When grown up to 24 h on glass surface the phenotypes of the cells were very similar after PMA stimulation, irrespective whether they expressed glycodelin or not. However, when the cells were cultured on top of Matrigel, PMA (10 or 2 nM) induced dramatic morphological changes in the control cells within 4 or 12 h incubation, respectively, where the cells formed net-like structures (Fig. 1D–G). However, the glycodelin-producing cells were virtually unresponsive. This effect of PMA in control cells could be blocked with a pool of four siRNAs that knocked down the expression of PKCδ and reduced PMA-induced ERK1/2 phosphorylation ($p = 0.01$) (Fig. 2A–C). Three of the siRNAs blocked the PMA-induced phenotypic differentiation also when used individually. Similarly, Rottlerin and Bryostatin 1, which inhibit PKCδ, and PDK1 inhibitor GSK2334470 blocked the phenotypic effect of PMA in the control cells, whereas they had no detectable effect on glycodelin-producing cells within 24 h observation (Fig. 2D–G and data not shown). Knockdown of both MEK1 and 2, or ERK1 and 2 by RNAi also prevented the effect of PMA in control cells (Fig. 2H–J), again indicating the involvement of MAPK pathway in PMA-induced phenotypic changes. To further study the involvement of MAPK pathway we used MAPK/ERK pathway activity reporter system. PMA increased the activity of the pathway 5.6 ± 0.5 fold in control cells, while in glycodelin-producing cells the increase was only 3.0 ± 1.1 fold ($p = 0.037$, $n = 3$ for both).

Addition of 5 ng/ml TGFβ to the cells grown on Matrigel induced similar morphological changes in the control cells as PMA (Fig. 3A). However, the changes were not seen until four days exposure to TGFβ. The effect of TGFβ was abolished by PKCδ siRNA (Fig. 3A) and

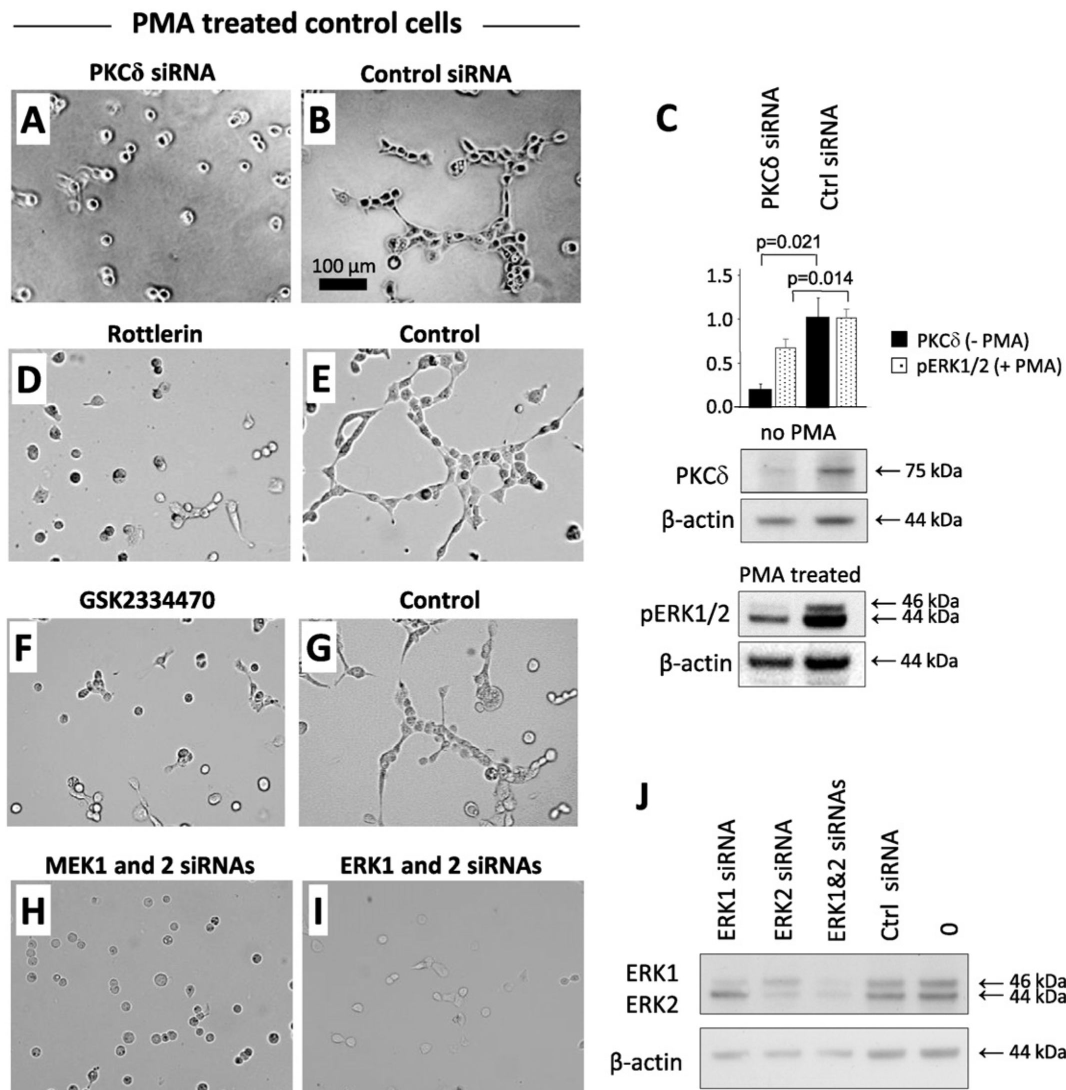

Fig. 2. PKCδ siRNA abolishes PMA-induced morphological differentiation in control cells grown on Matrigel (A, B). Downregulation of PKCδ using RNAi reduces also PMA-induced ERK1/2 phosphorylation (normalized against β-actin, mean + SD, quantitation is based on 3 cell clones in both groups) (C). The PMA-induced morphological differentiation was also abolished by preincubation of the cells with 10 μM Rottlerin (D, E) or 30 μM GSK2334470 (F, G), or RNAi knockdown of MEK1 and 2 (H), or ERK1 and 2 (I). Downregulation of ERK1 and 2 using RNAi (J). A–J are representative examples of 2–3 individual experiments with at least 3 different control cell clones.

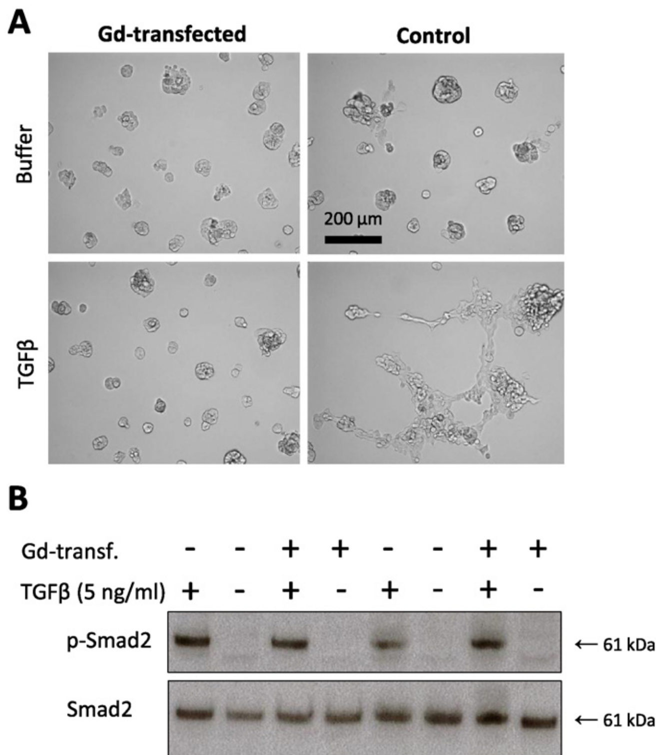

Fig. 3. (A) TGFβ induces morphological changes in HEC-1B control cells, while glycodelin-producing cells remain unresponsive. The cells were grown on Matrigel with or without 5 ng/ml TGFβ for four days. Representative examples from three different glycodelin-producing and control cell clones. (B) Similar phosphorylation of Smad2 after exposure of the cells to TGFβ for one hour was seen both in glycodelin-producing and control cells (two different clones for each).

Rottlerin (data not shown), strongly suggesting that PKCδ is also involved in mediating TGFβ-induced morphological changes. TGFβ-induced phosphorylation of Smad2 protein was found to be similar both in glycodelin-producing and control cells (Fig. 3B).

3.3. PMA-induced migration

In addition to morphological changes, PMA also increased the motility of the control cells grown on Matrigel, whereas the glycodelin-transfected cells remained unresponsive, spinning around themselves like the untreated cells (Fig. 4A and Supplementary data). In wound healing test, PMA caused a two-fold increase in the migration of control cells on glass surface, but had no effect on the migration of the glycodelin-transfected cells (Fig. 4B). PKCδ siRNA abolished the effect of PMA in control cells.

4. Discussion

Glycodelin has been shown to act as a driver of epithelial differentiation in two endometrial cancer cell lines, Ishikawa and HEC-1B cells [5–8]. Importantly, along with inducing epithelial differentiation, transfection of glycodelin into HEC-1B cells significantly reduced xenograft tumor growth in mice [7]. This together with clinical studies showing that high glycodelin expression is associated with more differentiated tumors and favorable prognosis in breast and ovarian cancer patients, respectively [2,3], suggest a tumor suppressing role for glycodelin. However, the underlying mechanisms mediating such an effect of glycodelin remain unraveled. In this study we show that glycodelin-producing HEC-1B endometrial adenocarcinoma cells have repressed response to the stimulation of PKCδ, which is likely to mediate the glycodelin-induced cell differentiation.

We found that in the glycodelin-transfected cells the level of phosphorylated (Thr505 in the activation loop), but not total PKCδ was significantly lower than in control cells. Phorbol esters, like PMA, have been found to stimulate the phosphorylation of Thr505 in PKCδ, which appears to be required for full activity of PKCδ and, thus, has been used as a surrogate marker for PKCδ activation [23]. When the cells were cultured in the presence of PMA, a well-known tumor promoter [12], repressed stimulation of phosphorylation of PKCδ was observed in glycodelin-producing cells as compared to control cells. Prolonged stimulation of cells with PMA has been found to induce downregulation of PKCs by proteolysis [24,25], a finding that is compatible with our observation on the downregulation of total PKCδ after long exposure of the carcinoma cells to PMA. When the cells were grown on Matrigel basement membrane preparation, PMA also induced dramatic morphological changes in the control cells, while the glycodelin-producing cells were virtually unresponsive. Rottlerin and Bryostatin 1, both of which inhibit PKCδ, blocked the effect of PMA in the control cells, while they had no effect on the glycodelin-producing cells. As the kinase inhibitors and activators, like Rottlerin and Bryostatin 1, are usually not highly specific, we also used the RNAi technique to knockdown the gene expression of PKCδ. Like Rottlerin and Bryostatin 1, PKCδ knockdown abolished the morphological changes caused by PMA. MEK1 and 2, and ERK1 and 2 siRNAs had the same effect, indicating that the effects of PMA are mediated via ERK pathway. Furthermore, MAPK/ERK pathway activity reporter system, based on ELK-1/SRF transcription factor, showed that PMA increases the activity of this pathway more in control cells than in glycodelin-producing cells. Noteworthy, this transcription factor is not entirely specific for MAPK/ERK pathway. These results suggest that glycodelin induces changes upstream of PKCδ, i.e., in the amount or activity of the factors involved in the phosphorylation of PKCδ.

The mechanisms of PKC activation are complex and, at least to some extent, dependent on the cell type. Before activation, PKCs undergo maturation, which involves series of phosphorylation steps, the first of which is mediated by PDK1 [10]. Mature novel PKCs, like PKCδ, can be activated by DAG or phorbol esters after which they are typically translocated to plasma membrane, which is considered as a hallmark of PKC activation [10,22]. Our results show that the levels of phosphorylated PDK1 tend to be reduced in glycodelin-producing cells. Furthermore, in control cells PDK1 inhibitor reduced PMA-induced PKCδ (Thr505) phosphorylation and phenotypic change. This strongly suggests that glycodelin-induced differentiation is mediated by reduced PDK1 levels, which results in reduced activation of PKCδ. However, when the cells overexpressing PKCδ-GFP fusion protein were exposed to PMA, translocation of the fusion protein to plasma membrane was observed both in glycodelin-producing and control cells, indicating that at least some activation of PKCδ can take place in both cell types. Noteworthy, it has been reported that the tight regulation of PKCδ phosphorylation at Thr505 is lost during PKCδ overexpression [26].

We found that PMA also increased the migration of the control cells, but not that of glycodelin-producing cells. Again this effect of PMA was abolished by PKCδ inhibitor and siRNA suggesting a crucial role for PKCδ also in mediating the migration of the cells. In keeping with this, glycodelin has been shown to reduce the migration of HEC-1B cells [27]. Contrary to HEC-1B cell studies, in Ishikawa endometrial adenocarcinoma cells HDACI-induced glycodelin expression has been reported to increase the migration [28]. Migration is one of the essential features for cancer cells required for metastatic dissemination [29]. However, it was speculated that glycodelin-induced migration of well-differentiated Ishikawa cells could rather be related to normal physiology of the endometrium than pathogenesis since glycodelin is expressed during the secretory phase when endometrial epithelial cells undergo morphological changes and migrate into the stroma. ERK pathway is one of the well-known pathways involved in cell migration [30] and PKCδ has been shown to mediate migration via ERK pathway in ovarian cancer cells [31]. PKCδ also enhances migration and motility of many other cell types [32–35].

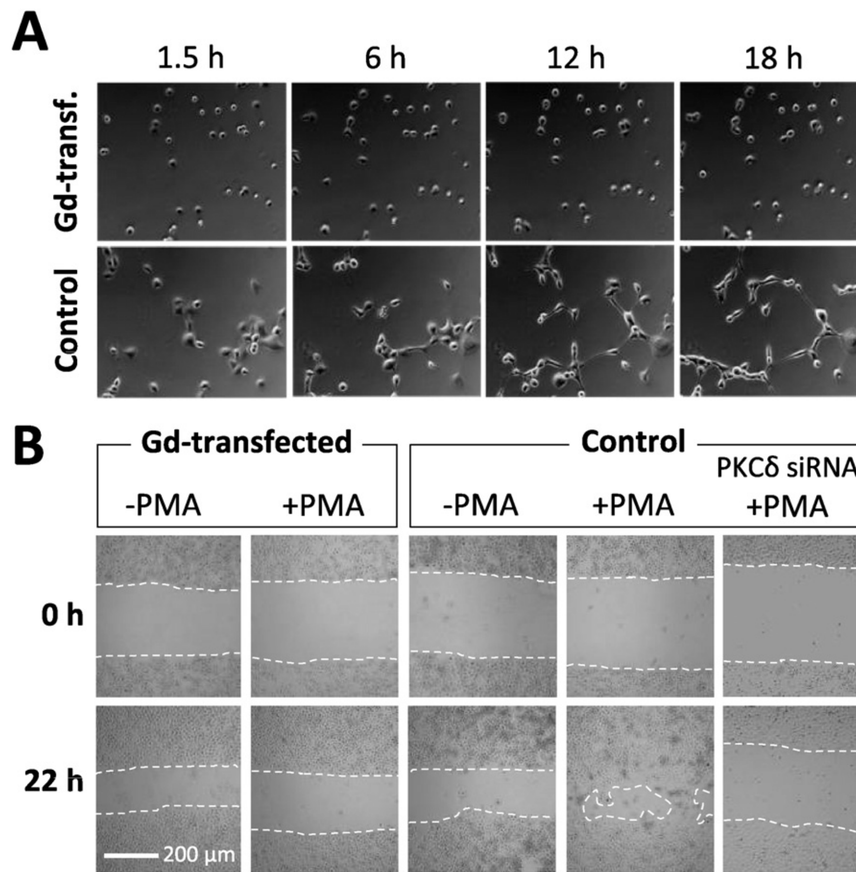

Fig. 4. (A) PMA induces changes in cell migration and morphology. Live cell images of glycodelin-producing and control cells grown on Matrigel and treated with 2 nM PMA for 18 h (for time-lapse see Supplementary film). (B) PMA increases the migration of control, but not glycodelin-producing cells. The effect was abolished using PKCδ siRNA. The cells were grown on chamber slides, scratched with a pipet tip and treated with and without 10 nM PMA for 22 h. Some of the control cells were pretreated with PKCδ siRNA for 48 h before the migration assay. Migration of the cells was continuously monitored under a light microscope. Quantitation was performed by measuring the area of the cell monolayer after 0 and 22 h using ImageJ software. Representative examples of at least two individual experiments with two different glycodelin-producing and control cell clones.

TGFβ, an endogenous cancer-related growth factor, had similar effect on cellular phenotype as PMA on control cells, while again glycodelin-producing cells remained unresponsive. The morphological changes induced by TGFβ took longer time, which is consistent with previous studies where TGFβ has been reported to have relatively weak potency to activate MAPK pathway [36]. TGFβ-driven change in cell phenotype is likely to be mediated by the same mechanism than the effect of PMA. This is supported by our finding that PKCδ knockdown abolished the effect of TGFβ on cell differentiation. We also found that TGFβ induced an equal phosphorylation of Smad2 in both the glycodelin-producing and control cells, suggesting that the effect of glycodelin is not mediated via canonical TGFβ pathway, which is associated with tumor suppressive activity of TGBβ in premalignant state [37]. This further supports the involvement of MAPK pathway and PKCδ i.e., noncanonical pathway, in the TGFβ-driven morphological differentiation. Interestingly, the acquisition of tumor promoting activities of TGFβ during tumor progression are associated with the impairment of canonical TGFβ signaling and shift to non-canonical signaling [37,38].

It is noteworthy that while the PMA- and TGFβ-induced changes in the morphology of the control cells were visible within hours or days, the differentiated phenotype of glycodelin-producing cells, without stimulation, has been observed only after the cells were grown on/in Matrigel for over a week [7]. Since such differentiation between the control and glycodelin-producing cells was not observed when the cells were grown on plastic, this indicates the importance of the microenvironment on glycodelin-induced cell differentiation.

PKCδ, as well as other PKCs, has multiple functions in cell signaling mediating the effects of several different molecules [10]. The effects of PKCδ are very different depending on the cell type and, thus, PKCδ has been reported to act both as a tumor promotor and suppressor [13]. Compared to normal endometrial tissue, decreased expression of PKCδ has been reported in endometrial cancer, correlating inversely with increasing tumor grade [19]. Decreased levels of PKCδ, particularly in nucleus where PKCδ is translocated during apoptosis in endometrial cancer, have been shown to diminish the sensitivity of Ishikawa endometrial adenocarcinoma cells to etoposide, suggesting that PKCδ may act as a tumor suppressor in endometrial carcinoma [19,39]. However, Yeramian and coworkers have reported elevated expression of *PRKCD* gene, encoding PKCδ, in endometrioid endometrial carcinoma as compared to normal endometrium [40]. To our knowledge, the activity and functions of PKCδ in normal and malignant endometrial tissue have not been addressed thoroughly.

Although the association of glycodelin with differentiated epithelia and its role in driving epithelial differentiation have been solidly established [5–8], the mechanism mediating this has been unknown. Our present results strongly suggest that repressed PKCδ activity is involved in the glycodelin-driven cell differentiation, which is likely to be relevant for both normal physiology of female reproductive tissues, like differentiation of secretory phase endometrium, and suppression of tumor growth. In the current study, glycodelin-producing cells were found to be unresponsive to phenotypic changes caused by tumor promoting molecules PMA and TGFβ. Therefore, it is feasible that previously observed reduced xenograft tumor growth of glycodelin-producing cells is related to repressed response to tumor growth promoting factors in tumor microenvironment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2016.06.020.

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