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Cadmium inhibits placental trophoblast cell migration via miRNA regulation of the transforming growth factor beta (TGF-β) pathway

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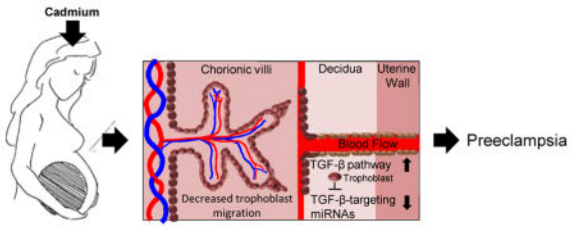
Graphical Abstract



1. Introduction

Previous studies have identified significant associations between exposures to the toxic metal cadmium (Cd) and the development of preeclampsia (PE) [1, 2]. PE is a pregnancy disorder that is characterized by hypertension and proteinuria that can cause adverse health effects for mother and child, and sometimes death [3]. The placenta has been identified as the potential etiologic factor in PE development, as well as a target organ for Cd accumulation[2]. Cd exposure can occur through various routes, such as cigarette smoke and ingesting contaminated food from Cd-containing soils [4]. In addition, Cd exposure through the consumption of contaminated drinking water remains a serious concern as millions of women in the U.S. and globally use unregulated drinking water that may contain elevated Cd levels[5]. Recently, supporting prior research on the links between Cd exposure and PE[1, 6–8], we demonstrated that elevated placental Cd levels significantly increased the odds of developing PE[1]. However, the underlying mechanism of Cd-associated PE is still unknown.

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Disruption in growth factor signaling has been postulated as a mechanism for PE development. The vascular endothelial growth factor (VEGF) and its receptor Fms-related tyrosin kinase 1 (Flt1) are important for placental vasculogenesis and have been suggested as potential biomarkers for early detection of PE [9]. Additionally, disrupted signaling of the transforming growth factor beta (TGF-β) pathway has been highlighted as a feature of PE. For example, elevated expression of the TGF-β pathway ligand TGFB1 was identified in the serum of preeclamptic women compared to women that were normotensive[10]. In prior studies, we observed that expression of genes belonging to the TGF-β pathway was enhanced in preeclamptic placentas [11]. Moreover, microRNAs (miRNAs) that target the TGF-β pathway were dysregulated in preeclamptic placentas in association with Cd [7]. In the same study, placental trophoblast cells treated with Cd displayed increased expression of the TGF-β pathway and dysregulation of TGF-β-targeting miRNAs following Cd treatment. These data support that the TGF-β pathway may be controlled epigenetically and showcase epigenetic dysfunction as a hallmark of PE [12]. Cd may also play a role in influencing cell migration, where treatment with Cd previously inhibited *in vitro* migration in immortalized human trophoblast cells (HTR-8/SVneo) through actin skeleton reorganization [13], a possible effect of Cd-induced expression of the TGF-β pathway.

We hypothesized that Cd exposure results in increased expression of the TGF-β pathway through miRNA-regulated mechanisms in placental trophoblast cells. The hypothesized impact of this dysregulation is inhibition of trophoblast cell migration and thus proper implantation of the placenta. To test this hypothesis, we utilized JEG-3 cells, a human placental trophoblast cell line that posesses similar biological characteristics of first trimester trophoblast cells and has been used in previous studies to evaluate the effects of environmental contaminants [7, 14] in relation to Cd exposure, pathway modulation and miRNA expression.

2. Methods

Cell culture methodology

JEG-3 cells, a human placental trophoblast cell line, were used for all cell culture analyses as previously published[7]. Cultured media contained Dulbecco's Eagle Minimum Essential Media with 10% FBS, sodium pyruvate, and penicillin/streptomycin. Cells were incubated at 37°C with 5% CO_2. Cd chloride ($CdCl_2$) was dissolved into the media by vortex for final concentrations of 0.028, 1, 10, and 25 uM and 5mL of Cd-containing media that was added to cells for 48 hours. TGFB1 (R&D Systems, Minneapolis, MN) and TGF-β receptor I/II Inhibitor LY2109761 (Selleckchem, Houston, TX) was added to cultured media for a final concentration of 10ng/mL and 5uM for 24 hours prior to Cd treatment, respectively.

Cell viability assay

JEG-3 cells were seeded in 96-well black clear bottom plates at 10,000/mL with a total of 200uL of cultured media. Dilutions of ($CdCl_2$) were carried out starting at 2,000 uM until a final concentration of 0.001 uM was reached. Experiments were carried out in biological triplicate. Following a 48-hour incubation, media was removed and cell viability measured

by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Luminescence was measured using a Promega GloMax Microplate Luminometer.

Transwells

Cells were seeded into a Corning 8.0 uM pore transwell chamber (Sigma-Aldrich) at 5 $\times 10^4$ with 200uL of Dulbecco's Eagle Minimum Essential Media. A total of 500uL of media was placed inside the bottom well. Cells were incubated at 37°C with 5% CO_2 for 48 hours and then fixed with formaldehyde. Once non-migrated cells were removed from inside the chamber, fixed cells were stained with crystal violet and membranes imaged using an Olympus IX81 Inverted Light Microscope. All images were taken at 10X magnification. 12 non-overlapping images from each membrane were captured and used with ImageJ64 (version 10.2) to measure the percentage area of migrated cells. Experiments were carried out in biological triplicate, and any outliers removed prior to statistical analyses.

Quantitative PCR analysis

RNA was collected using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia CA) according to manufacturer's instructions. The quantity of isolated RNA was measured with the Nanodrop 1000 spectrophotometer, and its integrity verified by the Agilent 2100 Bioanalyzer. cDNA was generated using either Qiagen's RT^2 First Strand kit or miScript II RT Kit for gene and miRNA analysis, respectively. miRNA expression was assessed using Qiagen's miScript Primer Assays and SYBR Green PCR kits (Valencia, CA). The miRNA U6 was used as a housekeeping gene. To evaluate the expression of genes within the TGF-β pathway, the TGF-β/BMP Signaling Pathway RT^2 Profiler PCR Array (Valencia, CA) was used, which tests 84 genes related to TGF-β signal transduction. Ct values from the RT^2 Profiler PCR Arrays were analyzed for subsequent statistical testing. Qiagen's one-step QuantiTect SYBR Green RT-PCR kit and QuantiTect Primer Assays (Valencia, CA) were used to evaluate expression of key members of the TGF-β pathway. Experiments were carried out in biological triplicate, and any outliers removed prior to statistical analyses.

Transfections

JEG-3 cells were seeded in 6-well cell culture plates with 2mL of cultured media. Once cells were ~75% confluent, cells were transfected with 5 nM of the miR26a inhibitor or mimic (Exiqon, Woburn, MA) using Invitrogen's Lipofectamine 2000 Reagent and protocol. Control cells were treated with Lipofectamine 2000 Reagent without RNA. Cells treated with Cd were transfected 24 hours after the 48-hour incubation.

Statistical Analysis

To assess gene expression and miRNA differences in cells, raw CT values from quantitative PCR were used to calculate delta CT values. Partek Genomics Suite 6.4 (St Louis, Missouri) was used to complete analysis of covariance (ANCOVA). Statistically differences in mRNA and miRNA expression was defined as a false discovery rate-corrected p-value (q-value) < 0.05.

Migration percentages from 12 non-overlapping images from each transwell were averaged and t-tests were performed to assess statistical significance. Cells without Cd treatment were

used as controls to assess migration differences of cells treated with Cd. To examine TGF-β-associated differences, cells treated with Cd and TGFB1 were compared to cells treated with TGFB1 only (control) and cells treated with Cd and LY2109761 were compared to cell treated with LY2109761 alone (control). Treatment with Lipofectamine only was used as a control to analyze alterations in migration between cells transfected with the miR26a Inhibitor and Mimic. P-values less than 0.05 were defined as statistically significant.

3. Results

3.1 Cd reduces placental trophoblast migration in JEG3 cells

JEG3 cells were treated with 0.028, 1, 10, and 25 uM CdCl2. These concentrations span a range of environmentally relevant low (i.e. <5% cytotoxity) to high (i.e. >35% cytotoxicity) Cd toxicity concentrations respectively (Supplemental Figure 1). Following a 48-hour incubation, cells treated with Cd displayed diminished ability to migrate compared to untreated cells, which was enhanced with higher concentrations of Cd (Figure 1A and B). Specifically, the 1 uM concentration resulted in a significant reduction in migration at 30% migration versus control. The 10 uM and 25uM concentrations resulted in a 50% and 90% reduction in migration respectively (Figure 1B).

3.2 Increased signaling through the TGF-β pathway results in decreased trophoblast migration

To assess whether Cd impacts the mRNA expression of key regulators of the TGF-β pathway in the placental trophoblast cells, qRT-PCR was used. Cd was found to induce the expression of transforming growth factor beta (*TGFB1*) at the lower concentrations (0.028 and 1uM) (Figure 1C and Supplemental Table 1). Higher concentrations (10uM and 25uM) increased SMAD family member 3 (*SMAD3*) and decreased *TGFB1*, transforming growth factor beta receptor 1 (*TGFBRI*), and SMAD family member 2 (*SMAD2*) expression (Figure 1C). Interestingly, transforming growth factor beta receptor 2 (*TGFBRII*) displayed increased mRNA expression with a range of low and high Cd concentrations.

Next, we investigated the role of the TGF-β pathway in Cd-associated placental cell migration. JEG-3 cells were first treated with either the TGFB1 ligand or the TGF-β receptorI/II inhibitor LY2109761 for 24 hours followed by a 48-hour treatment with 0.028 or 10uM Cd. Cells treated with the ligand TGFB1 and exposed to Cd displayed significantly decreased migration compared to control (Figure 2). In contrast, treatment with the inhibitor LY2109761 improved Cd-associated migration with 0.028 and 10uM Cd. Interestingly, cell migration was improved 10-fold with inhibition of the TGF-β pathway with low and high Cd concentrations, indicating that this pathway indeed influences Cd-associated placental cell migration (Figure 2B).

3.3 miRNA-mediated inhibition of the TGF-β pathway improves trophoblast migration

In prior work we identified a subset of 15 TGF-β pathway-targeting miRNAs that were associated with Cd in normotensive placenta, preeclamptic placenta, and placental trophoblast cells [7]. From the subset, miR-26a was selected as a miRNA that targets the TGF-β pathway [15] to assess epigenetic regulation of the TGF-β pathway and establish its

influence on placental cell migration. JEG-3 cells treated without or with 0.028uM Cd for 48 hours were subsequently transfected with either a miR-26a inhibitor (to reduce expression) or mimic (to increase expression) using Lipofectamine. Following transfection, cells treated with the inhibitor displayed significantly decreased migration without Cd ($p=6.48e^{-8}$) and with Cd ($p=0.03$) compared to control (Figure 3A and B). In contrast, transfection with the mimic enriched placental cell migration in cells without Cd ($p=0.001$) and with Cd (not significant).

Quantitative PCR (qPCR) of RNA isolated from JEG-3 cells transfected with the inhibitor confirmed that miR-26a expression was decreased in cells with reduced migration (Figure 3C and Supplemental Table 2) and increased in cells transfected with the mimic (Figure 3D) that displayed enhanced cellular migration. Moreover, 84 TGF-β pathway-associated genes were assessed in placental cells by qPCR following transfection with the miR-26a inhibitor. Out of the 84 assessed genes, 65 (77%) genes were upregulated (Supplemental Table 3), with 17 out of the 19 significantly altered genes displaying enhanced expression. Among these were *SMAD3, SMAD5*, transforming growth factor beta receptor associated protein 1 (*TGFBRAP1*), and *SMAD* specific E3 ubiquitin protein ligase 1 (*SMURF1*). Expression of *SMAD1* was increased, but failed to reach statistical significance (Supplemental Table 3).

4. Discussion

Increased levels of cadmium assessed in serum and placental tissue is established to increase the risk of preeclampsia[1, 2]. Additionally, increased expression of members of the TGF- β pathway has been established to be elevated in PE[2, 7, 10, 11, 16]. In prior work, we identified a subset of TGF-β pathway-targeting miRNAs that were associated with Cd in the preeclamptic placenta and placental trophoblast cells [7]. In the present study, we investigated the impact of Cd on signaling within the TGF-β pathway, the role of the TGF-β pathway as a mediator of trophoblast migration, and the effect of TGF-β pathway-associated miRNAs on placental cell migration capacity. The results demonstrate that even at environmentally relevant concentrations, Cd reduces placental trophoblast migration and that the TGF-β pathway enhances this effect via miRNA control serving as a molecular mediator. These results are critical in the study of environmental contaminants that impact placental function and disease and are highly relevant to understanding a mechanistic basis for the risk of developing PE.

The results from this study highlight the role and impact of the TGF-β pathway in relation to placental trophoblast migration, a mechanism that is likely tied to cytoskeleton redisposition[13]. The TGF-β pathway mediates the regulation of focal complexes and extracellular matrix reorganization[17], two factors that influence cellular migration. Increased signaling of the TGF-β pathway can induce mitogen-activated protein kinases (MAP kinases), extracellular signal-regulated kinase (ERK) 1 and 2, and c-jun N-terminal kinase (JNK) to reorganize cytoskeleton structure to regulate cell migration [18]. Thus, aberrant signaling in placental trophoblast can affect placental trophoblast migration to increase risk of PE. The inability of the placenta to properly implant to the uterus may also contribute to PE[3, 9]. Efficient placental implantation is dependent on the ability of trophoblast cells to invade the mother's vasculature to create proper blood flow to the placenta[19]. Differentiation

of progenitor cells to extravillous trophoblasts (EVTs) that are capable of migrating and invading has been established as a key upstream regulator of trophoblastic motility, with signaling of SMAD 1/5[20] and the Wnt pathway[21] highlighted as promoters. In contrast to the TGF-β pathway, activation of the Wnt pathway increased trophoblast migration, invasion, and the expression of pro-migratory genes in trophoblasts[22, 23], possibly through a T-cell factor-4-mediated mechanism[21], suggesting the Wnt pathway is an antagonist of the TGF-β pathway and could be used to counter the effects observed in Cd-associated PE.

We hypothesized that increased signaling of the TGF-β pathway would reduce trophoblast migration while decreased expression would increase migration. In support of this, placental trophoblast treated with Cd and the TGFB1 ligand displayed diminished cellular migration. TGFB1 treatment in Cd-treated trophoblast likely enhanced TGF-β pathway signaling, resulting in the significant reduction in migration compared to trophoblast treated with Cd and a TGF-β receptor inhibitor. These results showcase a role for the TGF-β pathway in Cd-mediated cellular migration in placental trophoblast cells.

To assess for the impact of epigenetic regulation of the TGF-β pathway on placental cell migration, miR-26a was selected for analysis. This miRNA has been shown to target and decrease the expression of the TGF-β pathway transcription factor SMAD family member 1 (SMAD1)[15]. The *a priori* hypothesis was that the decreased expression of miR-26a would result in increased expression of the TGF-β pathway members resulting in reduced migration of placental trophoblasts. The results highlighted that decreased expression of miR-26a achieved through transfection of the JEG3 cells with the miRNA inhibitor resulted in a modest increase in *SMAD1* expression, as well as significantly enhanced expression of *SMAD3* and *SMAD5.* This suggests possible crosstalk between TGF-β pathway-targeting miRNAs and other members of the pathway. Moreover, in line with the underlying hypothesis, reduction in the expression of miR-26a via the inhibitor resulted in significantly decreased placental trophoblast migration, corroborating a previous study that observed decreased cellular migration and expression of TGF-β pathway-targeting miRNAs in colorectal carcinoma cells following TGF-β treatment[24]. In contrast, migration was comparable to control cells in placental cells overexpressing miR-26a with or without Cd treatment. miRNA dysfunction is a hallmark of PE[12] and is postulated as a cause due to miRNAs being critical regulators of cell function by mediating gene expression. In addition, numerous members of the TGF-β pathway are targeted by miRNAs[25] further underlying the importance of understanding the mechanism of miRNA-mediated PE. These data highlight that similar to Cd treatment, reduced expression of TGF-β pathway-targeting miRNAs results in diminished trophoblast migration. Therefore, bolstering our hypothesis that Cd regulates the TGF-β pathway epigenetically through miRNA, impeding placental trophoblast migration.

In conclusion, there are three major findings from the present study. First, in support of prior work that showed an impact of Cd on migration[13], we confirm that exposure in placental trophoblast JEG3 cells decreases placental trophoblast migration. Second, the results demonstrate that an increase in signaling of the TGF-β pathway results in decreased trophoblast migration. Finally, the data support that miRNAs are critical for the TGF-β pathway-associated effect on cellular migration in placental trophoblast cells. In future,

these miRNAs can be examined as targets for epigenetic reprogramming to repair cellular mobility. Investigating the underlying mechanisms for PE and potential environmental influences can provide further insight into molecular targets for reducing disease risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Cadmium treatment decreases placental trophoblast migration.
- Increased TGF-β pathway signaling results in diminished trophoblast migration.
- TGF-β pathway-targeting miRNAs are critical in regulating trophoblast migration.
- miRNAs have the potential as therapeutic targets for cadmium-associated PE.

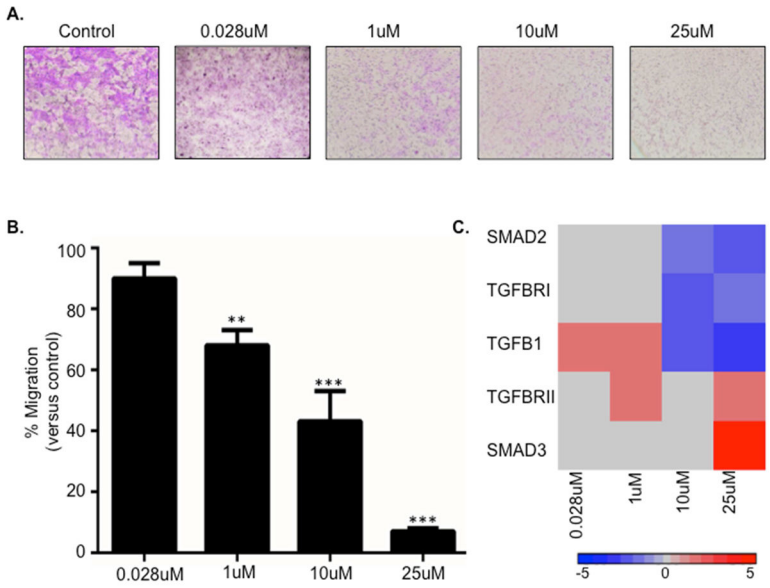

Figure 1. Cadmium (Cd) inhibits placental cell migration

(A) Representative images of crystal violet-stained transwell membranes from JEG-3 cells treated for 48 hours with 0.028, 1, 10, and 25uM Cd. (B) Quantitative results of the transwell analysis. Cd-treated cells were compared to untreated cells to determine significance. *=p<0.05; **=p<0.01; ***=p<.001 (C) Heatmap displaying quantitative PCR results of TGF-β pathway genes using RNA isolated from JEG-3 cells treated with 0.028, 1, 10, and 25uM Cd.

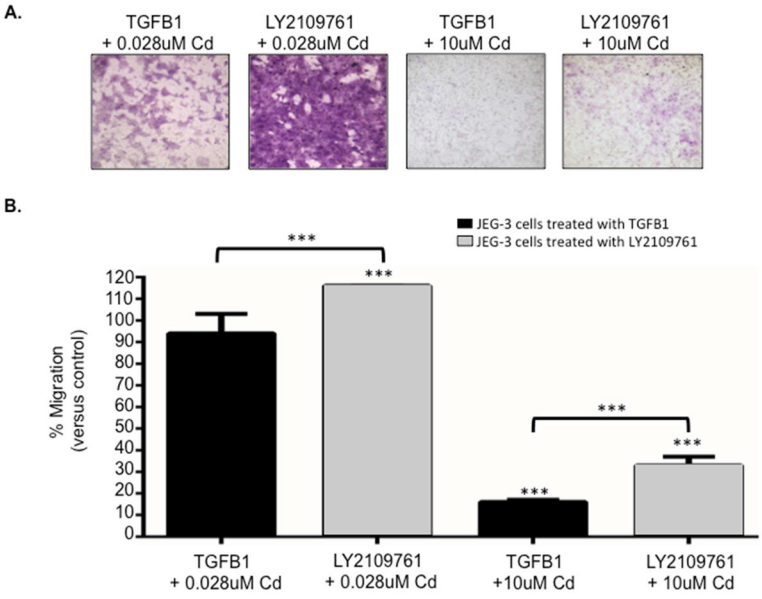

Figure 2. The TGF-β pathway facilitates Cd-induced migration inhibition in placental cells

(A) Representative images of crystal violet-stained transwell membranes from JEG-3 cells treated for 24 hours with either TGFB1 or the TGF-β receptorI/II inhibitor LY2109761 followed by a 48-hour treatment with 0.028 or 10uM Cd. (B) Quantitative results of the transwell analysis. Cells treated with TGFB1 and Cd was compared to cells treated with TGFB1 only (control) to determine significance, while cell treated with LY2109761 and Cd were compared to cells treated with LY2109761 only (control). *=p<0.05; **=p<0.01; ***=p<.001

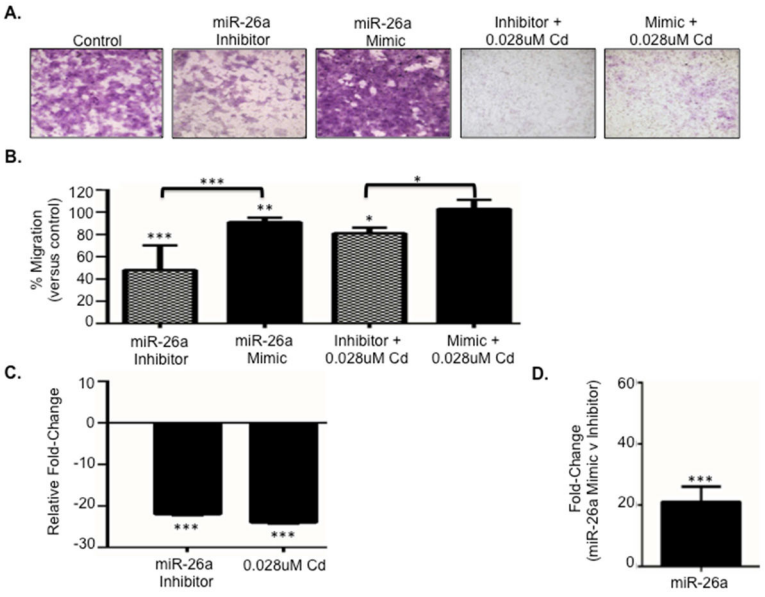

Figure 3. miR-26a expression regulates placental cell migration

(A) Representative images of crystal violet-stained transwell membranes from JEG-3 cells treated with or without Cd and transfected with a miR-26a inhibitor or mimic. (B) Bar graph depicting quantitative data from transwell analysis of JEG-3 cells with and without Cd treatment in combination with transfections with a miR-26a inhibitor or mimic. *=p<0.05; **=p<0.01; ***=p<.001 (C) Quantitative PCR (qPCR) of decreased miR-26a expression in cells transfected with the miR-26a Inhibitor and cells treated with 48-hour 0.028uM Cd compared to control. (D) qPCR of miR-26a expression in cells transfected with the miR-26a mimic compared to cells transfected with the inhibitor.