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Adrenomedullin Promotes Rat Trophoblast Stem Cell Differentiation[1]

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ABSTRACT

Accumulating data suggest that adrenomedullin (ADM) regulates the trophoblast cell growth, migration, and invasion. However, the effect of ADM on trophoblast differentiation is poorly understood. In this study, we hypothesized that ADM promotes the differentiation of trophoblast stem cells (TSCs) into trophoblast giant cells (TGCs). Using rat TSCs, Rcho-1 cells, we investigated the effect of ADM on TSC differentiation into TGCs in differentiation or stem cell media, respectively, and explored the effect of ADM on the mechanistic target of rapamycin (MTOR) signaling in trophoblast cell differentiation. The results include: 1) in the presence of differentiation medium, 10^{-7} M ADM, but not lower doses, elevated ($P < 0.05$) *Prl3b1/Esrrb* (i.e., the ratio of mRNA levels) by 1.7-fold compared to that in control; 2) the supplementation of ADM antagonist, regardless of the concentration of ADM, reduced ($P < 0.05$) *Prl3b1/Esrrb* by 2-fold, compared to control group, while the supplementation of CGRP antagonist, regardless of the concentration of ADM, did not change *Prl3b1/Esrrb*; 3) in the presence of stem cell medium, ADM did not alter the expression of TSC and TGC marker genes, however, the ratio of *Prl3b1/Esrrb* was reduced ($P < 0.05$) by ADM antagonist compared to that in control; and 4) ADM increased ($P < 0.05$) phosphorylated MTOR proteins and the ratio of phosphorylated to total MTOR proteins by 2.0- and 1.7-fold, respectively. The results indicate that ADM promotes but does not induce the differentiation of TSCs to TGCs in a dose-dependent manner and MTOR signaling may play a role in this process.

adrenomedullin, differentiation, MTOR, rat, Rcho-1 cell, trophoblast giant cell, trophoblast stem cell

INTRODUCTION

Trophoblast giant cells (TGCs) play versatile functions as pregnancy advances with secretion of a variety of agents, including steroid hormones and prolactin-related peptides at different stages [1], and thus are critical for implantation and placentation. TGCs, together with a variety of differentiated trophoblast lineages, are originally derived from the same progenitor: trophoblast stem cells (TSCs). In general, the differentiation of TSCs is complicated because multiple genes get involved in this process as determined by knockout, knockdown, and transgenic techniques [2–4]. To date, the differentiation of TSCs into TGCs has been intensively studied in trophoblast biology, with the application of mouse or rat TSC lines [5–7]. Recently, Kent et al. [8] reported that PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)-signaling pathway plays a critical role in the differentiation of TSCs, and this study sheds new light on the study of trophoblast cell differentiation because a large number of genes relevant to TSC differentiation were identified by DNA microarray analysis. Among those differentially expressed genes regulated by PI3K signaling, adrenomedullin (*Adm*) expression was highly increased in the differentiated trophoblast cells.

ADM, a member of calcitonin/calcitonin gene-related peptides (CALCA/CGRP) family, is produced by multiple tissues and organs, including placenta. The physiological or cellular effects of ADM are dependent on the type of tissues or organs, especially the distribution of its receptors consisting of CALCRL (calcitonin receptor-like) and receptor activity-modifying proteins (RAMP) [9]. Three known RAMPs facilitate ligands (ADM, CGRP, and ADM2) binding to CALCRL. Heterodimeration of RAMP2 or RAMP3 with CALCRL results in specific ADM receptor 1 and ADM receptor 2, respectively, while combination of RAMP1 with CALCRL forms CGRP receptors. The presence of specific receptor components determines signaling pathways induced by ADM binding. For instance, in the vascular relaxation, ADM receptor 2 in vascular smooth muscle is involved in stimulating endothelium-independent pathway and ADM receptor 1 in vascular endothelial cells is involved in stimulating endothelium (NO)-dependent pathway [9]. Moreover, ADM receptor 1 and 2 are selectively inhibited by truncated protein $ADM_{22–52}$ and $CGRP_{8–37}$, respectively [10]; thus, they are widely used as antagonists in the study of ADM functions and signaling pathways.

In both humans and rats, maternal plasma ADM levels progressively increase with pregnancy [11, 12], and feto-placental tissue is considered the major source of ADM during pregnancy[13]. During pregnancy, ADM has been suggested to play a critical role in the regulation of implantation and placentation because of its functions in angiogenesis, immune modulation, vasodilation, and other functions [14]. On the other hand, *Adm*-null mice are embryonic lethal [15], and a decrease in maternal expression of *Adm* results in impaired fertility, placentation, and fetal growth [16], supporting the importance of ADM in implantation and placentation. To date, the functions of ADM in implantation and placentation and associated mechanisms have not been completely understood.

Accumulating evidence indicates that ADM may regulate TSC differentiation by PI3K-AKT (thymoma viral proto-oncogene)-MTOR (mechanistic target of rapamycin) signaling pathway. ADM is known to exert its effects by stimulating the cAMP/PKA-signaling pathway in smooth muscle cells and cAMP/calcium/calmodulin signaling in endothelial cells [9]. PI3K-AKT signaling pathway has been demonstrated to play a crucial role in rodent trophoblast cell differentiation, and enhanced ADM expression is associated with the differentiation of TSCs [8]. In addition, it is well-known that PI3K is one of the upstream regulators of MTOR-signaling pathway in

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trophoblast cells in humans [17], mice [18], rats [19], and sheep [20]. However, the role of MTOR signaling has not been linked to TSC differentiation. In this study, we hypothesized that ADM enhances the differentiation of TSCs and the MTOR-signaling pathway is involved in this process. Using Rcho-1 rat TSCs, a well-characterized in vitro model for studying trophoblast [7], we investigated the effects of ADM on TSC differentiation. Marker genes for trophoblast lineages have been widely used to assess the differentiation status of TSCs in vivo, ex vivo, or in vitro [1, 21–24]. We assessed the ratios of differentiation marker genes (*Prl3b1*, *Prl2c1*) to stem cell marker genes (*Esrrb*, *Id1*) in the presence or absence of ADM and/or its antagonists in the culture media. We also investigated effects of ADM on phosphorylation of MTOR proteins, a crucial event in the activation of MTOR-signaling pathway.

MATERIALS AND METHODS

Cell Culture

In study one, we tried to optimize the culture conditions for TSCs and TGCs in our laboratory. Rat TSCs (Rcho-1 cells, generously provided by Dr. Thomas L. Brown) was seeded in 6-well culture plates, and cultured in stem cell medium (RPMI-1640 with L-glutamine supplemented with 20% fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 μg/ml penicillin/streptomycin, and 20 mM HEPES) overnight. Culture medium was replaced with differentiation medium (NCTC-135 medium with 1% horse serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 μg/ml penicillin/streptomycin, and 20mM HEPES), and the cells were cultured for 2, 4, or 6 days with culture medium changed every other day. Images were recorded on cultured cells under an Olympus U-TV1 microscope before harvest. On each harvesting day, culture medium was aspirated. Cells were washed in 1× PBS briefly, then lysed in Trizol reagent (15596-018; Invitrogen) for total RNA extraction.

In study two, we investigated the effect of ADM on TSC differentiation into TGCs in differentiation medium. Rat TSCs were seeded in 6-well culture plates and cultured in stem cell medium overnight. Culture medium was replaced with differentiation medium only or differentiation medium with ADM (10^{-9}, 10^{-8}, 10^{-7} M), ADM antagonist (ADM_{22-52}, 10^{-7} M), CGRP antagonist ($CGRP_{8-37}$, 10^{-7} M), and/or their combinations. Cells were cultured for 6 days with culture medium changed every other day. Upon harvest, culture medium was aspirated. Cells were washed in 1× PBS briefly, then lysed in Trizol reagent for total RNA extraction or lysed in RIPA buffer (9806; Cell Signaling Technologies) for protein extraction.

In study three, we investigated the effect of ADM on TSC differentiation into TGCs in stem cell medium. Rat TSCs were seeded in 6-well culture plates and cultured in stem cell medium overnight. Culture medium was replaced with stem cell medium only or stem cell medium with ADM (10^{-7} M) and/or ADM antagonist (10^{-7} M), and the cells were cultured for 4 days with culture medium changed every other day. Upon harvest, culture medium was aspirated. Cells were washed in 1× PBS briefly, then lysed in Trizol reagent for total RNA extraction.

In study four, we explored the role of MTOR protein in ADM-promoted TSC differentiation into TGCs. Rat TSCs were seeded in 6-well culture plates and cultured in stem cell medium overnight. Culture medium was replaced with differentiation medium only or differentiation medium with other treatments (ADM: 10^{-7} M; ADM antagonist, ADM_{22-52}, 10^{-7} M; COMB: ADM + ADM antagonist, 10^{-7} M), and the cells were cultured for 6 days with culture medium changed every other day. Upon harvest, culture medium was aspirated. Cells were washed in 1× PBS briefly and used for protein extraction.

RNA Extraction and RT-PCR

Total RNA was extracted from cultured cells by Trizol reagent according to the manufacturer's protocol. The possible genomic DNA in total RNAs was digested with RNA-free DNase I (79254; Qiagen Inc.), followed by clean-up procedures using a Qiagen RNeasy minikit (74104; Qiagen Inc.). In all these procedures, the manufacturer's instructions were followed. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by reverse transcription in a total volume of 20 μl by using a MyCycler Thermal Cycler (170-9703; Bio-Rad Laboratories) under the following conditions: one cycle at 28°C for 15 min, 42°C for 50 min, and 95°C for 5 min.

Quantitative Real-Time PCR

Real-time PCR detection was performed on a CFX96Real-Time PCR Detection System (184-5096; Bio-Rad Laboratories). Primers were prepared as described previously [25, 26]. Syber Green Supermix (170-8882; Bio-Rad Laboratories) was used for amplification of *esrrb*, *Id1*, *Prl3b1, Prl2c1,* and *Rn18s*. The reaction mixture was incubated at 95°C for 10 min and cycled according to the following parameters: 95°C for 30 sec and 60°C for 1 min for a total of 40 cycles. Negative control without cDNA was performed to test primer specificity. In study one, the relative gene expression was calculated by use of the threshold cycle (C_T) *Rn18s*/C_T target gene. In studies two and three, to determine the effect of differentiation medium or ADM on TSC differentiation, the ratio of C_T values of differentiation marker genes to C_T values of stem cell marker genes was calculated.

Protein Extraction from Cells

Cells were lysed in RIPA buffer. Cell lysates were centrifuged for 10 min at 1000 × *g* at 4°C, and the supernatant fractions were collected and stored at −80°C until Western blot analysis. Protein concentration was determined by using a Pierce BCA Protein Assay Kit (23225; Pierce Biotechnology).

Western Blot Analysis

Aliquots of 20 μg proteins were added with 4× sample buffer (NP0007; Invitrogen), followed by incubation at 70°C for 10 min. The separated proteins in SDS-PAGE were transferred onto a nitrocellulose membrane at 4°C overnight. After blocking in 5% nonfat milk, a rabbit anti-MTOR polyclonal immunoglobulin G (IgG) (2983; Cell Signaling) or a rabbit anti-phosphorylated MTOR (Ser^{2448}) polyclonal IgG (2971; Cell Signaling) at 1:2000 dilutions was added to nitrocellulose membrane and incubated at 4°C overnight. The blots were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (4030-05; Southern Biotech) at 1:2000 dilutions at room temperature for 1 h. TUBB (β-tubulin) was used as an internal control for Western blots in this study. Primary antibody, rabbit monoclonal antibody for TUBB (2128S; Cell Signaling), and secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (4030-05; Southern Biotech), were used at 1:5000 and 1:10 000 dilutions, respectively. Proteins in the blots were visualized with Pierce enhanced chemiluminescence detection (32209; Thermo Scientific) and Blue Lite Autorad Film (F9024; BioExpress) according to the manufacturer's recommendations. The signals in films representing the contents of the target proteins and the internal control protein TUBB were quantified by densitometry using Fluorchem 8000 software (Cell Biosciences). The relative amount of target protein was expressed as a ratio to TUBB analyzed by Western blot analysis.

Statistical Analysis

All the quantitative data were subjected to least-squares analysis of variance (ANOVA) by using the general linear models procedures of the Statistical Analysis System (SAS Institute). Data on gene expression and the relative abundance of proteins were analyzed for effects of ADM and its antagonist. In ANOVA, differences in treatments were determined by the Student-Newman-Keuls multiple comparison test. Log transformation of variables was performed when the variance of data were not homogenous among the treatment groups, as assessed by the Levene test. A P-value ≤ 0.05 was considered significant; a P-value > 0.05 and ≤ 0.10 was considered a trend toward significance. Data were presented as least-squares means with overall standard errors.

RESULTS

The Differentiation of TSCs occurs in Differentiation Medium

TSCs were small, and the nuclei were not very obvious under the microscope (Fig. 1A). After culturing in differentiation medium for 6 days, trophoblast cells demonstrated the typical phenotype of differentiated trophoblast cells, primarily TGCs. The cell size was largely increased; the area of the nucleus expanded and multiple nuclei were present, while TSCs continued to proliferate and aggregate together (Fig. 1B).

The differentiation status of cultured cells was indicated by the relative mRNA levels of TSC marker genes (*Esrrb*, *Id1*) and TGC marker genes (*Prl3b1, Prl2c1*). The presence of

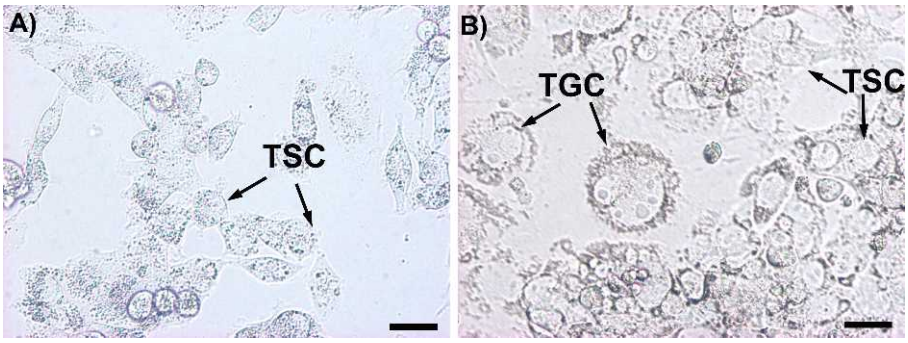

FIG. 1. Morphological changes in TSCs in differentiation medium. **A**) TSCs seeded in culture dish with stem cell medium. **B**) TGCs and TSCs after 6 days' culture in differentiation medium. TSC: trophoblast stem cell; TGC: trophoblast giant cell. Bar = 100 μm.

differentiation medium reduced ($P < 0.01$) the expressions of *Esrrb* and *Id1* by 3.4- and 2.1-fold, respectively, by 2 days, and the reduced expression of these genes were maintained thereafter. In contrast, the presence of differentiation medium increased ($P < 0.001$) the expressions of *Prl3b1* and *Prl2c1* by 5.2- and 7.0-fold, respectively, by 4 days in culture (Fig. 2). These indicate that the differentiation of TSCs into TGCs occurred progressively when TSCs were cultured in differentiation medium and the application of these TSC and TGC marker genes were reliable.

ADM Promotes TSC Differentiation in Differentiation Medium, While Its Antagonist Blocks This Effect

In the presence of differentiation medium, ADM (10^{-7} M) caused elevation ($P < 0.05$) in the *Prl3b1*/*Esrrb* ratio by 1.7-fold compared to that in control, but 10^{-9} and 10^{-8} M ADM did not change this ratio; the treatment with ADM antagonist (ADM_{22-52}), regardless of the concentration of ADM, reduced ($P < 0.05$) the *Prl3b1*/*Esrrb* ratio by 2-fold compared to that in the control group. However the CGRP antagonist ($CGRP_{8-37}$) treatment, regardless of the concentration of ADM, did not change the *Prl3b1*/*Esrrb* ratio (Fig. 3).

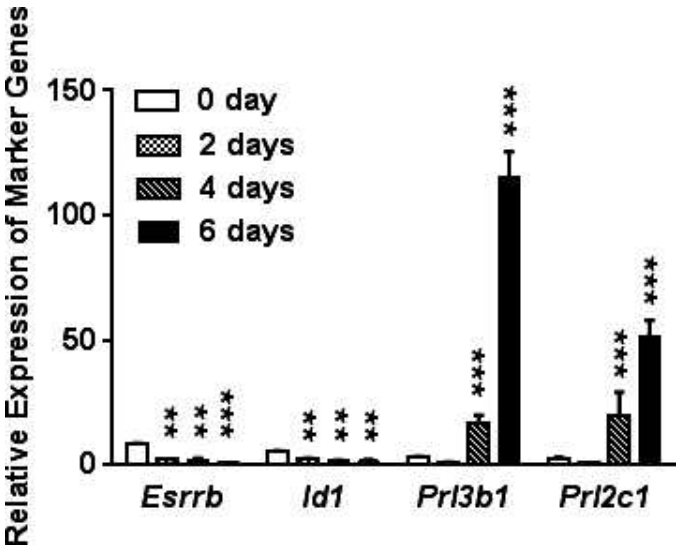

FIG. 2. Expressions of marker genes in TSC differentiation in differentiation medium. *Esrrb, Id1*: TSC marker genes; *Prl3b1, Prl2c1*: TGC maker genes. The error bar represents the mean ± SEM expressed as relative units of mRNA standardized against *Rn18s* (n = 3). $^{**}P < 0.01$; $^{***}P < 0.001$, all compared to normalized mRNA levels at Day 0.

ADM Does Not Induce TCS Differentiation in Stem Cell Medium

In the presence of stem cell medium, the supplementation of ADM, ADM antagonist, and their combination did not alter the expressions of both TSC and TGC marker genes. However, the *Prl3b1*/*Esrrb* ratio was reduced ($P < 0.05$) by ADM antagonist compared to that in the control group, and this ratio was comparable between ADM supplementation and control groups (Fig. 4).

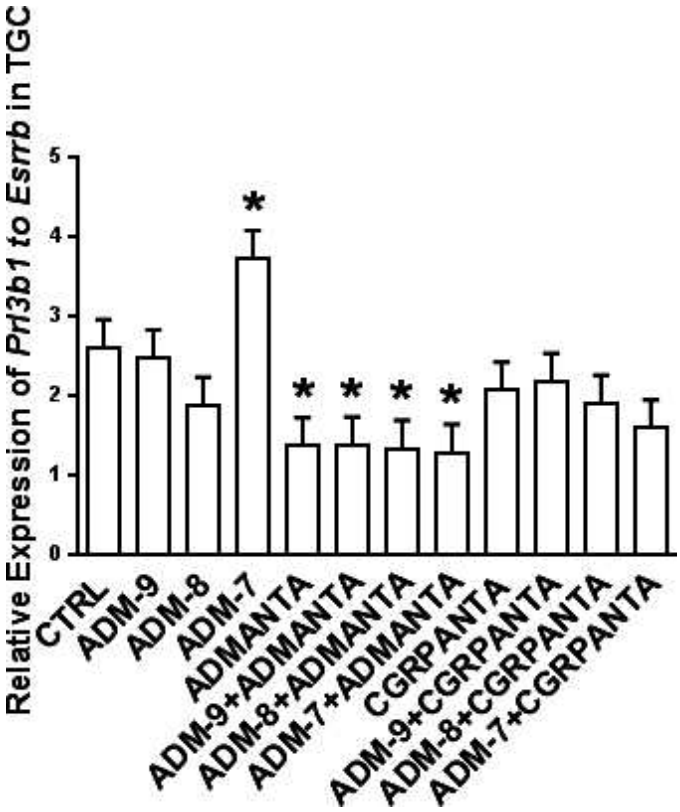

FIG. 3. Relative expression of *Prl3b1* to *Esrrb* in TGCs treated with ADM in differentiation medium. ADM: adrenomedullin (Doses: −7, 10^{-7} M; −8, 10^{-8} M; −9, 10^{-9} M); ADMANTA: ADM antagonist, ADM_{22-52}, 10^{-7} M; CGRPANTA: CGRP antagonist, $CGRP_{8-37}$, 10^{-7} M; CTRL: control, differentiation medium only. The error bar represents the mean ± SEM expressed as relative units of *Prl3b1* mRNA standardized against *Esrrb* (n = 3). The values of each treatment were compared to those of control. $^{*}P < 0.05$.

ADM Promotes TSC Differentiation by Activating MTOR

ADM treatment caused increases ($P < 0.05$) in the abundance of phosphorylated (Ser^{2448})MTOR proteins by 2.0-fold, and in the ratio of phosphorylated to total MTOR proteins by 1.7-fold. Treatment of ADM antagonist itself or the combination of ADM and ADM antagonist resulted in increases ($P < 0.05$) in the abundance of phosphorylated MTOR proteins and the ratio of phosphorylated to total MTOR proteins (Fig. 5).

DISCUSSION

This study for the first time demonstrates that ADM promotes TSC differentiation to TGCs. The effect of ADM appears to be through the ADM receptor consisting of CALCRL and RAMP2. Moreover, the activation of MTOR protein appears to be involved in ADM-enhanced TSC differentiation. This study together with our previous reports of placental and fetal growth restriction in the ADM antagonist-infused pregnant rats [27, 28] suggest a possible role of ADM in placental growth and trophoblast differentiation.

ADM promotes TSC differentiation in a dose-dependent manner (Fig. 3) but does not initiate TSC differentiation (Fig. 4). Among the three doses of ADM tested in this study, ADM at 10^{-9} M and 10^{-8} M was ineffective, while at 10^{-7} M, it promoted the differentiation of TSCs (Fig. 3). Thus, an elevated ADM levels to certain threshold are required to exert its effect on stem cell differentiation. The plasma levels of ADM were reported to increase with the progress of pregnancy [13], which could provide the stimulus in trophoblast differentiation. Placenta also produces large amounts of ADM and thus can be a major source of the elevated ADM during normal pregnancy [13]. This is consistent with a recent report that the expression of *Adm* in differentiated TGCs was increased by 5-fold [8]. In contrast, decreased maternal ADM levels are associated with adverse pregnancy outcomes, including reduced birth weight and increased risk for preeclampsia and gestational diabetes mellitus [29]. Infusion of ADM antagonist during early gestation [27] or late gestation [28] results in both fetal and placental growth reductions in the rat. Therefore, ADM of placental origin may be critical for normal pregnancy and perhaps may be involved in promoting TSC differentiation.

The effect of ADM on stem cell differentiation appeared to be mediated by ADM receptors consisting of CGRP receptor (i.e., CALCRL) and RAMP2. In this study, we used pharmacologic approaches to differentiate the involvement of RAMP2 or RAMP3 in ADM-induced enhancement of TSC differentiation. $ADM_{22–52}$, the ADM antagonist, primarily blocks the receptors consisting of CALCRL and RAMP2, and $CGRP_{8–37}$, a CGRP antagonist, has been shown to block ADM effects exerted through CALCRL and RAMP3 [9]. These antagonists were used in culture of stem cells in differentiation medium (Fig. 3) to assess specific RAMP involvement in this process. The stem cell differentiation-promoting ability of ADM was inhibited by $ADM_{22–52}$ but not by $CGRP_{8–37}$ (Fig. 3), suggesting that $RAMP_2$ rather than RAMP3 may be involved in TSC differentiation. Because the mRNA expression of *Ramp1* in rat TSCs as well as giant cells is extremely low, while expressions of *Ramp2* and *Ramp3* are relatively abundant (Gao and Yallampalli, unpublished data), we suggest any blocking effects of $CGRP_{8–37}$ on ADM-enhanced differentiation may involve $RAMP3$ and not RAMP1. In addition, several studies demonstrated that RAMP2 is required for normal pregnancy and fetal development, and genetic deletion of *Ramp2* is embryonically lethal [30, 31], while genetic deletion of *Ramp1* or *Ramp3* does not cause a grossly abnormal phenotype [30, 32]. The genetic deletion of Ramp2 results in cardiovascular defects in the fetus, resulting in fetal death. In the current study, we show an additional role for RAMP2 involvement during pregnancy in promoting TSC differentiation.

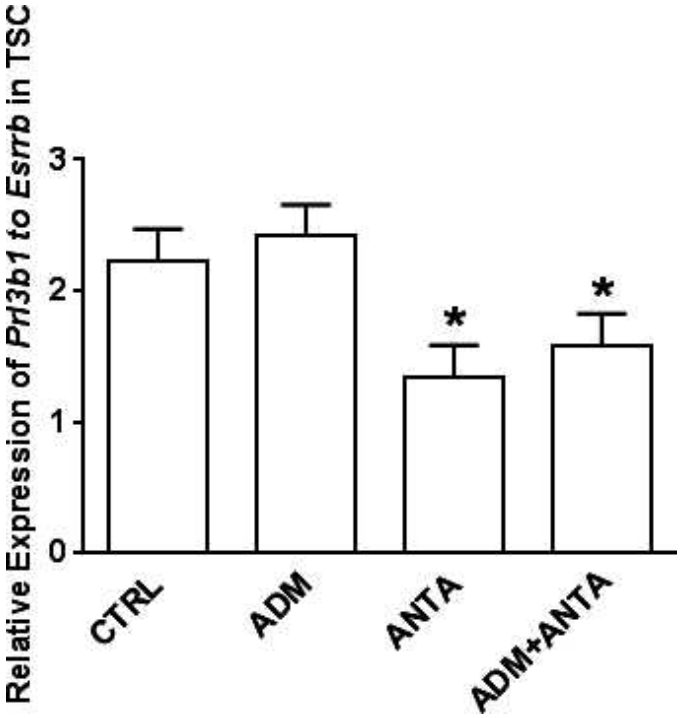

FIG. 4. Relative expression of *Prl3b1* to *Esrrb* in TSCs treated with ADM in stem cell medium. ADM: adrenomedullin, 10^{-7} M; ANTA: ADM antagonist, $ADM_{22–52}$, 10^{-7} M; CTRL: control, differentiation medium only. The error bar represents the mean ± SEM expressed as relative units of *Prl3b1* mRNA standardized against *Esrrb* (n = 3). *$P < 0.05$.

This study found that both the phosphorylated MTOR protein levels and ratio of phosphorylated to total MTOR protein levels were increased by ADM treatment compared with control (Fig. 5). We show for the first time that ADM can stimulate MTOR phosphorylation in the process of TSC differentiation. Phosphorylation of MTOR is a key step in the activation of MTOR-signaling pathway, which plays a critical role in regulating protein synthesis [33–35]. TGCs are active in protein synthesis in that a variety of protein hormones are synthesized and released from TGCs. As a result, these hormones may cause vascular remodeling and maternal adaptation to enhance the fetal growth [2, 36]. Thus, the versatile functions of ADM in pregnancy may be mediated, at least partly, by MTOR signaling in TGCs. This notion is supported by the following evidence. First, the PI3K- and AKT-involved pathway is critical for TSC differentiation [8, 26]. PI3K is an upstream component in the MTOR-signaling pathway, while AKT acts as either an upstream stimulator or downstream target of MTOR signaling [37]. Second, because the expression of *Adm* in differentiated TGCs was inhibited by PI3K inhibitors [8], it is possible that a positive feedback exists between ADM and MTOR signaling. Third, gestational protein insufficiency results in restricted placental growth [38], impaired TSC differentiation into TGCs [25], and down-regulated mTOR signaling [39], which are associated with decreased expression of placental ADM (Gao and Yallampalli, unpublished data).

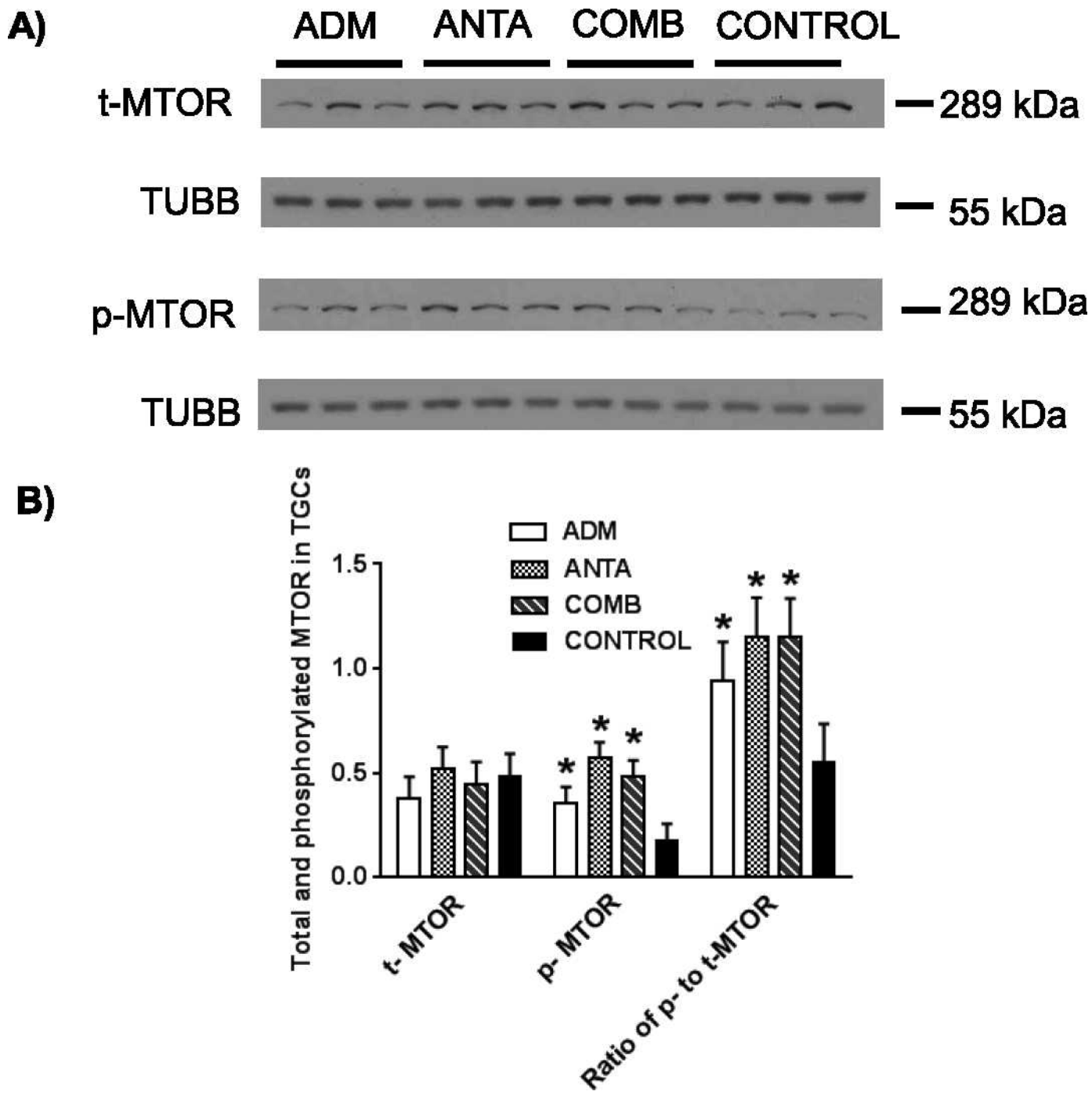

FIG. 5. Western blot analyses of MTOR in TGCs treated with ADM in differentiation medium. **A**) Total and phosphorylated MTOR proteins shown as 298-kDa bands. **B**) Relative abundance of total and phosphorylated MTOR proteins and ratio of phosphorylated to total MTOR. t-MTOR: total mechanistic target of rapamycin; p-MTOR: phosphorylated MTOR (Ser^{2448}); TUBB: beta-tubulin; ADM: adrenomedullin, 10^{-7} M; ANTA: ADM antagonist, ADM_{22-52}, 10^{-7} M; COMB: ADM+ADM antagonist, 10^{-7} M; CONTROL: differentiation medium only. For each parameter, the values of treatment were compared to those of control. The error bar represents the mean ± SEM expressed as the ratio of density of the total or phosphorylated MTOR band to that of TUBB or the ratio of density of phosphorylated to total MTOR proteins (n = 3). *$P < 0.05$.

Although ADM appears to promote MTOR phosphorylation, the ADM antagonist was unable to inhibit the ADM-induced MTOR phosphorylation. Moreover, ADM antagonist itself or the combination of ADM and its antagonist in differentiation medium (Fig. 5) caused increases in the phosphorylation of MTOR. These results are difficult to explain; however, some of the aspects to consider are: first, the phosphorylation and dephosphorylation are dynamic processes and can happen within a few minutes, but in this study, we are assessing the endpoint effect of ADM and its antagonist on phosphorylation after 4 days of treatment. Second, some of these antagonists may exert agonistic functions under certain conditions [40]. Third, differentiated trophoblast cells may produce a large quantity of endogenous ADM [8], which may have an effect on exogenous ADM antagonist.

In summary, we found that ADM enhanced the differentiation of TSCs to TGCs in the presence of differentiation

medium and this stimulatory effect of ADM on TSC differentiation was mediated by ADM receptor consisting of CALCRL and RAMP2 and phosphorylation of MTOR. The involvement of MTOR in ADM regulation of TSC differentiation will help to understand the underlying mechanisms of trophoblast differentiation and relevant pregnancy disorders.

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REFERENCES

1. Simmons DG, Fortier AL, Cross JC. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Dev Biol 2007; 304:567–578.
2. Hu D, Cross JC. Development and function of trophoblast giant cells in the rodent placenta. Int J Dev Biol 2010; 54:341–354.
3. Renaud SJ, Karim Rumi MA, Soares MJ. Review: genetic manipulation of the rodent placenta. Placenta 2011; 32(Suppl 2):S130–S135.
4. Simmons DG, Cross JC. Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. Dev Biol 2005; 284:12–24.
5. Selesniemi K, Reedy M, Gultice A, Guilbert LJ, Brown TL. Transforming growth factor-beta induces differentiation of the labyrinthine trophoblast stem cell line SM10. Stem Cells Dev 2005; 14:697–711.
6. Selesniemi KL, Reedy MA, Gultice AD, Brown TL. Identification of committed placental stem cell lines for studies of differentiation. Stem Cells Dev 2005; 14:535–547.
7. Sahgal N, Canham LN, Canham B, Soares MJ. Rcho-1 trophoblast stem cells: a model system for studying trophoblast cell differentiation. Methods Mol Med 2006; 121:159–178.
8. Kent LN, Konno T, Soares MJ. Phosphatidylinositol 3 kinase modulation of trophoblast cell differentiation. BMC Dev Biol 2010; 10:97.
9. Yallampalli C, Chauhan M, Sathishkumar K. Calcitonin gene-related family peptides in vascular adaptations, uteroplacental circulation, and fetal growth. Curr Vasc Pharmacol 2013; 11:641–654.
10. Miret JJ, Rakhilina L, Silverman L, Oehlen B. Functional expression of heteromeric calcitonin gene-related peptide and adrenomedullin receptors in yeast. J Biol Chem 2002; 277:6881–6887.
11. Kanenishi K, Kuwabara H, Ueno M, Sato C, Sakamoto H, Hata T. Change of adrenomedullin concentrations in plasma and amniotic fluid, and human placental adrenomedullin expression with advancing gestation. Placenta 2001; 22:244–250.
12. Kobayashi K, Kubota T, Aso T, Hirata Y, Imai T, Marumo F. Immunoreactive adrenomedullin (AM) concentration in maternal plasma during human pregnancy and AM expression in placenta. Eur J Endocrinol 2000; 142:683–687.
13. Minegishi T, Nakamura M, Abe K, Tano M, Andoh A, Yoshida M, Takagi T, Nishikimi T, Kojima M, Kangawa K. Adrenomedullin and atrial natriuretic peptide concentrations in normal pregnancy and pre-eclampsia. Mol Hum Reprod 1999; 5:767–770.
14. Wilson C, Nikitenko LL, Sargent IL, Rees MC. Adrenomedullin: multiple functions in human pregnancy. Angiogenesis 2004; 7:203–212.
15. Shindo T, Kurihara Y, Nishimatsu H, Moriyama N, Kakoki M, Wang Y, Imai Y, Ebihara A, Kuwaki T, Ju KH, Minamino N, Kangawa K, et al. Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. Circulation 2001; 104:1964–1971.
16. Li M, Yee D, Magnuson TR, Smithies O, Caron KM. Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. J Clin Invest 2006; 116:2653–2662.
17. Qiu Q, Yang M, Tsang BK, Gruslin A. Both mitogen-activated protein kinase and phosphatidylinositol 3-kinase signalling are required in epidermal growth factor-induced human trophoblast migration. Mol Hum Reprod 2004; 10:677–684.
18. Louden E, Chi MM, Moley KH. Crosstalk between the AMP-activated kinase and insulin signaling pathways rescues murine blastocyst cells from insulin resistance. Reproduction 2008; 136:335–344.
19. Zeng X, Huang Z, Mao X, Wang J, Wu G, Qiao S. N-carbamylglutamate enhances pregnancy outcome in rats through activation of the PI3K/PKB/mTOR signaling pathway. PLoS One 2012; 7:e41192.
20. Kim J, Song G, Gao H, Farmer JL, Satterfield MC, Burghardt RC, Wu G, Johnson GA, Spencer TE, Bazer FW. Insulin-like growth factor II activates phosphatidylinositol 3-kinase-protooncogenic protein kinase 1 and mitogen-activated protein kinase cell signaling pathways, and stimulates migration of ovine trophectoderm cells. Endocrinology 2008; 149:3085–3094.
21. Coan PM, Conroy N, Burton GJ, Ferguson-Smith AC. Origin and characteristics of glycogen cells in the developing murine placenta. Dev Dyn 2006; 235:3280–3294.
22. Anson-Cartwright L, Dawson K, Holmyard D, Fisher SJ, Lazzarini RA, Cross JC. The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. Nat Genet 2000; 25:311–314.
23. Simmons DG, Rawn S, Davies A, Hughes M, Cross JC. Spatial and temporal expression of the 23 murine prolactin/placental lactogen-related genes is not associated with their position in the locus. BMC Genomics 2008; 9:352.
24. Simmons DG, Natale DR, Begay V, Hughes M, Leutz A, Cross JC. Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. Development 2008; 135:2083–2091.
25. Gao H, Yallampalli U, Yallampalli C. Gestational protein restriction affects trophoblast differentiation. Front Biosci (Elite Ed) 2013; 5: 591–601.
26. Asanoma K, Rumi MA, Kent LN, Chakraborty D, Renaud SJ, Wake N, Lee DS, Kubota K, Soares MJ. FGF4-dependent stem cells derived from rat blastocysts differentiate along the trophoblast lineage. Dev Biol 2011; 351:110–119.
27. Penchalaneni J, Wimalawansa SJ, Yallampalli C. Adrenomedullin antagonist treatment during early gestation in rats causes fetoplacental growth restriction through apoptosis. Biol Reprod 2004; 71:1475–1483.
28. Witlin AG, Li ZY, Wimalawansa SJ, Grady JJ, Grafe MR, Yallampalli C. Placental and fetal growth and development in late rat gestation is dependent on adrenomedullin. Biol Reprod 2002; 67:1025–1031.
29. Lenhart PM, Nguyen T, Wise A, Caron KM, Herring AH, Stuebe AM. Adrenomedullin signaling pathway polymorphisms and adverse pregnancy outcomes. Am J Perinatol 2013; 31:327–334.
30. Dackor R, Fritz-Six K, Smithies O, Caron K. Receptor activity-modifying proteins 2 and 3 have distinct physiological functions from embryogenesis to old age. J Biol Chem 2007; 282:18094–18099.
31. Ichikawa-Shindo Y, Sakurai T, Kamiyoshi A, Kawate H, Iinuma N, Yoshizawa T, Koyama T, Fukuchi J, Iimuro S, Moriyama N, Kawakami H, Murata T, et al. The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. J Clin Invest 2008; 118:29–39.
32. Tsujikawa K, Yayama K, Hayashi T, Matsushita H, Yamaguchi T, Shigeno T, Ogitani Y, Hirayama M, Kato T, Fukada S, Takatori S, Kawasaki H, et al. Hypertension and dysregulated proinflammatory cytokine production in receptor activity-modifying protein 1-deficient mice. Proc Natl Acad Sci U S A 2007; 104:16702–16707.
33. Burton GJ, Jauniaux E, Charnock-Jones DS. The influence of the intrauterine environment on human placental development. Int J Dev Biol 2010; 54:303–312.
34. Kong X, Tan B, Yin Y, Gao H, Li X, Jaeger LA, Bazer FW, Wu G. L-Arginine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. J Nutr Biochem 2012; 23:1178–1183.
35. Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. Dev Biol 2001; 240:182–193.
36. Soares MJ, Konno T, Alam SM. The prolactin family: effectors of pregnancy-dependent adaptations. Trends Endocrinol Metab 2007; 18: 114–121.
37. Huang J, Manning BD. A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans 2009; 37:217–222.
38. Gao H, Sathishkumar KR, Yallampalli U, Balakrishnan M, Li X, Wu G, Yallampalli C. Maternal protein restriction regulates IGF2 system in placental labyrinth. Front Biosci (Elite Ed) 2012; 4:1434–1450.
39. Rosario FJ, Jansson N, Kanai Y, Prasad PD, Powell TL, Jansson T. Maternal protein restriction in the rat inhibits placental insulin, mTOR, and STAT3 signaling and down-regulates placental amino acid transporters. Endocrinology 2011; 152:1119–1129.
40. Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Morishita T, Inui T, Fukase M, Noda T, Fujita T. Calcitonin gene-related peptide receptor antagonist human CGRP-(8–37). Am J Physiol 1989; 256:E331–E335.