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MTA3 regulates differentiation of human cytotrophoblast stem cells

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Abstract

Introduction—Early placental development depends on the correct balance of cytotrophoblast (CTB) proliferation and differentiation, into either syncytiotrophoblast (STB) involved in nutrient/gas exchange, or invasive extravillous trophoblast (EVT) involved in establishment of blood flow to the placenta. Metastasis associated protein-3 (MTA3) is a transcriptional co-repressor known to regulate cell migration. In addition, MTA3 is reportedly decreased in preeclampsia. We set out to investigate the role of MTA3 in human trophoblast differentiation.

Methods—We co-stained first and third trimester placental sections with antibodies to MTA3 and other trophoblast markers. We also evaluated MTA3 expression following *in vitro* differentiation of primary isolated CTB. In order to evaluate the role of MTA3 in trophoblast differentiation, we used lentiviral constructs to overexpress and knock down its expression. Trophoblast differentiation was assessed by a combination of marker expression and functional assays, including hCG ELISA and cell migration.

Results—MTA3 was abundantly expressed in CTB and proximal cell column EVT in the human placenta and decreased with further differentiation into STB and mature EVT. MTA3 knockdown in JEG3 resulted in a 2–3 fold decrease in STB markers, CGB and GCM1, as well as in hCG secretion. In terms of EVT differentiation, MTA3 knockdown led to a 1.5–2 fold increase in HLA-G and cell migration, but decreased the mature EVT marker ITGA1.

Discussion—Taken together, our data suggest a role for MTA3 in terminal trophoblast differentiation into both hCG-secreting STB and mature EVT.

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Introduction

Normal placentation is very important for a successful pregnancy. Trophoblasts, the epithelial cells of the placenta, play a significant role in establishment and function of this important organ [1]. Abnormal placental development has been associated with placental dysfunction associated with the maternal hypertensive syndrome of preeclampsia (PE), particularly when this is associated with intrauterine growth restriction (IUGR) [2]. Part of the pathophysiology of these placental disorders is dysregulation of trophoblast proliferation and differentiation. This is seen in both the villous compartment, where accelerated villous maturity leads to premature loss of cytotrophoblast (CTB) and increased numbers of multinucleated syncytiotrophoblast (STB) [3,4], and in the extravillous compartment, where there is insufficient remodeling of maternal spiral arterioles by these extravillous trophoblast (EVT) [5].

Metastasis associated protein-3 (MTA3), a subunit of the nucleosome remodeling and histone deacetylase (NuRD) complex, is a transcriptional co-repressor shown to negatively regulate Snail (SNAIL), the master regulator of epithelial-mesenchymal transition or EMT [6]. MTA3 is most well-studied in breast cancer, where it has been identified as a key link between estrogen receptor status and tumor invasion [6]. MTA3 has also been implicated in regulation of trophoblast invasion, downstream of estrogen receptor signaling [7]. Expression of MTA3 is also reportedly decreased in preeclampsia [8]. To date, however, MTA3 has not been studied in the context of trophoblast differentiation, with only limited localization studies in human placental tissues in vivo [9].

Here we evaluated MTA3 expression in villous as well as extravillous trophoblast in normal human placenta at different points in gestation, and examined its role in trophoblast differentiation using both isolated primary cytotrophoblast and the trophoblast cell line JEG3. We find that MTA3 is localized to proliferative cytotrophoblast and immature extravillous trophoblast in the human placenta and that it plays a role in differentiation into the terminal trophoblast lineages.

Materials and Methods

Human placental samples, including isolation and culture of primary cytotrophoblast

Placental tissues were collected under a protocol approved by the Human Research Protections Program Committee of the University of California San Diego (UCSD) Institutional Review Board. All patients gave informed consent for collection and use of these tissues.

First-trimester CTBs were isolated from elective terminations between 7 and 14 weeks gestational age as previously described [10]. Cell purity was determined by EGFR flow cytometry; the majority of preps were >95% EGFR positive upon isolation. CTB were seeded at a density of 300,000 cells/cm² in 6-well plates coated with 20 µg/ml fibronectin. Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) (Sigma), 2% Penicillin/

Streptomycin, 0.2% Gentamicin, and 55 μ M 2-mercaptoethanol, with media changed every other day.

Isolated primary cells were collected on day 0 and day 4 and fixed with 4% PFA and evaluated by flow cytometry using APC-conjugated anti-EGFR (BioLegend) and PE-conjugated anti-HLA-G (MEMG9, Exbio). Cells were incubated with antibodies at room temperature for 1 hour, washed and resuspended in an appropriate volume of FACS buffer (10% FBS, 1% bovine serum albumin in 1X phosphate-buffered saline/PBS). FACS analysis was carried out using a BD FACS-Canto Flow Cytometer. Analysis was done by FlowJo software.

Immunohistochemistry of placental tissues

Placenta samples were fixed in neutral-buffered formalin for 3 days, processed, embedded in paraffin, and sectioned (5 μ M-thick) for immunohistochemistry. Sections were blocked with 2% goat serum and 0.2% fish skin gelatin (Sigma) in 1X PBS for an hour at room temperature, then incubated with primary antibodies (rabbit anti-MTA3 antibody, mouse anti-hCG antibody (clone 5H4-E2), mouse anti- HLAG antibody (clone 4H84), all from Abcam; mouse anti-Ki67 antibody from Dako; or nonspecific mouse or rabbit IgG as negative controls) at 4 °C overnight. After three washes with 1X PBS with 0.1% Tween-20, sections were incubated with secondary antibodies (Alexa Fluor 488- or 568-conjugated goat anti-mouse or anti-rabbit, from Invitrogen) and DAPI for one hour at room temperature, then mounted with anti-fade mounting solution (Invitrogen) and examined using a Leica fluorescence microscope. Adjacent sections were also stained using hematoxylin and eosin, according to standard protocols, and examined using an Olympus light microscope.

Culture and manipulation of JEG3 cells

The human choriocarcinoma cell line JEG3 was obtained from the American Type Culture Collection (Rockville, MD), grown in Dulbecco's modified Eagle's medium (DMEM) (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% Penicillin-Streptomycin (Life technologies) in 5% CO₂ at 37°C, and passaged every four to five days.

pLKO.1 based scrambled shRNA was obtained from Addgene (Cambridge, MA). Set of five Mission shRNA Lentiviral constructs targeting human MTA3 gene were purchased from Sigma. Full length MTA3 cDNA was PCR amplified and cloned into pCMV-lentiviral-based vector (kindly provided by Dr. Jack Bui, University of California San Diego, La Jolla, CA) using BamHI/SalI restriction sites. Lentiviruses were packaged for transduction according to the manufacture's instructions; lentiviral supernatants were concentrated with PEG-it virus precipitation solution (System Biosciences). The concentrated viral particles were then incubated with target cells in the presence of 8 μ g/ml polybrene (Sigma). Packaging and infection efficiency were tested using a GFP-expressing pLKO.1-based lentivirus. Cells were selected using 5 μ g/ml puromycin over 4–5 passages. Knockdown efficiency was determined by western blot. The combination of all five MTA3 shRNAs worked best in HEK293 cells (data not shown); therefore, subsequent experiments were performed with these five shRNAs pooled together (see Fig. 3).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the NucleoSpin RNAII Kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's protocol. Purity and concentrations of samples were measured using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Science). cDNA was prepared with 500 ng RNA using iScript (Bio-Rad) and diluted five fold in water. SYBR Green (Lifetechnology) qRT-PCR reactions were carried out in biological triplicates. Relative mRNA expression levels, normalized to 18S rRNA, were determined using the C_T method. Primers are listed in Table 1.

Transwell migration assay

Transwell membranes (8.0 μ m pore inserts in 24-well BioCoat Chambers, BD Biosciences) were used to study cell migration in vitro. Cells were trypsinized, washed in cold PBS, and resuspended in serum-free media with 5×10^4 cells per well in the upper chamber and medium with 10% fetal bovine serum in the lower chamber. After 18 h of incubation, cells on the upper surface were removed by scrubbing with a cotton swab; filters were then stained with Diff-Quik Stain Set (Dade Behring, Newark, DE, USA). Five random 20 $\times$ fields per well were counted and combined. Five independent experiments were performed.

Measurement of secreted total hCG

Supernatants were collected from cells cultured over 48 hours. Levels of total hCG were quantified using an hCG ELISA Kit (Calbiotech) following the manufacturer's protocol. The results were normalized to DNA content of cells from the same well, extracted using the DNeasy Kit (Qiagen).

Western blot

Total protein was obtained following cell lysis in 1% TritonX-100 and 0.5% SDS in 1X Tris-buffered saline (TBS) and sonication. Protein concentration was quantified by Pierce BCA protein assay (Thermo Scientific). 25–30 μ g of protein were separated on a 10% SDS-PAGE gel. After transfer to a polyvinylidene fluoride (PVDF) membrane (BioRad), western blotting was performed as previously described [10] using rabbit anti-MTA3 antibody (Abcam) and mouse anti- β -actin (Sigma).

Statistical analysis

All JEG3 data (qPCR, migration, and hCG secretion) are combined from a range of three to five independent experiments, each performed using a different preparation of cells. Paired t-test or Wilcoxon signed-rank test was performed, as appropriate, and P values below 0.05 were taken to indicate a statistically significant difference between the indicated populations.

Results

Expression of MTA3 in the human placenta and isolated primary trophoblast

To examine the role of MTA3 in human trophoblast, we first stained tissues from both early gestation and term (Fig. 1). Four early gestation (6–15 week) placentas and three term

placentas were stained. We used hCG as a marker of syncytiotrophoblast (STB) and HLAG as a marker of extravillous trophoblast (EVT), and co-stained sections with a specific antibody to MTA3. First trimester floating villi showed nuclear staining in cytotrophoblast (CTB), the inner layer of trophoblast adjacent to villous stroma, but not in the multinucleated syncytiotrophoblast (STB), the outer layer of trophoblast adjacent to maternal blood (Fig. 1A, top panels). MTA3 was also present in cell column trophoblast and HLA-G⁺ EVT of anchoring villi in early gestation tissues (Fig. 1A, middle panels), but in the basal plate of the term placenta, it remained only in a small number of HLA-G⁺ cells closer to the villi (Fig. 1A, bottom panels). Adjacent sections of each placenta were also stained using H&E (as shown in Fig. 1A), to confirm localization in specific trophoblast compartments, and using nonspecific rabbit and mouse IgG (as shown in Supplementary Fig. 1), to confirm specificity of antibodies. We also evaluated the relationship between MTA3 expression and proliferation, using the MIB-1 antibody against the Ki67 antigen. All Ki67⁺ trophoblast were MTA3 positive, but the converse was not true, with some MTA3⁺ cell column trophoblast being negative for Ki67 (Fig. 1B). Next, we isolated CTBs from first trimester placentas and cultured them for 4 days *in vitro*. The results presented are representative of experiments with three different CTB preparations. These cells differentiate into both HLA-G⁺ EVT and hCG-secreting multinucleated STB (Fig. 2A–C). Western blot showed a dramatic decrease in MTA3 expression following differentiation (Fig. 2D).

Effect of MTA3 knockdown on trophoblast proliferation and differentiation

We next used the JEG3 choriocarcinoma cell line to examine the role of MTA3 in trophoblast proliferation and differentiation. Of the widely available human trophoblast cell lines (JAR, BeWo, HTR8, and JEG3), JEG3 cells are thought to be most representative of first trimester trophoblast based on appropriate expression of HLA antigens [11]. Following confirmation of 90% or greater MTA3 knockdown in stable cell lines (Fig. 3A), we evaluated differentiation based on expression of lineage-specific markers, including GCM1 and CGB as markers of STB [12], HLAG as a marker of cell column trophoblast and EVT, ITGA5 as a marker of proximal cell column trophoblast, and ITGA1 as a marker of mature, invasive EVT [13]. Compared to the scramble-shRNA line, the knockdown line showed suppression of both STB markers, CGB (50% reduction, $p=0.02$) and GCM1 (40% reduction, $p=0.02$) (Fig. 3B). HLAG was up-regulated 1.8 fold ($p=0.047$). ITGA5 expression was not changed (data not shown), but ITGA1, the marker of mature EVT was reduced (40%, $p=0.01$) (Fig. 3B).

We next turned to assays for functional analysis of MTA3 knockdown in JEG3 cells. Assays for cell proliferation did not show any significant differences in cell number or EdU incorporation following MTA3 knockdown (data not shown). hCG secretion, a functional marker of STB, was measured by ELISA and found to be reduced (60%, $p=0.04$) in MTA3-shRNA-expressing cells compared to those expressing scramble-shRNA (Fig. 4A). Conversely, migration, a phenotype characteristic of cells differentiating down the invasive EVT lineage, was increased 1.8-fold ($p=0.04$) (Fig. 4B).

We next overexpressed MTA3 in JEG3 cells, using a lentiviral vector with a CMV promoter (Fig. 5). qPCR for the same lineage-specific markers showed no statistically significant changes (Fig. 5, and data not shown).

Discussion

Proper development of any organ is the result of a fine balance between proliferation and differentiation of tissue-specific stem cells. In the placenta, cytotrophoblast (CTB) are considered to be such stem cells for the epithelial compartment. These cells are proliferative (Ki67⁺) but are also able to differentiate into both STB and EVT [14]. We previously evaluated the role of p63, a nuclear protein in the p53 family, and its role in the maintenance of the CTB stem cell state [10,15]. In this study, we evaluated MTA3, as we found that this protein also localizes primarily to CTB in the human placenta.

Interestingly, however, the localization of MTA3 is slightly different. Unlike p63, whose expression diminishes abruptly from CTB to the cell column trophoblast [10,14], MTA3 expression appears to be maintained in the cell column and in at least a subset of HLA-G⁺ EVT; it is only lost in mature EVT at the basal plate, and is also absent from multinucleated STB. This pattern resembles that of the proliferation marker, Ki67, for which both CTB and proximal cell column trophoblast are positive [14]. For this reason, we evaluated MTA3 and Ki67 co-staining and found that all Ki67⁺ trophoblast (both CTB and proximal cell column trophoblast) were also MTA3⁺. However, MTA3 was also expressed in some distal column, non-proliferative trophoblast cells. These results suggested a possible role for MTA3 in both proliferation and differentiation of CTB.

In fact, MTA3 has been shown to modulate proliferation in other cell types, including mouse granulose cells [16]. However, unlike p63, whose knockdown decreased proliferation in JEG3 cells [10], MTA3 knockdown revealed no such effect in these cells. This may be because as choriocarcinoma cells, these cells are not representative of primary trophoblast, particularly in terms of regulation of cell proliferation; unfortunately, since primary CTB do not proliferate in culture, assessment of proliferation following genetic manipulation is not possible.

Alteration of MTA3 expression did affect trophoblast differentiation but in a different manner than p63. While p63 knockdown led to increased differentiation [10], MTA3 appeared to be required for terminal differentiation, for both STB and EVT lineages: knockdown of MTA3 reduced GCM1, CGB, and ITGA1, as well as secretion of hCG. Nevertheless, MTA3 knockdown led to slight, but statistically significant, increase in HLAG, suggesting that differentiation into proximal cell column-type trophoblast was not inhibited. This is also supported by an increase in cell migration in MTA3 knockdown cells. It therefore appears that, unlike p63, which is required for maintenance of the CTB stem cell state, MTA3 facilitates terminal differentiation of CTB: in anchoring villi, it is required for differentiation of proximal column trophoblast into mature EVT, and in floating villi, it is required for differentiation into hCG-secreting STB.

In addition to MTA3 knockdown, we also overexpressed MTA3 in JEG3 cells. However, we did not note any effect on lineage-specific marker expression or hCG secretion (data not shown). While this may be due to a suboptimal induction of MTA3 expression, it is also possible that MTA3 is required, but not sufficient, for alterations in trophoblast differentiation.

Our study is consistent with findings of Chen et al. (2013), including expression of MTA3 in CTB, and its loss in STB. However, following knockdown of MTA3 in BeWo cells, they noted an *increase* in hCG protein and induction of a CGB5 promoter-driven luciferase. We always noted a decrease, both in the CGB RNA and in hCG secretion, upon MTA3 knockdown, both in JEG3 cells (as shown above) and BeWo cells (data not shown). These differences may be due to MTA3 knockdown having opposite effects on expression of hCG components and its secretion. Since STB are functionally defined by their secretion of this hormone, and not simply the synthesis of its components, we propose that MTA3 in fact plays a role in induction of STB, based on the fact that its knockdown leads to decreased expression of GCM1 and CGB as well as decreased secretion of hCG.

In conclusion, our data suggest a role for MTA3 in facilitating terminal trophoblast differentiation, specifically showing that it is required for the transition to terminally differentiated EVT and STB. Future studies will focus on how MTA3 interacts with other factors (both transcription factors and chromatin modifiers) to modulate these differentiation states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- MTA3 is expressed in cytotrophoblast cell column trophoblast.
- MTA3 expression is lost in syncytiotrophoblast and mature extravillous trophoblast.
- MTA3 knockdown inhibits terminal differentiation of trophoblast.

Figure 1.

Expression of MTA3 in the human placenta. **A)** Immunohistochemistry of early gestation and term placental sections with antibodies to MTA3, hCG, and HLAG. Note MTA3 expression in cytotrophoblast (arrows in top panels) is lost in hCG-positive syncytiotrophoblast (arrowheads in top panels). MTA3 is also expressed in extravillous trophoblast (EVT), but is most commonly seen in EVT proximal to the chorionic villi (arrows in middle and bottom panels), decreasing as these cells mature and move away from the villi (arrowheads in middle and bottom panels). **B)** MTA3 co-staining with Ki67. Note expression of MTA3 in all cell column trophoblast (arrows), including all Ki67⁺ cells in this region.

Figure 2.

MTA3 expression in the cultured primary cytotrophoblast (CTB). **A–C**) After 4 days in culture, first trimester CTB differentiate, as noted by morphologic changes, hCG secretion, and HLAG expression. **D**) MTA3 protein expression is high in isolated CTB (day 0) and decreased following differentiation (day 4).

Figure 3.

MTA3 knockdown in JEG3 trophoblast cell line. **A)** Confirmation of MTA3 knockdown (>90%) based on protein expression. **B)** qRT-PCR for *HLAG*, *GCM1*, *CGB*, and *ITGA1* in JEG3 MTA3 knockdown cells, compared to scramble control. Data are normalized to 18S and expressed as fold change relative to the scramble control cells as mean $\pm$ standard deviation (from four independent experiments). *Indicates $p < 0.05$ relative to the scramble control.

Figure 4.

Assessment of trophoblast function following MTA3 knockdown. **A)** hCG ELISA of scramble control and MTA3 knockdown JEG3 cells. hCG secretion was normalized to DNA concentration. Data are from three independent experiments, expressed as mean $\pm$ standard deviation. **B)** Transwell migration assay. Five independent 20X fields for each well were combined used for quantification. Data are from five independent experiments, expressed as mean $\pm$ standard deviation. For both A and B, *indicates $p < 0.05$ relative to the scramble control.

Relative mRNA expression

Figure 5. MTA3 overexpression in JEG3 trophoblast cell line. qRT-PCR for *MTA3*, *CGB*, *ITGA1*, and *HLAG*. Data are normalized to 18S and expressed as fold change relative to the mock (empty vector) control cells as mean $\pm$ standard deviation (from four independent experiments). *Indicates $p < 0.05$ relative to the mock control.

Table 1

List of Quantitative PCR Primers

Primer Name	Primer sequence
MTA3	F 5'- TCC TCC AGC AAC CCA TAC CT -3' R 5'- TCG GTC AAG TCA GCC TCA AC -3'
HLA3	F 5'- CAG ATA CCT GGA GAA CGG GA -3' R 5'- CAG TAT GAT CTC CGC AGG GT -3'
GCM1	F 5'- CTC GCA GAG GAG GGG CGC AA -3' R 5'- ATA AAG CGT CCG TCG TGC CTC -3'
CGB	F 5'- ACC CTG GCT GTG GAG AAG G -3' R 5'- ATG GAC TCG AAG CGC ACA -3'
ITGA1	F 5'- CTG GAC ATA GTC ATA GTG CTG GA -3' R 5'- ACC TGT GTC TGT TTA GGA CCA -3'
ITGA5	F 5'- GGC TTC AAC TTA GAC GCG GAG -3' R 5'- TGG CTG GTA TTA GCC TTG GGT -3'
18S	F 5'-CGC CGC TAG AGG TGA AAT TCT-3' R 5'-CGA ACC TCC GAC TTT CGT TCT-3'