

OVO-like 1 regulates progenitor cell fate in human trophoblast development

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Epithelial barrier integrity is dependent on progenitor cells that either divide to replenish themselves or differentiate into a specialized epithelium. This paradigm exists in human placenta, where cytotrophoblast cells either propagate or undergo a unique differentiation program: fusion into an overlying syncytiotrophoblast. Syncytiotrophoblast is the primary barrier regulating the exchange of nutrients and gases between maternal and fetal blood and is the principal site for synthesizing hormones vital for human pregnancy. How trophoblast cells regulate their differentiation into a syncytium is not well understood. In this study, we show that the transcription factor OVO-like 1 (OVOL1), a homolog of *Drosophila ovo*, regulates the transition from progenitor to differentiated trophoblast cells. OVOL1 is expressed in human placenta and was robustly induced following stimulation of trophoblast differentiation. Disruption of OVOL1 abrogated cytotrophoblast fusion and inhibited the expression of a broad set of genes required for trophoblast cell fusion and hormonogenesis. OVOL1 was required to suppress genes that maintain cytotrophoblast cells in a progenitor state, including *MYC*, *ID1*, *TP63*, and *ASCL2*, and bound specifically to regions upstream of each of these genes. Our results reveal an important function of OVOL1 as a regulator of trophoblast progenitor cell fate during human trophoblast development.

epithelial barrier | placenta | trophoblast | OVO-like 1 | differentiation

Epithelial cells turn over regularly and are reliant on a pool of cells that either replenish the reservoir of progenitor cells or differentiate into the specialized epithelium required for that tissue's function. An excellent paradigm of epithelial turnover exists in the human placenta, where mononuclear cytotrophoblast cells lining the inner portion of the chorionic villi comprise the progenitor cells of the placental epithelium. These cells either propagate to maintain an adequate reservoir of progenitor cells or undergo a differentiation program that results in fusion with an overlying syncytium (1). This syncytium, termed "syncytiotrophoblast," forms the principal epithelial barrier separating maternal and fetal blood. Syncytiotrophoblast plays a vital role in regulating nutrient, water, waste, and gas exchange between maternal and fetal circulations and produces various hormones vital for fetal development and the maintenance of human pregnancy (2). Because of its importance for fetal health and development, disruptions in syncytiotrophoblast formation or functionality can have devastating consequences for pregnancy (3–5).

Syncytiotrophoblast has a limited lifespan and is shed into the maternal circulation throughout pregnancy (6, 7). Therefore, to maintain the integrity of the maternal–fetal exchange surface, syncytiotrophoblast is continually replenished by select populations of cytotrophoblast cells that forego self-renewal and instead fuse into the overlying syncytiotrophoblast. This feature, analogous to paradigms established in other epithelial barrier systems in which differentiated cells are replenished by underlying stem/progenitor cell populations but unique in the nature of the differentiation program (cell fusion), is fundamental to human placentation (1). A key question that remains to be addressed is how cytotrophoblast cells "decide" to exit their progenitor state and commence cell fusion.

Activation of cytotrophoblast fusion is a highly synchronized event, requiring that (i) the genes involved with maintenance of progenitor states are turned off and (ii) genes characteristic of differentiated phenotypes are turned on. To date, several regulatory factors and signaling pathways have been linked with the promotion of human cytotrophoblast fusion (reviewed in refs. 8–10). The most notable of these factors include the *syncytin* genes *ERVW-1* and *ERVFRD-1*, which are co-opted retroviral *env* genes embedded in the human genome. These genes, uniquely expressed by trophoblast cells, encode proteins that act as cellular fusogens (11, 12). Transcriptional activation of both *ERVW-1* and *ERVFRD-1* is promoted by the chorion-specific transcription factor glial cells missing-1 (GCM1) (13, 14). However, there is a dearth of knowledge about how regulatory factors promoting the maintenance of the cytotrophoblast progenitor state are suppressed to facilitate cell differentiation.

To gain insight into potential transcriptional regulators of trophoblast differentiation, we performed a DNA microarray using a well-characterized in vitro model of human trophoblast fusion. Using this approach, we found that OVO-like 1 (OVOL1) was the most highly induced transcription factor associated with trophoblast syncytialization. The robust increase of OVOL1 expression is intriguing, given its known role as an early inducer of terminal differentiation in distinct epithelial cell lineages of a wide spectrum

Significance

Epithelial barrier integrity is dependent on progenitor cells that either divide to replenish themselves or differentiate into a functional epithelium. In the placenta, cytotrophoblast cells comprise this progenitor population, but the differentiation program they undertake is unlike any other in human tissues: acquisition of hormonogenesis and cell fusion to form a syncytialized (syncytio)trophoblast. Syncytiotrophoblast forms the primary epithelial barrier separating maternal and fetal tissue and performs functions vital for pregnancy. In the present study, we found that OVO-like 1 (OVOL1), a transcription factor homolog of *Drosophila ovo*, regulates the transition between progenitor and differentiated cytotrophoblast. It does so by repressing genes that maintain cytotrophoblast progenitor traits. This study provides insight into the role of OVOL1 in human trophoblast development.

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of organisms [e.g., flies, worms, and mice (15–20)]. *OVOL1* is a highly conserved C2H2 zinc finger transcription factor homologous to *Drosophila ovo*. An initial characterization of *OVOL1* expression in human tissues revealed high levels in placenta and weaker expression in only one other organ, fetal kidney (21), although studies in mice indicate that it may be expressed in some other epithelial tissues (e.g., epidermis and male germinal epithelium) (17). Given the evidence that *OVOL1* is involved in the regulation of epithelial differentiation during early development, and because trophoblast cells are epithelial in nature, we postulated that *OVOL1* is involved in human trophoblast differentiation. In this study, we examined *OVOL1* expression in human placenta and used a loss-of-function approach using several models of human trophoblast cell differentiation to determine the importance of *OVOL1* in syncytiotrophoblast formation. We show that *OVOL1* is required to restrict the expression of key factors that maintain cytotrophoblast cells in a progenitor state, thereby facilitating the induction of differentiation-associated transcripts, including major genes required for syncytiotrophoblast hormonogenesis and both human fusogenic *syncytin* genes.

Results

Gene-Expression Changes Associated with Syncytiotrophoblast Development.

In human placenta, trophoblast cells lining chorionic villi are segregated into two layers: a basal layer of mononuclear cytotrophoblast cells that express E-cadherin (CDH1) and an outer multinucleated syncytiotrophoblast layer that lacks CDH1 but robustly expresses the pregnancy hormone chorionic gonadotropin [CG; immunostaining for the CG β subunit (CGB) is shown in Fig. 1A]. This cellular transition from CDH1-expressing mononuclear trophoblast cells to CG-expressing (CDH1-lacking) multinucleated syncytiotrophoblast can be modeled in vitro by exposing BeWo trophoblast cells to cAMP analogs (Fig. 1B–E) (22). Induction of differentiation in this model requires that trophoblast cells switch from a progenitor state to a committed cytotrophoblast state before ultimately undergoing cell fusion. We noted a progressive, time-dependent increase in syncytialization events (clusters of cells that lost their CDH1 skeleton and gained expression of CGB) following exposure to 250 μ M 8-bromo-cAMP (henceforth referred to as “differentiating conditions” unless otherwise indicated). When these cells were exposed to differentiating conditions for 48 h, ~20% of cell nuclei were contained within syncytia (Fig. 1F). Therefore, to profile early changes in gene expression before the initiation of cell fusion, we performed a DNA microarray comparing cells cultured under control conditions or following incubation for 24 h in differentiating conditions. Overall, from a total of 15,098 transcripts examined, 369 were changed significantly (more than twofold) between the two groups ($P < 0.05$). Of these, 150 transcripts were decreased, and 219 transcripts were increased (Fig. S1A). The top 45 transcripts exhibiting an increase or decrease following exposure to differentiating conditions are listed in Table S1. Transcripts that were induced following differentiation included those involved in hormonogenesis (*CGA*, *CGB*, *HSD3B1*, *HSD11B2*, and *CYP19A1*), as is consistent with transitioning to an endocrinologically competent syncytiotrophoblast (Fig. S1B and Table S1). From this DNA microarray analysis, we determined that the conserved C2H2 zinc finger transcription factor *OVOL1* was the most highly up-regulated transcript encoding a transcription factor (5.95-fold increase) (Fig. 2).

Expression of *OVOL1* in Trophoblast Cells and in Human Placenta.

OVOL1 was induced robustly at both RNA and protein levels following stimulation of differentiation (Fig. 3A and B). *OVOL1* protein expression was detectable as early as 6 h following exposure to differentiation conditions and was maximal at 24 and 48 h postinduction. Both nuclear/cytoplasmic cell fractionation and immunofluorescence showed that *OVOL1* accumulates in the nucleus following stimulation of differentiation (Fig. 3B and

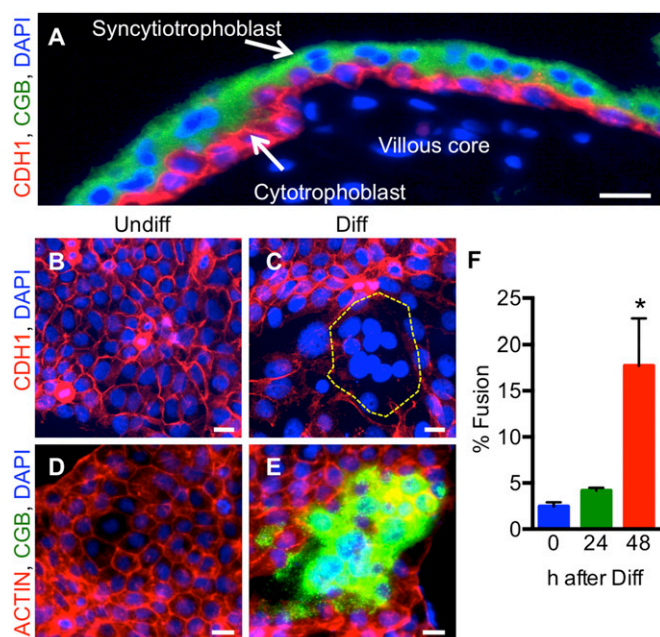


Fig. 1. In situ and in vitro analysis of syncytiotrophoblast. (A) Eight-week human placenta immunostained for CDH1 (red) and CGB (green). Nuclei are counterstained blue using DAPI. Note that CDH1 is expressed specifically by cytotrophoblast cells, whereas CGB localizes exclusively to syncytiotrophoblast. (B–E) BeWo trophoblast cells cultured under undifferentiated (Undiff; B and D) or differentiating (Diff; C and E) conditions. Cells subsequently were immunostained with CDH1 (B and C) or CGB (D and E). Cells were counterstained with phalloidin in D and E to demarcate the actin cytoskeleton and with DAPI to identify nuclei in all panels. Note the presence of cell clusters that have lost CDH1 expression and express CGB following exposure to differentiating conditions. (Scale bars, 25 μ m.) (F) Graphical depiction showing the number of nuclei associated with fused cells following 0, 24, and 48 h exposure to differentiating conditions. * $P < 0.05$, $n = 4$.

C). *OVOL1* is detected at strong levels in mononuclear cells, indicating that it is induced before cell fusion; expression also was detected to a lesser extent in fused cells. We also demonstrated that *OVOL1* transcript was stimulated by 8-Br-cAMP in a dose-responsive manner (Fig. 3D). Higher doses of 8-Br-cAMP increased the extent of trophoblast differentiation (0 μ g/mL 8-Br-cAMP: <1% of nuclei in syncytia; 250 μ g/mL 8-Br-cAMP: 13.9% of nuclei in syncytia; 1,500 μ g/mL 8-Br-cAMP: 29.4% of nuclei in syncytia, $P < 0.05$; representative images are shown in Fig. 3E).

To determine whether *OVOL1* is expressed in human placenta, we performed RT-PCR analysis at four gestational ages: 6, 8, 12, and 39 wk of gestation. *OVOL1* was expressed in human placenta throughout pregnancy (Fig. 4A). In situ hybridization analysis revealed that *OVOL1* was expressed by a subset of *CDH1*⁺ cytotrophoblast cells (Fig. 4B–G). The frequency of *OVOL1*⁺ cells was higher in the early stages of pregnancy, when many cytotrophoblast cells are present, and waned by term, when the relative number of cytotrophoblast cells declines. We also isolated primary cytotrophoblast cells from term placenta. Under our culture conditions, primary cytotrophoblast cells rapidly lose their progenitor characteristics and thus do not proliferate ex vivo; instead they fuse spontaneously to form syncytiotrophoblast (Fig. 4H). We identified high expression of *OVOL1* in freshly isolated cytotrophoblast cells (6 h plated), but expression declined once cells syncytialized (Fig. 4H–K; *CGA* and *CGB* transcript expression profiles are presented in Fig. S2). These results are consistent with a predominantly cytotrophoblastic expression pattern of *OVOL1*.

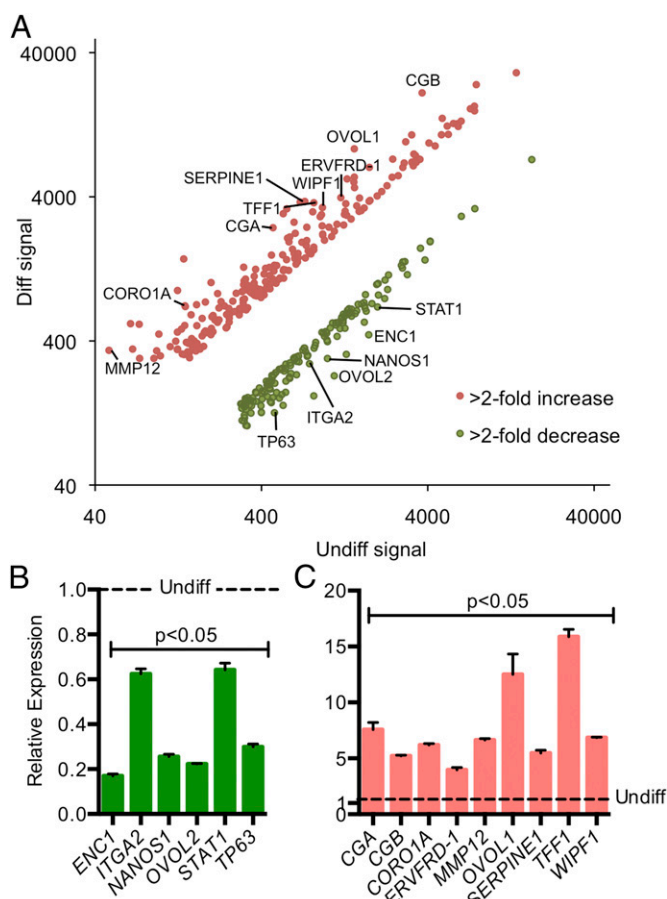


Fig. 2. DNA microarray analysis was conducted on BeWo trophoblast cells cultured under undifferentiated (Undiff) or differentiating (Diff) conditions for 24 h. (A) Scatter plot showing the signal intensity of probe sets. Transcripts highlighted in pink are increased significantly (twofold or more); transcripts highlighted in green are decreased significantly (twofold or more). Selected transcripts are identified. (B and C) qRT-PCR validation of selected transcripts that decreased (B; $n = 3$; all $P < 0.05$) and increased (C; $n = 3$; all $P < 0.05$) following differentiation. Data are normalized to values obtained from trophoblast cells under undifferentiated conditions denoted with a dashed line.

OVOL Factors in Rodent Placenta. Because mice and rats exhibit hemochorial placentation and have a fetal–maternal exchange surface lined by syncytialized trophoblast, features shared with the human, we sought to determine whether *Ovol1* is expressed in rodent placenta. RT-PCR analysis on mouse and rat placentae revealed that *Ovol1* is expressed at low levels in both tissues. Mouse and rat skin were used as positive controls. *Ovol1* mRNA was not detectable in mouse trophoblast stem (TS) cells or rat TS cells in either stem or differentiated states. However, a related transcription factor, *Ovol2*, was readily detectable in both mouse and rat placentae as well as in mouse and rat TS cells following induction of differentiation (Fig. S34). *OVOL2* expression was readily apparent in trophoblast cells located in both labyrinth and junctional zones of mouse and rat placenta, but blood cells, deciduas, and most embryonic tissues were negative (Fig. S3B; decidua is shown at the top of each panel and represents a negative control).

OVOL1 Is Required for Trophoblast Syncytialization. To determine the biological significance of *OVOL1* in human trophoblast cells, we used a loss-of-function strategy to decrease *OVOL1* expression. Lentiviral vectors were used to deliver shRNAs targeting *OVOL1* in BeWo trophoblast cells. Two shRNAs targeting *OVOL1* were used: sh*OVOL1*-a and sh*OVOL1*-b. Controls consisted of cells

infected with virus carrying an shRNA with no known mammalian target. Incorporation of shRNAs had no discernable effect on cell proliferation or viability under undifferentiated conditions. Following stimulation of differentiation for 48 h, we observed a marked reduction of *OVOL1* mRNA and protein expression in cells expressing either *OVOL1*-a or *OVOL1*-b shRNAs (Fig. 5A and B, respectively). Cells expressing *OVOL1*-a or *OVOL1*-b shRNAs exhibited a 69% and 79% reduction, respectively, in cell fusion compared with cells expressing the control shRNA (Fig. 5C; representative images are shown in Fig. 5D). These results demonstrate that *OVOL1* contributes to the regulation of trophoblast syncytialization.

Whole Transcriptome Analysis Reveals the Importance of *OVOL1* for the Promotion of Trophoblast Differentiation. To ascertain globally the transcriptional targets dependent on *OVOL1* expression, we performed high-throughput RNA sequencing (RNA-seq) comparing BeWo trophoblast cells expressing control shRNA versus cells deficient in *OVOL1* (expressing sh*OVOL1*-b), cultured under differentiating conditions. Overall, 4,452 transcripts met or exceeded the threshold reads per kilobase of exon per million reads mapped (RPKM) in at least one of the two groups (Fig. 6A). Of these, 79 transcripts were changed significantly (twofold or more; $P < 0.05$) between the two groups (Table S2). Fourteen transcripts, 13 that were differentially expressed between the two groups and one that was not significantly changed (*ERVH48-1*), were validated by quantitative RT-PCR (qRT-PCR) (Fig. 6B).

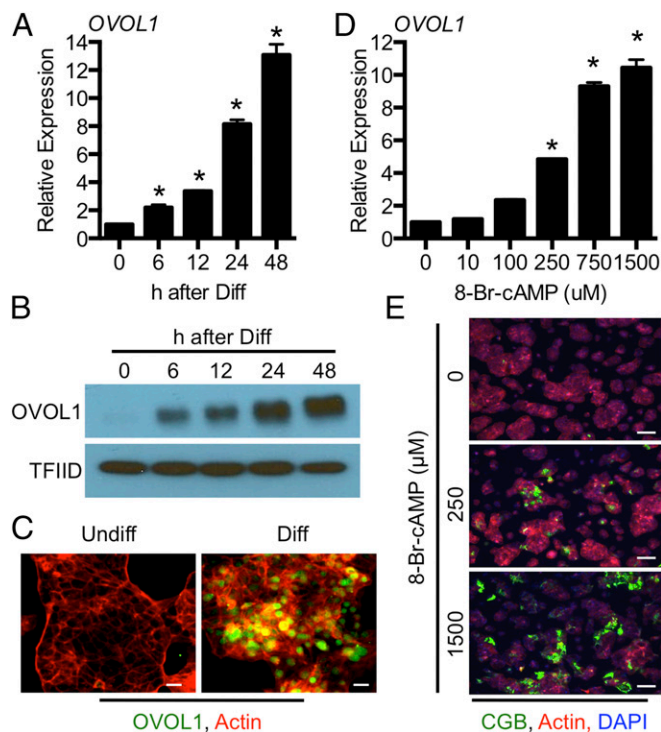


Fig. 3. *OVOL1* is induced under differentiating conditions. BeWo trophoblast cells were exposed to differentiating (Diff) conditions for 6, 12, 24, or 48 h. Undifferentiated (Undiff) cells (0 h) were used as controls. (A and B) qRT-PCR (A) and Western blotting (B) showing expression of *OVOL1* mRNA and nuclear protein accumulation, respectively. (C) *OVOL1* immunolocalization in cells maintained in undifferentiated or differentiating conditions for 48 h. (D) qRT-PCR for *OVOL1* expression following exposure to various concentrations of 8-Br-cAMP for 48 h. (E) Immunofluorescence images of CGB (green) showing differentiated BeWo trophoblast cells cultured under control conditions or in the presence of 250 or 1,500 μM 8-Br-cAMP for 48 h. Phalloidin was used as a counterstain in C and E to demarcate the actin cytoskeleton. * $P < 0.05$; $n = 3$. (Scale bars, 50 μm .)

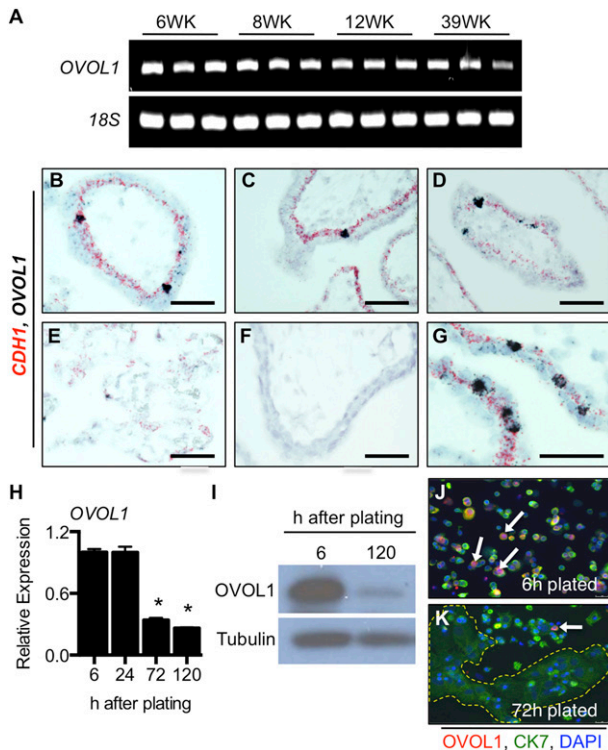


Fig. 4. OVOL1 is expressed in human placenta. (A) RT-PCR analysis of human placenta collected at 6, 8, 12, and 39 wk of gestation. 18S rRNA was used as a loading control. (B–G) Two-plex in situ hybridization of *OVOL1* (black) and *CDH1* (red) in 6-wk (B), 8-wk (C), 12-wk (D), and 39-wk (E) placentae. Note that *OVOL1* is expressed specifically in select cells within the *CDH1*-positive cytotrophoblast layer. (F) Hybridization using a negative control probe (*DapB*). (G) Higher magnification of *OVOL1* and *CDH1* at 6-wk gestation. (H) Term cytotrophoblast cells were plated for 6, 24, 72, or 120 h. Relative transcript expression of *OVOL1* was assessed at each time point. * $P < 0.05$; $n = 3$. (I) Protein expression of *OVOL1* in primary trophoblast cell lysates following 6-h and 120-h plating, as determined by Western blotting. Tubulin was used as a loading control. (J and K) Immunofluorescent images of *OVOL1* (red) and cyokeratin 7 (green) are shown at 6-h plating (J) and 72-h plating (K). Nuclei are counterstained with DAPI. The dashed line in K demarcates a large syncytial cluster; white arrows indicate cells expressing high levels of *OVOL1*. (Scale bars, 50 μm .)

Transcripts that were significantly decreased in *OVOL1*-deficient trophoblast cells included those encoding hormone subunits and hormone biosynthetic machinery robustly expressed in syncytiotrophoblast (*CGA*, *CGB*, *CGB7*, *CGB5*, *CGB8*, *CTB-60B18.6*, *HSD3B1*, *HSD11B2*, and *CYP19A1*), as is consistent with *OVOL1* having an essential role in facilitating syncytiotrophoblast development (Fig. 6 B and C). Intriguingly, we also noted that *OVOL1* deficiency caused a marked reduction in the expression of four endogenous retrovirus-derived genes, including transcripts that encode both *syncytin* fusogens [*ERVW-1* (*syncytin-1*), 60% decrease; *ERVFRD-1* (*syncytin-2*), 83% decrease; *ERVV-1*, 69% decrease; *ERVV-2*, 66% decrease; Fig. 6 B and C]. We also confirmed that the expression of all four of these endogenous retrovirus-derived genes was induced following stimulation of differentiation (*ERVW-1*: 2.8-fold; *ERVFRD-1*: 24.4-fold; *ERVV-1*: 10.1-fold; *ERVV-2*: 11.0-fold) and that trophoblast cells deficient in *OVOL1* (i.e., expressing either *shOVOL1-a* or *shOVOL1-b*) exhibited markedly reduced expression of each gene (Fig. 6D). Expression of a fifth endogenous retrovirus-derived gene, *ERVH48-1*, which was highly expressed in trophoblast cells, was not altered during the course of trophoblast differentiation, nor was it affected by *OVOL1* depletion, indicating that *OVOL1* does not generically target genes with retroviral ori-

gins. We also performed Western blotting for *ERVFRD-1* to determine whether protein levels correlated with the changes we observed in mRNA levels. *ERVFRD-1* protein was robustly increased in control cells cultured in differentiating conditions but not in *OVOL1*-deficient cells (Fig. 6E). Finally, to determine the effect of *OVOL1* deficiency on the expression of endogenous retroviral-derived genes in primary cytotrophoblast cells, we transduced these cells with lentivirus encoding control shRNA, *shOVOL1-b*, or GFP (to examine transduction efficiency; Fig. 6F) and 48 h later assessed transcript levels of these retroviral genes. Using this strategy, we achieved ~40% knockdown of *OVOL1*. Intriguingly, despite the limited degree of *OVOL1* knockdown, we still observed reduced expression of two endogenous retrovirus-derived genes in cells with *OVOL1* knockdown: *ERVFRD-1* (34% decrease) and *ERVV-1* (30% decrease) (Fig. 6G). Collectively, these results demonstrate that *OVOL1* regulates genes associated with syncytiotrophoblast formation and function.

OVOL1 Regulates Development of the Syncytiotrophoblast Phenotype in Bone Morphogenetic Protein-Treated Human Pluripotent Stem Cells.

To gain insight into the role of *OVOL1* in the regulation of syncytiotrophoblast development in a complementary model of human trophoblast differentiation, we treated human ES cells with bone morphogenetic protein 4 (BMP4). Treatment of human ES cells with BMP4 promotes the acquisition of a trophoblast cell fate, including expression of genes associated with syncytiotrophoblast differentiation (23–26). After stimulating ES cells with BMP4, we noted a substantial induction of transcripts expressed by differentiated trophoblast cells, including *CGA* (40,360-fold), *CGB* (2,316-fold), and the aforementioned endogenous retrovirus-derived genes *ERVW-1* (650-fold), *ERVFRD-1* (110-fold), *ERVV-1* (1,400-fold), and *ERVV-2* (1,828-fold) (all $P < 0.05$) (Fig. 7A). *CGB* immunofluorescence is shown in Fig. 7B. Compellingly, *OVOL1* was increased substantially at both RNA (25-fold; $P < 0.05$) and protein levels following differentiation. Similar results were obtained using human induced pluripotent stem (iPS) cells (Fig. S4). Knockdown of *OVOL1* in human ES cells before differentiation abrogated the expression of *CGA* (89% decrease), *CGB* (79% decrease), and all four endogenous retrovirus-derived genes (*ERVW-1*: 73% decrease; *ERVFRD-1*: 57% decrease; *ERVV-1*: 78% decrease; *ERVV-2*: 82% decrease; all $P < 0.05$) (Fig. 7C). Again, similar results were obtained using *OVOL1*-deficient human iPS cells (*ERVW-1*: 66% decrease; *ERVFRD-1*: 84% decrease; *ERVV-1*: 58% decrease; *ERVV-2*: 60% decrease, all $P < 0.05$) (Fig. S4).

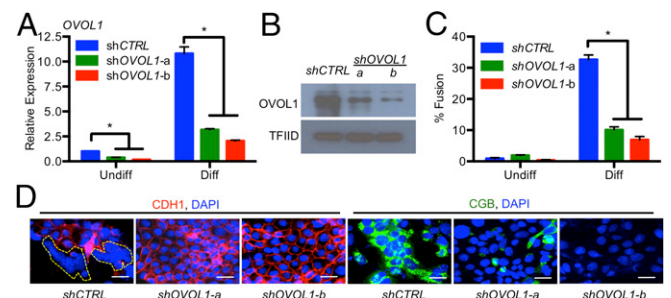


Fig. 5. *OVOL1* regulates trophoblast cell fusion. BeWo trophoblast cells expressing control shRNA (*shCTRL*) or *OVOL1* shRNAs (*shOVOL1-a* and *shOVOL1-b*) were cultured under standard (Undiff) or differentiating (Diff) conditions for 48 h. (A and B) Efficiency of *OVOL1* knockdown was determined by qRT-PCR (A) and Western blotting (B). (C) The number of nuclei associated with fused cells. (D) Representative images of *CDH1* and *CGB* expression in trophoblast cells expressing *shCTRL*, *shOVOL1-a*, or *shOVOL1-b* cultured under differentiating conditions. Nuclei are counterstained blue using DAPI. * $P < 0.05$; $n \geq 3$. (Scale bars, 50 μm .)

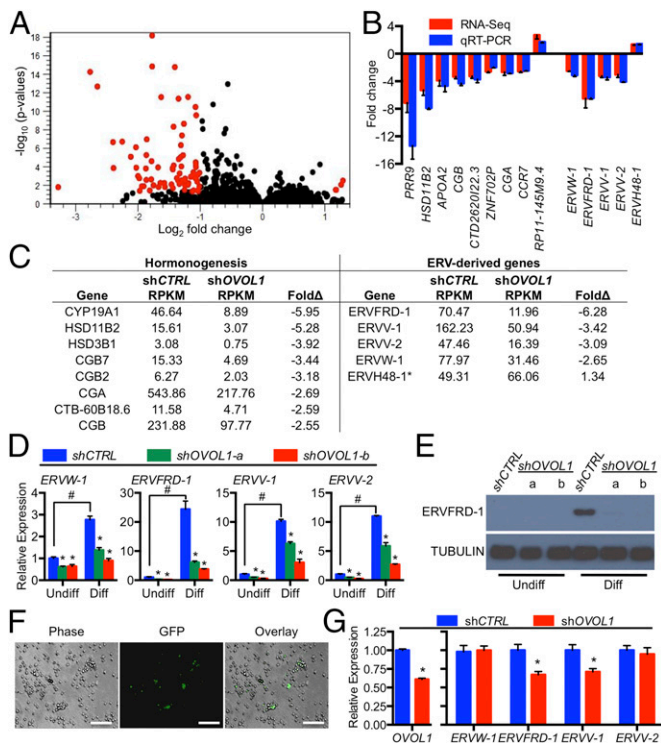


Fig. 6. Analysis of transcripts affected by OVOL1 knockdown in trophoblast cells. RNA-seq was performed on BeWo trophoblast cells expressing either control shRNA (shCTRL) or shOVOL1-b under differentiating (Diff) conditions. (A) Volcano plot showing 4,552 unique transcripts expressed in trophoblast cells. Transcripts altered twofold or more ($P < 0.05$) in cells expressing shOVOL1-b are shown in red. (B) Correlative analysis comparing mRNA fold changes of 14 transcripts in trophoblast cells expressing shOVOL1-b relative to cells expressing shCTRL, as determined by RNA-seq (red bars) and qRT-PCR (blue bars). (C) Fold differences in RPKM in selected transcripts associated with hormonogenesis (Left) and endogenous retrovirus (ERV)-derived genes (Right) between trophoblast cells expressing shOVOL1-b compared with cells expressing shCTRL. (D) qRT-PCR analysis of ERV-derived genes in trophoblast cells expressing shCTRL, shOVOL1-a, or shOVOL1-b under undifferentiated (Undiff) or differentiating conditions. * $P < 0.05$, comparing cells cultured in undifferentiated and differentiating conditions. (E) Protein expression of ERVFRD-1 in trophoblast cells expressing shCTRL, shOVOL1-a, or shOVOL1-b under undifferentiated or differentiating conditions. α -Tubulin was used as a loading control. (F) Primary cytotrophoblast cells were infected with a GFP-containing pLKO.3G vector. The number of GFP-expressing trophoblast cells was assessed 48 h later. (Scale bars, 100 μ m.) (G) Transcript expression of *OVOL1*, *ERVV-1*, *ERVFRD-1*, *ERVV-1*, and *ERVV-2* in primary cytotrophoblast cells transduced with shRNAs targeting *OVOL1* (shOVOL1-b). * $P < 0.05$ comparing cells expressing shCTRL or shOVOL1. $n = 3$ except in G, in which $n = 6$.

OVOL1 Suppresses Factors Involved in Maintaining the Cytotrophoblast Progenitor State. We reasoned that the effects of OVOL1 deficiency on the expression of genes associated with syncytiotrophoblast differentiation could be explained best by (i) OVOL1 directly repressing genes that maintain the cytotrophoblast progenitor state or (ii) OVOL1 facilitating the activation of genes that promote syncytiotrophoblast formation. The first option is consistent with the recognized role of OVOL1 as a transcriptional repressor (27). Therefore, to screen for potential intermediary targets of OVOL1 involved in maintaining the progenitor status of cytotrophoblast cells, we perused our DNA microarray for known transcriptional regulators whose expression was down-regulated early during differentiation. Through this analysis we found that *MYC*, *ID1*, and *TP63* were all significantly down-regulated following 24-h stimulation of differentiation (*MYC*: 38% decrease; *ID1*: 57% decrease; *TP63*: 68% decrease; all $P < 0.05$). We also examined the expression of *ASCL2*, which encodes a basic helix-loop-helix transcription

factor essential for diploid trophoblast development in mice (28), because its expression trended upward following OVOL1 knockdown (1.9-fold increase following OVOL1 knockdown). We confirmed by both qRT-PCR and Western blotting that the expression of each of these factors declined progressively during BeWo trophoblast differentiation in a time-dependent manner (Fig. 8A and B). Immunofluorescence staining for phospho-histone H3 revealed a loss of proliferative potential in cells during differentiation (75% fewer cells expressed phospho-histone H3 following stimulation of differentiation; $P < 0.05$) (Fig. 8C). To determine whether *MYC*, *TP63*, *ID1*, and *ASCL2* are expressed by proliferating cytotrophoblast cells in vivo, we assessed the expression of these proteins in human placenta using immunohistochemistry. Proliferating cell nuclear antigen (PCNA) was used to detect cytotrophoblast cells maintained in a progenitor state; CGB was used as a marker for syncytiotrophoblast. *MYC*, *TP63*, *ID1*, and *ASCL2* were detected exclusively in cytotrophoblast cells in situ. *MYC* and *TP63* were

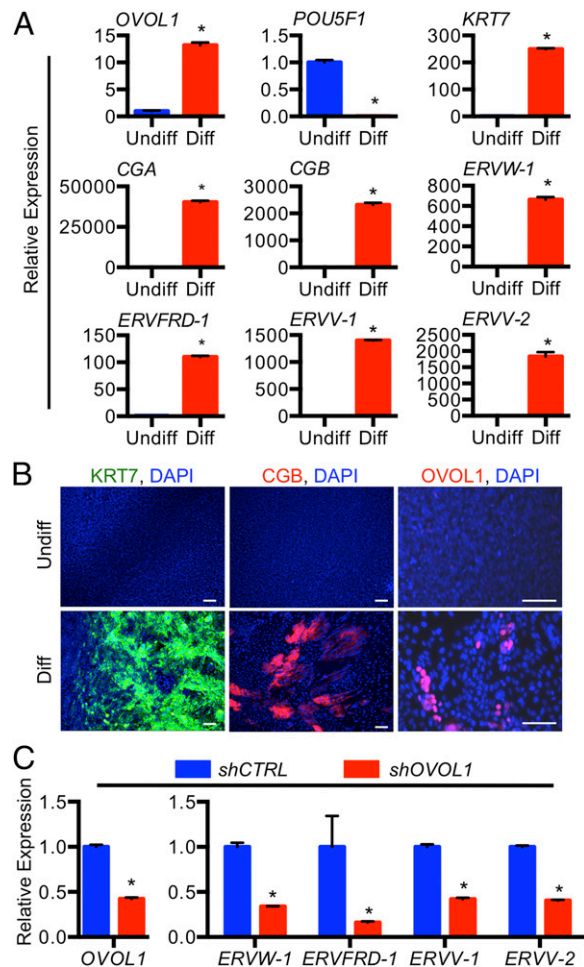


Fig. 7. OVOL1 regulates syncytiotrophoblast gene expression in human ES cells induced to differentiate along the trophoblast lineage. Human ES cells were maintained in undifferentiated (Undiff) conditions or were exposed to 10 ng/mL BMP4 (Diff) for 7 d. (A) Transcript expression of *OVOL1*, *POU5F1*, *KRT7*, *CGA*, *CGB*, *ERVV-1*, *ERVFRD-1*, *ERVV-1*, and *ERVV-2* in human ES cells maintained in undifferentiated or differentiating conditions. (B) Immunofluorescence for *KRT7*, *CGB*, and *OVOL1* in human ES cells maintained in undifferentiated or differentiating conditions. DAPI was used to identify nuclei. (Scale bars, 100 μ m.) (C) Transcript expression of *OVOL1*, *CGA*, *CGB*, *ERVV-1*, *ERVFRD-1*, *ERVV-1*, and *ERVV-2* in *OVOL1* shRNA-expressing human ES cells maintained in differentiated conditions. Human ES cells expressing control shRNA (shCTRL) were used as controls. * $P < 0.05$; all $n = 3$.

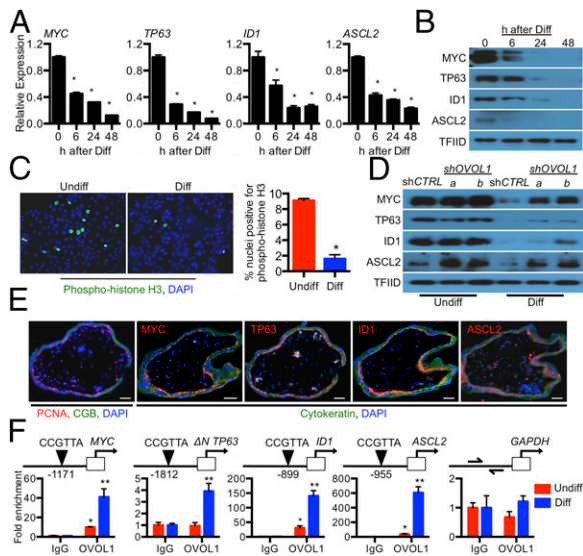


Fig. 8. OVOL1 represses genes that promote the trophoblast progenitor state. (A and B) qRT-PCR (A) and Western blot (B) analysis of MYC, TP63, ID1, and ASCL2 transcript and protein levels, respectively, in BeWo trophoblast cells cultured under standard conditions (Undiff, 0 h) or following 6-, 24-, or 48-h exposure to differentiating (Diff) conditions. TFIIID was used as a loading control. (C) Phospho-histone H3 immunofluorescence in BeWo trophoblast cells cultured in undifferentiated or differentiating conditions. DAPI was used to identify nuclei. The percentage of phospho-histone H3-positive nuclei is shown on the right. (D) Protein expression of MYC, TP63, ID1, and ASCL2 in BeWo trophoblast cells expressing shCTRL, shOVOL1-a, or shOVOL1-b maintained in undifferentiated or differentiating conditions. (E) Immunofluorescence of MYC, ID1, TP63, and ASCL2 in 8-wk human placenta. Antibodies that detect PCNA and CGB were used to identify proliferating cytotrophoblast cells and syncytiotrophoblast, respectively. (Scale bars, 50 μ m.) (F) ChIP analysis using antibodies for OVOL1 or IgG (negative control) in lysates of BeWo trophoblast cells maintained in undifferentiated or differentiating conditions. * $P < 0.05$, ** $P < 0.05$, $n \geq 3$.

detected in cytotrophoblast nuclei; ID1 and ASCL2 exhibited both cytoplasmic and nuclear staining (Fig. 8E). These factors were not expressed in syncytiotrophoblast. Therefore, decreased expression of MYC, TP63, ID1, and ASCL2 correlates with loss of trophoblast cell capacity for self-renewal and promotion of differentiation.

We next examined whether expression of MYC, TP63, ID1, or ASCL2 was altered following OVOL1 knockdown. To do so, we assessed these proteins in nuclear lysates of control or OVOL1-deficient BeWo trophoblast cells under undifferentiated or differentiating conditions. In control shRNA-expressing trophoblast cells, stimulation of differentiation caused a marked reduction in the expression of all four factors, similar to the time-dependent reduction in the levels of these proteins following trophoblast differentiation described above. In contrast, in cells deficient in OVOL1, high levels of MYC, TP63, ID1, and ASCL2 were retained even as cells were cultured under differentiating conditions (Fig. 8D). Similarly, in primary cytotrophoblast cells, we observed a 20% increase in ASCL2 expression following knockdown of OVOL1 ($P < 0.05$) (Fig. S5).

We next sought to determine whether OVOL1 bound directly within the upstream genomic region of MYC, ID1, TP63, and ASCL2. Putative OVOL1-binding sites were identified within the proximal promoter regions of MYC (CCGTGA, 1,171 bp upstream of the transcription start site), ID1 (CCGTGA, 899 bp upstream), TP63 (CCGTGA, 1,812 bp upstream of the alternative initiation site Δ NP63), and ASCL2 (CCGTGA, 955 bp upstream). We also identified several putative OVOL1-binding sites proximate to and within (10 kb upstream to 5 kb downstream) ERVFRD-1 (Fig. S6A). We chose to examine OVOL1 binding near ERVFRD-1 because ERVFRD-1 is a potent inducer

of trophoblast fusion (29) and because the encoded transcript for this gene was one of the most robustly affected following disruption of OVOL1 in our RNA-seq analysis and in primary cytotrophoblast cells. Thus, ERVFRD-1 served as a prototype to determine whether OVOL1 is capable of directly activating genes that promote syncytiotrophoblast formation. Using ChIP, we observed enrichment of OVOL1 in the upstream genomic regions of MYC (40-fold), TP63 (threefold), ID1 (135-fold), and ASCL2 (600-fold) (all $P < 0.05$ compared with nonspecific IgG binding) (Fig. 8F). No enrichment was identified in the proximal promoter region of GAPDH lacking putative OVOL1-binding sites (negative control) or at any of the potential OVOL1-binding sites surrounding ERVFRD-1. We also did not observe any changes in nuclear GCM1 levels between control and OVOL1-deficient trophoblast cells, despite a robust induction of GCM1 when trophoblast cells were exposed to differentiating conditions (Fig. S6). Therefore, we were unable to demonstrate that the actions of OVOL1 on trophoblast differentiation are dependent on directly targeting genes that promote syncytiotrophoblast formation (such as GCM1 or ERVFRD-1). Rather, our findings show that the likely function of OVOL1 in human placenta is to restrict the expression of key factors such as MYC, ID1, TP63, and ASCL2 that maintain cytotrophoblast cells in a progenitor state.

Discussion

We have found that OVOL1 is a critical determinant of cytotrophoblast differentiation in human placenta. OVOL1 promotes syncytiotrophoblast formation by restricting progenitor cell traits associated with cytotrophoblast cells and facilitating the activation of genes involved in both cell fusion and hormonogenesis. This study sheds light on the transcriptional regulation of human trophoblast differentiation, emphasizing the previously unrecognized importance of transcriptional repression in guiding cytotrophoblast cell fate.

For our experiments, we used three well-established models of human trophoblast syncytialization: cAMP-stimulated BeWo trophoblast cells, BMP4-treated human pluripotent stem cells, and primary term cytotrophoblast cells. Each of these models has its own merits and limitations, but combined they provide a compelling strategy for assessing molecular mechanisms that regulate human trophoblast differentiation, especially when used in addition to in situ assessment of the human placenta. In particular, the use of BeWo trophoblast cells and BMP4-treated human pluripotent stem cells is advantageous, because they can be induced to cease proliferation and commence differentiation by exogenous agents, thereby enabling the identification of factors controlling the development of more committed trophoblast lineages. BeWo trophoblast cells have been used as an in vitro model of cell fusion and hormonogenesis for decades (30). These cells are maintained as progenitor-like cells but can be induced to differentiate following exposure to cAMP analogs. How elevated intracellular cAMP facilitates fusion in these trophoblast cells is still not well understood. Stimulation of human pluripotent stem cells with BMP4 in the presence of fibroblast conditioned medium, causing the induction of genes consistent with trophoblast cells, was first reported by Xu et al. (23). The advantage of this system is that trophoblast differentiation can be studied through its entirety, i.e., from pluripotent stem cells to multipotent trophoblast precursors to terminally differentiated trophoblast-derived lineages (25). We have demonstrated a vital role for OVOL1 in trophoblast differentiation in each model.

Cytotrophoblast cells enzymatically isolated from placenta fail to proliferate ex vivo and differentiate spontaneously in culture. The use of primary cytotrophoblast cells has provided invaluable information about factors promoting syncytiotrophoblast derivation and function, although using isolated cytotrophoblast cells to delineate factors involved in the maintenance of the

cytotrophoblast progenitor state is difficult because these cells initiate terminal differentiation following isolation. These cells thus are inherently different from their *in vivo* counterparts, in that culture conditions that facilitate the progression of these cells through the cell cycle have not been established. Therefore we identified robust *OVOL1* expression in cytotrophoblast cells immediately after isolation, consistent with these cells having initiated differentiation. We were successful in achieving a degree of *OVOL1* knockdown in these cells using a lentiviral strategy; however, because of the transient nature of the experiment, selection of transduced cells was not possible, and the degree of knockdown was modest. Despite these limitations, we were able to demonstrate significant differences in the transcript levels of genes required for fusion (such as *ERVFRD-1*) and self-renewal (such as *ASCL2*) in primary trophoblast cells following *OVOL1* knockdown; these results are similar to our observations in other trophoblast differentiation models.

Our finding that *OVOL1* acts as a “switch” enabling commencement of differentiation is consistent with its role in several other epithelial lineages of other organisms. For example, in flies, *ovo* (a transcription factor homologous to *OVOL1*) is required for both oogenesis and cuticular development. Mutations in this gene result in egg chambers filled with undifferentiated germ cells and impairment of differentiated epidermal appendages (16, 31, 32). In mice, *OVOL1* deficiency causes reduced meiotic progression of spermatogonia as well as basal epidermal hyperproliferation caused by ineffective transition to the initial stages of terminal differentiation (17–19). Thus, *OVOL1* may be fundamental for the regulation of differentiation of several distinct mammalian epithelial lineages.

Despite the seemingly conserved function of *OVOL1* as a core regulator of epithelial differentiation, cytotrophoblast fusion to generate syncytiotrophoblast is rather exceptional. Unlike any other epithelial differentiation program in human tissues, formation of syncytiotrophoblast is both an endocrinological event and a cell-fusion event. The onset of hormonogenesis and of fusion are coupled during differentiation but are regulated by distinct signaling pathways (30). The endocrinological machinery of syncytiotrophoblast includes the proteins encoded by the CG subunits *CGA* and *CGB*, which maintain ovarian corpus luteum function; *HSD3B1*, which is necessary for progesterone synthesis; *CYP19A1*, which catalyzes the conversion of androgens to estrogens; and *HSD11B2*, which protects the embryo from the potentially detrimental effects of cortisol (33). Collectively, these genes encode proteins that prevent pregnancy failure and promote fetal development and thus are vital for human reproduction. Our finding that all these genes are downstream of *OVOL1* actions emphasizes the importance of *OVOL1* in the regulation of placental hormonogenesis and, thus, in human pregnancy.

With respect to the cell-fusion component of syncytiotrophoblast derivation, the expression of both *ERVW-1* (*syncytin-1*) and *ERVFRD-1* (*syncytin-2*)—the remnants of ancient retroviral *env* genes that are uniquely expressed by trophoblast cells and which encode the only two known fusogenic genes in human tissues—was strongly increased during *in vitro* syncytiotrophoblast derivation, as is consistent with other reports (29). We also found that two other endogenous retrovirus-derived genes, *ERVV-1* and *ERVV-2*, also were induced robustly during trophoblast differentiation. The proteins encoded by these genes do not possess fusogenic properties in human trophoblast cells, but their orthologs are capable of promoting fusion in the placentae of more distantly related primates (34). We discovered that the expression of all four of these retrovirus-derived genes in trophoblast cells is downstream of *OVOL1* actions. Reduced expression of the *syncytin* genes is a likely reason for the decreased cell fusion apparent in *OVOL1*-deficient trophoblast cells.

There are two probable explanations for how *OVOL1* promotes the expression of endocrinologically relevant genes, endogenous

retrovirus-derived genes, and syncytiotrophoblast development in general. The first is that *OVOL1* represses genes that maintain the progenitor status of cytotrophoblast cells, thereby indirectly facilitating the activation of differentiation-associated transcripts. Alternatively, it is possible that *OVOL1* stimulates the expression of a differentiation-associated factor (or factors) directly. The former option is consistent with the published role of *OVOL1* in other epithelial systems.

OVOL1 represses key genes involved in the maintenance of epithelial stem-like properties to promote differentiation (18, 19, 35). It does so either by competing for DNA binding with other transcription factors or by actively recruiting corepressor complexes (27). We identified four potential targets of *OVOL1*-mediated repression in trophoblast cells: *MYC*, *TP63*, *ID1*, and *ASCL2*. The products encoded by each of these genes decreased progressively during *in vitro* syncytiotrophoblast development, inversely correlating with *OVOL1* levels. Each factor also was strongly expressed in proliferating cytotrophoblast cells *in situ* and was absent in syncytiotrophoblast, as is consistent with other reports (36–38). Importantly, each of these factors has been linked to maintaining a proliferation and/or antidifferentiation state in various epithelial lineages (39–41). In cytotrophoblast cells, ectopic expression of either *TP63* or *ASCL2* impairs trophoblast differentiation and prevents fusion (26, 42–44); similarly, *MYC* maintains trophoblast cells in an undifferentiated state by inducing microRNAs that specifically target and repress differentiation-inducing factors (45). Although less is known about how *ID1* is involved in human placental development, all four ID proteins (*ID1*, *ID2*, *ID3*, and *ID4*) have been immunolocalized to cytotrophoblast cells (37), as is fitting, given their primary role in preventing differentiation of progenitor cells (39). The potential importance of *ID1* for cytotrophoblast proliferation is underscored by the excessive production of this transcriptional modulator in gestational trophoblastic disease (37). We found that *OVOL1* bound directly upstream of each of *MYC*, *TP63*, *ID1*, and *ASCL2* and that cells deficient in *OVOL1* were incapable of suppressing each factor following induction of differentiation. Our finding in trophoblast cells that *MYC* is a target for *OVOL1*-mediated repression is consistent with another report in mouse epidermis, in which *OVOL1* directly and autonomously repressed *Myc* expression during epidermal differentiation by binding to a conserved site upstream of the *Myc* transcription start site (19). Thus, *OVOL1*-mediated repression of *MYC* may be a conserved regulatory feature of epithelial differentiation. We also have shown that *OVOL1* binds upstream of *ID1*, *ASCL2*, and *TP63* and in each case is associated with gene repression. *OVOL*-binding sites upstream of the ΔN alternative promoter of mouse *Trp63* were reported previously in embryonic epidermal progenitor cells (46). Although our study focused on *MYC*, *TP63*, *ID1*, and *ASCL2* because of their known roles in maintaining the progenitor status of epithelial cells and more specifically in cytotrophoblast cells, we fully expect that there are other genes expressed by cytotrophoblast cells that are subject to *OVOL1*-mediated repression and contribute to the maintenance of the progenitor state.

We also addressed the possibility that, in addition to repressing genes that maintain cytotrophoblast progenitor traits, *OVOL1* directly activates genes that promote syncytiotrophoblast development. However, we did not detect *OVOL1* binding within the immediate vicinity of *ERVFRD-1*, despite the dramatic effect on transcript levels following *OVOL1* knockdown. Thus, we have no evidence for direct activation of this gene by *OVOL1*. We also determined that *GCM1*, a key activator of syncytiotrophoblast development and *syncytin* gene transcription (47, 48), was not substantially modified in cells deficient in *OVOL1* despite profound effects on expression of *syncytin* genes and cell fusion. This finding emphasizes that *GCM1* levels are not the only factor to be considered when determining the expression of *syncytin* genes and cell fusion in the human placenta. *GCM1* is expressed heterogeneously in the human placenta *in vivo*, including throughout

trophoblast cell columns where no fusion occurs (49). Although *OVOL1* did not affect *GCM1* expression significantly, it certainly is feasible that genes directly targeted by *OVOL1* ultimately affect *GCM1* activity or DNA-binding affinity, thereby facilitating the expression of *syncytin* genes and consequent cell fusion. It also is possible that *OVOL1* is induced directly by *GCM1* and that *GCM1*-induced *OVOL1* is at least partially responsible for mediating the prodifferentiation actions of *GCM1*. Regardless, in human placenta, we propose that *OVOL1* executes the essential function of repressing major regulators that maintain cytotrophoblast progenitor traits, thereby facilitating syncytiotrophoblast development.

Syncytiotrophoblast is not a cell type unique to human placenta. The primary epithelial barrier separating maternal and fetal blood in many other mammals, including mice and rats, is comprised of syncytialized trophoblast. *Ovol1* was detected at low levels in mouse and rat placentae and was not detected in mouse or rat TS cells in stem or differentiated conditions. However, an *Ovol1* paralog, *Ovol2*, is highly expressed in mouse and rat placentae (this study and ref. 50), and we found that *Ovol2* is induced in mouse and rat TS cells following differentiation in vitro. Intriguingly, *OVOL1*-deficient mice are viable until birth, and no evident defects in placentation have been reported (20), but *OVOL2*-deficient mice die by midgestation with severe deficits in the development of the labyrinth placenta, the murine equivalent of the chorionic villi (50). *OVOL2* binds to the same DNA-binding site as *OVOL1* and can exert functions redundant to those of *OVOL1* (46, 51, 52). Thus, it could be proposed that *OVOL2* regulates placentation in mice and rats in a manner similar to the actions of *OVOL1* in humans.

In conclusion, we have found that *OVOL1* is a major regulator of cytotrophoblast differentiation in the human placenta. We also have uncovered a previously unidentified mechanism of action whereby *OVOL1* acts to repress four key transcriptional regulators known to maintain cytotrophoblast cells in a progenitor state. The importance of the “brake” provided by *OVOL1* on these upstream factors is manifest, given the broad and powerful effects of manipulating *OVOL1* levels on diverse sets of genes required for both hormonogenesis and fusion (e.g., *syncytins*). Our research paves the way for future studies, including the identification of additional *OVOL1* target genes and their roles in maintaining the cytotrophoblast progenitor state, determination of whether *OVOL1* is also important for extravillous trophoblast development, and assessment of whether *OVOL1* expression or activity is dysregulated in diseases associated with a suboptimal syncytiotrophoblast barrier (e.g., preeclampsia or intrauterine growth restriction) or in pathologies associated with cytotrophoblast hyperproliferation (e.g., gestational trophoblastic disease).

Methods

Human Placenta Collection and Trophoblast Cell Isolation. Paraffin-embedded and frozen human placental tissue was obtained from the Research Centre for Women’s and Children’s Health Biobank (Mount Sinai Hospital, Toronto). All collections were performed with appropriate consent and were approved by the University of Toronto and the University of Kansas Medical Center (KUMC) human research ethics boards. Term trophoblast cells were processed from placental villous tissue obtained with consent from the University of Kansas Hospital under a protocol approved by the KUMC Institutional Review Board. Trophoblast cells were processed according to the method of Kliman et al. (53).

Animals and Tissue Collection. C57BL/6 mice were obtained from The Jackson Laboratory. Holtzman Sprague–Dawley rats were obtained from Harlan Laboratories. Males and females of the appropriate species were caged together overnight, and the presence of a seminal plug or sperm in the vaginal smear was designated day 0.5 of pregnancy. Placental and epidermal tissues were collected on gestation days 13.5 for rats and 15.5 for mice. Tissues were frozen in dry ice-cooled heptane for immunohistochemical analysis or were snap-frozen in liquid nitrogen and were stored at -80°C until processing. The KUMC Animal Care and Use Committee approved the protocols for the care and use of animals.

Cells. All cells were maintained at 37°C , 5% CO_2 . BeWo trophoblast cells and HEK-293FT cells were obtained from the American Type Culture Collection. To stimulate BeWo trophoblast differentiation, cells were treated with 8-Br-cAMP (Sigma-Aldrich), which is commonly used to stimulate syncytialization in these cells (22).

Human ES cells were obtained from WiCell (clone WA09, feeder-independent). Cells were maintained on growth factor-reduced Matrigel (Corning-Fisher Scientific) in mTESR1 medium (STEMCELL Technologies). To induce trophoblast differentiation, mTESR1 medium was removed, and cells were exposed to 10 ng/mL BMP4 (R&D Systems) diluted in mouse embryonic fibroblast-conditioned medium containing 80% knockout DMEM/20% knockout serum replacement (Life Technologies) with 1 mM L-glutamine, nonessential amino acid supplement, and 0.1 mM 2-mercaptoethanol (all from Sigma-Aldrich). Cells were exposed to BMP4 for 7 d, similar to published reports (23–25, 54).

Human iPSCs were obtained from the Harvard Stem Cell Institute iPSC cell core facility (Line 11b derived from skin fibroblasts of a healthy female) (55). Cells were maintained on growth factor-reduced Matrigel in 80% DMEM/F12 supplemented with 20% knockout serum replacement, 1 mM L-glutamine, nonessential amino acid supplement, 0.1 mM 2-mercaptoethanol, and 4 ng/mL FGF2 at subconfluent conditions. To induce trophoblast differentiation, the medium was supplemented with 10 ng/mL BMP4 in place of FGF2.

Mouse TS cells were a kind gift from Janet Rossant of The Hospital for Sick Children, Toronto, and were cultured as previously described (56). Rat TS cells were generated and characterized previously (57). Differentiation of the TS cells was induced by the removal of FGF4, heparin, and the embryonic fibroblast-conditioned medium (56). Medium was changed every 2 d for a total of 8 d.

RT-PCR. Total cellular RNA was extracted using Tri-Reagent (Sigma-Aldrich). cDNA was synthesized through reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Primers used for PCR are detailed in Table S3. Conventional PCR was performed for 30–35 cycles (94°C for 30 s; 55 – 60°C for 30 s; 72°C for 30 s). qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Amplification and fluorescence detection were carried out using an ABI Prism 7500 Real Time PCR system (Applied Biosystems). Relative mRNA expression was calculated by the $\Delta\Delta\text{Ct}$ method, using 18S rRNA as a reference RNA.

In Situ Hybridization. In situ hybridization for *OVOL1* and *CDH1* expression was performed on paraffin-embedded sections using the RNAScope 2-plex chromogenic assay (Advanced Cell Diagnostics 320494), following the protocols provided by the manufacturer. Probes were designed to detect *OVOL1* (NM_004561.3, probe region 893–2,313), *CDH1* (NM_004360.3, probe region 263–1,255), *POLR2A* (positive control, NM_000937.4, probe region 2,514–3,433), *PP1B* (positive control, NM_000942.4, probe region 139–989), and *DapB* (negative control, EF191515, probe region 414–862).

Western Blotting. Whole-cell lysates were prepared using 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 50 mM DTT; nuclear lysates were prepared using the NE-PER nuclear/cytoplasmic protein extraction kit (Thermo Fisher Scientific). Protein was electrophoretically separated on SDS/PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were subsequently probed using antibodies for *OVOL1* (1 $\mu\text{g/mL}$; clone ARP38500; Aviva Systems Biology), ERVFRD-1 (1 $\mu\text{g/mL}$; ARP56008_050; Aviva Systems Biology), MYC (1:1,000; clone D84C12; Cell Signaling Technology), TP63 α (1:1,000; 4892, Cell Signaling Technology), ID1 (1:1,000; clone EPR7098; Abcam), ASCL2 (1 $\mu\text{g/mL}$; AF6539; R&D Systems), *GCM1* (0.1 $\mu\text{g/mL}$; HPA011343; Sigma-Aldrich), transcription factor II D (TFIID) (0.2 $\mu\text{g/mL}$; sc-56794; Santa Cruz Biotechnology), and α -tubulin (0.05 $\mu\text{g/mL}$; clone CP06; EMD-Millipore Corp.). Membranes then were incubated with HRP-conjugated antibodies, immersed in Luminata Classico chemiluminescence reagent (EMD-Millipore Corp.), and detected using GeneMate basic blue autoradiography film (BioExpress).

Immunofluorescence and Immunohistochemistry. For immunofluorescence studies, cells were fixed using 4% paraformaldehyde and were permeabilized. Paraffin-embedded tissues were sectioned at 5- μm thickness, deparaffinized, rehydrated in a graded series of ethanol washes, and subjected to antigen retrieval at 95°C in deoaking agent (RV1000M, Biocare Medical). Subsequently, cells or tissues were probed with antibodies specific for *OVOL1* (1:50; clone HPA003984; Sigma-Aldrich), CGB (1:200; clone RB-059A; DAKO), *CDH1* (1:200; clone 24E10; Cell Signaling Technology), cytokeratin 7 (1:100; clone OV-TL 12/30; DAKO), phospho-histone H3 Ser10 (1:400; 9701; Cell Signaling Technology), MYC (1:200; clone Y69; Abcam), TP63 (1:100; clone 4A4; Biocare Medical), ID1 (1:1,000; Abcam), ASCL2 (5 $\mu\text{g/mL}$; R&D Systems), PCNA (1:200; clone PC10; Santa Cruz Biotechnology), *OVOL2* (1:100; clone NBP1-88754; Novus

Biologicals), and FITC-conjugated pan-cytokeratin antibody (1:400; clone C-11; Sigma-Aldrich). Following a 1-h incubation with species-appropriate fluorescent-conjugated antibodies, cells were incubated with DAPI (Molecular Probes) to detect nuclei or with rhodamine-phalloidin (1:100; Life Technologies) to detect actin fibers (F-actin) and were visualized using a Leica DMI 4000 microscope (Leica Microsystems GmbH). For analysis of cell fusion, images were taken from four randomly selected fields per well in triplicate at 40× magnification. Lack of CDH1 or actin staining combined with CGB fluorescence was used to denote clusters of cells that had syncytialized. The total number of nuclei in these syncytialized clusters was divided by the number of nuclei within the field and multiplied by 100 to calculate a “% fusion” index. For analysis of phospho-histone H3 staining, two fields per well in four wells were examined for each group at 40× magnification. The number of nuclei that stained positively for phospho-histone H3 was divided by the total number of nuclei within that field and multiplied by 100 to calculate the percent of nuclei positive for phospho-histone H3.

DNA Microarray. Affymetrix human U133 2.0 DNA microarray chips (Affymetrix) were probed with cDNAs generated from BeWo trophoblast cells grown under control conditions or following treatment with 8-Br-cAMP. Each group was analyzed in triplicate. Chips were subsequently scanned using the Affymetrix GeneChip Scanner 3000 (Affymetrix) with autoloader by the KUMC Biotechnology Support Facility. Hybridization signals were normalized to internal controls using the MAS5 algorithm in the Expression Console (Affymetrix), and fold change was calculated. Significant differences were determined by Student's *t* tests. Analysis was limited to probe sets changing at least twofold between groups, with signal strengths ≥ 300 for the maximal value. Genes significantly altered were categorized using the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (58). Data are available on the Gene Expression Omnibus website (accession no. GSE66840).

RNA-Seq. Deep sequencing was conducted on mRNA isolated from 8-Br-cAMP-exposed BeWo trophoblast cells expressing control shRNA versus *OVOL1* shRNA (in triplicate). CDNA libraries from total RNA samples (500 ng input) were prepared from Illumina TruSeq RNA sample preparation kits (Illumina), and protocols followed the manufacturer's recommendations. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) before sequencing. Libraries were clustered onto a TruSeq paired-end flow cell and were sequenced (100-bp paired-end reads) using a TruSeq 200-cycle SBS kit (Illumina). Samples were run on an Illumina HiSeq2000 sequencer located at the KUMC Genome Sequencing Facility. Reads from *.fastq files were mapped to the human reference genome (hg19) using CLC Bio Genomics Workbench 7.0 (CLC Bio), and transcript abundance was expressed as RPKM. Analysis was restricted to transcripts in which all replicates > 0 RPKM and with a weighted difference between groups $\geq 1 \times 10^{-6}$. Statistical significance was calculated by empirical analysis of digital gene expression, followed by Bonferroni's correc-

tion. Data are available on the Gene Expression Omnibus website (accession no. GSE66962).

shRNA Constructs and Production of Lentiviruses. Human *OVOL1* shRNA constructs in pLKO.1 vectors were obtained from Sigma-Aldrich. Control shRNA that does not target any known mammalian gene was obtained from Addgene (pLKO.1-shScramble, plasmid 1864); a plasmid with the same backbone as pLKO.1 but with a GFP cassette replacing the puromycin resistance cassette was obtained from Addgene (pLKO.3G, plasmid 14748). The sense target sites for the shRNAs are *OVOL1*-a, AGTGTACACACGACGTCGAAGA; *OVOL1*-b, CCGCCACATGAAG-TGTCAACA; Control, CCTAAGGTTAAGTCGCCCTCG. Lentiviruses were generated from Lipofectamine 2000-based transfection of HEK-293FT cells as previously described (59, 60).

ChIP. ChIP was performed as previously described (59). Briefly, fixed, sonicated lysates were incubated with *OVOL1* antibody (2.5 μ g) (Clone HPA003984; Sigma-Aldrich) or affinity-purified rabbit IgG (negative control; 550875; BD Biosciences). Immunoprecipitated chromatin fragments were captured using protein A-conjugated Sepharose beads (GE Healthcare); eluted and purified DNA fragments were assessed by qPCR using SYBR Green PCR master mix. Primers were designed to span the region surrounding consensus *OVOL1* DNA-binding motifs *C*₁-GTTA (Table S4) (19, 46, 61). Relative occupancy was normalized to input samples by use of the $\Delta\Delta$ Ct method.

Statistical Analysis. Statistical analyses of data obtained by DNA microarray and RNA-seq are discussed above. For other measures, statistical comparisons between two means were performed with Student's *t* test. Comparisons of multiple groups were evaluated with ANOVA. The source of variation from significant *F*-ratios was determined with Bonferroni's multiple comparison test. Results were deemed statistically significant when $P < 0.05$. All experiments were conducted at least in triplicate and were replicated at least two to three times. Graphing and statistical analyses were performed using GraphPad Prism 6.0.

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