Title

Generation of cytotrophoblast-like cells from human embryonic stem cells in defined media

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P1.111.
ACTIVIN A, B AND AB INCREASE HUMAN TROPHOBLAST CELL INVASION BY UP-REGULATING N-CADHERIN

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Objective: Though activin A has been shown to enhance human trophoblast cell invasion, whether two additional activin isoforms, activin B and AB, exert similar effects remains unknown. While the mechanisms underlying these effects are unclear, we have recently demonstrated that expression of the mesenchymal adhesion molecule N-cadherin is associated with invasive behaviour in trophoblasts. Thus, the purpose of our study was to examine the effects of all three activin isoforms on human trophoblast cell invasion, and to define the molecular mechanisms involved, in particular the role of N-cadherin.
Methods: The HTR-8/SVneo immortalized human trophoblast cell line was used as an in vitro model. The TGF-β type I receptor inhibitor SB431542 was used to block signaling from activin receptor complexes. Small interfering RNA (siRNA)-mediated knockdown approaches were used to investigate the molecular determinants of activin-mediated functions. RT-quantitative real-time PCR and Western blot analysis were used to examine mRNA and protein levels, respectively. Cell invasiveness was assessed by Matrigel-coated transwell assays.
Results: Treatment of HTR-8/SVneo cells with activin A, B or AB produced comparable increases in cell invasion as well as N-cadherin mRNA and protein levels. Interestingly, basal and activin-induced cell invasion were attenuated by siRNA-mediated down-regulation of N-cadherin. All activin isoforms induced equivalent phosphorylation of SMAD2 and SMAD3. In addition, activin treatment up-regulated the mRNA levels of Snail and Slug, but not TWIST, RUNX2, ZEB1 or ZEB2. Co-treatment with SB431542 abolished activin-induced cell invasion, up-regulation of N-cadherin, activation of SMAD2/SMAD3 and up-regulation of Snail and Slug. Interestingly, treatment with siRNA for common SMAD4, but not for Snail or Slug, abolished the effects of all three activin isoforms on N-cadherin.
Conclusion: Activin A, B and AB increase human trophoblast cell invasion by up-regulating N-cadherin expression in a Smad-dependent manner.

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P1.112.
TRANSFECTION OF BEWO CELLS WITH A TRUNCATED HUMAN ENDOGENOUS RETROVIRUS ERV3 *ENV* INDUCES β-HCG

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Objectives: Human endogenous retroviral element ERV3 *env* is highly expressed during differentiation of villous cytotrophoblast to syncytiotrophoblast. We reported previously that, unlike other exogenous and endogenous retroviral *env* regions that encode fusion proteins, ERV3 regulated the induction of β-hCG. The apparent biological relevance of ERV3 *env* was greatly diminished by a report of adults with homozygous stop mutations leading to a natural knockout of ERV3 *env*. A trunicated (p25) molecule was translated, but lacked the typical biologically active regions of retroviral Env proteins. However, the p25 region has never been tested for capacity to induce expression of β-hCG.
Methods: We cloned and inserted the entire ERV3 *env* open reading frame (ERV3), the ERV3 SU region, and the ERV3 p25 region into pCMV6-AC expression vectors, stably transfected BeWo cells, and monitored for levels of intracellular β-hCG by quantitative western blot analysis, normalized to levels of actin, and data expressed as means (SD) of the ratio of β-hCG to actin of three independent experiments.
Results: β-hCG expression was not detectable in untreated BeWo (negative control) and maximum in forskolin-treated cells (positive control; 1.83 ± 0.83). Transfection with vector alone did not affect β-hCG expression (0.07 ± 0.13), whereas ERV3, ERV3 SU, and p25 induced significantly ($P<0.01$) greater levels of β-hCG expression. BeWo cells were also stably transfected with plasmids expressing siRNAs targeted to ERV3 *env*, and the cell lines treated with forskolin or vehicle alone for 24, 48, and 72 hr. ERV3 env contains two proposed start sites at nt 595 and nt 715. Transfection with si670, targeted to nt 670-688, between the two proposed start sites, completely prevented the induction of β-hCG by forskolin at all time points.
Conclusions: ERV3 *env* is an atypical retroviral element with a unique trophoblast hormone regulatory site in the p25 component of the SU region.

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P1.113.
PLACENTAL PERFUSION AS A MODEL OF SYNCYTIOTROPHOBLAST VESICLE RELEASE

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Objectives: Placental perfusion allows collection of physiologically relevant syncytiotrophoblast derived vesicles (STBM) in large numbers. These preparations contain vesicles of varying size (ie apoptotic and necrotic debris ~>1μm, microvesicles ~100nm-1μm and exosomes ~100nm) and origin (ie syncytiotrophoblast, red blood cells (RBC) and platelets). There is growing evidence that different categories of vesicles exhibit diverse functions (Redman et al 2012. Placenta 33 S48-S54). Therefore better purification and fractionation is required to elucidate the role of syncytiotrophoblast derived exosomes and microvesicles in normal and pathological pregnancies.
Methods: A protocol using sequential centrifugation, bead depletion of contaminating vesicles and a final filtration step was developed to produce highly pure STBM preparations that were enriched for either exosomes or microvesicles from placental lobe perfusate. Perfusates were prepared from normal term placentas by dual placental lobe perfusion. Five colour flow cytometry, Nanoparticle Tracking Analysis (NTA) and Western blotting were used to assess purity and size distribution of vesicles.
Results: Freshly collected placental perfusates contained high numbers of contaminating RBC (~68%) and platelets (~2.4%). Centrifugation at 1500xg (2x10min) and incubation of the resultant supernatant (SN) with magnetic dynabeads coated with anti-glycophorin A/B (RBC), anti-CD41 (platelets) and anti-pan HLA (platelets/leukocytes) antibodies removed contaminating platelets (>86%), RBC (>99%) and platelet and RBC vesicles (>32% and >95% respectively). Following centrifugation at 10,000xg (30min), microvesicles of sizes ~360nm (mean) and 310nm (mode) were enriched in the pellet. Finally 150,000xg centrifugation (2h) of the microvesicle depleted SN and 0.22μm filtration of the resultant pellet gave an exosome fraction (sizes ~150nm (mean) and 200nm (mode)), enriched for exosome markers Alix and CD63.
Conclusion: Five colour flow cytometry and NTA has enabled the development of a protocol that yields highly pure STBM preparations that can be fractionated to give enriched preparations of exosomes and microvesicles from placental lobe perfusate.

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P1.114.
GENERATION OF CYTOTROPHOBLAST-LIKE CELLS FROM HUMAN EMBRYONIC STEM CELLS IN DEFINED MEDIA

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Objectives: Human trophoblast lineage-specific differentiation is difficult to study, due to a lack of a multipotent trophoblast stem cell (TSC) model. First trimester human cytotrophoblast (CTB) are bipotential, with the ability to differentiate into both hCG-secreting syncytiotrophoblast (STB) and HLAG-positive extravillous trophoblast (EVT); however, access to large numbers of such cells is limited. Human embryonic stem cells (hESCs) can be differentiated into trophoblast using BMP4 in presence of feeder-conditioned media (FCM), but these conditions are inconsistent and often result in heterogeneous populations of cells. We set out to develop defined media conditions for differentiation of hESCs into bipotential CTBs.
Methods: Feeder-free hESCs (WA09/H9) were cultured in StemPro+bFGF. To differentiate the cells into CTB, cells were switched to minimal media for two days, then treated with BMP4 (10 ng/ml) for an additional four days. Microarray gene expression data from these cells were compared to data from first trimester primary CTB, placental stroma, amnion epithelial cells, JEG3 and BeWo cells, and human dermal fibroblasts. Treated hESCs were also evaluated by immunostaining for markers of pluripotency and CTB.
Results: After four days in minimal media plus BMP4, cells downregulated the pluripotency marker OCT4, and induced CTB markers CK7, EGFR, and TP63. Microarray analysis revealed that these cells cluster most closely with JEG3 and BeWo and, in turn, are more closely related to the first trimester CTB. These cells could be replated, and when cultured in FCM+BMP4, could be induced to differentiate into both STB and EVT.
Conclusions: In summary, we have successfully established a reproducible in vitro system, using defined media, to derive CTB-like cells from hESCs. These cells offer a superior alternative to the existing aneuploid human trophoblast cell lines, and, with the ability to differentiate into both STB and EVT, can be used to study trophoblast lineage-specific differentiation.

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P1.115.
MIR-21 REGULATES PROLIFERATION AND INVASION IN TROPHOBLASTIC CELLS AND TARGETS PTEN

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Objective: MicroRNAs (miRNAs) are small non coding RNAs that post-transcriptional regulate gene expression by inducing translational inhibition or transcript degradation. MiR-21 is an oncomir and has been associated with various types of cancer. Interestingly, our group has been reported that miR-21 is the highest expressed miRNA, out of 762 analyzed, in first trimester isolated trophoblast cells. In this study, we investigated the functions of miR-21 in trophoblastic cell lines and its possible target genes.
Methods: The immortalized human trophoblast cell line HTR-8/svneo and the choriocarcinoma cell line JEG-3 were transfected with miR-21 inhibitor or mimic. Total RNA was extracted and RNA quality and quantity were determined by spectrophotometer (Nanodrop 1000). MiRNA-21 expression was quantified by qPCR using Taqman®miRNA single assay. Cell growth and invasion were followed after transfection with miR-21 inhibitor or mimic for up to 72 hr by BrdU assay and Matrigel invasion assay. Potential targets of miR-21 were analyzed by RT-PCR.
Results: MiR-21 expression was higher in HTR-8/svneo cells than in JEG-3 cells.
In JEG-3 cells, miR-21 inhibition significantly increased proliferation while over-expression resulted in a decreased proliferation when compared to untreated cells and negative control cells. Moreover, JEG-3 cells demonstrated significant reduction of invasion after inhibition of miR-21 expression. Knockdown of miR-21 in HTR-8/svneo cells significantly suppressed proliferation compared to untreated cells and negative control cells, but no change was observed in invasion. MiR-21 inhibitor inhibited expression of PTEN in JEG-3 cells, while increased it in HTR-8/svneo cells.
Conclusions: Both cell lines express miR-21 but in different levels. MiR-21 plays an important role on regulating cell growth and invasion in trophoblast cells possibly by a mechanism that involves control of PTEN expression.

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P1.116.
KRUPPEL-LIKE FACTOR-6 ACTIVATES PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR AND PROMOTES TROPHOBLAST DIFFERENTIATION IN BEWO CELL LINES

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Normal pregnancy depends mainly on normal placental development. More importantly trophoblast differentiation is thought to be at the root of many pregnancy-related diseases such as pre-eclampsia and intra uterine growth restriction. Therefore, the understanding of trophoblast differentiation process is crucial. Trophoblast differentiation is characterised by the formation of a specific multinuclear structure, the syncytiotrophoblast. This structure arises by fusion and differentiation of the relatively undifferentiated, mitotically active cytotrophoblast cells. Many signaling pathways and transcription factors have been reported to control this process. Kruppel-like factor (KLF) family contains many members and they have been reported to play a role in many cellular process including cell differentiation, cell cycle control and proliferation. However, little is known about the role of KLF6 in trophoblast differentiation. Therefore, we examined the expression of KLF6 isoform in BeWo cells and their role in trophoblast differentiation. BeWo cells were treated with 50 micro molar forskolin for 72 hours. Trophoblast differentiation was evaluated by measuring the level of hCG by real time PCR. Our results show that BeWo cells express all 4 isoforms of KLF6 and that their expression is not modified during BeWo differentiation using forskolin. Moreover, we showed that KLF6 transfection transactivated the peroxysome proliferator response element (PPRE) in BeWo cells. Our results suggest a role for KLF6 in trophoblast differentiation by activating PPAR signaling pathway.

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P1.117.
THE GAP JUNCTION CHANNEL ENCODING GENES CONNEXIN31 (*GJB3*) AND CONNEXIN31.1 (*GJB5*) HAVE DIRECTLY OPPOSED EFFECTS ON TROPHOBLAST STEM CELL DIFFERENTIATION DURING MOUSE PLACENTAL DEVELOPMENT

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Objective: The expression of gap junction forming connexins (Cx) is subject to exacting spatio-temporal regulations in the trophoblast lineage. Inactivation of either the Cx31 (*Gjb3*) or the Cx31.1 (*Gjb5*) gene in the mouse results in placental phenotypes leading to embryonic loss between ED10.5 to ED12.5. Whereas Cx31 is widely known as a modulator of trophoblast stem cell differentiation and repressor of trophoblast giant cell differentiation, the molecular role of the co-expressed Cx31.1 remained unclear. Here we analyze the impact of Cx31.1 in trophoblast differentiation and discriminate its function compared to Cx31.
Methods: We generated trophoblast stem cell (TSC) lines from Cx31.1-deficient blastocysts and analyzed their capacity to differentiate into the placental trophoblast sub-populations. In addition, we analyzed the expression of placental marker genes between ED9.5 to ED17.5 by qPCR,