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Homeobox gene *Distal-less* 3 (DLX3) is a regulator of villous cytotrophoblast differentiation

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ABSTRACT

Dlx3, a member of the large homeobox gene family of transcription factors, is important for murine placental development. Targeted deletion of *Dlx3* in the mouse results in embryonic death due to placental failure. This study investigated the role of human *DLX3* in villous cytotrophoblast (VCT) differentiation in the placenta. Primary VCT from human term placentae, which spontaneously differentiate when maintained in culture over 72 h, showed a significant increase in mRNA and protein expression of *DLX3* and 3βHSD. The functional role of *DLX*3 was determined using trophoblast derived-cell line, BeWo. Forskolin treated BeWo cells showed significantly increased *DLX3* mRNA and protein expression. Forskolin stimulation also showed a significant increase in syncytin and 3βHSD mRNA expression, and increased release of βhCG into the cell culture supernatant. To determine whether *DLX3* had a direct or indirect effect on VCT differentiation, mRNA and protein expression of *DLX3* was increased using a plasmid *DLX3* over-expression construct. Over-expression of *DLX3* resulted in increased mRNA expression of 3βHSD and syncytin, as well as increased secretion of β-hCG protein in the cell culture medium. In conclusion, we provide evidence that *DLX3* acts upstream of syncytin, 3βHSD and βhCG and that DLX3 has a regulatory role in VCT differentiation.

1. Introduction

The placenta is a structural and physiological barrier between the fetal and maternal circulations, providing an exchange interface for nutrients, gases and wastes [1]. During early human pregnancy, mononuclear villous cytotrophoblast cells fuse to form a multinucleated syncytium. For continual exchange, the syncytiotrophoblast layer must grow and be refreshed by differentiation and fusion of underlying, proliferating villous cytotrophoblast cells. It is important that villous cytotrophoblast differentiation is tightly controlled because inadequate differentiation would compromise the production and function of the syncytiotrophoblast and reduce the efficiency of the maternal-fetal exchange system.

Villous cytotrophoblast differentiation is accompanied by increased expression of critical hormones and factors such as the human chorionic gonadotropin β-subunit (β-hCG), a marker of *in vitro* differentiation [2], 3β-hydroxysteroid dehydrogenase (3βHSD), an enzyme essential for the maintenance of pregnancy [3], and syncytin-1, a retroviral envelope protein that appears to be primarily expressed in the syncytiotrophoblast [4].

Villous cytotrophoblast cell functions are regulated by a variety of growth factors, cytokines and transcription factors [5–10]. This study focuses on a member of the large family of transcription factors, called the homeobox genes. Homeobox genes play important roles in mammalian embryonic development [11] and are characterised by a conserved 60 amino acid homeodomain which is necessary for DNA binding [12,13]. Mouse knockout studies of homeobox genes provide evidence for critical roles of these regulatory genes in placental development [14–16]. Our specific interest lies in a sub-family of homeobox genes called *Distal-less*.

Members of the *Distal-less* gene (*Dlx*) organised in pairs as 1–6 on chromosome 17q21, play important roles in cellular differentiation [17–20]. Targeted deletion of mouse *Dlx3* results in embryonic death by day 10 due to placental vascularisation defects that alter the development of the labyrinth, which is thought to be equivalent in functions to the syncytiotrophoblast in humans [21,22]. Furthermore, *Dlx3* is involved in 3βHSD placental hormone

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regulation [23] and in the regulation of other critical hormones such as the α subunit of chorionic gonadotropin hormone and placental lactogen, both of which are important for villous cytotrophoblast differentiation and are required for a successful pregnancy [3,24–26]. Recently, we described the detailed expression pattern of DLX3 in the human placenta [27] and found DLX3 to be primarily expressed in the villous cytotrophoblasts and syncytiotrophoblast. The localization of DLX3 to these cell types is suggestive of a role for *DLX3* in villous cytotrophoblast differentiation. However, direct evidence for this has not yet been provided.

In this study, we measured *DLX3* expression in spontaneously differentiating primary villous cytotrophoblast cells of term human placentae. BeWo cells were used as a model for villous cytotrophoblasts *in vitro*. Differentiation of BeWo cells was induced by adenylate cyclase activator, forskolin [28,29], and the effect on *DLX3* and villous cytotrophoblast differentiation was determined. Finally to determine whether *DLX3* was sufficient for villous cytotrophoblast differentiation, a plasmid *DLX3* over-expression construct was used in BeWo cells.

2. Materials and methods

2.1. Term placental trophoblast cells

This study was approved by the Human Research and Ethics Committees of the Royal Womens' Hospital, Melbourne, Australia and the Northern Regional Ethics Committee, Auckland, New Zealand. Placentae ($n = 10$) were obtained with informed consent from women after delivery by Caesarean section at term. Cytotrophoblasts were isolated using trypsin digestion as described previously [30]. In brief, villous tissue from term placenta was subjected to eight consecutive digestions in 0.25% trypsin and cells were isolated by centrifugation at 300 g for 7 min. Erythrocytes were removed by incubation of the cell pellet in red cell lysis buffer (50 mM NH4Cl, 10 mM NaHCO3, and 0.1 mM EDTA) and cytotrophoblasts were purified by centrifugation at 1,200 g for 20 min on a discontinuous Percoll gradient (20–60%). Cells were grown for 24 h in M199 media, supplemented with 10% FCS, EGF (10 ng/ml), insulin (5 ng/ml), transferrin (10 ng/ml), sodium selenite (0.2 nM), and penicillin/streptomycin (100 U/ml) in a 5% CO_2 humidified atmosphere at 37 °C. Cytotrophoblast purity was confirmed where the proportion of cytokeratin-7 positive cells in the culture was 95.7 ± 3.14% (n = 3) [30].

2.2. Cell culture

The human trophoblast derived-choriocarcinoma cell line, BeWo, was a kind gift from A/Prof. Stephen Rogerson (University of Melbourne Department of Medicine, Royal Melbourne Hospital, Victoria, Australia). Cells were grown in RPMI-1640, supplemented with 10% fetal calf serum (FCS) (w/v), 200 U/ml penicillin and 200 μg/ml streptomycin (Invitrogen, Australia) and were incubated at 37 °C, 5% CO_2 and 95% air in a humidified chamber.

2.3. Forskolin mediated trophoblast differentiation

BeWo cells were seeded in 6-well culture dishes at a density of 2×10^5 cells/well and serum starved with RPMI-1640 media supplemented with 1% BSA (w/v) overnight. Forskolin (Invitrogen/LifeTechnologies, Australia) was added at a concentration of 10 μM [28,29] to induce differentiation with subsequent incubations for 24, 48 and 72 h. At the end of each incubation period, media was collected and stored at −20 °C and the cells were processed for RNA or protein analysis. Untreated control cells were seeded in RPMI 1640 media/1% BSA media only.

2.4. β-hCG protein assay

For determination of β-hCG protein levels, an enzyme linked immunosorbent assay (ELISA, Alpha Diagnostic International, Australia) was performed following the manufacturer's instructions, using conditioned media collected from cell culture treatments. The minimum concentration of human HCG detected using this assay was 1.5 mIU/ml. The standards used for this assay were from 0 to 200 mIU/ml, calibrated against the World Health Organisation (WHO 3rd 75/537) standard.

2.5. Over-expression of DLX3 expression by cDNA plasmid transfection

A human cDNA clone of the homeobox gene *DLX3* was purchased together with TurboFectin 8.0 Transfection Reagent (both from Origene Technologies, USA) and used to over-express *DLX3* in confluent cultures of BeWo cells. Experiments were carried out following the manufacturer's instructions. Briefly, BeWo cells were seeded at a density of 2×10^5 cells/well in 6-well plates and maintained in culture with *DLX3* cDNA plasmid (1 μg/μl) in TurboFectin 8.0 transfection reagent (cDNA plasmid:TurboFection 8.0, 1:3) for 48 h. A vector or no cDNA insert is used as a control.

2.6. RNA extraction and cDNA preparation

Total RNA was extracted using RNeasy Microkits according to the manufacturer's instructions (Qiagen, Australia). Total RNA was reverse-transcribed using Superscript III ribonuclease H-reverse transcriptase (Invitrogen, Australia) and cDNA was prepared in a two-step reaction as previously described [31,32].

2.7. Real-time PCR

Quantitation of *DLX3*, 3βHSD and syncytin mRNA in BeWo cells was performed in an ABI Prism 7700 (Perkin-Elmer-Applied Biosystems) using inventoried assay mix, which consisted of unlabelled PCR primers and a TaqMan FAM-labelled MGB probe (*DLX3*: Hs00270938_m1; 3βHSD: Hs00426435_m1; syncytin: Hs02341 206_m1, Applied Biosystems). Gene expression quantification was performed as the

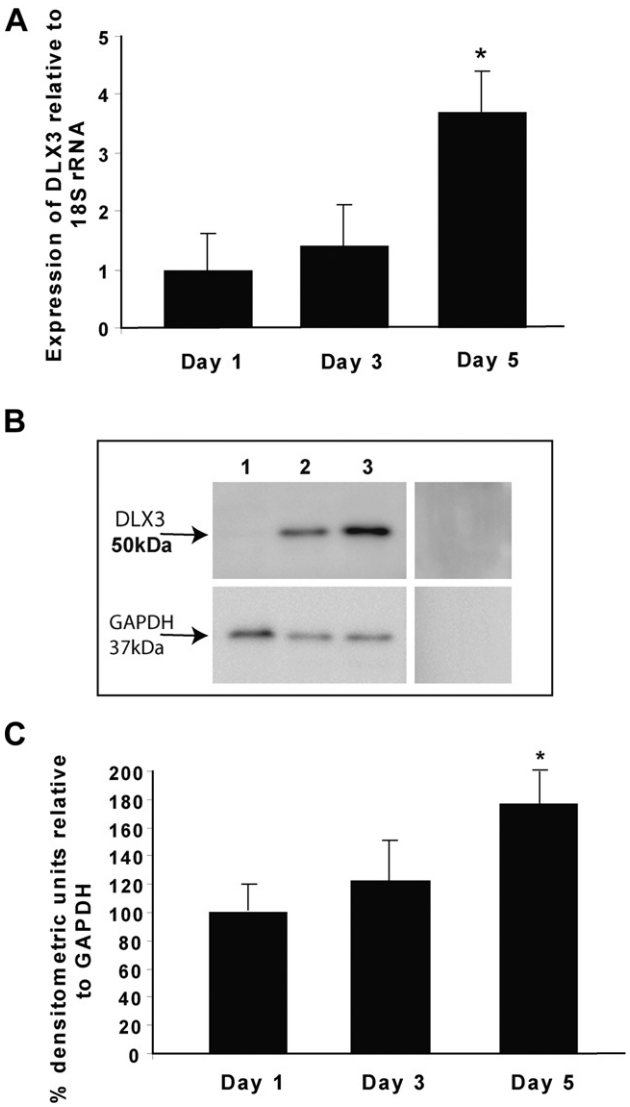

Fig. 1. DLX3 mRNA expression in term primary VCT. A. The relative mRNA expression of *DLX3* in primary term VCTs cultured from day 1–5 was determined by real-time PCR. Relative quantification of *DLX3* mRNA level was normalised to a Day 1 calibrator. * Denotes $p < 0.05$, $n = 10$ (Mann–Whitney Test). B. Representative immunoblot of the DLX3 protein in primary VCTs cultured over 1 (Lane 1), 3 (Lane 2) and 5 (Lane 3) days is shown ($n = 3$). A 50 kDa immunoreactive DLX3 protein was observed. Lane 4 shows the DLX3 competition control. GAPDH immunoblotting shows the protein load for all samples. C. Semi-quantitative analyses of DLX3 protein were performed by densitometry relative to GAPDH. The *Y*-axis represents the percentage densitometric units of DLX3 expression normalised to GAPDH. * Denotes $p < 0.05$, $n = 3$ (Mann–Whitney Test).