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# Netrin-4 Expression and Regulation by Hypoxia in Human Placenta

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## Abstract

Netrin-4 is a well-known actor involved in axonal guidance. Recently we have reported the cellular localization of netrin-4 in human placenta. Furthermore we demonstrated its role in placental angiogenesis.

Here we determined netrin-4 mRNA expression in first trimester placenta. First, we showed an increase in netrin-4 mRNA expression at 11-13 weeks of gestation (wg), suggesting a negative regulation of netrin-4 expression by hypoxia. In fact, the human placenta undergoes a transition from a low oxygenated to a highly oxygenated environment during the first trimester of pregnancy. This physiological switch in oxygen tension is a prerequisite for proper placental development and involves the hypoxia-inducible factor (HIF-1), a protein that is up-regulated under hypoxic conditions. Then, we used the trophoblast cell line BeWo to test this hypothesis. BeWo cells cultured under hypoxic conditions exhibited decreased levels of netrin-4 mRNA expression. Using siRNA strategy, hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) knock-down induced a significant increase in netrin-4 mRNA expression. Altogether these results indicate that netrin-4 expression in the trophoblast cells is regulated by hypoxia *via* the HIF-1 pathway.

**Keywords:** Netrin-4; Hypoxia; cytotrophoblast; human placenta; BeWo cells

## Introduction

Netrin-4 is one of the most extensively studied members of the netrin family. Netrin-4 is a secreted protein involved in neurite growth and migration orientation during the development of the central nervous system [1]. Beside the central nervous system, netrin-4 has been shown to regulate

epithelial branching and morphogenesis in the lung [2], pancreas [3], salivary gland branching [4], lymphangiogenesis, angiogenesis, and tumour growth [5]. We have recently investigated and characterized the expression of netrin-4 in the human placenta [6] and determined its role in placental angiogenesis [7].

During the first trimester of pregnancy, human placenta undergoes a transition from a low oxygenated to a highly oxygenated environment. This physiological switch in oxygen tension is a prerequisite for proper placental development and involves the hypoxia-inducible factor (HIF-1), a protein that is up-regulated under hypoxic conditions. HIF-1 modulates gene transcription by binding to a specific DNA sequence known as the hypoxic response element (HRE). HIF-1 is a heterodimer composed of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits. HIF-1 $\beta$  is generally constitutively expressed and insensitive to changes in O<sub>2</sub> availability, whereas HIF-1 $\alpha$  is acutely regulated in response to hypoxia [8].

Before 11 weeks of gestation (wg), placental oxygen remains low and is equivalent to 2-3%, which appears to be necessary to allow for specific placental metabolic activities, and to protect both placental and fetal tissues against toxic oxygen metabolites [9,10]. An increase in the oxygen level occur around 10 to 12 wg [11], when a continuous maternal blood flow is established in the intervillous space.

In the present study we investigated the effect of hypoxia on the expression of netrin-4 in isolated cytotrophoblasts from human first trimester placenta and direct involvement of HIF-1 signaling pathway in BeWo cells.

## Materials and Methods

### Tissues collection

Placental collection was approved by the district and local hospital ethical committees, and performed according to the Poissy Hospital code of practice. All subjects gave written informed consent. For the ontogeny study nine placentas were obtained from healthy women with viable singleton ultrasound-dated pregnancies, who were undergoing an elective termination of pregnancy procedure at first trimester between 7 and 13 wg.

**Cytotrophoblast isolation:** Isolation and treatment of cytotrophoblasts were performed as described by Dakouane-Giudicelli and collaborators [12]. Then cytotrophoblasts were RNA extracted. Culture of choriocarcinoma BeWo cell line under hypoxic and normoxic conditions and Invalidation of HIF-1 $\alpha$ .

BeWo choriocarcinoma cells (ATCC Rockville, MD, USA) were plated at a density of 2.105 cells on a 35 mm dish in 2 ml DMEM/F-12 Ham (Sigma-Aldrich) containing 15% fetal bovine serum, 100 IU penicillin and 10 mg/mL streptomycin. After 24 h, cells were cultured under normoxic (20% O<sub>2</sub>) or hypoxic (2% O<sub>2</sub>) conditions for 6, 24 or 48 h.

HIF-1 $\alpha$  knockdown induced by small interfering RNA (siRNA) in choriocarcinoma BeWo cell line, 2.105 BeWo cells were plated

in a 35 mm dish in 2 ml DMEM/F-12 Ham containing 15% fetal bovine serum without antibiotics. Cells were grown up to 40–50% confluency and then transfected with HIF-1 $\alpha$  specific siRNA oligonucleotides (Santa Cruz biotechnology, Santa Cruz CA, USA) or with fluorescent-labeled negative control siRNAs, using Lipofectamine™ RNAiMAX (Invitrogen Carlsbad, CA, USA) for 24 h, 48 h and 72 h. Then BeWo were RNA extracted.

### RNA isolation and RT-PCR analysis

RNA extraction, RNA quantification and reverse Transcription were performed, followed by quantitative PCR of duplicate sample, as previously described by Dakouane-Giudicelli et al. [12] using syber green detection on a light cycler 480 (Roche Light Cycler 480, Mannheim, Germany). The primer pair used (5'-ggcctggaagatgatgtgt-3' and 5'-ttgaggctcttcgttcaggt-3' amplified a 235-bp fragment of netrin-4 cDNA.

Netrin-4 mRNA relative quantification using the 2- $\Delta\Delta$ Cp method relative to level of mean Cp values of both housekeeping genes *tbp* and  $\beta$ 2m in villous cytotrophoblast cells isolated from first trimester placenta, in BeWo cells cultured under conditions of normoxia and in HIF-1 $\alpha$

Knock down induced by small interfering RNA (siRNA) in choriocarcinoma BeWo cell line (see table for primer reference).

**Table:** primer sequences

Gene	Accession number and location	Primer	Sequence	Product size (base pair)	Tm°C
Netrin-4	NM_021229 954-1188	Forward	GGCCTGGAAGATGATGTTGT	235	60
		Reverse	TTGAGGCTCTTCGTTTCAGGT		
HIF-1 $\alpha$	NM_181054.2 <b>815-1065</b>	Forward	TCCATGTGACCATGAGGAAA	251	58
		Reverse	CCAAGCAGGTCATAGGTGGT		
TBP	NM_003194.3 893-1024	Forward	TGACAGGAGCCAAGAGTGAA	132	60
		Reverse	CACATCACAGCTCCCCACCA		
B2M	NM_004048.2 589-674	Forward	TGCTGTCTCCATGTTTGATGTATCT	86	60
		Reverse	TCTCTGCTCCCCACCTCTAAGT		

### Statistics

All statistical analyses were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA, USA). Data were analyzed by Student's t-test

## Results and Discussion

Netrin-4 mRNA expression in human placenta was low around 7-10 wg, during the gestational period of low oxygen tension, and then increased at 11-13 wg (Figure 1), when the blood flow is established and oxygen tension increases in the intervillous

space. The present findings also demonstrate for the first time that netrin-4 expression is down-regulated by hypoxia in cultured BeWo cells. BeWo cells cultured under hypoxic conditions exhibited significant low levels of netrin-4 transcript when compared to normoxia (Figure 2a). Our in vitro experiments established that hypoxia is an inhibitor of netrin-4 expression. Here, we demonstrated in the human placenta a similar pattern of netrin-4 gene expression with a dependence on oxygen tension throughout the first trimester of pregnancy. We found that the changes in netrin-4 expression correlate with the changes in oxygen tension.

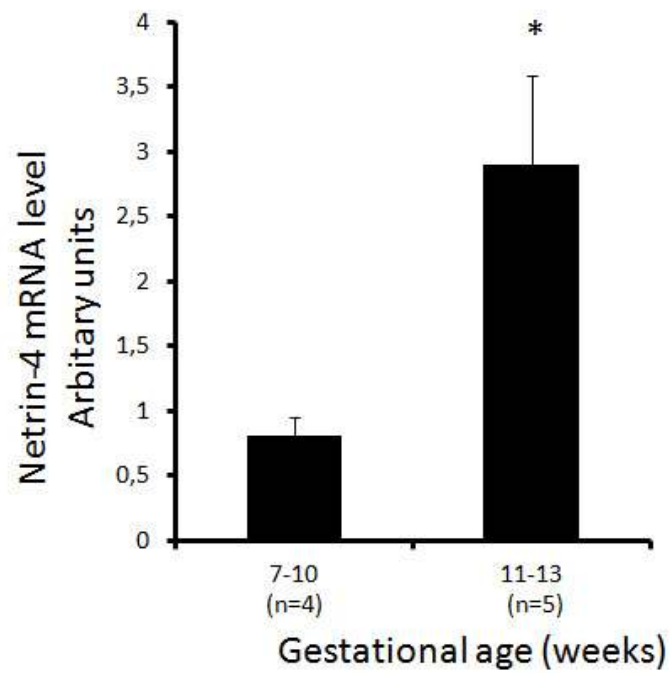


Figure1

**Figure 1:** Expression and ontogeny of netrin-4 mRNA in isolated cytotrophoblast from human first trimester placenta. (A) netrin-4 expression was normalized to placenta at 7 wg. Netrin-4 mRNA expression is significantly increased in 11-13 wg compared to 7-10 wg. The graph shows mean  $\pm$  SEM, where n represents the number of placentas analyzed for each gestational age and  $*p < 0.05$ .

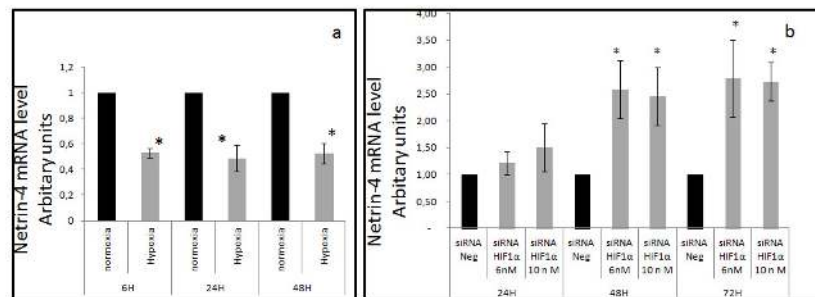


Figure 2

**Figure 2:** Effect of hypoxia on netrin-4 expression, (a) In BeWo cells hypoxia induced a decrease in netrin-4 expression after 24 h of incubation. (b) Significant increase in netrin-4 expression was observed in response to HIF-1 $\alpha$  silencing using siRNA against HIF-1 $\alpha$ . Netrin-4 mRNA was increased by 3 fold. Control cells were transfected with non-silencing siRNA. Data represent the mean of 4 experiments  $\pm$  SEM \* $p$ <0.05.

Importantly, we also found a negative correlation between the rates of expression of netrin-4 and netrin-1 [13]. These data suggest a strong resemblance between villous cytotrophoblast from early first trimester placenta and solid tumors in terms of netrin-1 and netrin-4 transcriptional activities [14]. In the placenta, the angiogenic activity is known to be increased before its oxygenation, which correlates with the decrease in netrin-4 expression. The increase in netrin-4 levels coincides with the oxygenation of the placenta [15]. This suggests a potential role of netrin-4 in the control of angiogenic processes as demonstrated in our recent study [7].

In this work, we also demonstrated that a knockdown of HIF-1 $\alpha$  using a siRNA strategy under hypoxic environment induced an increase in netrin-4 mRNA expression (figure 2b), providing evidence that netrin-4 down-regulation is mediated through the HIF-1 pathway. Therefore, additional research may provide new insights into the role of netrin-4 in normal human placenta and associated diseases, such as preeclampsia and growth restriction, which are both characterized by hypoxia [16].

In conclusion, our results elucidate the pattern of expression of netrin-4 and demonstrate for the first time that netrin-4 expression is down-regulated by hypoxia in the human placenta during the first trimester of pregnancy, and that its regulation is under the direct control of HIF-1. These data bring new insights into the control of Netrin-4 in the placenta and report a new mechanism for the hypoxic inactivation of netrin-4 through the HIF-1 pathway.

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