Hypoxia-inducible factor-dependent histone deacetylase activity determines stem cell fate in the placenta

Emin Maltepe[1], Geoffrey W. Krampitz[2], Kelly M. Okazaki[2], Kristy Red-Horse[2], Winifred Mak[3], M. Celeste Simon[4,5,6] and Susan J. Fisher[2,7,8,9,*]

[1]Department of Pediatrics and Molecular Medicine Program, University of California, Parnassus Avenue, San Francisco, CA 94143, USA
[2]Department of Cell and Tissue Biology, University of California, Parnassus Avenue, San Francisco, CA 94143, USA
[3]Obstetrics and Gynecology, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, West Hollywood, CA 90048, USA
[4]Howard Hughes Medical Institute, Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA 19104, USA
[5]Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104, USA
[6]Abramson Cancer Research Institute, University of Pennsylvania, Philadelphia, PA 19104, USA
[7]Obstetrics, Gynecology and Reproductive Sciences, University of California, Parnassus Avenue. San Francisco, CA 94143, USA
[8]Department of Pharmaceutical Chemistry, University of California, Parnassus Avenue, San Francisco, CA 94143, USA
[9]Department of Anatomy, University of California, Parnassus Avenue, San Francisco, CA 94143, USA
*Author for correspondence (e-mail: sfisher@cgl.ucsf.edu)

Accepted 31 May 2005

Development 132, 3393-3403
Published by The Company of Biologists 2005
doi:10.1242/dev.01923

Summary

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor composed of HIFα and the arylhydrocarbon receptor nuclear translocator (ARNT/HIF1β). Previously, we have reported that ARNT function is required for murine placental development. Here, we used cultured trophoblast stem (TS) cells to investigate the molecular basis of this requirement. In vitro, wild-type TS cell differentiation is largely restricted to spongiotrophoblasts and giant cells. Interestingly, *Arnt*-null TS cells differentiated into chorionic trophoblasts and syncytiotrophoblasts, as demonstrated by their expression of Tfeb, glial cells missing 1 (Gcm1) and the HIV receptor CXCR4. During this process, a region of the differentiating *Arnt*-null TS cells underwent granzyme B-mediated apoptosis, suggesting a role for this pathway in murine syncytiotrophoblast turnover. Surprisingly, HIF1α and HIF2α were induced during TS cell differentiation in 20% O_2; additionally, pVHL levels were modulated during the same time period. These results suggest that oxygen-independent HIF functions are crucial to this differentiation process. As histone deacetylase (HDAC) activity has been linked to HIF-dependent gene expression, we investigated whether ARNT deficiency affects this epigenetic regulator. Interestingly, *Arnt*-null TS cells had reduced HDAC activity, increased global histone acetylation, and altered class II HDAC subcellular localization. In wild-type TS cells, inhibition of HDAC activity recapitulated the *Arnt*-null phenotype, suggesting that crosstalk between the HIFs and the HDACs is required for normal trophoblast differentiation. Thus, the HIFs play important roles in modulating the developmental plasticity of stem cells by integrating physiological, transcriptional and epigenetic inputs.

Key words: HIF, ARNT, HDAC, Stem cell, Syncytiotrophoblast, Placenta, Mouse

Introduction

As the primary interface between mother and embryo/fetus, the placenta plays a myriad of functions, including anchoring of the conceptus to the uterus, transport of nutrients and waste products, hormone secretion, and allotransplantation of the genetically foreign embryo/fetus (Cross et al., 1994). Thus, it is not surprising that numerous studies in transgenic mice have shown that placentation is a crucial regulator of embryonic and fetal development (Paria et al., 2002; Cross et al., 2003). The series of events that culminate in formation of a mature placenta begins when cells at the surface of the morula adopt a trophoblast fate. Implantation of the developing embryo requires their further differentiation to trophectoderm, which emerges as a polarized outer layer of cells surrounding the blastocyst. The inner layer of the blastocyst, which remains apolar, gives rise to the inner cell mass, which goes on to form the embryo. Further maturation of the trophectoderm lineage produces multiple specialized cell types: spongiotrophoblasts, trophoblast giant cells, chorionic trophoblasts and syncytiotrophoblasts (reviewed by Rossant and Cross, 2001).

Murine and human trophoblasts exhibit many unique characteristics. For example, spongiotrophoblasts, the murine equivalent of human cytotrophoblast progenitors, proliferate in response to hypoxia – a characteristic they share with hematopoietic precursors (Adelman et al., 1999). This unique feature is likely to be one important component of the mechanisms that contribute to the explosive growth of the

placenta as compared with the early embryo (Genbacev et al., 1997; Adelman et al., 2000). Murine trophoblast giant cells are among the few mammalian cells to undergo endoreduplication – repeated DNA replication without intervening mitoses that produces a polyploid state (Cross et al., 1994; Zybina and Zybina, 2000). Likewise, differentiation of human invasive extravillous cytotrophoblasts produces aneuploid cells (Weier et al., 2005). In addition, trophoblast giant cells and invasive cytotrophoblasts carry out endovascular invasion, a process that includes transdifferentiation into endothelial-like cells that line the maternal vasculature (Cross et al., 1994; Zhou et al., 1997). The multinucleated syncytiotrophoblasts, which surround fetal blood vessels and maternal blood sinuses in mice, and form the surface of vascularized chorionic villi in humans, are responsible for transport. These cells arise from the underlying chorionic plate in mice or villous cytotrophoblasts in humans, where they are also largely responsible for the endocrine functions of the placenta. Thus, the mature placenta is a complex organ with multiple specialized cell types produced through an equally complex series of differentiation processes.

Hypoxia-inducible factor 1 (HIF1) is an important regulator of the responses of a cell to oxygen tension. HIF1 is a widely expressed basic helix-loop-helix (bHLH)-PAS transcription factor composed of two subunits: HIF1α and ARNT/HIF1β (Wang et al., 1995). This family is responsible for mediating the response of cells and organisms to various environmental stimuli, including xenobiotic exposure, hypoxia and light (Gu et al., 2000). We have previously demonstrated a requirement for ARNT in murine development (Maltepe et al., 1997; Adelman et al., 1999). As the heterodimerization partner for HIF1α and the related HIF2α in most tissues, ARNT mediates transcriptional responses to oxygen deprivation (Wang et al., 1995; Ema et al., 1997; Tian et al., 1997). In the mature organism, this pathway is often activated in response to pathology. By contrast, mammalian development normally transpires in a hypoxic environment (Maltepe and Simon, 1998). We initially focused on the role of ARNT in embryonic vascularization and hematopoiesis (Maltepe et al., 1997; Adelman et al., 1999). It later became apparent that the primary cause of lethality of *Arnt*-null embryos was placental failure caused by a loss of the spongiotrophoblast population (Kozak et al., 1997; Adelman et al., 2000). As a result, the placental labyrinth, which is crucial for maternal-fetal gas and nutrient exchange, fails to form.

In vitro models of differentiation are important adjuncts to in vivo analyses. The recent derivation of TS cells from mouse blastocysts (Tanaka et al., 1998) created a reliable system to study trophoblast giant cell and spongiotrophoblast differentiation in vitro. Using the TS model, we now report an unexpected role for ARNT in placental development. Specifically, differentiation of *Arnt*-null TS cells in vitro produced syncytiotrophoblasts and chorionic trophoblasts. As to the mechanisms involved, our data suggest that the interplay between HIFs and histone deacetylases (HDACs) is crucial for TS cell differentiation, linking HIF function with epigenetic effectors. These results are evidence that the HIFs can coordinate diverse epigenetic mechanisms with profound effects on intrauterine development.

Materials and methods

Cell culture

The production of $Arnt^{+/+}$ and $Arnt^{-/-}$ TS cells has been described previously (Adelman et al., 2000). TS cells were initially derived on mouse embryonic fibroblasts as originally described (Tanaka et al., 1998). However, their later expansion and maintenance used human placental fibroblast feeders as described by Genbacev et al. (Genbacev et al., 2005). Briefly, TS cells were maintained in RPMI-1640 with 25 mM HEPES and 2.0 g/l $NaHCO_3$ with 20% fetal calf serum, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, 2 mM L-glutamine and penicillin/streptomycin at 50 μg/ml each (TS medium; UCSF Cell Culture Facility) in the presence of 25 ng/ml FGF4 and 1 μg/ml heparin (Sigma, St Louis, MO). Before differentiation or for replating under feeder-free conditions, TS cells on placental fibroblasts were trypsinized and plated for 1 hour before the nonadherent TS cells were transferred to a different tissue culture plate. Approximately 80 to 90% of the fibroblasts were removed by this method. For the expansion of an undifferentiated population in the absence of feeders, the TS cells were replated in 70% placental fibroblast-conditioned medium with FGF4 and heparin at the concentrations noted above. To initiate differentiation, the TS cells were replated at near confluence in TS medium without FGF4 and heparin. Then, the cells were allowed to differentiate for 6 days, with medium changes every second day, prior to further analysis. In some cases, the cells were maintained at 2% O_2 (37°C) for various periods of time in a humidified Bactron Anaerobic/Environmental Chamber (Sheldon Manufacturing, Cornelius, OR). Differentiation was monitored by phase-contrast microscopy. For differentiation in the presence of HDAC or HSP90 inhibitors, TS cells were cultured in plain TS medium containing either 100 nM TSA, 2.5 mM sodium butyrate or 100 nM GA for 6 days.

Labeling of apoptotic cells

Differentiated wild-type or *Arnt*-null cells grown on glass coverslips were fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.4, for 1 hour at room temperature. DNA breaks were labeled with the In Situ Cell Death Detection kit (Roche Applied Science, Penzberg, Germany) and apoptotic cells were visualized by fluorescence microscopy.

Time-lapse Video Microscopy

Arnt-null TS cells at near confluence were allowed to differentiate in an environmental chamber mounted on a motorized microscope stage (Carl Zeiss MicroImaging). Time-lapse images were collected every 15 minutes beginning 96 hours after the initiation of differentiation using a SPOT-RT CCD camera (Molecular Dynamics).

RNA FISH

TS cells were cultured on gelatin-coated glass coverslips, fixed in 4% formaldehyde/PBS for 15 minutes, permeabilized on ice in PBS containing 0.5% Triton X-100 for 4 minutes, washed with PBS and then rinsed twice in 2× SSC. RNA FISH and washes were performed as described (Heard et al., 1999) using DNA probes labeled with Spectrum Red or Green dUTP (Vysis, Downer Grove, IL). DNA was visualized by staining for 2 minutes with DAPI.

Northern blot analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The Errβ2, placental lactogen I and 4311 probes (Adelman et al., 2000) as well as VEGF, PGK1 and GLUT1 probes have been described previously (Maltepe et al., 1997). The Gcm1 probe was the kind gift of Dr James Cross (U Calgary). A Tfeb probe corresponding to the terminal 300 bp of the coding region along with 300 bp of the 3′UTR was generated by RT-PCR. The product was cloned into the PCR 2.1 vector (Invitrogen, Carlsbad, CA). Northern blot hybridization was

performed using standard methodologies. Briefly, 20 μg of total RNA was loaded per lane and resolved on 1.5% agarose gels in a MOPS buffer containing formaldehyde. Then the samples were transferred to Hybond N+ membranes (Amersham Biosciences, Piscataway, NJ) and hybridized in QuickHyb hybridization solution (Stratagene, La Jolla, CA) to ^{32}P-labeled DNA probes generated with High Prime reagent (Roche Diagnostics GMBH, Mannheim, Germany). Binding of labeled probes was detected using BIOMAX MS film (Kodak, Rochester, NY).

RNase protection assay

Total RNA, isolated by using Trizol reagent as described above, was analyzed using the RiboQuant Multi-Probe RNase protection assay kit with the hCR-8 Multi-Probe Template purchased from Pharmingen (San Diego, CA) according to the manufacturer's instructions. Briefly, a probe set containing the C-X-C chemokine receptor 4 was labeled with ^{32}P-CTP and hybridized to 10 μg total RNA isolated from either undifferentiated or differentiated wild-type or mutant TS cells. After RNase digestion, the protected products were resolved on sequencing gels and identified by size. Glyceraldehyde-3-phosphate dehydrogenase transcripts were used to assess sample loading.

Immunoblotting

Cytoplasmic and nuclear extracts were obtained according to the manufacturer's instructions using the NE-PER Nuclear and Cytoplasmic Extract Reagents kit (Pierce Biotechnology, Rockford, IL) to which the Halt protease inhibitor cocktail was added (Pierce Biotechnology). Whole cell lysates were prepared using a buffer consisting of 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), containing 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1% SDS and 10% glycerol. Samples were run on precast BioRad readygels and transferred to nitrocellulose using standard techniques. Positive immunoreactivity was visualized with the ECL Plus Western Blotting Detection Reagent (Amersham).

Histone deacetylase activity

Nuclear extracts of undifferentiated or differentiated wild-type and *Arnt*-null TS cells (obtained as described above) were analyzed for HDAC activity via the HDAC Fluorometric Assay kit (Upstate, Lake Placid, NY) according to the manufacturer's instructions.

Chemicals and reagents

Trichostatin A (TSA), geldanamycin A (GA) and sodium butyrate (NaB) were obtained from Sigma-Aldrich (St Louis, MO). The following antibodies were used for immunoblotting: HIF1α NB 100-105 (Novus Biologicals, Littleton, CO), anti human/mouse HIF1α (R&D Systems, Minneapolis, MN), HIF2α NB 100-122 (Novus Biologicals, Littleton, CO), ARNT 2B10 (Abcam, Cambridge, MA), E-cadherin (BD Transduction Pharmingen, San Diego, CA), Hsp90β Ab-1 (Neomarkers, Fremont, CA), HDAC1 (Upstate, Lake Placid, NY), HDAC2 (Zymed, South San Francisco, CA), HDACs 3-7 (Cell Signaling Technology, Beverly, MA), HDAC9 (Biovision Research Products, Mountain View, CA), Ac-H4 (Serotec, Raleigh, NC), PARP clone C-2-10 (Biomol, Plymouth Meeting, PA) and Granzyme B (Labvision, Fremont, CA).

Results

Arnt-null TS cells cultured in 20% oxygen differentiate into chorionic trophoblasts and syncytiotrophoblasts

Initial experiments showed that undifferentiated wild-type (control) and Arnt-null (experimental) TS cells maintained on feeder fibroblasts exhibited similar morphological features irrespective of genotype (Fig. 1A,B). As previously demonstrated, wild-type TS cells differentiated into trophoblast giant cells and spongiotrophoblasts (Fig. 1C). By contrast, cultures of differentiated Arnt-null TS cells, which lacked trophoblast giant cells, fused forming multinucleated syncytiotrophoblasts that were interspersed

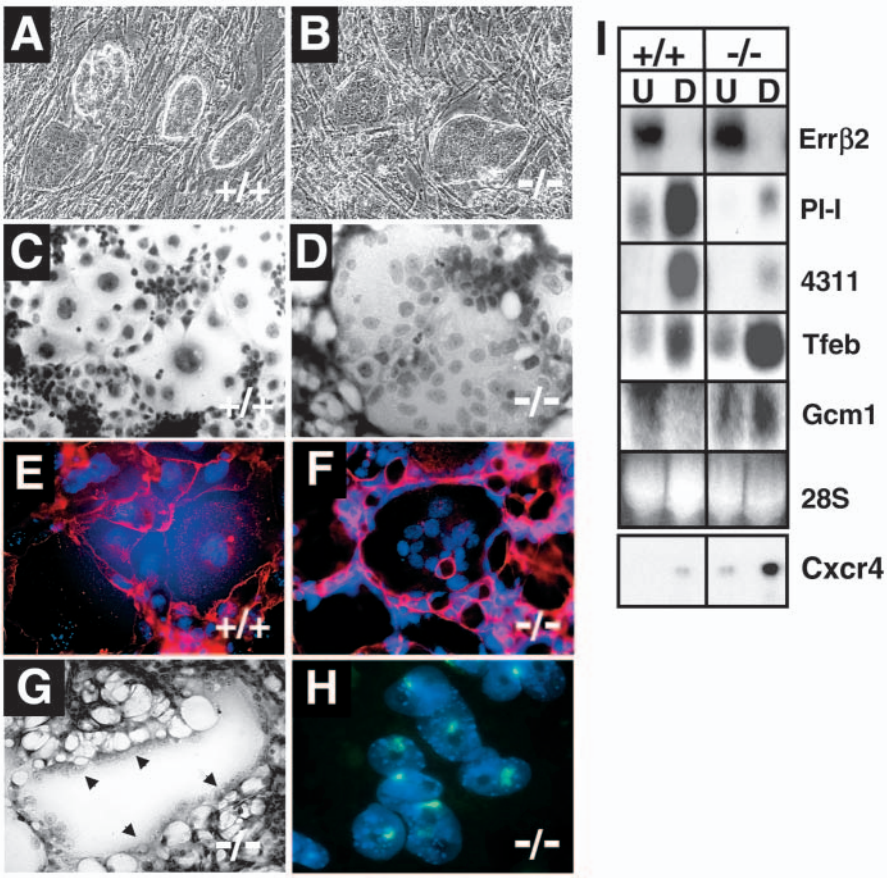

Fig. 1. *Arnt*-null TS cells differentiate into chorionic trophoblasts and syncytiotrophoblasts. Undifferentiated wild-type (A) and *Arnt*-null (B) TS cells grown on fibroblast feeders exhibited identical morphological features. Differentiated wild-type cells (C) formed large trophoblast giant cells and smaller spongiotrophoblasts. Differentiated *Arnt*-null TS cells (D) produced large multinucleated syncytiotrophoblasts and smaller chorionic trophoblasts. E-cadherin staining of differentiated wild-type cells revealed one or occasionally two nuclei per cell (E), whereas *Arnt*-null cells formed syncytia containing multiple nuclei (F). In some cases, multinucleated cells with peripherally distributed nuclei (G, arrowheads) and central clearing appeared to form epithelial-lined spaces. (H) RNA FISH analysis of Xist RNA revealed a single inactive X chromosome per nucleus in differentiated *Arnt*-null TS cells, additional evidence for lack of endoreduplication. (I) Analysis of lineage-specific markers by northern blot hybridization and RNase protection assays confirmed these findings.

among smaller cells (Fig. 1D). Following differentiation, E-cadherin staining revealed control cells with one or occasionally two large nuclei (Fig. 1E), whereas differentiated Arnt-null cells frequently formed syncytia that contained many nuclei (Fig. 1F). Occasionally, multinucleated cells with peripherally distributed nuclei and central clearing appeared (Fig. 1G, arrowheads) that formed large epithelial-lined spaces.

To confirm these morphological observations, we exploited two unique features of trophoblast cells. First, TS cell differentiation along the giant cell lineage is accompanied by endoreduplication in which the normal chromosome complement increases by severalfold (Tanaka et al., 1998). Second, allocation of the trophoblast lineage entails X inactivation (Mak et al., 2004): Xist RNA expression spreads in cis from the Xic locus, which inactivates the paternal X chromosome (Brockdorff, 1998). To determine if differentiated Arnt-null TS cells have a normal karyotype, we subjected them to fluorescence in situ hybridization (FISH) analysis for Xist RNA. As shown in Fig. 1H, 90% of the cells, which are female (data not shown), contained a single inactive X chromosome. This was consistent with the conclusion that differentiating Arnt-null TS cells, like syncytiotrophoblasts, do not undergo endoreduplication, the hallmark of trophoblast giant cell formation.

To obtain additional information about the identity of differentiated Arnt-null TS cells, we compared their expression of lineage-specific markers with that of their wild-type counterparts. In accordance with published results, expression of estrogen related receptor β 2 (ERRβ2) was limited to undifferentiated TS cells (Fig. 1I). After differentiation, the wild-type cells expressed predominantly giant cell (placental lactogen I) and spongiotrophoblast (4311) markers. By contrast, Arnt-null cells, which expressed very low levels of these markers upon differentiation, strongly upregulated the chorionic trophoblast marker Tfeb (Steingrimsson et al., 1998) and the syncytiotrophoblast marker Gcm1 (Basyuk et al., 1999) (Fig. 1I). Quantitative RT-PCR analysis showed an approximately 16-fold higher level of Gcm-1 mRNA in differentiated Arnt-null cells as compared with their wild-type counterparts (data not shown). Additionally, differentiation of the mutant cells was associated with strong upregulation of the C-X-C chemokine receptor 4 (CXCR4), a human immunodeficiency virus 1 (HIV 1) receptor that is expressed on human trophoblast syncytium (Douglas and Thirkill, 2001).

Arnt-null TS cells undergo granzyme B-mediated apoptosis during differentiation

In humans, syncytial turnover mediated by a region of the apoptotic cascade has been proposed as an important component of placental development in vivo (Huppertz and Kingdom, 2004). Thus, we investigated whether differentiation of Arnt-null TS cells is associated with apoptosis. At a morphological level, DAPI-staining of Arnt-null TS cell nuclei 48 hours after initiation of differentiation revealed many pyknotic nuclei (frequently a sign of apoptosis) that were not commonly observed in cultures of wild-type TS cells (Fig. 2A). This observation was confirmed by TUNEL staining (Fig. 2A, inset). Quantification of these results

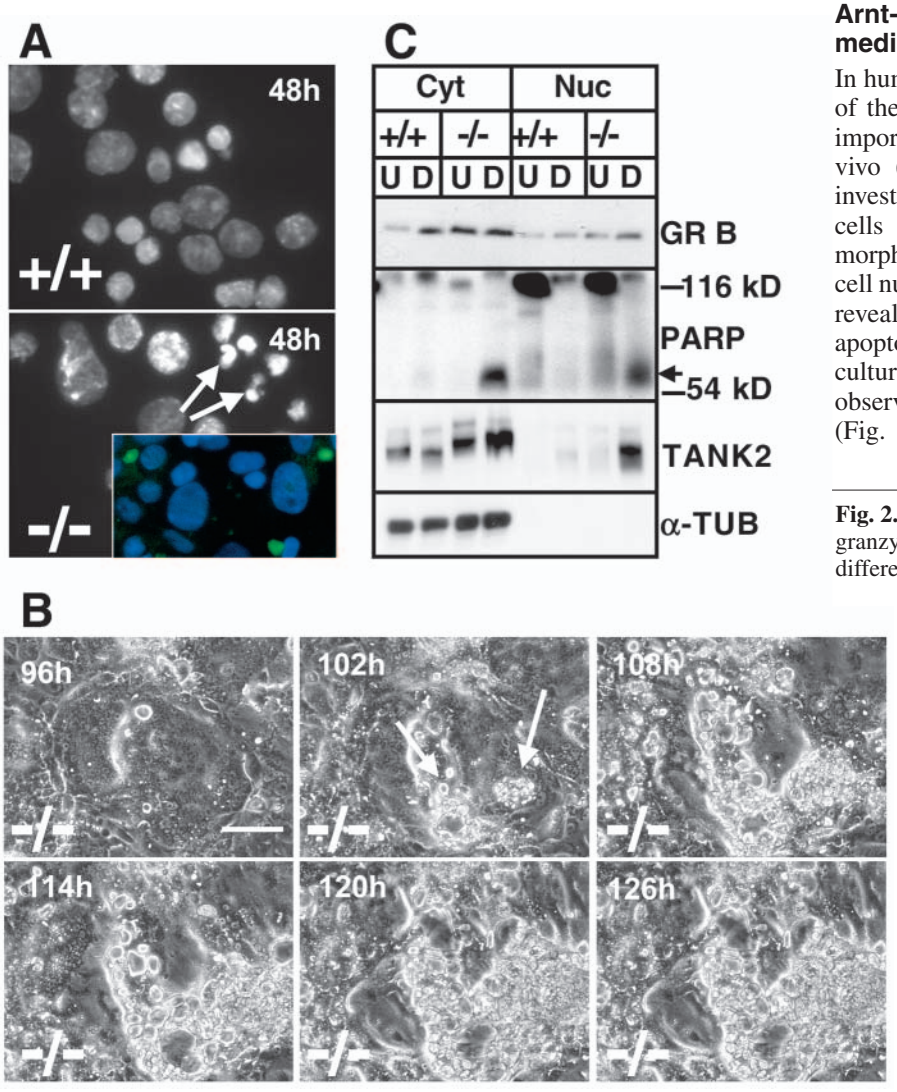

Fig. 2. *Arnt*-null TS cell differentiation can lead to granzyme B-mediated apoptosis. (A) DAPI-staining of differentiated wild-type (upper panel) and mutant (lower panel) TS cells revealed a significant number of pyknotic nuclei indicative of apoptosis only in *Arnt*-null cells (arrows, lower panel). This impression was confirmed by FITC labeling of nicked DNA (inset). (B) Time-lapse video microscopy of *Arnt*-null cells was performed starting 4 days after differentiation. The results showed wide-scale breakdown of the plasma membrane and cytoplasmic vacuolization (arrows) that culminated in cell sloughing from the tissue culture plate (data not shown). (C) Cytoplasmic (Cyt) and nuclear (Nuc) extracts of undifferentiated (U) and differentiated (D) wild-type and *Arnt*-null TS cells were subjected to immunoblot analysis using antibodies specific for granzyme B (GR B), poly (ADP-ribose) polymerase (PARP), tankyrase 2 (TANK2) and α–tubulin (α-TUB).

revealed an approximate sevenfold increase (14% versus 2%) in the number of apoptotic cells in Arnt-null versus wild-type cultures. To better understand the consequences, we used timelapse video microscopy to analyze this process. As shown in Fig. 2B, in approximately half of the differentiating Arnt-null cells, syncytialization was followed at 6 days by large-scale membrane breakdown and cytoplasmic vacuolization that commonly resulted in cell sloughing (not shown). The remaining cells had normal morphological characteristics.

Next, we used poly (ADP-ribose) polymerase (PARP) cleavage into specific fragments as a readout of the pro-apoptotic pathways involved. In addition to classic caspase family members, granzyme B – which after birth is largely restricted to cytotoxic T-lymphocytes and natural killer (NK) cells – is highly expressed in human villous trophoblast (Bladergroen et al., 2001; Hirst et al., 2001). Consistent with this observation, immunoblot analyses showed that TS cells expressed granzyme B protein (Fig. 2C). In wild-type cells, expression of this molecule, which was predominantly cytoplasmic, was markedly induced during differentiation. By comparison, mutant cells expressed constitutively elevated levels that did not change upon withdrawal of FGF4 and heparin. In addition, undifferentiated wild-type and Arnt-null TS cells expressed PARP protein, which was largely confined to the nucleus (Fig. 2C). Upon differentiation, nuclear PARP levels were dramatically decreased in both cell types, with a 64 kDa fragment that is indicative of Granzyme B-mediated proteolysis apparent in both the nuclear and cytoplasmic fractions of Arnt-null cells (Fig. 2C, arrow). This was in contrast to an 89 kDa fragment that is generated by caspase activity (Soldani and Scovassi, 2002). TS cells also expressed the PARP-related enzyme tankyrase 2 (TANK2). Overexpression of this molecule has been shown to lead to cell death (Kaminker et al., 2001). Differentiated Arnt-null cells expressed much higher cytoplasmic and nuclear levels of TANK2 than were observed in wild-type cells. Taken together, these results indicated that, in some cases, differentiating Arnt-null TS cells underwent programmed cell death via mechanisms that included the actions of granzyme B and TANK2.

Oxygen-independent modulation of HIFα expression during TS cell differentiation

To more clearly define the function of HIF family members in TS cells, we analyzed their expression in wild-type and *Arnt*-null cells during differentiation. As expected, only wild-type cells expressed Arnt mRNA (Fig. 3A). HIF1α, but not HIF2α, mRNA was expressed in undifferentiated TS cells. Interestingly, levels of both transcripts increased dramatically with differentiation (Fig. 3A).

HIF activity is coupled to oxygen tension by mechanisms that operate at the post-translational level. Specifically, the constitutive hydroxylation of critical proline residues within the oxygen-dependent degradation domain of HIFα by the PHD family of prolyl hydroxylases enables recognition and subsequent ubiquitination by the Von Hippel-Lindau (pVHL) tumor suppressor protein (Epstein et al., 2001; Ivan et al., 2001). Under standard tissue culture conditions of 20% O_2, ubiquitinated HIFα is degraded in the proteasome. This process, which is inhibited by hypoxia, enables accumulation of the active protein under conditions of reduced oxygen tension.

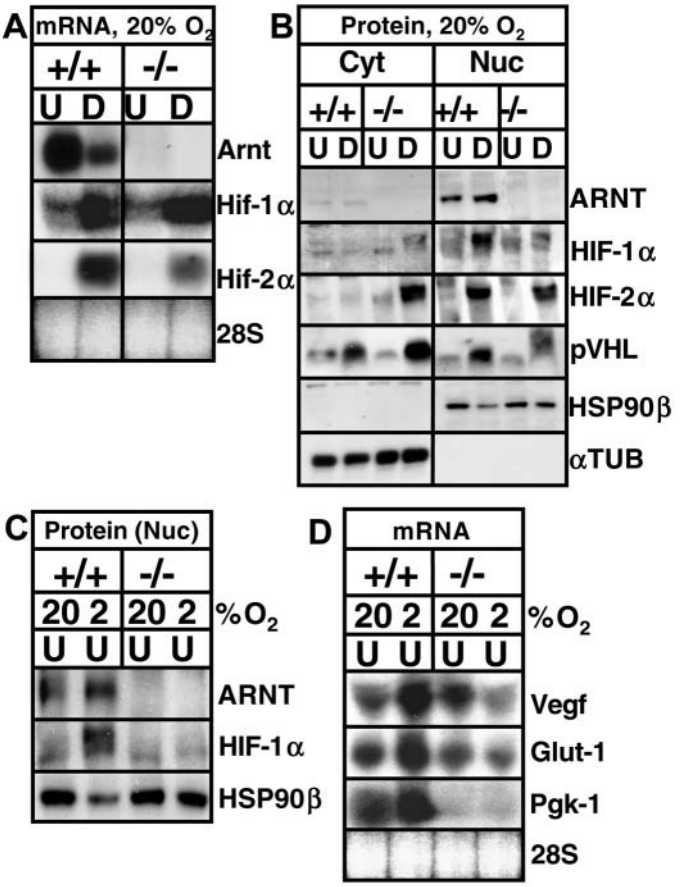

Fig. 3. Oxygen-independent modulation of HIFα expression during TS cell differentiation. (A) Levels of mRNAs encoding Arnt, HIF1α and HIF2α were assayed by Northern blot hybridization after differentiation under standard tissue culture conditions for 1 week. (B) Protein levels of the HIFs and related factors were assayed by immunoblot. ARNT protein was solely detected in the nuclei of wild-type TS cells and levels did not change with differentiation. In normoxia (20% O_2), HIF1α expression was strongly induced in wild-type but not mutant TS cells. With regard to HIF2α, high cytoplasmic expression was observed only after differentiation of *Arnt*-null TS cells, with comparable levels of nuclear expression in both wild-type and mutant cells after FGF4 and heparin withdrawal. pVHL levels, in both the cytoplasmic and nuclear fractions, were induced during differentiation. HSP90β expression, which was confined to the nucleus, did not change significantly during either wild-type or mutant TS cell differentiation. (C) The effects of hypoxia (2% O_2) on ARNT, HIF1α and HSP90β expression in undifferentiated wild-type and mutant TS cells mirrored the effects of differentiation. (D) As expected, mRNA levels of the HIF1 target genes VEGF and GLUT1 were upregulated when wild type, but not mutant TS cells were cultured under hypoxic conditions. PGK1, which was upregulated by hypoxia in wild-type TS cells, was expressed only at low levels in the *Arnt*-null mutants.

Therefore, we used an immunoblot approach to assess HIF protein abundance during TS cell differentiation under standard culture conditions, to eliminate hypoxia as a variable. As expected, the mutant cells lacked ARNT protein, whereas a strong immunoreactive band was detected in nuclear extracts prepared from wild-type TS cells (Fig. 3B), the intensity of which did not change significantly with differentiation. As to

HIF1α, prior to differentiation, nuclear extracts prepared from both TS cell lines contained either undetectable or low levels of this protein (compare Fig. 3B with 3C). In accordance with the mRNA expression data (Fig. 3A), HIF2α protein was either not detected or expressed at very low levels in undifferentiated wild-type or mutant TS cells (Fig. 3B). Surprisingly, when the cells were differentiated in 20% O_2, nuclear HIF1α was induced dramatically in wild-type cells, but not in the *Arnt*-null mutants (Fig. 3B). With regard to HIF2α, very low or no expression was detected in the cytoplasmic or nuclear fractions of either cell line prior to differentiation. Unexpectedly, upon differentiation cytoplasmic levels of HIF2α dramatically increased in mutant but not wild-type cells, whereas nuclear expression was highly upregulated in both lines (Fig. 3B). Taken together these results highlight the differences in regulation of HIF1α and HIF2α stabilization and subcellular localization that have been previously noted (Genbacev et al., 2001; Park et al., 2003). Interestingly, cytoplasmic and nuclear pVHL expression was also induced with differentiation in both wild-type and mutant cells (Fig. 3B). Comparable levels of heat shock protein 90β (HSP90β), which also stabilizes HIF1α in a pVHL- and hypoxia-independent fashion (Isaacs et al., 2002; Katschinski et al., 2002), were expressed in the nuclear compartments of both undifferentiated and differentiated TS cells (Fig. 3B). Next, we studied the effects of hypoxia on the nuclear expression of a subset of these proteins in undifferentiated TS cells (Fig. 3C). In general, culturing the cells in 2% O_2 mimicked the changes in ARNT, HIF1α and HSP90β protein abundance that occurred as a function of differentiation (compare Fig. 3C with 3B).

To determine the functional significance of these observations, we analyzed mRNA abundance of HIF1 target genes in undifferentiated TS cells by Northern blot hybridization. As expected, exposure to 2% O_2 increased expression of vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT1) only in wild-type TS cells (Fig. 3D). Unexpectedly, there was a dramatic difference in phosphoglycerate kinase 1 (PGK1) expression between wild-type and *Arnt*-null TS cells. In wild-type cells, PGK1 expression was regulated similarly to the other HIF1 targets we analyzed. In sharp contrast, the abundance of mRNA encoding this molecule was significantly diminished in mutant cells maintained in 20% O_2, and transcript abundance did not change when the cells were cultured in hypoxic conditions (Fig. 3D). This observation is consistent with the fact that PGK1 mRNA expression is crucially dependent on HIF1α during embryonic development in the mouse (Iyer et al., 1998).

In this context, we assessed the effects of physiological hypoxia on TS cell differentiation in vitro. Similar to the transition that occurs during weeks 10-12 of human development, the murine placenta gains access to a supply of maternal blood at approximately embryonic day 9.5 (E9.5), which drastically increases oxygen availability to this organ. In these experiments, we attempted to mimic this crucial period in vitro. As shown in Fig. 4, differentiation in physiological hypoxia (2% O_2) did not redirect the fate of either wild-type or mutant cells. To model the hypoxia/normoxia transition, differentiating TS cells that had been cultured in 2% O_2 for 3 days, were transferred to a 20% O_2 environment for 3 days. Reoxygenation did not change the differentiative capacity of *Arnt*-null TS cells. By contrast, this transition promoted wild-type TS cell differentiation to spongiotrophoblasts, as shown by the dramatic increase in 4311 mRNA levels, with no discernible effects on giant cell formation. This observation is in keeping with a required role for ARNT in maintenance of the spongiotrophoblast population during placental development (Adelman et al., 2000) and supports the concept that physiological hypoxia acts as an important developmental morphogen.

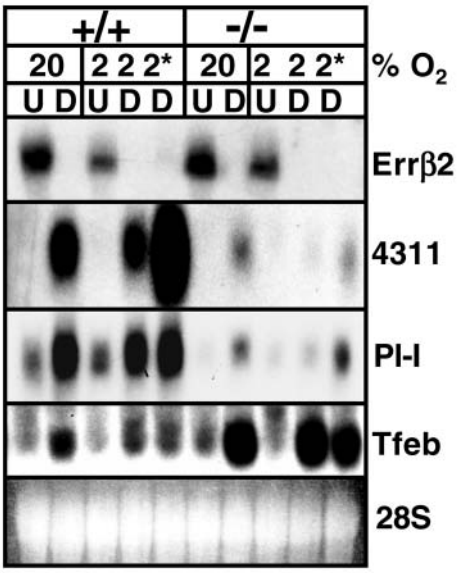

Fig. 4. The hypoxia-normoxia transition promotes spongiotrophoblast expansion. Hypoxia fails to redirect wild-type or mutant TS cell fate in vitro. In contrast, hypoxia followed by reoxygenation (2*) led to an expansion of the spongiotrophoblast lineage, as shown by the dramatic enhancement of 4311 expression in wild-type TS cells.

Impaired HDAC activity and nuclear localization in *Arnt*-null TS cells

Multiple proteins have been shown to interact with HIF1α. Among these, the HDACs are of particular interest. HIF1α interacts with HDAC1, HDAC2 and HDAC3 via pVHL (Mahon et al., 2001), as well as HDAC4 and HDAC7 (Kato et al., 2004). The HDAC inhibitor trichostatin A (TSA) inhibits hypoxia-induced gene expression. Conversely, hypoxia induces HDAC activity (Kim et al., 2001; Mie Lee et al., 2003). To determine whether the latter activity was altered in the absence of functional HIF, nuclear extracts isolated from either wild-type or *Arnt*-null TS cells were assayed using a fluorometric approach. The results showed that undifferentiated wild-type TS cells contained significantly more nuclear HDAC activity than their *Arnt*-null counterparts (64,520±1893 RFU versus 58,664±3483 RFU; n=5, P<0.05). Based on these results, we estimated histone acetylation as a function of differentiation. Consistent with the activity assays, undifferentiated *Arnt*-null TS cells had higher levels of the acetylated form of histone H4 (Ac-H4) than did wild-type cells. In both cases, acetylation increased during differentiation, but the pattern of higher levels in *Arnt*-null cells was maintained (Fig. 5A).

Targeted disruption of individual HDAC family members in

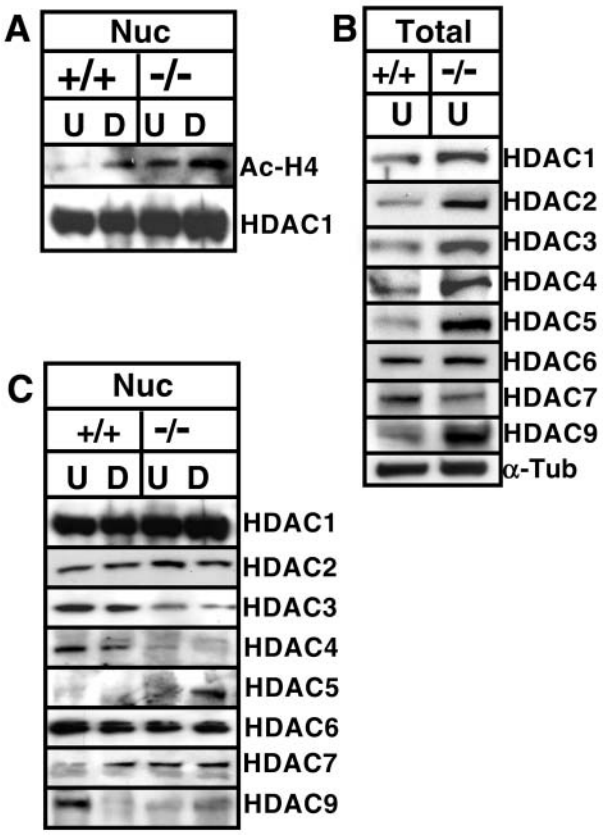

Fig. 5. HDAC activity is altered in *Arnt*-null TS cells. (A) Differentiation of wild-type TS cells was associated with increased levels of acetylated H4 (Ac-H4). A similar increase was observed during differentiation of the *Arnt*-null TS cells, although baseline levels in the undifferentiated state were much higher than those observed in wild-type cells. HDAC1 expression, which does not change during differentiation, was used as a loading control. (B) Compensatory upregulation of multiple HDACs was observed in whole-cell lysates prepared from undifferentiated *Arnt*-null TS cells. (C) Analysis of nuclear extracts revealed altered translocation of multiple HDACs in mutant TS cells.

mouse embryonic stem cells reveals compensatory increases in the expression of other family members (Lagger et al., 2002). To determine whether a similar phenomenon occurs in *Arnt*-null TS cells, which have diminished HDAC activity, the relative levels of multiple HDACs were assayed using an immunoblot approach. As shown in Fig. 5B, whole-cell lysates derived from undifferentiated cells showed a pattern of increased HDAC levels (HDAC1, HDAC2, HDAC3, HDAC4, HDAC5 and HDAC9) in the absence of ARNT. Because the nuclear localization of individual HDACs largely determines their biological functions, nuclear extracts from wild-type and *Arnt*-null TS cells were also assayed (Fig. 5C). In addition to the class I HDAC HDAC3, the class II HDACs (HDAC4, HDAC5, HDAC7 and HDAC9), the activities of which are implicated in differentiation, showed the highest degree of misregulated nuclear expression in *Arnt*-null cells.

HDAC inhibition phenocopies ARNT deletion

To determine whether HDAC activity plays a role in HIF-dependent gene expression and trophoblast differentiation, we cultured TS cells in the presence of the HDAC inhibitor TSA. With regard to undifferentiated cells, PGK1 mRNA levels were dramatically reduced in *Arnt*-null TS cells grown at 20% O_2 (see Fig. 3D and Fig. 6A). Likewise, TSA significantly repressed the expression of PGK1 mRNA by the cells, indicating that HDAC activity plays an important role in induction of HIF1 target gene expression. Similarly, HSP90 inhibition with geldanamycin A (GA), which also inhibits HIF1 target gene expression, repressed PGK1 gene expression in undifferentiated wild-type TS cells.

Finally, we tested the effects of TSA and GA on TS cell differentiation. As seen in Fig. 6B, TSA treatment of differentiating wild-type cells abolished expression of placental lactogen I (Pl-I), indicative of giant cell formation, and greatly reduced expression of the spongiotrophoblast marker 4311. Instead, as with *Arnt*-null TS cells, *Tfeb* mRNA expression, indicative of chorionic trophoblast formation, was upregulated. TSA treatment of differentiating wild-type TS cells prevented the formation of trophoblast giant cells and spongiotrophoblasts and instead promoted syncytialization (Fig. 6C, arrows) as well as cytoplasmic vacuolization and membrane breakdown (Fig. 6C, arrowheads) akin to that observed with *Arnt*-null cells. Similar results were obtained in multiple independently derived wild-type TS cell lines, as well as with another HDAC inhibitor, sodium butyrate (data not shown). Like TSA, GA treatment inhibited trophoblast giant cell differentiation and augmented Tfeb-positive chorionic trophoblast expansion in wild-type cells (Fig. 6B). However, HSP90 inhibition with GA preserved formation of 4311-positive spongiotrophoblast during wild-type TS cell differentiation (Fig. 6B). Thus, while both HDAC and HSP90 activity are required for HIF1 induction of target gene expression, only HDAC inhibition phenocopies the effects of ARNT deletion on TS cell differentiation.

Discussion

Our results demonstrate a novel role for ARNT in trophoblast differentiation. Specifically, TS cells derived from *Arnt*-null blastocysts preferentially differentiated into chorionic trophoblasts and syncytiotrophoblasts, while their wild-type counterparts formed spongiotrophoblasts and trophoblast giant cells. In addition to providing an in vitro model for studying syncytiotrophoblast differentiation and function, we discovered an important relationship between ARNT and HDAC activity during development. Specifically, the absence of ARNT protein resulted in the altered expression and subcellular distribution of multiple HDAC family members and decreased HDAC activity. As HDAC inhibition phenocopied ARNT deletion, these results suggested that HIFHDAC interactions play crucial roles in regulating TS cell fate.

Together, our data provide evidence for a model of trophoblast stem cell differentiation in which the progenitors either proliferate in an undifferentiated state or enter one of two differentiation pathways (Fig. 7). In vitro, TS cell differentiation is skewed down the pathway that gives rise to spongiotrophoblasts and trophoblast giant cells. Our previous work showed that ARNT deficiency in vivo results in impaired maintenance of the spongiotrophoblast population, and a concomitant increase in the number of trophoblast giant cells, suggesting that spongiotrophoblasts act as precursors to

trophoblast giant cells. We show that when wild-type TS cells are subjected to a cycle of hypoxia (2% O_2) and reoxygenation (20% O_2), conditions that mimic the stage when blood flow to the placenta begins in vivo, the spongiotrophoblast population also expands. Thus, oxygen plays a crucial role in regulating TS cell differentiation both in vivo and in vitro.

Trophoblast differentiation in vivo also gives rise to chorionic trophoblasts and syncytiotrophoblasts. TS cell differentiation in vitro fails to recapitulate this process as only a small fraction (~5%) of the cells adopt this fate, suggesting that the culture conditions do not support differentiation along this pathway. The reasons may involve culture-induced differences in the epigenetic mechanisms involved in this process. As evidence for this theory, we found that global HDAC inhibition in wild-type TS cells promoted differentiation to chorionic trophoblasts and syncytiotrophoblasts in vitro. In addition, ARNT-deficiency resulted in altered HDAC nuclear localization and activity, along with global alterations in histone acetylation patterns, and redirected TS cell differentiation. To our knowledge this is the first time that TS cell differentiation into chorionic trophoblast and syncytiotrophoblast has been induced at the expense of spongiotrophoblast and trophoblast giant cell formation in vitro.

The lack of robust models of trophoblast differentiation into syncytium has made it difficult to study this process, which is crucial to placental function. The chorionic plate of the murine placenta begins to produce *Gcm1*-expressing syncytiotrophoblasts on embryonic day E7.5 (Basyuk et al., 1999). Formation of the mature labyrinthine layer is initiated when invading embryonic endothelial cells from the allantois are surrounded by a bilayer of multinucleated syncytiotrophoblasts. As an important interface between mother and embryo/fetus, the syncytium plays crucial barrier and transport roles. The derivation of syncytiotrophoblasts from trophoblast stem cells provides an in vitro system for studying these functions. In addition, the syncytium, which is bathed in maternal blood, must play important immunomodulatory roles. For example, human syncytiotrophoblasts lack MHC class I and II expression (Faulk et al., 1977). In addition, these cells express other molecules that have immune functions such as CXCR4 (Douglas et al., 2001), which our data show is also expressed by murine syncytiotrophoblasts in vitro. As this molecule is an important HIV co-receptor (Coakley et al., 2005), *Arnt*-null TS cells could be a model for studying viral transmission during pregnancy.

In addition, apoptosis is thought to play a crucial role in tissue remodeling and cell turnover during ontogeny (Baehrecke, 2002). For example, human syncytiotrophoblast turnover uses apoptotic pathways (Huppertz and Kingdom, 2004). In accordance with this observation, we found a significant degree of apoptosis during *Arnt*-null TS cell differentiation that was mediated, in part, by the caspase-like serine proteinase granzyme B. After plasma membrane permeabilization with perforin, cytotoxic T lymphocytes and natural killer cells use granzyme B to induce target cell killing. Multiple studies have localized granzyme B and its inhibitor, the serpin proteinase inhibitor 9 (PI9), to the human placenta (Bladergroen et al., 2001; Hirst et al., 2001). Here, we show that murine TS cells also expressed granzyme B, which is upregulated and activated in the absence of ARNT. This fact probably accounts for the observation that a region of differentiating *Arnt*-null TS cells underwent programmed cell death. Thus, *Arnt*-null TS cells provide a model for studying the role of intrinsic granzyme B-mediated apoptosis in regulating the natural lifecycle of syncytiotrophoblasts in vitro.

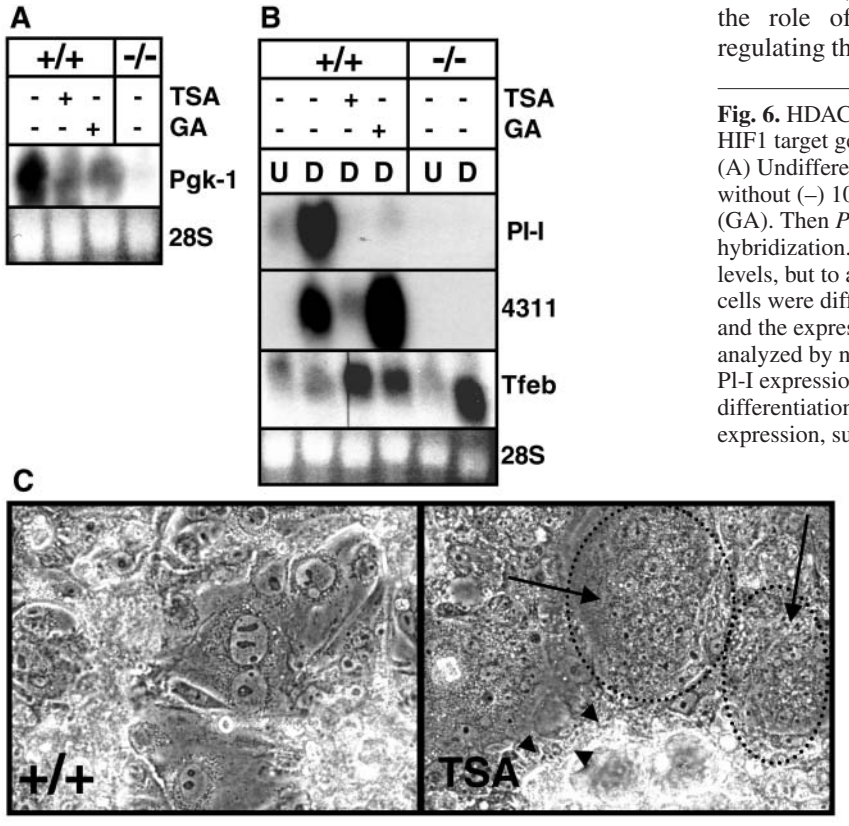

Fig. 6. HDAC inhibition phenocopies ARNT deletion with respect to HIF1 target gene expression and TS cell differentiation. (A) Undifferentiated wild-type TS cells were cultured with (+) or without (–) 100 nM trichostatin A (TSA) or 100 nM geldanamycin A (GA). Then *Pgk1* gene expression was analyzed by northern blot hybridization. Both compounds significantly reduced *Pgk1* mRNA levels, but to a lesser extent than ARNT deletion. (B) Wild-type TS cells were differentiated in the presence or absence of TSA or GA and the expression of mRNAs encoding lineage-markers was analyzed by northern blot hybridization. Both compounds inhibited Pl-I expression, suggesting impaired trophoblast giant cell differentiation. Interestingly, TSA but not GA, inhibited 4311 expression, suggesting differential effects of these compounds on spongiotrophoblast differentiation. Enhanced expression of Tfeb suggested that TS cell differentiation was redirected to chorionic trophoblast formation. (C) Phase-contrast microscopy of differentiated wild-type TS cells cultured with or without 100 nM TSA for the duration of the differentiation period. Although many large trophoblast giant cells and clusters of spongiotrophoblasts were seen in untreated cultures, TSA treatment promoted syncytialization (broken circles indicated by arrows) along with cytoplasmic vacuolization and membrane breakdown (arrowheads) as seen with differentiating *Arnt*-null cells.

Differentiating TS cells also exhibited other unique phenomena. Whereas ARNT protein levels remained relatively constant, differentiation of wild-type TS cells cultured in 20% O_2 induced both HIF1α and HIF2α mRNA and protein expression. The mechanism, as yet unknown, represents a novel mode of regulating the abundance of these transcription factors, which in most tissues are responsible for modulating gene expression in response to hypoxia. Whether or not the response pathways include IGF receptor signaling, which has been implicated in the oxygen-independent activation of HIF1α (Feldser et al., 1999), or estrogen- and progesterone-mediated HIFα induction, as described in the peri-implantation mouse embryo, remains to be determined (Daikoku et al., 2003). Interestingly, the expression of pVHL, the E3 ubiquitin ligase responsible for the degradation of HIFα, was also induced with differentiation, suggesting novel regulatory mechanisms. In human cytotrophoblasts, differentiation is also associated with oxygen-independent upregulation of HIFα and pVHL expression (Genbacev et al., 2001), another example of important parallels between human and murine placentation.

HSP90 also plays a role in regulating HIF stabilization (Isaacs et al., 2002; Katschinski et al., 2002). In addition, disruption of HSP90β in mice leads to midgestational embryonic lethality because of impaired placentation (Voss et al., 2000). Interestingly, the HSP90 inhibitor GA, which selectively targets HIF1α over HIF2α (Park et al., 2003), is a useful reagent for dissecting the individual roles of these transcription factors. Here, we show that this inhibitor impairs HIF1 dependent gene expression, which in turn inhibits trophoblast giant cell differentiation in vitro.

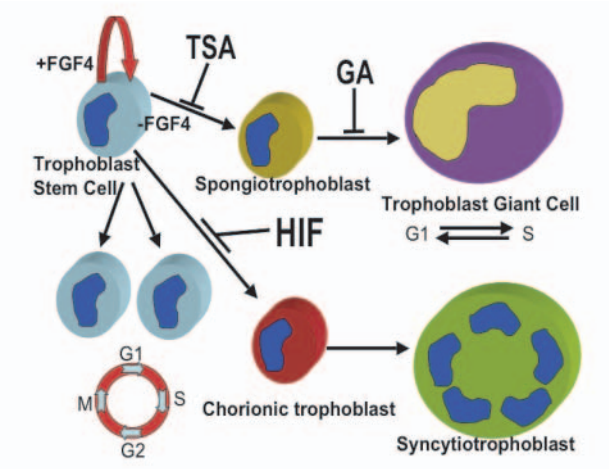

Fig. 7. Model of trophoblast stem cell differentiation: the oxygen-independent role of HIF-regulated HDAC activity. In the presence of FGF4, TS cells are maintained in a replicative pluripotent state. Upon growth factor withdrawal, wild-type TS cells differentiate along the pathway that leads to giant cell formation. Although portrayed as a linear sequence, it is also possible that this differentiation pathway bifurcates, with either spongiotrophoblasts or trophoblast giant cells being produced. HIF activity suppresses generation of chorionic trophoblasts and syncytiotrophoblasts, whereas HDAC and HSP90 activity is required for trophoblast giant cell formation in vitro. In support of this model, *Arnt*-null TS cells predominantly differentiated into chorionic trophoblasts and syncytiotrophoblasts, with the HDAC inhibitor TSA having similar effects. Interestingly, the HSP90 inhibitor geldanamycin selectively inhibited trophoblast giant cell differentiation.

However, HSP90 inhibition only partially phenocopied ARNT deficiency, suggesting that HIF1-dependent gene expression is only one aspect of ARNT function in the placenta. As TS cells upregulate expression of both HIF1α and HIF2α during differentiation, these results also suggest an important role for the latter transcription factor in the maintenance of the spongiotrophoblast population. Although neither *Hif1a* nor *Hif2a*-null mouse embryos exhibit placental phenotypes, disruption of both genes phenocopies the placental defects observed in *Arnt*-null mice and TS cells (K. Cowden-Dahl and M.C.S.., unpublished). Thus, the two HIFα family members have a significant degree of functional redundancy as regulators of placental development.

Because histone deacetylase activity can be induced by hypoxia (Kim et al., 2001), we investigated possible links between HIF functions in the placenta and epigenetic phenomena. This association could have important ramifications because histone acetylation is a crucial regulator of chromatin structure and hence gene expression. Many transcription factors interact with both histone acetyltransferases (HAT) and HDACs, which are components of large multimolecular complexes (Legube and Trouche, 2003). In general, histone acetylation relaxes chromatin structure, whereas deacetylation has the opposite effect. Consequently, the balance of HAT and HDAC activities helps regulate gene expression.

HDACs belong to one of three classes that were first described in yeast (Kurdistani and Grunstein, 2003). Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) are ubiquitous regulators of transcription. Interestingly, HIF1α interacts with the HAT p300/CBP and several HDACs. Class II HDACs play important roles in regulating tissue-specific gene expression and hence differentiation (Verdin et al., 2003). For example, the class II HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) mediate the myoblast-to-myocyte transition by modifying MEF2C transcriptional activity (McKinsey et al., 2000; Dressel et al., 2001; Miska et al., 2001). Interestingly, subcellular localization is an important determinant of class II HDAC activity. For example, HDAC5 phosphorylation unmasks docking sites for molecular chaperones of the 14-3-3 family, which results in nuclear export and subsequent inhibition of HDAC activity. Class III HDACs, which bear homology to the NAD-dependent SIR2 family in yeasts, do not appear to be involved in hypoxia responses. Thus, our results with regard to ARNT effects on HDAC expression and localization are consistent with the known effects of these molecules during differentiation and in response to physiological stimuli.

Our findings are also consistent with the molecular details of HIF and HDAC interactions. For example, we have shown that ARNT is involved in the nuclear translocation of HIF1α and HDACs, one possible mechanism whereby hypoxia induces HDAC activity. Conversely, HDAC inhibition represses hypoxia-induced gene expression and subsequent angiogenesis in murine tumor models (Kim et al., 2001). Here, we have shown that both undifferentiated and differentiated *Arnt*-null TS cells express less HDAC activity than their wild-type counterparts. These cells, similar to HDAC1-deficient ES cells, exhibited compensatory upregulation of many other family members. Additionally, the absence of ARNT dramatically impaired the nuclear localization of multiple

HDACs, which was consistent with the reduction we observed in the cells' HDAC activity. The fact that in wild-type TS cells HDAC inhibition recapitulated the *Arnt*-null phenotype provides a functional link between impaired HDAC localization/activity and changes in TS cell fate. Additionally, differentiating TS cells upregulated both HIFα and pVHL expression. As physical interactions between HIF1α and multiple HDACs occur in a complex that also includes pVHL (Mahon et al., 2001), parallel regulation of these molecules would be required for changes in gene expression. Thus, ARNT interaction with HDACs regulated by both oxygen-dependent and -independent mechanisms is crucial to placental development.

In conclusion, the results of this study significantly broaden our understanding of the functions of ARNT. In accordance with its known actions, ARNT mediates oxygen-dependent gene expression and expansion of the spongiotrophoblast lineage in vivo and in vitro. However, in TS cells, ARNT also influences HDAC expression, localization and activity in an oxygen-independent fashion. Thus, during differentiation this transcription factor integrates multiple epigenetic inputs, such as oxygen tension and histone acetylation, with classic transcriptional regulatory mechanisms. The study of ARNT-deficient TS cells will allow the further dissection of this complex regulatory network into its component parts. Whether or not similar networks function during the development of other organs remains to be determined. If these principles apply to embryonic development, they could shed light on the mechanisms involved in fetal programming of adult disease, a process by which the physiological environment, perhaps working through epigenetic pathways, eventually alters gene expression.

This work was supported by NIH grant RO1-HL64597. E.M. is a UCSF Molecular Medicine Fellow supported by NIH grants T32-DK07418 and T32-HD07162. We are grateful to Drs Jay Cross and Adrian Erlebacher for critical review of this manuscript and for providing reagents, and to Dr Olga Genbacev, Eduardo Caceres and Aneel Nath for providing human placental fibroblasts. We also thank Sami Essayem for excellent technical assistance and laboratory management.

References

Adelman, D. M., Maltepe, E. and Simon, M. C. (1999). Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. *Genes Dev.* **13**, 2478-2483.

Adelman, D. M., Gertsenstein, M., Nagy, A., Simon, M. C. and Maltepe, E. (2000). Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. *Genes Dev.* **14**, 3191-3203.

Baehrecke, E. H. (2002). How death shapes life during development. *Nat. Rev. Mol. Cell Biol.* **3**, 779-787.

Basyuk, E., Cross, J. C., Corbin, J., Nakayama, H., Hunter, P., Nait-Oumesmar, B. and Lazzarini, R. A. (1999). Murine Gcm1 gene is expressed in a subset of placental trophoblast cells. *Dev. Dyn.* **214**, 303-311.

Bladergroen, B. A., Strik, M. C., Bovenschen, N., van Berkum, O., Scheffer, G. L., Meijer, C. J., Hack, C. E. and Kummer, J. A. (2001). The granzyme B inhibitor, protease inhibitor **9**, is mainly expressed by dendritic cells and at immune-privileged sites. *J. Immunol.* **166**, 3218-3225.

Brockdorff, N. (1998). The role of Xist in X-inactivation. *Curr. Opin. Genet. Dev.* **8**, 328-333.

Coakley, E., Petropoulos, C. J. and Whitcomb, J. M. (2005). Assessing chemokine co-receptor usage in HIV. *Curr. Opin. Infect. Dis.* **18**, 9-15.

Cross, J. C., Werb, Z. and Fisher, S. J. (1994). Implantation and the placenta: key pieces of the development puzzle. *Science* **266**, 1508-1518.

Cross, J. C., Baczyk, D., Dobric, N., Hemberger, M., Hughes, M., Simmons, D. G., Yamamoto, H. and Kingdom, J. C. (2003). Genes, development and evolution of the placenta. *Placenta* **24**, 123-130.

Daikoku, T., Matsumoto, H., Gupta, R. A., Das, S. K., Gassmann, M., DuBois, R. N. and Dey, S. K. (2003). Expression of hypoxia-inducible factors in the peri-implantation mouse uterus is regulated in a cell-specific and ovarian steroid hormone-dependent manner. Evidence for differential function of HIFs during early pregnancy. *J. Biol. Chem.* **278**, 7683-7691.

Douglas, G. C. and Thirkill, T. L. (2001). Chemokine receptor expression by human syncytiotrophoblast – a review. *Placenta* **22**, S24-S28.

Douglas, G. C., Thirkill, T. L., Sideris, V., Rabieh, M., Trollinger, D. and Nuccitelli, R. (2001). Chemokine receptor expression by human syncytiotrophoblast. *J. Reprod. Immunol.* **49**, 97-114.

Dressel, U., Bailey, P. J., Wang, S. C., Downes, M., Evans, R. M. and Muscat, G. E. (2001). A dynamic role for HDAC7 in MEF2-mediated muscle differentiation. *J. Biol. Chem.* **276**, 17007-17013.

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y. and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc. Natl. Acad. Sci. USA* **94**, 4273-4278.

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A. et al. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54.

Faulk, W. P., Sanderson, A. R. and Temple, A. (1977). Distribution of MHC antigens in human placental chorionic villi. *Transplant Proc.* **9**, 1379-1384.

Feldser, D., Agani, F., Iyer, N. V., Pak, B., Ferreira, G. and Semenza, G. L. (1999). Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res.* **59**, 3915-3918.

Genbacev, O., Zhou, Y., Ludlow, J. W. and Fisher, S. J. (1997). Regulation of human placental development by oxygen tension. *Science* **277**, 1669-1672.

Genbacev, O., Krtolica, A., Kaelin, W. and Fisher, S. J. (2001). Human cytotrophoblast expression of the von Hippel-Lindau protein is downregulated during uterine invasion in situ and upregulated by hypoxia in vitro. *Dev. Biol.* **233**, 526-536.

Genbacev, O., Krtolica, A., Zdravkovic, T., Brunette, E., Powell, S., Nath, A., Caceres, E., McMaster, M., McDonaugh, S., Li, Y. et al. (2005). Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders. *Fertil. Steril.* **83**, 1517-1529.

Gu, Y. Z., Hogenesch, J. B. and Bradfield, C. A. (2000). The PAS superfamily: sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* **40**, 519-561.

Heard, E., Mongelard, F., Arnaud, D., Chureau, C., Vourc'h, C. and Avner, P. (1999). Human XIST yeast artificial chromosome transgenes show partial X inactivation center function in mouse embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **96**, 6841-6846.

Hirst, C. E., Buzza, M. S., Sutton, V. R., Trapani, J. A., Loveland, K. L. and Bird, P. I. (2001). Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis and placenta suggests a role for granzyme B-mediated proteolysis in reproduction. *Mol. Hum. Reprod.* **7**, 1133-1142.

Huppertz, B. and Kingdom, J. C. (2004). Apoptosis in the trophoblast–role of apoptosis in placental morphogenesis. *J. Soc. Gynecol. Investig.* **11**, 353-362.

Isaacs, J. S., Jung, Y. J., Mimnaugh, E. G., Martinez, A., Cuttitta, F. and Neckers, L. M. (2002). Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. *J. Biol. Chem.* **277**, 29936-29944.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G., Jr (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* **292**, 464-468.

Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y. et al. (1998). Cellular and Developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev.* **12**, 149-162.

Kaminker, P. G., Kim, S. H., Taylor, R. D., Zebarjadian, Y., Funk, W. D., Morin, G. B., Yaswen, P. and Campisi, J. (2001). TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression. *J. Biol. Chem.* **276**, 35891-35899.

Kato, H., Tamamizu-Kato, S. and Shibasaki, F. (2004). Histone deacetylase 7 associates with hypoxia-inducible factor-1alpha and increases transcriptional activity. *J. Biol. Chem.* **279**, 41966-41974.

Katschinski, D. M., Le, L., Heinrich, D., Wagner, K. F., Hofer, T., Schindler, S. G. and Wenger, R. H. (2002). Heat induction of the unphosphorylated form of hypoxia-inducible factor-1alpha is dependent on heat shock protein-90 activity. *J. Biol. Chem.* **277**, 9262-9267.

Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y. et al. (2001). Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat. Med.* **7**, 437-443.

Kozak, K. R., Abbott, B. and Hankinson, O. (1997). ARNT-deficient mice and placental differentiation. *Dev. Biol.* **191**, 297-305.

Kurdistani, S. K. and Grunstein, M. (2003). Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell Biol.* **4**, 276-284.

Lagger, G., O'Carroll, D., Rembold, M., Khier, H., Tischler, J., Weitzer, G., Schuettengruber, B., Hauser, C., Brunmeir, R., Jenuwein, T. et al. (2002). Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. *EMBO J.* **21**, 2672-2681.

Legube, G. and Trouche, D. (2003). Regulating histone acetyltransferases and deacetylases. *EMBO Rep.* **4**, 944-947.

Mahon, P. C., Hirota, K. and Semenza, G. L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.* **15**, 2675-2686.

Mak, W., Nesterova, T. B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A. P. and Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. *Science* **303**, 666-669.

Maltepe, E. and Simon, M. C. (1998). Oxygen, genes, and development: an analysis of the role of hypoxic gene regulation during murine vascular development. *J. Mol. Med.* **76**, 391-401.

Maltepe, E., Schmidt, J. V., Baunoch, D., Bradfield, C. A. and Simon, M. C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* **386**, 403-407.

McKinsey, T. A., Zhang, C. L., Lu, J. and Olson, E. N. (2000). Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* **408**, 106-111.

Mie Lee, Y., Kim, S. H., Kim, H. S., Jin Son, M., Nakajima, H., Jeong Kwon, H. and Kim, K. W. (2003). Inhibition of hypoxia-induced angiogenesis by FK228, a specific histone deacetylase inhibitor, *via* suppression of HIF-1alpha activity. *Biochem. Biophys. Res. Commun.* **300**, 241-246.

Miska, E. A., Langley, E., Wolf, D., Karlsson, C., Pines, J. and Kouzarides, T. (2001). Differential localization of HDAC4 orchestrates muscle differentiation. *Nucleic Acids Res.* **29**, 3439-3447.

Paria, B. C., Reese, J., Das, S. K. and Dey, S. K. (2002). Deciphering the cross-talk of implantation: advances and challenges. *Science* **296**, 2185-2188.

Park, S. K., Dadak, A. M., Haase, V. H., Fontana, L., Giaccia, A. J. and Johnson, R. S. (2003). Hypoxia-induced gene expression occurs solely through the action of hypoxia-inducible factor 1alpha (HIF-1alpha): role of cytoplasmic trapping of HIF-2alpha. *Mol. Cell Biol.* **23**, 4959-4971.

Rossant, J. and Cross, J. C. (2001). Placental development: lessons from mouse mutants. *Nat. Rev. Genet.* **2**, 538-548.

Soldani, C. and Scovassi, A. I. (2002). Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. *Apoptosis* **7**, 321-328.

Steingrimsson, E., Tessarollo, L., Reid, S. W., Jenkins, N. A. and Copeland, N. G. (1998). The bHLH-Zip transcription factor Tfeb is essential for placental vascularization. *Development* **125**, 4607-4616.

Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A. and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. *Science* **282**, 2072-2075.

Tian, H., McKnight, S. L. and Russell, D. W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* **11**, 72-82.

Verdin, E., Dequiedt, F. and Kasler, H. G. (2003). Class II histone deacetylases: versatile regulators. *Trends Genet.* **19**, 286-293.

Voss, A. K., Thomas, T. and Gruss, P. (2000). Mice lacking HSP90beta fail to develop a placental labyrinth. *Development* **127**, 1-11.

Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl. Acad. Sci. USA* **92**, 5510-5514.

Weier, J. F., Weier, H. U. G., Jung, C. J., Gormley, M., Zhou, Y., Chu, L. W., Genbacev, O., Wright, A. A. and Fisher, S. J. (2005). Human cytotrophoblasts acquire aneuploidies as they differentiate to an invasive phenotype. *Dev. Biol.* **279**, 420-432.

Zhou, Y., Fisher, S. J., Janatpour, M., Genbacev, O., Dejana, E., Wheelock, M. and Damsky, C. H. (1997). Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J. Clin. Invest.* **99**, 2139-2151.

Zybina, T. G. and Zybina, E. V. (2000). Genome multiplication in the tertiary giant trophoblast cells in the course of their endovascular and interstitial invasion into the rat placenta decidua basalis. *Early Pregnancy* **4**, 99-109.