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ORIGINAL ARTICLE

Ahmed H.K. El-Hashash · Pedro Esbrit · Susan J. Kimber

PTHrP promotes murine secondary trophoblast giant cell differentiation through induction of endocycle, upregulation of giant-cell-promoting transcription factors and suppression of other trophoblast cell types

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Abstract The murine trophoblast cell lineage represents an intriguing experimental cell model as it is composed of four trophoblast stem (TS)-derived cell types: trophoblast giant cells (TGCs), spongiotrophoblast, syncytiotrophoblast, and glycogen trophoblast cells. To investigate the role of parathyroid hormone-related protein (PTHrP) in TGC differentiation, we analyzed the effect of exogenous PTHrP on secondary TGCs of day 8.5 p.c. ectoplacental cone explant culture. Secondary TGCs expressed PTHrP and PTHR1 receptor *in vivo* and *in vitro*. TGCs treated with PTHrP had reduced proliferation and decreased apoptosis starting from day 2 in culture, and enhanced properties of giant cell differentiation: increased DNA synthesis, number of cells with giant nuclei and expression of placental lactogen-II (PL-II). The induction of TGC formation by PTHrP correlated with downregulation of cyclin B1 and mSNA expression, but upregulation of cyclin D1, thus allowing mitotic-endocycle transition. Moreover, PTHrP treatment influenced TGC differentiation by inducing the expression of transcription factors known to stimulate giant cell formation: Stra13 and AP-2γ, and inhibiting the formation of other trophoblast cell types by suppressing trophoblast progenitors and spongiotrophoblast-promoting factors, Eomes, Mash-2, and mSNA. Taken together with the spatial and temporal patterns of TGC formation and PTHrP synthesis *in vivo*, these findings indicate an important role for PTHrP in the differentiation of secondary TGCs during placentation.

Key words secondary trophoblast giant cell · PTHrP · cyclins · apoptosis · PL-II · AP-2γ

Ahmed H.K. El-Hashash · Susan J. Kimber (✉)
Faculty of Life Sciences
University of Manchester
3.239 Stopford Building
Oxford Road, Manchester M13 9PT
U.K.
Tel: +44 0161 275 5773
Fax: +44 0161 275 3915
E-mail: Sue.Kimber@manchester.ac.uk

Pedro Esbrit
Bone and Mineral Metabolism Laboratory
Research Unit
Fundación Jiménez Díaz
28040 Madrid
Spain

Introduction

The earliest lineage to form in the mammalian conceptus is the trophoblast, which contributes exclusively to the extraembryonic structures that form the placenta (Cross et al., 1994). This lineage arises at the late morula stage when the outer cells become specified as trophectoderm (TE), the external epithelial layer surrounding the inner cell mass (ICM) and blastocyst cavity. Shortly after implantation, the mural TE, which lacks ICM contact, differentiates into the primary trophoblast giant cells (TGCs) that invade the uterine tissues to mediate initial implantation, while polar TE differentiates later into the extraembryonic ectoderm (EXE) that forms the ectoplacental cone (EPC). The EPC consists of cells that become committed to both secondary giant cells and spongiotrophoblasts (Carney et al., 1993).

The murine placenta consists of three distinctive subtypes of trophoblast cells: innermost mesodermal labyrinthine layer, intermediate spongiotrophoblast and the outermost layer of secondary TGCs. At primitive streak stage (6.5–7.5 p.c.), the outermost layer of the EPC gives rise to secondary TGCs, which later control

the exchange of nutrients and gasses between maternal and fetal circulation forming an indispensable functional component of the mature placenta. The spongiotrophoblast layer forms after day 7.5 p.c. as the result of the expansion and flattening of the EPC (reviewed in Rinkenberger et al., 1997).

The two distinct cell populations within the EPC, giant and diploid, non-giant cells exhibit different cellular programs. Mitotic cycles of non-giant spongiotrophoblast cells provide a continuous source of cells for the giant cell compartment (Cross et al., 1994). In contrast, endoreduplication leads to repeated rounds of DNA synthesis without cell division and the formation of polyploid TGCs (Zybina and Zybina, 1996; MacAuley et al., 1998; Ohgane et al., 1998). Endoreduplication is the common mode of TGC growth in eutherian mammals (Therman et al., 1983; D'Amato, 1989; Zybina and Zybina, 1996), increases cell differentiation and chromosome number (MacAuley et al., 1998; Goncalves et al., 2003) and involves changes in cyclin expression and checkpoint controls (Nakayama et al., 1998; Palazon et al., 1998; Hattori et al., 2000; Soloveva and Linzer, 2004). TGC differentiation is also characterized by the expression of growth factors and hormones such as PL-I, PL-II, and proliferin (Faria et al., 1991; Lin and Linzer, 1998; Peters et al., 2000). PL-II, a nonglycosylated single-chain polypeptide, has frequently been used as a specific marker for secondary TGCs (Robertson et al., 1982; Carney et al., 1993). The differentiation of TGCs is critical for implantation and to the maintenance of pregnancy.

Several transcription factors have been identified to progressively regulate trophoblast differentiation (reviewed by Cross, 2000; Cross et al., 2003). *Eomes* and *cdx2* are implicated in FGF-4-regulated trophoblast stem (TS) cell maintenance (Chawengsaksophak et al., 1997; Tanaka et al., 1998; Russ et al., 2000). Another TS-maintaining factor, activator protein-2γ (AP-2γ), is expressed by trophoblast cell lineages (Shi and Kellems, 1998; Sapin et al., 2000; Auman et al., 2002). Mash2 and Hand1, two members of the basic helix–loop–helix (bHLH) family are essential regulators of TGC differentiation, but with antagonistic actions (Cross et al., 1995; Scott et al., 2000). The formation of TGCs depends on Hand1 (Riley et al., 1998; Scott et al., 2000); while Mash2 and mSNA are essential for maintaining spongiotrophoblast cells and blocking giant cell differentiation (Guillemot et al., 1994; Tanaka et al., 1997; Nakayama et al., 1998). *Stra13*, another bHLH, is expressed by TGCs *in vivo* (Boudjelal et al., 1997), and stimulates their differentiation *in vitro* (Hughes et al., 2004).

Many of the extracellular signals controlling TGC differentiation remain to be clarified. A few ligands have been found to induce secondary TGC differentiation using the EPC explant assay. For instance, nerve growth factor stimulated the differentiation of EPC trophoblast into giant cells (Kanai-Azuma et al., 1997). Interaction with ECM molecules, through α6 and α7 integrins is also important for EPC trophoblast development (Sutherland et al., 1993). One recent possibility is regulation of TGC differentiation by parathyroid hormone-related protein (PTHrP). PTHrP, a 139–173 amino acid protein, was first isolated from tumors of patients with humoral hypercalcaemia (Suva et al., 1987). PTHrP gene is widely expressed in a various normal cells and tissues, in which PTHrP appears to act as a paracrine, autocrine, or intracrine growth factor (Philbrick et al., 1996; Wysolmerski and Stewart, 1998; Fiaschi-Taesch and Stewart, 2003). Like parathyroid hormone, PTHrP signals through a widely distributed G protein-coupled receptor, PTHR1, which is often expressed by cells adjacent to those producing PTHrP (Abou-Samra et al., 1992; Lee et al., 1995).

Three principal developmental roles for PTHrP have been well documented in mammals. PTHrP signalling via the PTHR1 has been found to regulate chondrocyte differentiation (Lanske et al., 1996; Weir et al., 1996), and participate in the epithelial–mesenchymal interactions during the formation of certain epithelial organs (Wysolmerski et al., 1995; Philbrick et al., 1998). It also induces parietal endoderm outgrowths from the ICM *in vitro* (Behrendtsen et al., 1995). Recently another developmental role for PTHrP, stimulation of murine primary TGC outgrowth, has been shown *in vitro* (Nowak et al., 1999). However the involvement of PTHrP signalling during secondary TGC differentiation still remains enigmatic.

Using our established culture model for secondary trophoblast outgrowth and differentiation (El-Hashash and Kimber, 2004), we have examined the role of PTHrP in secondary TGC development. We found that PTHrP stimulates secondary TGC proliferation, DNA endoreduplication and differentiation *in vitro*. The EPC culture model enabled us to monitor the expression of a number of genes involved in the regulation of TGC differentiation and cell cycle regulation. Our results strongly indicate a role for PTHrP in inducing trophoblast differentiation into a giant cell fate by regulating the programme of transcription factor expression.

Methods

Animals

All embryos were produced by natural mating of MF1 female mice with stud males (Harlan Olac Ltd., Bicester, Oxon, U.K.) and husbandry was as reported (El-Hashash and Kimber, 2004). Mating was confirmed by the presence of a vaginal plug the following morning (day 0.5 of pregnancy).