Chair's introduction

Anne McLaren

The origin of this meeting was a rather good dinner in King's College Cambridge, at which Charlie Loke, Ashley Moffett, Barry Keverne, Azim Surani and myself got together, and it occurred to us that the trophoblast as a tissue was shamefully neglected. Since the definition of the word 'trophoblast' clearly means 'original feeding tissue' it is perhaps appropriate that the meeting had its origins in a good dinner!

Because I have spent a lot of time looking at sections of mouse implantation, seeing the giant mouse trophoblast cells, I have always found trophoblast rather scary. These cells are huge: they are so big they can be seen with the naked eye. In sections they seem to engulf the uterine epithelium and then they engulf the stromal cells. They are very aggressive cells, but they do a remarkable job. Those primary trophoblast cells are directly responsible for the very explosive growth that occurs in mouse implantation during gastrulation. It has been estimated that from the inner cell mass of the 3-day blastocyst (i.e. 3–4 days *post coitum* (dpc)) up to the 7-day embryo there is a more than 500-fold increase in tissue volume. This is all due to the yolk sac placenta, which does a remarkable job in nourishing and supporting this explosive growth. At 8 days the allantois is growing: it hasn't quite reached the chorion, so we don't have a chorioallantoic placenta, but it is the chorioallantoic placenta which is in a way more remarkable because this supports the entire human fetal growth up to full term – in most cases, fortunately, rather successfully. Animals of course eat their placentas and derive considerable nutritional benefit from doing so. This is rare in humans, but I believe recipes have been published.

The chorioallantoic placenta has certainly been a source of wonder in many parts of the world for centuries. I have read in Maureen Young's book (Young 2001) that the Balinese, for example, wash the placenta in perfumed water after birth, wrap it in a cloth and then bury it on the threshold of the family home in a carefully

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prepared coconut. Think of that next time you see a placenta! The Japanese used to bury it in a cedar wood placental pot. If it was from a boy then it had Indian ink and a writing brush with it; from a girl it would have a needle and thread. The Egyptians were also very keen on the placenta. It was considered to be the seat of the eternal soul and after a pharaoh died his placenta was preserved in a jar.

In our first talk, Jay Cross is going to tell us how the various trophoblast cell lineages contribute to this seat of the eternal soul – the placenta.

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Trophoblast cell fate specification

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The trophoblast cell lineage is the first cell type to be specified during mammalian development – as the trophectoderm layer in the blastocyst – and is fated to form the epithelial cell compartment of the placenta (Cross *et al.* 1994, Rossant 1995). Trophoblast cells can be derived from blastocysts, at least in mice, that show properties expected of trophoblast stem (TS) cells in that they can differentiate into a range of differentiated trophoblast cell subtypes both in vitro and in vivo (Tanaka *et al.* 1998, Hughes *et al.* 2004). The trophoblast cell lineage is relatively simple in mice, in that TS cells differentiate into only four major differentiated cell types: trophoblast giant cells, spongiotrophoblast, glycogen trophoblast cells and syncytiotrophoblast (Cross *et al.* 2003) (Fig 1.1). Considerable progress has been made in the last few years in defining the molecular mechanisms that regulate the maintenance of the stem cell fate as well as the formation of the alternative differentiated cell types. This review focuses on the key transcription factors that specify trophoblast cell fates and the emerging evidence as to how signalling pathways interact with these transcription factors ultimately to regulate alternative cell fate decisions.

Trophoblast stem cells

Trophoblast stem cell lines can be derived from mice by culturing blastocysts or dissected extraembryonic ectoderm (chorionic trophoblast) in the presence of fibroblast growth factor (FGF)4 and feeder-cell conditioned medium (Tanaka *et al.* 1998). The identification of FGF4 as a critical factor was based on the findings that mutations in both the *Fgf4* gene, which is expressed by embryonic ectoderm (Feldman *et al.* 1995, Goldin & Papaioannou 2003), or the FGF receptor gene *Fgfr2*, which is expressed in trophectoderm (Arman *et al.* 1998), result in early post-implantation lethality due to a failure in trophoblast proliferation. The homeobox transcription factor genes *Cdx2* (Chawengsaksophak *et al.* 1997) and *Eomes* (Russ *et al.* 2000) are

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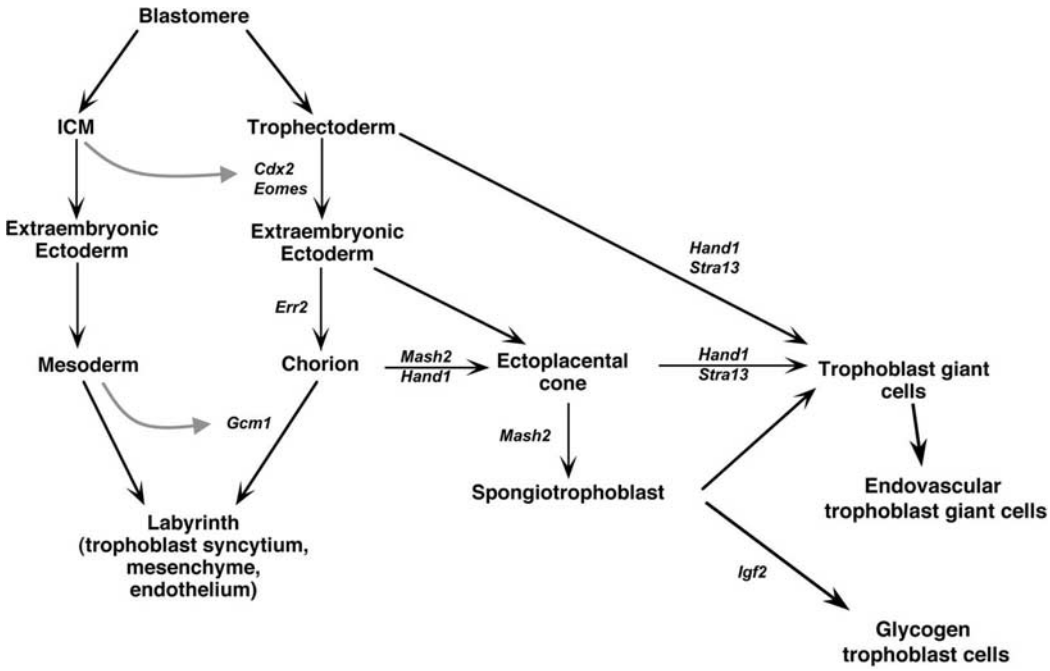

Figure 1.1 Outline of murine trophoblast cell lineage and regulatory genes. ICM, inner cell mass.

activated by FGF signalling and are clearly somehow required in turn for maintenance of TS cell fate, as mutations in either gene result in early post-implantation lethality similar to the *Fgf4* and *Fgfr2* mutants. Removal of FGF4 from cultured TS cells results in rapid down-regulation of *Cdx2* and *Eomes* expression and the cells stop dividing soon thereafter (Tanaka *et al.* 1998), and the vast majority of cells differentiate into trophoblast giant cells (Hughes *et al.* 2004). This implies that differentiation of trophoblast giant cells does not require specific external cues. The *Err2* (Luo *et al.* 1997, Tremblay *et al.* 2001) and *AP*γ (Auman *et al.* 2002) transcription factor genes are also required for maintenance of the trophoblast stem cell fate, but are only required at a slightly later stage.

Trophoblast giant cells

Trophoblast giant cells are large polyploid cells that mediate implantation and invasion of the conceptus into the uterus. They also produce several growth factors and hormones that promote both local and systemic physiological adaptations in the mother that are necessary for embryonic growth and survival (Linzer & Fisher 1999, Cross *et al.* 2002). Primary trophoblast giant cells arise directly from the mural trophectoderm at the blastocyst stage (Cross *et al.* 1994, Cross 2000). These cells

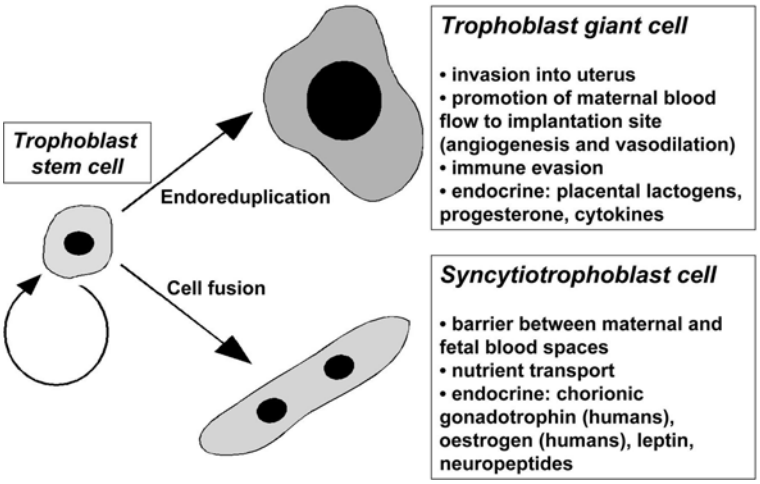

Figure 1.2 Comparison of trophoblast giant cells and syncytiotrophoblast cells.

exit the mitotic cell cycle and stop dividing, but continue to go through rounds of DNA replication without intervening mitoses (endoreduplication) to become polyploid (MacAuley *et al.* 1998, Nakayama *et al.* 1998) (Fig 1.2). While there are only ~50 mural trophectoderm/primary giant cells in the peri-implantation blastocyst, the number of trophoblast giant cells increases to over 400 over the next few days through the process of secondary giant cell differentiation in which cells of the ectoplacental cone (precursor to the spongiotrophoblast layer) differentiate into giant cells (Cross *et al.* 1994, Cross 2000).

The differentiation of trophoblast giant cells is promoted by two basic helix-loop-helix (bHLH) transcription factor genes, *Hand1* and *Stra13*. Expression of *Hand1* mRNA is low or undetectable in TS cells but is induced as they differentiate into giant cells (Cross *et al.* 1995, Scott *et al.* 2000, Hughes *et al.* 2004). Ectopic expression of *Hand1* in growing TS cells is sufficient to promote their differentiation into giant cells, even if the cells are maintained in FGF4 (Hughes *et al.* 2004). By contrast, while *Hand1*-deficient embryos are able to implant, they arrest their development within a few days and both primary and secondary giant cell differentiation is blocked (Riley *et al.* 1998, Scott *et al.* 2000). The *Stra13* gene is a retinoic acid-inducible bHLH transcription factor gene that is also induced during giant cell differentiation (Hughes *et al.* 2004). Both retinoic acid treatment (Yan *et al.* 2001) and ectopic expression of *Stra13* (Hughes *et al.* 2004) in TS cells promote rapid arrest of cell proliferation and giant cell differentiation. While bHLH factors form dimers in general in order to bind DNA, the Hand1 and Stra13 proteins do not appear to interact directly (Hughes *et al.* 2004).

The functions of the *Hand1* and *Stra13* genes in regulating trophoblast differentiation in mice are likely conserved in other species as well, at least on the basis of gene expression studies. The human *HAND1* and *STRA13* genes are both expressed in early trophoblast derivatives. Expression of *HAND1* mRNA and protein has been detected in the trophectoderm of blastocysts (Knofler *et al.* 1998, 2002), but not in villous tissue or cytotrophoblast cells isolated from villous tissue (Knofler *et al.* 1998, 2002, Janatpour *et al.* 1999). Whether human HAND1 is expressed in trophoblast cells that invade the placental bed is not clear. The *STRA13* gene mRNA expression has been detected in both placental tissue and in isolated cytotrophoblast cells that are differentiated into 'extravillous-like' cells in vitro (Janatpour *et al.* 1999).

While *Hand1* and *Stra13* are the only transcription factor genes that have been identified to date that promote trophoblast giant cell differentiation, there is mounting evidence that the giant cell layer is actually heterogeneous, implying that other regulators must be involved. First, the Stra13 protein is detectable in only a small subset of the Hand1-positive giant cells (Hughes *et al.* 2004). Second, several giant-cell restricted genes such as *Mrj* (Hunter *et al.* 1999), *Ctps7* (Hemberger *et al.* 2000) and *Mps1* (Hemberger *et al.* 2000) show non-uniform expression within the giant cell layer in vivo. Finally, some giant-cell specific genes such as *Pl1* and *Pl2* are temporally regulated, such that *Pl1* expression is restricted to giant cells within the first few days after implantation, whereas *Pl2* expression begins only after embryonic day 9 (Yamaguchi *et al.* 1994).

Endovascular trophoblast giant cells

After implantation, a subtype of trophoblast giant cell invades into the spiral arteries that bring maternal blood to the implantation site, thereby promoting the transition from endothelial-lined arteries to the haemochorial blood spaces typical of rodent and primate placentas (Adamson *et al.* 2002). Although these endovascular trophoblast cells are not as large as primary giant cells surrounding the implantation site, nor do they express genes like *Pl1*, they do have enlarged nuclei compared with other uterine bed cells and they also express the *Plf* gene, a gene that in the placenta is otherwise specifically expressed in trophoblast giant cells. The factors that mediate the differentiation of endovascular trophoblast giant cells are unknown.

Spongiotrophoblast

The spongiotrophoblast layer forms the middle layer of the placenta between the outermost giant cells and the innermost labyrinth layer. The function of the spongiotrophoblast layer is unknown, although it probably has a structural role

and also produces several layer-specific secreted factors. For example, spongiotrophoblast cells express anti-angiogenic factors that may prevent the growth of maternal blood vessels into the fetal placenta, including soluble Flt1 (a vascular endothelial growth factor (VEGF) antagonist) and Prp (an antagonist of Plf) (Cross *et al.* 2002).

The formation and/or maintenance of the spongiotrophoblast layer is dependent on the *Mash2* bHLH transcription factor gene. The *Mash2* gene mRNA is expressed in the ectoplacental cone and later the spongiotrophoblast layer in mice, and expression is normally down-regulated in trophoblast giant cells (Guillemot *et al.* 1994, Scott *et al.* 2000). In *Mash2* mutants, the spongiotrophoblast layer is lost by embryonic day 10 and more trophoblast giant cells are formed (Guillemot *et al.* 1994, Tanaka *et al.* 1997). The latter finding implies that spongiotrophoblast cells can differentiate into giant cells and that Mash2 regulates this step. Consistent with this, ectopic expression of Mash2 in Rcho-1 cells, which are derived from a rat trophoblast tumour (choriocarcinoma), blocks giant cell differentiation (Cross *et al.* 1995, Kraut *et al.* 1998, Scott *et al.* 2000). The ability of Mash2 to suppress trophoblast giant cell differentiation may be mediated in part by its ability to suppress Hand1-induced differentiation (Scott *et al.* 2000, Hughes *et al.* 2004). Interestingly, however, Mash2 overexpression cannot block Stra13-induced differentiation (Hughes *et al.* 2004). Given these functions of Mash2, the *Mash2* mutant phenotype could be explained by premature differentiation of spongiotrophoblast to trophoblast giant cells. In addition, though, recent evidence suggests that Mash2 may also directly promote proliferation of trophoblast cells (Hughes *et al.* 2004).

The actions of Mash2 may be modified by two other (non-bHLH) transcriptional regulators, I-mfa and Sna. The I-mfa protein inhibits the ability of some bHLH proteins including Mash2 to bind DNA. Consistent with an essential function in suppressing Mash2 function, I-mfa-deficient mice show a defect in the differentiation of trophoblast giant cells (Kraut *et al.*, 1998), albeit not as severe as Hand1 mutants. The zinc-finger transcription factor Sna has a consensus DNA recognition sequence similar to Mash2, and like Mash2 can block trophoblast giant cell differentiation (Nakayama *et al.*, 1998).

Glycogen trophoblast cells

Glycogen trophoblast cells appear only late in gestation, first within the spongiotrophoblast layer. After embryonic day 12, the glycogen trophoblast cells then invade into the uterus in a diffuse interstitial pattern that is quite distinct from the endovascular trophoblast giant cells (Adamson *et al.* 2002). Indeed, the glycogen cells appear to invade everywhere except for within or even close to the spiral arteries. The distinctive feature of glycogen cells is their accumulation of glycogen-rich granules, but its function is unknown. The developmental origin of these cells is not entirely

clear, though their appearance first within the spongiotrophoblast layer and their expression of spongiotrophoblast-specific genes (e.g. *Tpbpa*) implies that glycogen trophoblast cells are a specialised subtype of spongiotrophoblast cell (Adamson *et al.* 2002). The only molecular insight that we have into the regulation of their development is that the number of glycogen trophoblast cells is reduced in *Igf2* mutants, implying a potential role for Igf2 in promoting either their differentiation or glycogen storage (Lopez *et al.* 1996).

Syncytiotrophoblast and chorionic villi

Syncytiotrophoblast cells form the major nutrient transport surfaces within the labyrinth layer of the rodent placenta and covering the chorionic villi in the primate placenta. Whereas trophoblast giant cells are mononuclear (occasionally binucleate) polyploid cells that arise as a result of endoreduplication, syncytiotrophoblast cells arise from the fusion of cells that have left the cell cycle (Cross 2000) (Fig 1.2). As a result, syncytiotrophoblast cells contain multiple diploid nuclei. In rodents, syncytiotrophoblast cell formation is first detected at the time that villous morphogenesis begins and indeed the two processes are dependent on a single transcription factor gene, *Gcm1* (Anson-Cartwright *et al.* 2000). Expression of *Gcm1* mRNA appears in small clusters of cells within the chorion layer that is otherwise comprised of cells with trophoblast stem cell potential (Basyuk *et al.* 1999, Anson-Cartwright *et al.* 2000). Ectopic expression of the *Gcm1* gene in TS cells is sufficient to force them out of the cell cycle, and to block their ability to differentiate into trophoblast giant cells but instead poise them to initiate morphogenesis and fuse into a syncytium (Hughes *et al.* 2004). In humans, GCM1 directly activates the transcription of the *Syncytin* gene (Yu *et al.* 2002), a gene encoding a cell surface, fusogenic protein (Mi *et al.* 2000). In *Gcm1* mutant mice, the chorioallantoic interface fails to initiate morphogenesis and syncytiotrophoblast cells fail to form (Anson-Cartwright *et al.* 2000).

The restricted and clustered pattern of *Gcm1/GCM1* expression in the developing villi of the mouse (Anson-Cartwright *et al.* 2000) and human (Baczyk *et al.* 2004) placenta implies very tight control over its expression. In mice, at least, the pattern appears within the chorion layer cell autonomously, but maintenance of the expression is dependent on attachment of the allantois to the chorion at embryonic day 8.5 (Hunter *et al.* 1999, Stecca *et al.* 2002). Indeed, syncytiotrophoblast differentiation does not normally begin until after the allantois makes contact. Likewise, TS cells do not normally develop into syncytiotrophoblast very efficiently in vitro (Hughes *et al.* 2004). However, addition of an allantois to an explanted chorion or to monolayers of TS cultured cells promotes Gcm1 expression (J. Cross, unpublished observation). The signal(s) from the allantois that regulates Gcm1 expression is unknown but is likely to be cell surface associated.

Conclusions

The molecular and genetic studies of the last few years have identified several critical regulators of placental development such that we now know of key regulators for most of the major cell differentiation steps in the trophoblast cell lineage. The identification of these factors will allow the more complete regulatory network to be described as upstream and downstream genes are identified. However, even as it stands now several important general principles of trophoblast development have emerged. First, the formation of distinct trophoblast cell subtypes appears to be specified by distinct molecular mechanisms. This implies that the spectrum of placental changes observed in human diseases like pre-eclampsia and intrauterine growth restriction, in which both villous and extravillous trophoblast defects have been described (Pijnenborg *et al.* 1981, Cross 1996, Pijnenborg 1996), cannot be explained by a single, direct molecular mechanism. Second, paracrine interactions regulate the development of the trophoblast lineage, at least in part, by modifying the expression if not the activity of key transcriptional regulators (e.g. FGF4 expression by embryonic ectoderm promoting TS cell proliferation/maintenance through Cdx2 and Eomes; allantoic factor promoting Gcm1 expression). Third, the ability of the Hand1, Stra13 and Gcm1 transcription factors to promote differentiation of TS cells, and even override the ability of FGF4 to maintain their stem cell character, implies that normally suppressing their expression and function within the stem cell compartment is critical for maintenance of the TS cell phenotype.

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