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The Hand1, Stra13 and Gcm1 transcription factors override FGF signaling to promote terminal differentiation of trophoblast stem cells

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Abstract

The trophoblast cell lineage is an interesting model system because it is composed of a limited number of cell types that are spatially patterned. Trophoblast stem (TS) cells reside within a layer called the chorion and either remain as stem cells or differentiate into spongiotrophoblast (SpT), trophoblast giant (TG) cells or syncytiotrophoblast cells (SynT) of the labyrinth. Maintenance of the TS phenotype is dependent on stimulation by FGF4, whereas differentiation and/or maintenance of the differentiated derivatives are dependent on key transcription factors: Mash2 for SpT, Hand1 for TG cells and Gcm1 for SynT cells. TS cells proliferate and retain their stem cell phenotype in culture in response to FGF4 and an additional factor(s) that can be provided by conditioned medium from embryonic fibroblast feeder cells (CM). To understand the functions of Hand1, Mash2 and Gcm1 at a cellular level, we tested the effects of their ectopic and over-expression on the ability of TS cells to either continue to proliferate or differentiate into their alternative fates. Expression of Mash2 alone had no effects on TS cell differentiation. However, *Mash2*-transfected cells continued to divide longer after withdrawal of FGF/CM. *Hand1* promoted TGC differentiation, even in the continued presence of FGF4/CM. *Stra13*, another bHLH factor gene that is expressed in TG cells, also induced TG differentiation. *Gcm1* induced a rapid arrest of TS proliferation but, in contrast to *Hand1* and *Stra13*, blocked TG cell differentiation. Although *Gcm1* was not sufficient to promote SynT formation, expression of an antisense *Gcm1* transcript blocked SynT differentiation. These data suggest that *Mash2* functions to promote transient FGF4-independent amplification of trophoblast cells that are progressing towards the SpT and TG cell phenotype. By contrast, *Hand1* and *Stra13* promote cell cycle exit and restrict cells towards the TG fate, whereas *Gcm1* promotes cell cycle exit and restriction towards the SynT fate.
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Keywords: Trophoblast cells; Cell cycle; Differentiation

Introduction

The trophoblast cell lineage is specified at the blastocyst stage of development and contributes exclusively to the epithelial compartment of the placenta. The cell lineage is relatively simple in that there are only four major differentiated cell types that can be derived from trophoblast stem (TS) cells in mice: trophoblast giant cells (TG), spongiotrophoblast (SpT), syncytiotrophoblast (SynT) and glycogen trophoblast cells (GlyT) (Cross et al., 2002b; Rossant and Cross, 2001). TG cells are large polyploid cells that appear soon after embryo implantation and mediate invasion into

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the uterus and also promote local and systemic adaptations in the mother that are necessary for embryonic growth and survival (Cross et al., 1994, 2002c). A subtype of TG cells invades into the maternal spiral arteries that bring blood to the implantation site (Adamson et al., 2002). The SpT layer forms the middle part of the placenta between the outermost giant cells and the innermost labyrinth layer. It probably has a structural role but in addition, SpT cells produce some unique secreted proteins and are also precursors of TG cells and GlyT cells (Rossant, 1995). GlyT cells have an unknown function but they appear only late in gestation, first within the SpT layer and later they invade diffusely into the interstitium of the uterus (Adamson et al., 2002). SynT cells are multi-nucleated cells that form as a result of cell–cell fusion and function as the major transport surface for nutrient and gas exchange between the maternal and fetal circulation in the villous part of the placenta (called the labyrinth in mice) (Cross, 2000; Cross et al., 2002a).

Genetic studies in mice have revealed the major pathways controlling development of the trophoblast cell lineage. The maintenance of TS cells in the early embryo is dependent on FGF signaling involving the ligand FGF4 (Feldman et al., 1995), the FGFR2 receptor (Arman et al., 1998) and the downstream transcription factors Cdx2 and Eomes (for review, see Rossant and Cross, 2001). The orphan nuclear receptor Err2 is required for stem cell maintenance at a slightly later stage (Luo et al., 1997; Tremblay et al., 2001). The formation and/or maintenance of the major differentiated trophoblast subtypes can be traced to transcription factor pathways that ultimately regulate cell fate decisions. The formation of TG cells is dependent on Hand1, a member of the basic helix-loop-helix (bHLH) transcription factor family (Riley et al., 1998; Scott et al., 2000). Another bHLH factor, Mash2, is essential for maintaining SpT cells, as in its absence, the SpT layer is lost and more TG cells form (Guillemot et al., 1994; Tanaka et al., 1997). Consistent with the later finding, over-expression of Mash2 in Rcho-1 cells, a rat trophoblast tumor (choriocarcinoma) cell line, prevents TG cell differentiation (Cross et al., 1995; Kraut et al., 1998; Scott et al., 2000). Other bHLH factors are implicated in trophoblast development based on specific expression patterns. *Stra13* mRNA expression has been suggested in TG cells in mice, though not well documented (Boudjelal et al., 1997), and the dominant-negative HLH factor Id2 is expressed in the TS cell compartment (Jen et al., 1997). Regulated expression of STRA13 and ID2 in the human placenta is consistent with potential roles in trophoblast differentiation (Janatpour et al., 1999, 2000). No bHLH factors have been implicated in SynT differentiation. However, the formation of SynT cells is dependent on a distinct type of transcription factor called Gcm1 (Anson-Cartwright et al., 2000).

Although it is clear from the studies to date that key transcription factors are required for formation and/or maintenance of differentiated trophoblast subtypes, it is unclear how their activities are controlled and coordinated with the signals that regulate the maintenance of the TS cell phenotype. One of the interesting features of the trophoblast lineage is that early after implantation, the TS cell population is restricted to a discrete compartment called the chorion (also called extraembryonic ectoderm) (Rossant and Cross, 2001; Uy et al., 2002). In situ hybridization studies have failed to detect *Hand1* mRNA in the chorion (Cross et al., 1995; Scott et al., 2000), although expression from a LacZ ‘knock in’ reporter can be detected (Firulli et al., 1998), implying that *Hand1* transcription is either very low and/or that the transcript is unstable in chorionic trophoblast cells. *Gcm1* mRNA expression is limited to only those specific cells that are selected out to contribute to the labyrinth and differentiate into SynT cells (Anson-Cartwright et al., 2000; Stecca et al., 2002). *Mash2* mRNA is expressed in the chorion, but also in the ectoplacental cone (which later becomes the SpT layer) (Guillemot et al., 1994; Nakayama et al., 1997). These data suggest that suppressing the expression of these differentiating transcription factors in the chorion may be essential for the maintenance of TS cells. Rcho-1 cells have been previously used to test the effect of ectopic expression of *Hand1* and *Mash2* on TG cell differentiation (Cross et al., 1995; Kraut et al., 1998; Scott et al., 2000). However, these cells cannot be regulated in the same way as TS cells, nor do they develop into the diverse range of differentiated cell types. For this reason, we conducted studies using a murine TS cell line in which the differentiation potential of the cells is modulated by culture in the presence or absence of FGF4 (Tanaka et al., 1998). We found that ectopic expression of *Hand1*, *Stra13* and *Gcm1* were able to arrest cell proliferation and promote differentiation even when the cells were maintained in FGF4. *Mash2* had no significant effect on the behavior of TS cells in the presence of FGF4, but promoted transient proliferation after FGF4 withdrawal.

Materials and methods

Plasmids

For TS cell transfection experiments, the cDNAs for *Hand1*, *Mash2* and *Gcm1* were subcloned in the sense orientation into the pTRACER plasmid (Invitrogen) downstream of the CMV promoter/enhancer. The full-length Gcm1 cDNA was also subcloned in an antisense orientation that was used for some experiments. The plasmid also carries an EF-1α promoter driving expression of a GFP/blasticidin fusion protein that allows detection of transfected cells by visualization of GFP. The *Stra13* expression vector (pSG5-STRA13) was obtained from Dr. Pierre Chambon (Strasbourg), and was co-transfected into TS cells together with the empty pTRACER vector (ratio of 5:1). CMV expression vectors for *Hand1* and *Mash2*, used for the Rcho-1 experiments, have been previously described (Cross et al., 1995; Scott et al., 2000).

Trophoblast cell culture and transfections

A TS cell line derived from *Rosa26* mice was used for all experiments (from Dr. Janet Rossant, Toronto) and was maintained as described previously (Tanaka et al., 1998). Briefly, the cells were grown in medium supplemented with 25 ng/ml FGF4 (Sigma Chemical Company) and 70% conditioned medium from mouse embryonic fibroblasts (CM). To promote differentiation, the growth medium was removed, the cells were washed once with phosphate-buffered saline and re-fed with medium lacking the FGF4 and CM. Cells seeded in six-well plates were transfected with Lipofectamine using a total of 4 μg of plasmid per well. Rcho-1 cells were grown and transfected as described previously (Cross et al., 1995; Scott et al., 2000). For cell differentiation experiments, transfected cells were plated at low density (1:50) and observed for up to 72 h. Transfected cells were detected by their expression of EGFP, scored for morphology, and their ability to proliferate was based on the emergence of a multi-cell clone. Fifty to one hundred clones were assessed for each transfection group, and experiments were repeated two to three times.

RNA analysis

Total RNA was isolated from TS cells using TRIzol® (Invitrogen) and 10 μg per sample was analyzed by Northern blotting. Complementary DNA probes for *Gcm1* (Altshuller et al., 1996), *Mash2* (Guillemot et al., 1994), *Stra13* (Boudjelal et al., 1997), *Hand1* (Cross et al., 1995) and *Pl1* (Colosi et al., 1987) have been described previously. Northern blots were quantitated by densitometry using NIH Image 1.62 on scanned blots, and signals were normalized for loading by using an 18S rRNA probe. Two identical blots were prepared to deal with all of the different probes. In situ hybridization was performed on paraffin sections of conceptuses in deciduo from embryonic day 8.5 using nonradioactive RNA in situ hybridization as described previously (Anson-Cartwright et al., 2000).

Antibodies, Western blotting and immunostaining

An affinity-purified rabbit polyclonal antibody was made against a Stra13-specific peptide (ELEKGDLRSEQ-PYFKSDH) by Research Genetics Inc., and has been previously described (St-Pierre et al., 2002). Cell lysates from TS cells or their differentiated derivatives were analyzed by Western blotting using standard procedures. A mouse monoclonal antibody against Stra13 was prepared by the laboratory of Dr. Paul Hamel (Department of Laboratory Medicine and Pathobiology, University of Toronto) by immunizing mice against a GST-Stra13 fusion protein. Stra13-specific antibodies were selected by Western blot analysis for specific recognition of HIS-Stra13 in Dr. Hamel's laboratory. To assess the specificity of the monoclonal antibody, COS-7 cells were transfected with pcDNA3, pcDNA3-Stra13 or pcDNA3-FLAG-Stra13 using Qiagen Superfect reagent, then lysed 36–48 h post-transfection. The presence of ectopically expressed Stra13 in soluble protein lysate was detected by Western blot using rabbit polyclonal anti-Stra13, mouse monoclonal anti-Stra13 or mouse monoclonal anti-FLAG (M2 antibody from Sigma).

Immunoperoxidase staining was performed on 4% paraformaldehyde-fixed monolayers of differentiated cells derived from TS cell cultures, using the Stra13 monoclonal antibody. Dual immunofluorescent staining for Hand1 and Stra13 was performed using a rabbit anti-Hand1 (Firulli et al., 2003) and the mouse anti-Stra13 antibodies. TS cells were differentiated in 'TS cell medium' lacking FGF/CM for 5 days before immunostaining. Cells were then fixed with cold 4% paraformaldehyde, and permeabilized with methanol and then 0.5% Triton X-100 in PBS on ice. The cells were double stained with rabbit anti-Hand1 antiserum (provided by Dr. Cserjesi) and mouse anti-Stra 13 monoclonal antibody for either overnight at 4 °C or 1 h at room temperature, followed by the staining with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Molecular Probes) and

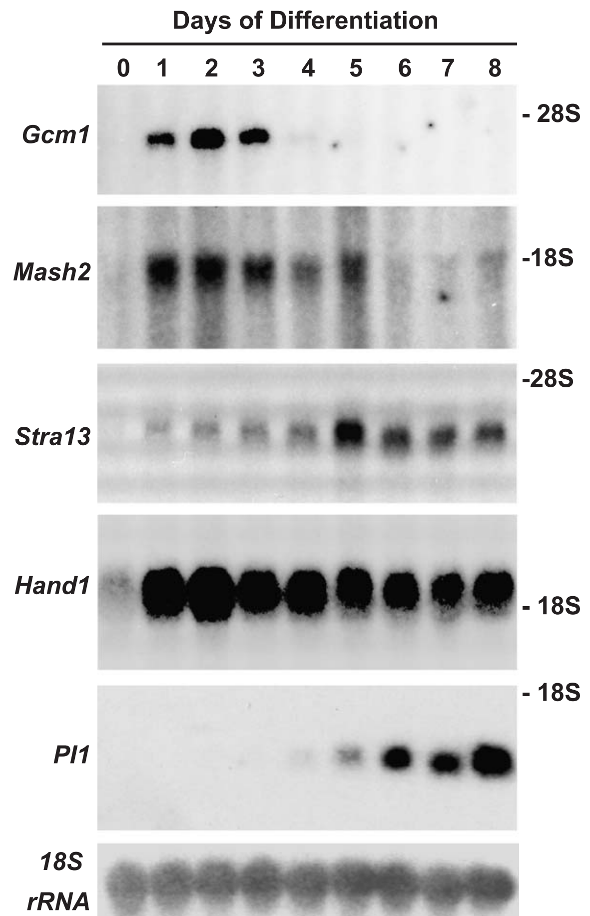

Fig. 1. Expression of *Hand1*, *Mash2*, *Gcm1* and *Stra13* mRNAs during TS cell differentiation. Total RNA was collected from TS cells maintained in the presence of FGF4/CM (time 0) or at different days after withdrawal of FGF4/CM and analyzed by Northern blotting. *Pl1* is a TG cell-specific gene.

Cy™ 3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) for 1 h at room temperature. The cells were counterstained with DAPI and then scored for immunoreactivity (n = 245).

Results

Expression of Gcm1, Hand1, Mash2 and Stra13 during trophoblast differentiation

We performed Northern blots to assess the expression of *Hand1*, *Mash2* and *Gcm1* in cultured TS cells before and after differentiation. TS cells that are cultured in the presence of FGF4 and conditioned medium from feeder cells (CM) retain characteristics of TS cells in vivo, but after withdrawal of FGF4/CM differentiate primarily into TG cells (Tanaka et al., 1998). We collected RNA from cells at various days after withdrawal of FGF4/CM (Fig. 1). The onset of *Pl1* gene expression, which is specific to TG cells (Colosi et al., 1987), was detectable only after day 4 of differentiation (Fig. 1). The mRNAs for *Hand1*, *Mash2* and *Gcm1* were expressed at either low (*Gcm1*, *Hand1*) or undetectable (*Mash2*) levels in undifferentiated TS cells but were readily detectable within 24 h after withdrawal of FGF4/CM (Fig. 1). Weak expression of these genes in undifferentiated cells may be explained by the fact that a few differentiated derivatives always appear in TS cell cultures, even under optimal stem cell growth conditions (see Fig. 3). After withdrawal of FGF4/CM, the expression of Gcm1 mRNA increased approximately 12-fold to peak around day 2, declined by day 3 and was undetectable after day 6 (Fig. 1). The expression pattern for *Mash2* was similar except that the decline was less precipitous. Expression of *Hand1* mRNA was high throughout the differentiation time course (Fig. 1), consistent with its expression in both ectoplacental cone and TG cells in vivo (Cross et al., 1995; Firulli et al., 1998; Scott et al., 2000), though expression did decline about 3-fold between days 2 and 8 of differentiation.

In addition to *Hand1*, *Mash2* and *Gcm1*, we also studied the expression of *Stra13*, a gene encoding a bHLH transcription factor. Expression of this gene has been described in the human placenta, with expression increasing as cytotrophoblast cells differentiate into invasive extravillous cytotrophoblast cells (Janatpour et al., 1999). *Stra13* expression in the mouse placenta has been only crudely described (Boudjelal et al., 1997). During TS cell differentiation in vitro, *Stra13* mRNA was detectable only after withdrawal of FGF4, increased to a peak around day 5 of differentiation and remained detectable thereafter (Fig. 1). This pattern was consistent with expression in TG cells but potentially also in precursor cells. To localize the mRNA expression, we first performed RNA in situ hybridization on tissue sections, but *Stra13* expression was only weakly detected (data not shown). We therefore used immunostaining with two independent Stra13-specific antibodies. Both antibodies recognize an approximately 45-kDa band in cell lysates similar to the size of the predicted protein (Fig. 2A). The Stra13 protein was up-regulated

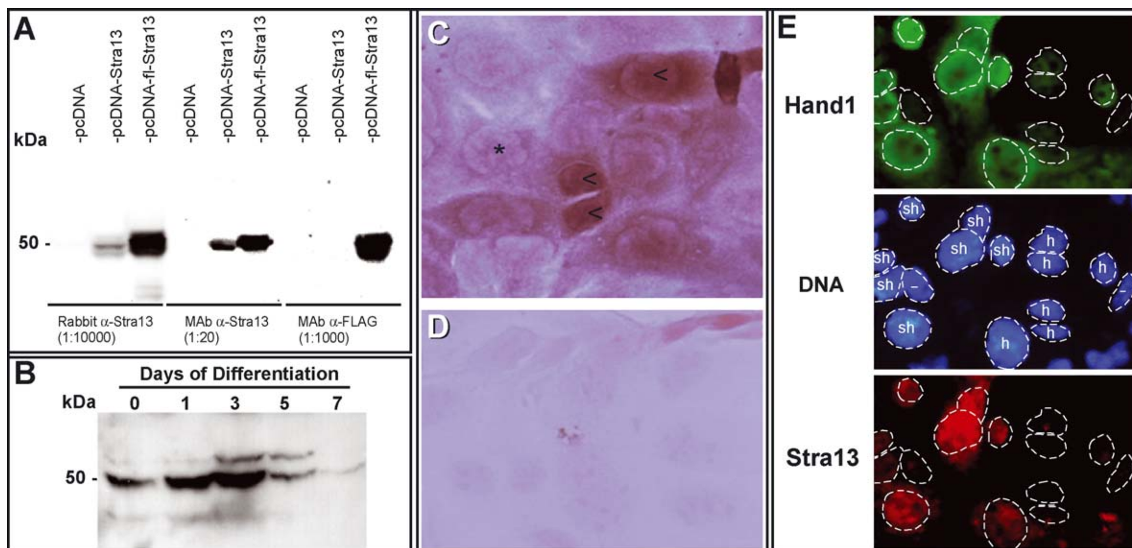

Fig. 2. Expression of the Stra13 protein in TG cells. (A) Characterization of the Stra13 monoclonal antibody. Cell lysates from COS-7 cells transfected with Stra13 or FLAG-epitope tagged Stra13, or empty vector as negative control were immunoblotted with either the rabbit polyclonal antibody, mouse monoclonal or anti-FLAG antibodies. (B) Western blot of cell lysates from TS cells and their differentiated derivatives at various days after withdrawal of FGF4/CM. (C, D) TS cells grown on coverslips and differentiated for 5 days were fixed and processed for immunoperoxidase staining using either the mouse anti-Stra13 monoclonal (C) or control hybridoma supernatant (D). Arrowhead denotes immunopositive and asterisk indicates an immunonegative cells. (E) Differentiated TS cells dual stained with anti-Hand1 and anti-Stra13 antibodies. Cells were scored for staining and counted as either immunonegative (−), or positive for Hand1 (h), Stra13 (s), or both (sh).

during early TS cell differentiation and was then down-regulated similar to the mRNA (Fig. 2B). Stra13 immunoreactivity was detected in TG cells derived from the differentiation of cultured TS cells (Fig. 2C). We found that the Stra13 protein was localized in the nucleus, as would be expected for a transcription factor, but was only detected in <50% of the cells. The distinct patterns of localization may relate to the fact that Stra13 protein levels are regulated by the Vhl and UBC9/ubiquitin proteasome degradation pathway (Ivanova et al., 2001). To determine how the expression of the Stra13 protein compared with Hand1, we performed dual immunofluorescence analysis on monolayers of TS cells that had been allowed to differentiate in the absence of FGF4/CM for 5 days (Fig. 2E). By 5 days of differentiation, the majority of cells in the culture are polyploid, but only cells that had a typical TG cell morphology (expansive flat cytoplasm, extensive ER and large nucleus) were scored for expression. We detected Stra13 immunoreactivity in only 30.8% of TG cells, compared with 62.4% of cells that were immunopositive for Hand1. The Stra13-positive cells were a subset of the Hand1-positive cells, as 96% of the Stra13-postive TG cells were also positive for Hand1, whereas only 47% of Hand1-positive cells were also immunopositive for Stra13. Roughly a third (36.9%) of the TG cells failed to stain with either antibody.

Trophoblast stem cells differentiate into both giant cells and syncytiotrophoblast after FGF4 withdrawal

Before studying the effect of altering the expression patterns for *Hand1*, *Mash2*, *Gcm1* and *Stra13* on the developmental potential of TS cells, we first quantified the normal changes in cell populations as TS cells differentiate in vitro. TS, TG and SynT cells have distinct cell morphologies that can be readily distinguished in vitro (Fig. 3A). TS cells are cuboidal and have a large nucleus-to-cytoplasmic ratio. TG cells have a large expansive cytoplasm and large polyploid nuclei. SynT cells have multiple nuclei (usually only two in vitro) and more

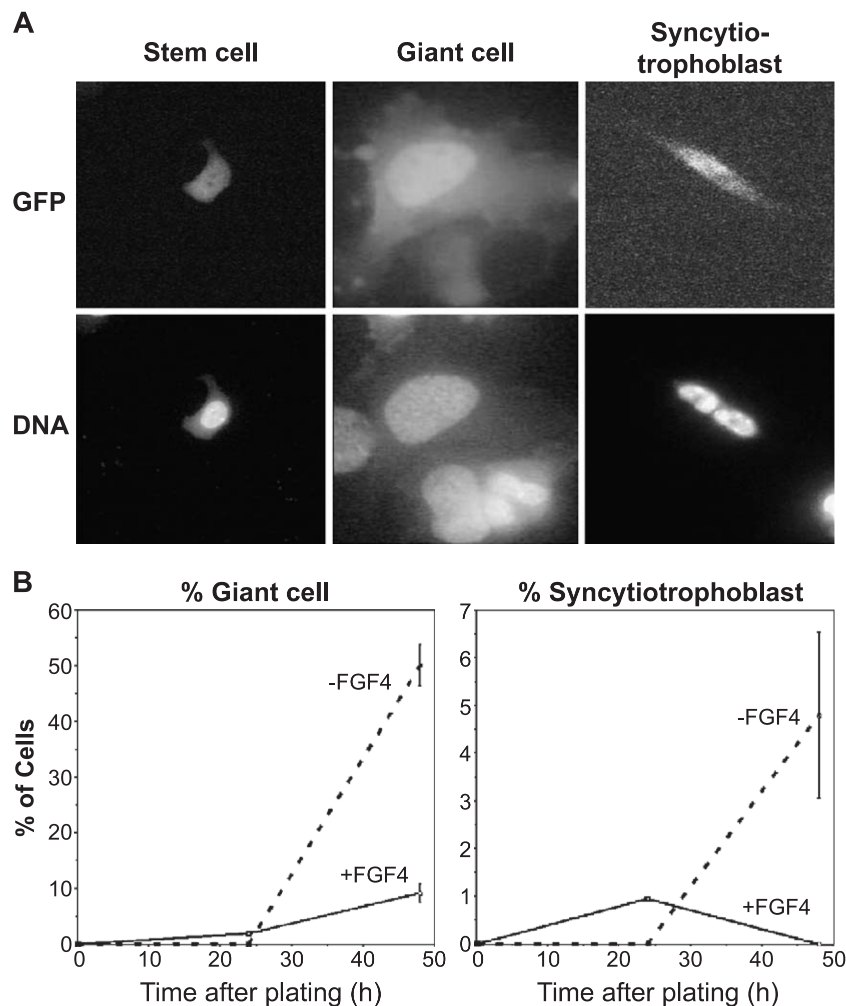

Fig. 3. Differentiation of TS cells in vitro. (A) Morphology of TS cells and their differentiated cell derivatives. (B) Time course of trophoblast stem cell differentiation. Cells were plated at low density in either the presence or absence of FGF4/CM and the relative proportions of each cell type were assessed by scoring cells under 200× magnification. Fifty to one hundred fields were counted for each time point. Plotted values represent mean ± SEM.

elongated cytoplasm. The size of the nuclei in SynT cells is similar to that of TS cells, consistent with them remaining diploid. When cultures of TS cells are trypsinized briefly, the differentiated cell types are more resistant and remain attached to the dish. Therefore, by re-plating only trypsin-sensitive cells, one starts with a relatively homogeneous group of stem cells. To assess the time course of cell differentiation, we plated trypsin-sensitive TS cells at low density (1:20) and then counted the numbers of cells conforming to the three primary cell morphologies in TS cell cultures either left growing in the presence of FGF4/CM, or following their withdrawal (Fig. 3B). We found that within 48 h of FGF4/CM withdrawal, the numbers of TG and SynT cells increased significantly compared with cells remaining in FGF4/CM ($P < 0.05$). Notably, approximately 50% of the cells had transformed into TG cells, whereas the number of SynT cells never reached levels above approximately 5%. Withdrawal of FGF4/CM is associated with cell division arrest (Tanaka et al., 1998), but no obvious increase in cell death, indicating that the increase in relative numbers of TG and SynT cells reflects a true increase in cell differentiation and not simply a loss of stem cells.

Mash2 maintains trophoblast TS cell proliferation after withdrawal from FGF4

To test if ectopic or over-expression of transcription factors had any effect on TS cell proliferation or differentiation, we transfected Rosa26 TS cells with expression vectors encoding Hand1, Mash2, Gcm1 and Stra13. TS cells were plated at very low density to allow analysis of the fate of individual transfected cells identified by expression of a co-transfected EGFP reporter. Transfection of TS cells with an EGFP expression vector alone did not alter the patterns or rates of TS cell proliferation or differentiation compared with non-transfected cells (data not shown). Transfection of TS cells with a *Mash2* expression vector had no effect on the percentage of cells that differentiated to the SynT fate (data not shown). Likewise, *Mash2* over-expression did not affect the number of cells able to differentiate into TG cells (Figs. 4B, D), in contrast to previous findings with transfected Rcho-1 cells (Cross et al., 1995; Kraut et al., 1998; Scott et al., 2000). *Mash2*-expressing cells that were maintained in the presence of FGF4/CM did have a slightly faster rate of proliferation; however, as assessed by cell clone size by 48 h after transfection, the difference was not statistically

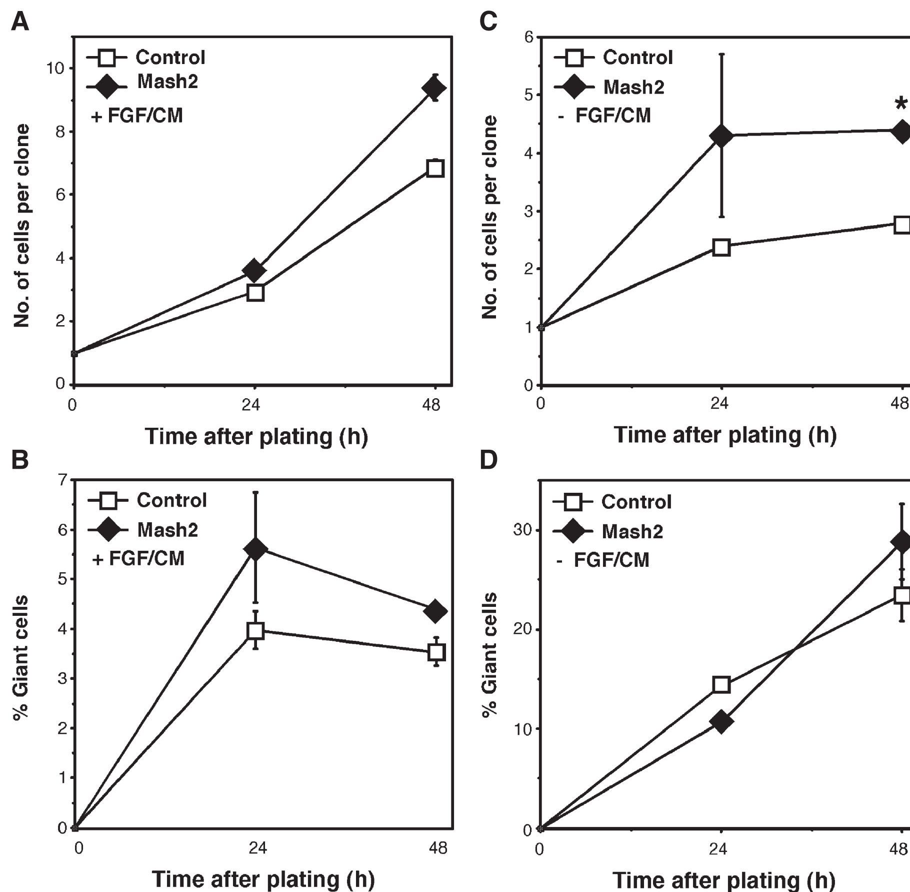

Fig. 4. Effect of *Mash2* over-expression on trophoblast differentiation. TS cells were transfected with empty vector or *Mash2* expression vector and scored for their ability to proliferate (A, C) or differentiate into TG cells (B, D), either in the presence (A, B) or absence (C, D) of FGF4/CM. Plotted values represent mean ± SEM. Asterisk (*) indicates that control and experimental values are significantly different by *t* test ($P < 0.05$).

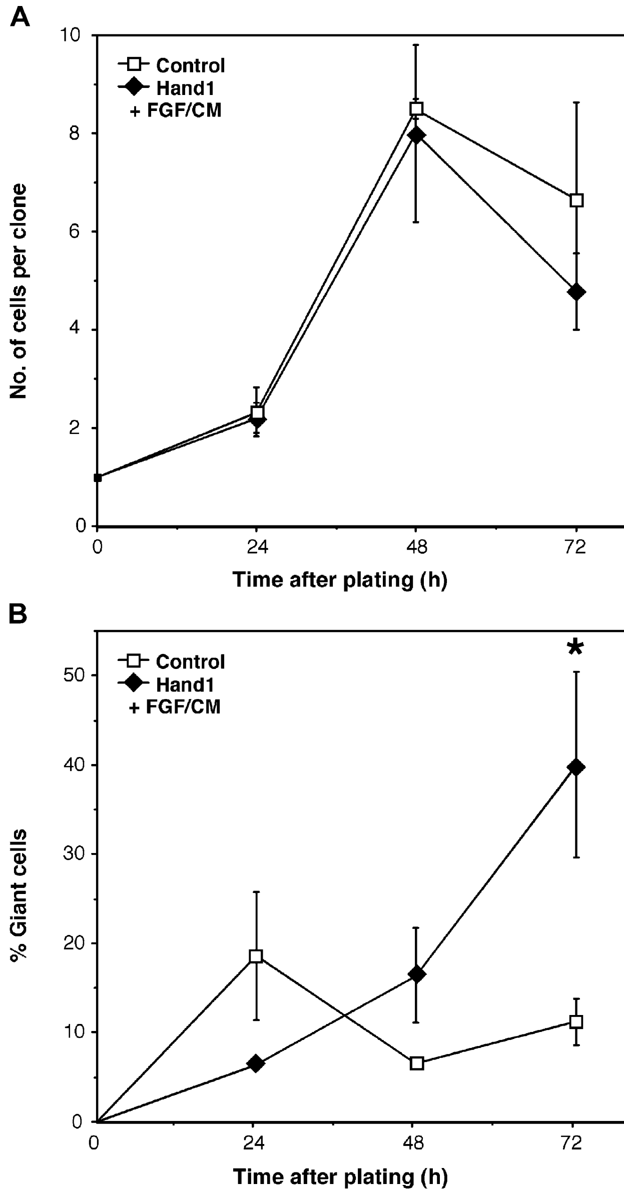

Fig. 5. Effect of *Hand1* over-expression on trophoblast differentiation. TS cells maintained in FGF4/CM were transfected with empty vector or *Hand1* expression vector and scored for their ability to proliferate (A) or differentiate into TG cells (B). Plotted values represent mean ± SEM. Asterisk (*) indicates that control and experimental values are significantly different by *t* test ($P < 0.05$).

significant (Fig. 4A). Interestingly, whereas control TS cells divided only once after FGF4/CM was withdrawn from the medium, *Mash2*-transfected cells divided twice (Fig. 4C). These data suggest that *Mash2* can stimulate FGF4-independent proliferation of TS cells, although the effect is transient.

Hand1 and Stra13 promote differentiation of TS cells into giant cells even in the presence of FGF4

Ectopic expression of *Hand1* in TS cells that were maintained in the presence of FGF4/CM resulted in a significant increase in the formation of TG cells by 72 h after transfection (Fig. 5B), as well as eventually slowing cell proliferation (Fig. 5A). In cells where differentiation was promoted by withdrawal of FGF4/CM, *Hand1* over-expression had no additional effect (data not shown). Over-expression of *Stra13* in TS cells also promoted TG cell differentiation. However, the effect occurred more rapidly and was more pronounced than with *Hand1* (Fig. 6B). *Stra13* expression promoted a rapid arrest of cell proliferation, such that transfected cells only divided once within 48

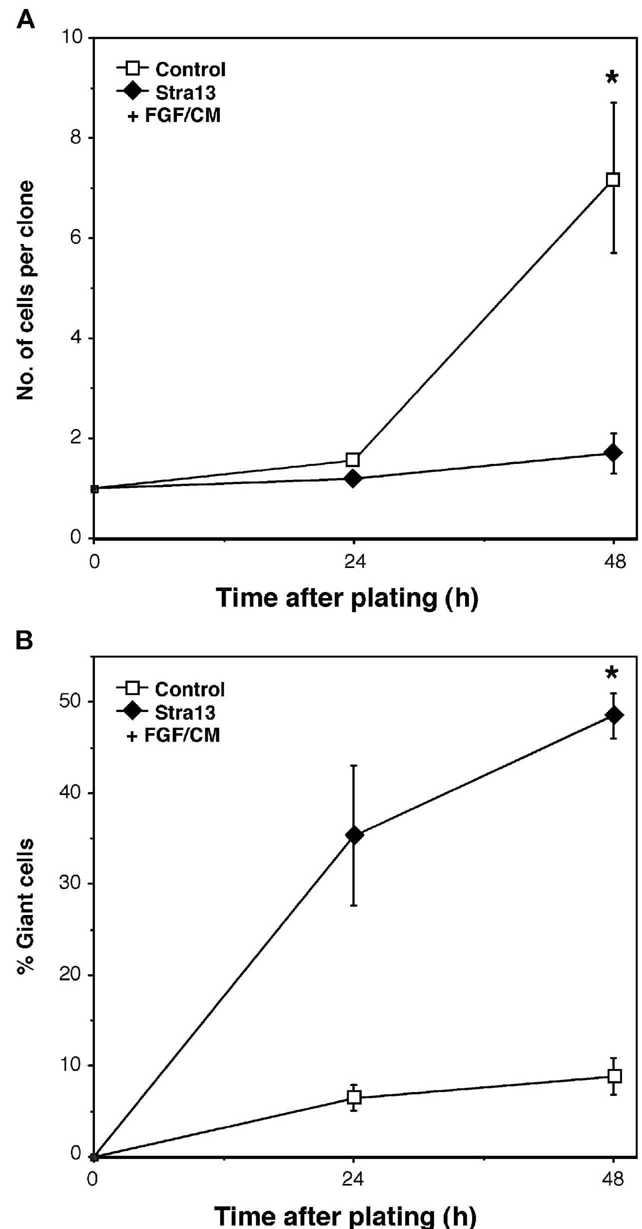

Fig. 6. Effect of *Stra13* over-expression on trophoblast differentiation. TS cells maintained in FGF4/CM were transfected with empty vector or Stra13 expression vector and scored for their ability to proliferate (A) or differentiate into TG cells (B). Plotted values represent mean ± SEM. Asterisk (*) indicates that control and experimental values are significantly different by *t* test ($P < 0.05$).

h (Fig. 6A). Notably, over 50% of transfected cells had become post-mitotic TG cells by 48 h (Fig. 6B). The lesser effect of *Hand1* was not due to a failure in transfection, the expression cassette or ability of *Hand1* to be expressed in TS cells, as judged by immunostaining for an epitope-tagged version of the *Hand1* protein (data not shown) or Western blot analysis (Fig. 7). The proportion of TS cells transfected with *Hand1* that were transformed into TG cells was similar to that observed previously in Rcho-1 cell transfection experiments (Cross et al., 1995; Kraut et al., 1998; Scott et al., 2000).

To define *Stra13* function within the context of the other bHLH factors, we tested for interactions among *Stra13*, *Hand1* and *Mash2*. We have previously found that the ability of *Hand1* over-expression to promote the differentiation of TG cells in transfected Rcho-1 cells is inhibited by *Mash2* over-expression (Scott et al., 2000), possibly through either direct or indirect protein interactions (Firulli et al., 2000; Scott et al., 2000). Rcho-1 cells were therefore co-transfected with *Stra13* and *Mash2* expression constructs. In contrast to its effects on *Hand1* activity, *Mash2* over-expression did not inhibit *Stra13* activity (Fig. 8A). Similar results were observed in TS cells (Fig. 8B). The Stra13 protein does not appear to act as a heterodimer with Hand1, as a Hand1–Stra13 interaction has not been detected in mammalian two-hybrid experiments (data not shown), and co-transfection of expression vectors for *Stra13* and *Hand1* did not further stimulate TG cell differentiation (Fig. 8B).

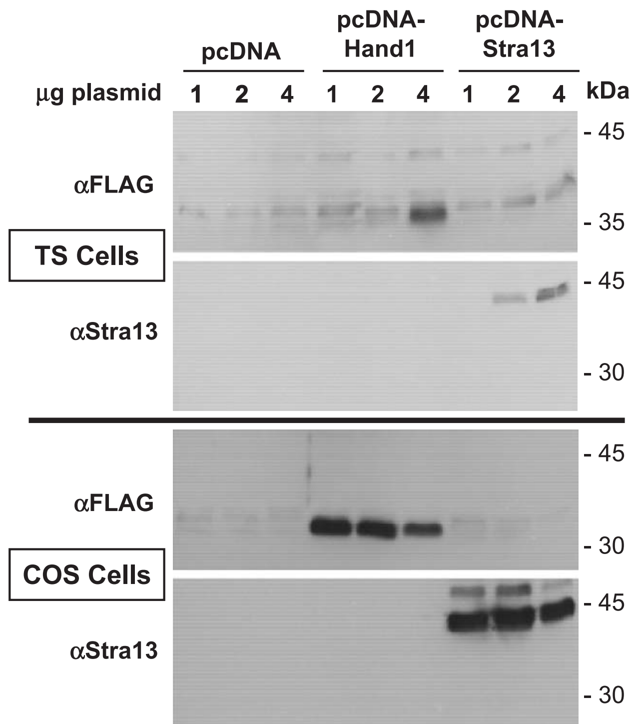

Fig. 7. Expression of Hand1 and Stra13 proteins in transfected cells. TS and COS (used as a positive control) cells were transfected with increasing amounts of expression vector for FLAG epitope-tagged Hand1 and for Stra13, harvested 24 h later and subjected to Western blotting.

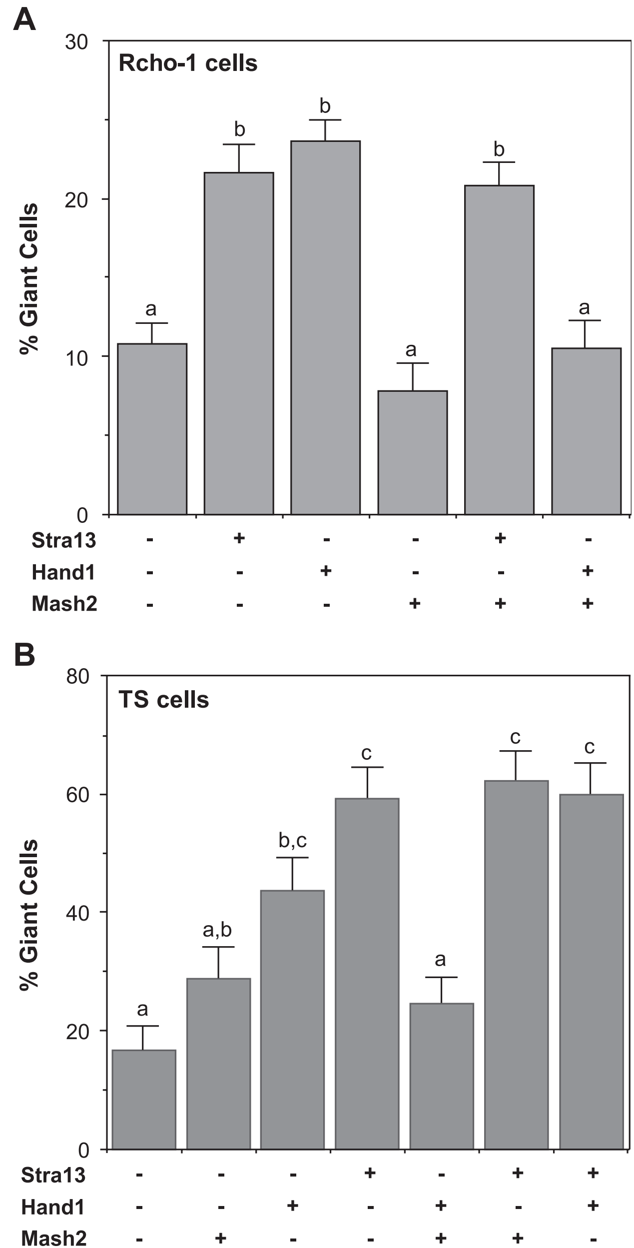

Fig. 8. *Stra13*-stimulated TG cell differentiation is not inhibited by *Mash2*. Rcho-1 stem cells (A) and TS cells (B) were transfected with either empty vector, or expression vectors for Hand1, Mash2 and Stra13 either alone or in combination. TG cell differentiation of transfected cells was scored 48 h later. Scale bars represent mean ± SEM. Values with different superscripts are significantly different ($P < 0.05$).

Gcm1 promotes cell division arrest and blocks giant cell differentiation

Ectopic expression of Gcm1 in TS cells maintained in the presence of FGF4/CM resulted in a dramatic arrest of cell proliferation, such that the cells stopped dividing immediately and remained as single cell clones (Figs. 9A, B). In contrast with *Hand1*- and *Stra13*-transfected cells, the *Gcm1*-transfected cells did not transform into TG cells and retained a TS-like morphology (Fig. 9B). Indeed, when

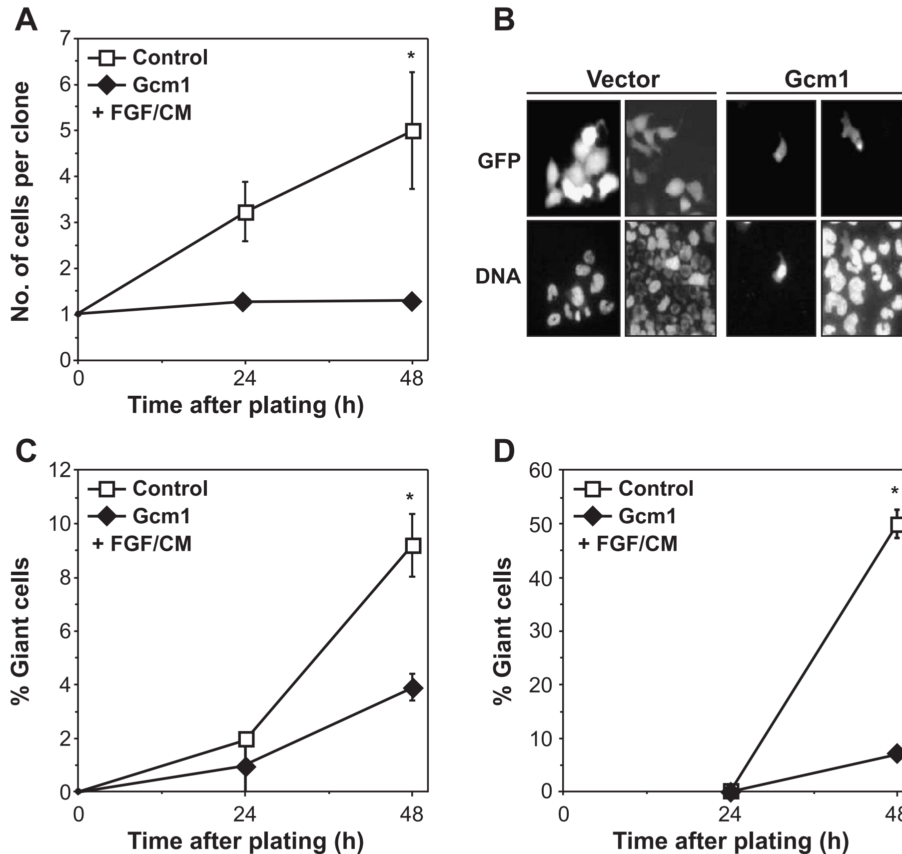

Fig. 9. Effect of *Gcm1* on proliferation, TG and SynT differentiation. TS cells were transfected with empty vector or *Gcm1* expression vector and scored for their ability to proliferate (A, B) or differentiate into TG cells (C, D), either in the presence (A, B, C) or absence (D) of FGF4/CM. Panel B shows the morphology of the *Gcm1*-transfected cells 72 h after transfection, and that they maintain a small, TS cell-like morphology. Plotted values represent mean ± SEM. Asterisk (*) indicates that control and experimental values are significantly different by *t* test ($P < 0.05$).

compared with controls, the *Gcm1*-transfected cells differentiated into TG cells at a significantly lower rate (Figs. 9C, D). Even when TG differentiation was maximally stimulated by withdrawal of FGF4/CM (up to 50% of cells are TG cells by 48 h), only approximately 5% of *Gcm1*-transfected cells were able to differentiate into TG cells. Over-expression of *Gcm1* had no effect on the rate at which SynT cells formed, though it should be noted that, due to the rapid arrest of cell proliferation induced by *Gcm1*, these cells were only rarely close enough together to undergo fusion. However, cells transfected with a vector encoding *Gcm1* in an antisense orientation did show a block to SynT differentiation (Control: 2.6%; Gcm1 antisense: 0%; chi-square: $P < 0.05$), consistent with the phenotype of Gcm1-deficient mice (Anson-Cartwright et al., 2000).

Discussion

The results of the TS cell transfection studies presented here complement previous work analyzing mouse mutants to give insights into the functions of *Hand1*, *Mash2*, *Stra13* and *Gcm1* at a cellular level (see Fig. 10). Importantly, because the ongoing proliferation of TS cells and the maintenance of their stem cell character is dependent on FGF4 and uncharacterized factors from feeder cells (Tanaka et al., 1998), we were able to explore the interactions between these pathways. Withdrawal of FGF4/CM from TS cells in vitro was associated with rapid up-regulation in

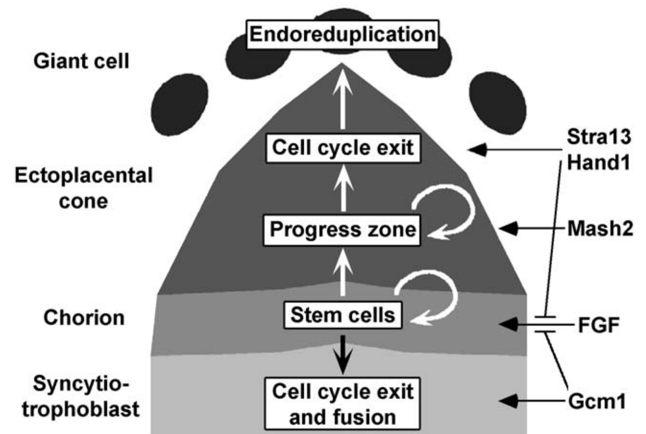

Fig. 10. Model of how the Hand1, Stra13, Mash2 and Gcm1 transcription factors, and FGF4/CM signaling interact to regulate alternative trophoblast cell fates.

the expression of *Hand1*, *Mash2*, *Stra13* and *Gcm1*. Coincident with this, TS cells lost the ability to proliferate and they terminally differentiated into either TG cells (at a high rate) or SynT cells (to a more limited extent). The most important general conclusion is that if *Stra13* or *Gcm1* (to a lesser extent *Hand1*) are ectopically expressed in TS cells still being stimulated by FGF4/CM, they each have dominant effects in being able to promote cell cycle arrest and differentiation into either TG cells or SynT.

Whereas *Hand1*, *Stra13* and *Gcm1* action is associated with cell cycle arrest, *Mash2* was able to sustain the proliferation of TS cells after FGF4/CM withdrawal, albeit only transiently. In vivo, *Mash2* mRNA is expressed weakly in the chorion and more strongly in the ectoplacental cone and later the SpT. Cells within these regions express mitotic markers (M.S. and J.C.C., unpublished observations) but, unlike the chorion, do not express FGFR2, the primary FGF receptor mediating the FGF4 effect in TS cells of the chorion (Arman et al., 1998; Tanaka et al., 1998). Therefore, proliferation within the ectoplacental cone and SpT may be FGF-independent and based on our experiments, only transient. These data fit the model that trophoblast cells in the ectoplacental cone and SpT are a transiently amplifying "progress zone" that serves as an expanded reservoir of cells that have lost their stem cell potential and are ultimately dedicated to form TG cells. In contrast to findings in transfected Rcho-1 cells (Cross et al., 1995; Kraut et al., 1998; Scott et al., 2000), over-expression of *Mash2* in TS cells did not suppress TG cell differentiation. Rcho-1 cells, which were derived from a trophoblast tumor, grow in an FGF4-independent manner although they do not express *Mash2* and do not apparently differentiate into any trophoblast derivative other than TG cells (Cross et al., 1995; Faria and Soares, 1991; MacAuley et al., 1998; Soares et al., 1996). As such, they are poised to only execute the TG differentiation program. The effect of ectopic *Mash2* expression in Rcho-1 cells may therefore simply be to convert them to a cell type more like the transiently amplifying population of TS cells. This fits with the slowly progressive phenotype of *Mash2* mutant mice (Guillemot et al., 1994; Tanaka et al., 1997).

Previous experiments have shown that *Hand1* is both necessary (Riley et al., 1998; Scott et al., 2000) and sufficient (Cross et al., 1995; Kraut et al., 1998; Scott et al., 2000) for promotion of TG cell differentiation. Our results in transfected TS cells support these conclusions. We found that another bHLH factor gene, *Stra13*, also promotes TG cell differentiation. *Stra13* expression is inducible by retinoic acid (Boudjelal et al., 1997), which has been shown to also induce TG differentiation (Yan et al., 2001). Although both *Stra13* and *Hand1* mRNAs are detected in TG cells, their expression actually begins in precursor cells (this study; Cross et al., 1995; Scott et al., 2000). Similarly, during differentiation of TS cells in culture, the *Stra13* and *Hand1* mRNAs were detected several days before the onset of expression of the giant cell-specific gene *Pl1*. Expression was not detected in undifferentiated TS cells, but ectopic expression of either *Hand1* or *Stra13* was sufficient to stimulate TG cell differentiation. Notably, if TG cell differentiation was stimulated by withdrawal of FGF4/CM from the cultures, over-expression of either *Hand1* or *Stra13* had no additional effect, implying that both factors act primarily to promote mitotic cell cycle exit and poise the cells to endoreduplicate. The effects of *Hand1* and *Stra13* were distinct in that *Stra13* had a much more dramatic and rapid effect both in arrest of cell proliferation and in stimulation of TG differentiation. This difference in results may be because, whereas *Hand1* action can be interrupted by *Mash2* (Scott et al., 2000), the *Stra13* effects were not. The effect of *Mash2* on *Hand1* is likely due to either direct or indirect interactions affecting DNA binding (Firulli et al., 2000; Scott et al., 2000). Stra13 has been shown to dimerize in vitro with E47 and Mash1, as well as form homodimers (Boudjelal et al., 1997; St-Pierre et al., 2002), similar to the types of possible interactions for Hand1 (Firulli et al., 2000, 2003; Scott et al., 2000). However, the difference in behavior of Hand1 and Stra13 in response to Mash2 implies that the dimerization characteristics of the Hand1 and Stra13 proteins are different in vivo.

Gcm1 mRNA is expressed in a subset of trophoblast cells at the chorioallantoic interface at embryonic day 8.5 in mice that corresponds to sites where morphogenesis begins (Anson-Cartwright et al., 2000; Basyuk et al., 1999). The placentas in *Gcm1*-deficient embryos show a defect in both chorioallantoic morphogenesis and SynT cell differentiation (Anson-Cartwright et al., 2000). Our experiments here showed that expression of antisense *Gcm1* RNA blocked SynT differentiation, without affecting cell proliferation or TG differentiation. This indicates that the absence of SynT cells in the *Gcm1* mutants is a primary phenotype and not simply secondary to the morphogenetic defect. The first step in SynT formation is exit of cells from the mitotic cell cycle and, therefore, the effect of *Gcm1* mutation may simply be to prevent cell cycle exit, a possibility supported by the ability of ectopic *Gcm1* expression to arrest TS cell proliferation. *Gcm1* also appears to regulate the fusogenic process (Yu et al., 2002). The other major effect of ectopic *Gcm1* expression was to restrict the ability of the cells to form TG cells. The fundamental decision of a TS cell to remain as a stem cell, or to progress towards the alternative fates of TG and SynT cells is therefore regulated, rather simply, by regulated expression of *Gcm1*.

The striking effects of *Hand1*, *Stra13* and particularly *Gcm1* on suppressing TS cell proliferation raise two interesting issues. First, it will be interesting to understand at a molecular level how *Hand1*, *Stra13* and *Gcm1* modify the responses of the cells to FGF4/CM. One possibility is that these transcription factors directly suppress the signaling ability of FGF4/CM by altering expression of a signaling component. Withdrawal of FGF4/CM from the culture medium results in cell proliferation arrest that takes about 2 days to complete. This is similar to the time frame for the

effects of *Hand1* and *Stra13*. *Stra13* is associated with arrest of cell proliferation in other systems (Sun and Taneja, 2000). Notably though, *Gcm1* has a much more rapid effect in that ectopic expression results in immediate cell proliferation arrest. Therefore, it is likely that *Gcm1* has an effect on cell proliferation that is more than simply shutting off FGF4/CM signaling. Induction of a cyclin-dependent kinase inhibitor is one possibility. The second issue concerns the importance of regulated expression of the *Hand1*, *Stra13* and *Gcm1* genes for normal trophoblast development. None of these factors are widely expressed in the chorion and the implication of the antiproliferative effects is that suppressing their expression within the chorion is actually rather critical for maintenance of the TS cell phenotype.

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