

Transcription Factors Underlying the Development and Endocrine Functions of the Placenta

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ABSTRACT

The placenta has been the subject of extensive basic research efforts in two distinct fields. The developmental biology of placenta has been studied because it is the first organ to develop during embryogenesis and because a number of different gene mutations in mice result in embryonic lethality due to placental defects. The trophoblast cell lineage is relatively simple such that only two major, terminally differentiated cell types appear: an “invasive trophoblast” cell subtype such as extravillous cytotrophoblast cells in humans and trophoblast giant cells in mice, and a “transport trophoblast” cell subtype that is a syncytium (syncytiotrophoblast) in humans and mice. These two cell types also have been the focus of endocrinologists because they are the source of major placental hormones. Understanding the transcriptional regulation of placental hormone genes has given insights into the control of specificity of gene expression. Because most placental hormones are produced by very specific trophoblast cell subtypes, the transcriptional details promise to give insights into cell-subtype specification. The fields of developmental biology and molecular endocrinology appear to be meeting on this common ground with the recent discovery of key transcription factors. Specifically, the basic helix-loop-helix (bHLH) transcription factor Hand1 is essential for differentiation of trophoblast giant cells in mice and also regulates the promoter for the giant cell-specific hormone, placental lactogen I gene (*PLI*). In contrast, formation of syncytiotrophoblast cells in mice is controlled by a distinct genetic pathway that is governed by the Gcm1 transcription factor, a homologue of the *Drosophila glial cells missing* gene. Human GCM1 has been shown to regulate the activity of the placental-specific enhancer of the aromatase gene (*CYP19*), which is specifically expressed in syncytiotrophoblast. Together, these findings imply that some key transcription factors have the dual functions of controlling both critical cell fate decisions in the trophoblast cell lineage and later the transcription of cell subtype-specific genes unrelated to development.

I. Introduction

Although we leave it behind at birth, the placenta is an amazing organ that is essential for intrauterine development. It allows the embryo to implant into the uterus and transports the nutrients and oxygen necessary for fetal growth. In addition, it has a major endocrine function that helps to subvert and orchestrate several maternal physiological systems that, together, further promote fetal

growth and survival. These functions include promoting the growth of maternal blood vessels to the implantation site and their dilation, suppression of the local immune system, promoting mammary gland development and continued production of progesterone from the corpus luteum (Linzer and Fisher, 1999; Cross *et al.*, in press). Failures in any one of these functions are associated with a range of complications of human pregnancy, including missed abortion, miscarriage, intrauterine growth restriction, and pre-eclampsia (Cross, 1996; Kingdom and Kaufmann, 1997; Cross, 1999). Aside from these specific functional aspects, study of the placenta gives insights into general aspects of developmental biology that are applicable to other systems. Indeed, because of the relative simplicity of the placental cell lineages, combined with the fact that the placenta is so sensitive to genetic perturbation, the placenta is an attractive model system for understanding the control of stem cells, cell lineage, and cell-cell interactions (Cross, 2000; Hemberger and Cross, 2001).

The placenta is derived from two major cell lineages (Cross *et al.*, 1994; Cross, 2000). Trophoblast of the blastocyst is the precursor to the trophoblast cell lineage that will give rise to the epithelial parts of the placenta. Extra-embryonic mesoderm gives rise to the stromal cells and blood vessels of the placenta. In rodents and primates, the outer layer of the mature fetal placenta consists of cells that are inherently invasive and associate with maternal blood vessels (called extravillous cytotrophoblast cells in primates and trophoblast giant cells in rodents). The innermost layer of the placenta consists of villous, tree-like branches that provide a large surface area for nutrient and gas exchange (called chorionic villi in primates and “the labyrinth” in rodents). The villi are covered with multinucleated syncytiotrophoblast (two layers in rodents) and also contain trophoblast stem cells, stromal cells, and blood vessels. The middle layer of the rodent and primate placenta consists of densely packed trophoblast cells called cytotrophoblast cell columns in primates and the spongiotrophoblast layer in rodents. Its function may simply be structural to support the underlying villi, although the spongiotrophoblast cells in rodents secrete a number of polypeptide hormones (Soares *et al.*, 1996,1998; Linzer and Fisher, 1999). In addition, the cells may represent a reserve of precursors of the invasive trophoblast population. This idea is based on the observation, made in both human (Damsky *et al.*, 1992,1994) and murine (Carney *et al.*, 1993) systems, that while trophoblast stem cells spontaneously differentiate into their invasive derivatives *in vitro*, along the way, they progress through a stage typical of the intermediate cell layer.

The differentiation of trophoblast stem cells to either “invasive trophoblast” or syncytiotrophoblast represents the fundamental, alternative cell fate decision in the trophoblast lineage (Figure 1). Along the way to forming these two different cell types, different genetic programs are initiated and include differences in hormone gene expression. One approach to understanding differences in cell-differentiation programs at a genetic level has been to study the transcrip-

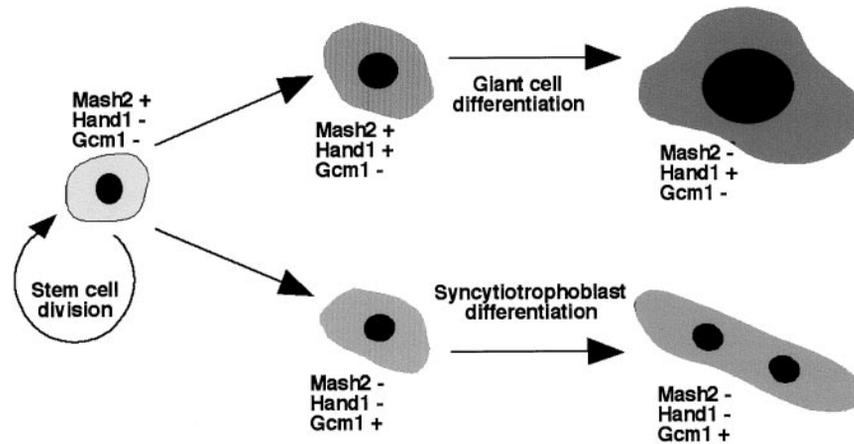


FIG. 1. Summary of the trophoblast cell lineage outlining the alternative differentiated trophoblast cell fates and the expression of key regulatory transcription factors. + indicates that the gene is expressed, whereas - indicates that the gene is not expressed.

tional mechanisms that regulate these trophoblast cell subtype-specific hormones. This approach has been very successful in other systems. An example is Pit-1, which was discovered for its ability to regulate pituitary hormone gene expression and later turned out to be essential for organ development as well (Andersen and Rosenfeld, 2001). Several different hormone genes have been intensively studied. For example, placental lactogen (*Pl1* and *Pl2*) and several prolactin-related protein genes are expressed exclusively by the trophoblast giant cell subtype in rodents (Linzer and Fisher, 1999). Their promoters have been intensively characterized in both transfected cells and transgenic mice (Shida *et al.*, 1992,1993; Vuille *et al.*, 1993; Ma *et al.*, 1997; Lin and Linzer, 1998; Sun and Duckworth, 1999; Ma and Linzer, 2000). The *Cyp19* gene, which encodes the aromatase enzyme involved in estrogen biosynthesis, is an example of a gene that is exclusively expressed in the syncytiotrophoblast cell subtype (Hinshelwood *et al.*, 1995; Yamada *et al.*, 1995,1999; Kamat *et al.*, 1998,1999). Curiously, while estrogen is produced by syncytiotrophoblast in the human placenta, the mouse placenta does not make estrogen or express *Cyp19*. However, when transgenic mice are made using the human gene promoter, the transgene is expressed in syncytiotrophoblast cells of the labyrinth (Kamat *et al.*, 1999). This implies that the mouse cells must have the transcription factors that are necessary to regulate the gene appropriately. Why the endogenous mouse *Cyp19* gene is not expressed likely reflects differences with the human gene in the gene sequence itself.

The alternative approach to finding transcription factors necessary for development has been to simply clone new members of transcription factor families that have an “evolutionary history” of regulating cell fate decisions during development. Some of these gene families were first identified in lower organisms such as *Drosophila melanogaster*. Such families include homeobox and basic helix-loop-helix (bHLH) genes that encode “master” regulators of development. We have taken this latter approach and have, as a result, identified a transcription factor that underlies the development of trophoblast giant cells (*Hand1*) and another critical for syncytiotrophoblast development (*Gcm1*) in mice.

II. Molecular Basis of Placental Trophoblast Cell Development

A. TROPHOBLAST GIANT CELLS

Considerable genetic evidence indicates that bHLH transcription factors function as cell-lineage determinants in a variety of cell lineages. This was first demonstrated by studies of skeletal muscle development in mammals (*MyoD*, *Myogenin*, *Mrf4*, *Myf5*), and in mesoderm (*twist*) and neuronal cell differentiation in *Drosophila* (*achaete-scute*) (Olson, 1990; Jan and Jan, 1993; Olson and Klein, 1994). Members of the bHLH family are thought to function as heterodimers, typically between the cell subtype-specific factors and the widely expressed E proteins, such as E12/E47 (which are products of the *E2A* gene) (Murre *et al.*, 1991), HEB (Hu *et al.*, 1992), and ITF2 (Henthorn *et al.*, 1990). The ability of cell subtype-specific factors to heterodimerize with E factors provided a functional means of cloning new members of the family. Cloning of genes based on such functional characteristics was revolutionized 10 years ago with the development of two techniques that relied on protein-protein interaction properties of gene products. Yeast two-hybrid screening relies on interactions of proteins in transformed yeast cells. An alternative method based on phage expression of target proteins, called “interaction cloning,” uses a labeled bait protein to screen the library (Blanar and Rutter, 1992). The advantage of the latter is that the approach could be used with existing λ gt11 or λ -based cDNA libraries.

The *Hand1* cDNA was cloned independently by three different groups and was originally given the different names *Hxt*, *eHand*, and *Thing1* (Cross *et al.*, 1995; Cserjesi *et al.*, 1995; Hollenberg *et al.*, 1995). Using an interaction cloning approach with an E47 bait protein, *Hand1* was isolated from a blastocyst stage cDNA library (Cross *et al.*, 1995). Later, it was shown that *Hand1* mRNA is expressed in trophoblast cells of the placenta and also in several structures of the embryo proper (Cross *et al.*, 1995; Cserjesi *et al.*, 1995; Hollenberg *et al.*, 1995). The original blastocyst library was made using sheep blastocysts, yet a mouse homologue was found to be expressed in mouse blastocysts and later the placenta

(Cross *et al.*, 1995). The choice of library was partly convenience, as a similar mouse library was not available at the time. However, given the differences in placental morphology among mammalian species (Wooding and Flint, 1994), the selection of a bHLH factor that was expressed in both mouse and sheep increased the level of stringency of the screen for factors important for fundamentally conserved aspects of placental development. Most of the subsequent work has concentrated on dissecting the functions of *Hand1* using the mouse as a model system.

The initial evidence that *Hand1* might regulate the differentiation of trophoblast giant cells came from the expression pattern of the gene during early mouse development. At early post-implantation stages, *Hand1* mRNA is not detectable by *in situ* hybridization in trophoblast stem cells of the chorion layer. It appears to be upregulated in the ectoplacental cone (the precursor to the spongiotrophoblast) and is most highly expressed in the trophoblast giant cell layer surrounding the implanted conceptus (Cross *et al.*, 1995; Scott *et al.*, 2000). This early pattern persists through later stages of development such that *Hand1* mRNA is detectable in spongiotrophoblast and trophoblast giant cells. To test the function of *Hand1*, the gene was overexpressed in Rcho-1 cells, a rat trophoblast tumor (choriocarcinoma) cell line. It has the interesting property that the dividing cells will spontaneously differentiate into postmitotic cells that eventually take on the morphological, cell-cycle, and gene-expression profile characteristics of giant cells (Faria and Soares, 1991; Hamlin *et al.*, 1994; Cross *et al.*, 1995; MacAuley *et al.*, 1998). Because the rate of giant cell transformation is normally relatively low when the cells are maintained under growth conditions, it affords the opportunity to detect the effect of factors that promote giant cell differentiation. Overexpression of *Hand1* promotes cell proliferation arrest and giant cell differentiation in transfected Rcho-1 cells (Cross *et al.*, 1995). A similar effect has been observed in primary trophoblast stem cell lines (I.C. Scott and J.C. Cross, unpublished data).

The real test of *Hand1* function was the generation of *Hand1*-deficient mice through gene targeting. Embryos that are homozygous for a *Hand1* null mutation do not survive beyond embryonic day (E) 8.5–9.0 and, in terms of their overall size, do not progress beyond E7.5–8.0 (Firulli *et al.*, 1998; Riley *et al.*, 1998). The mutants are recognizable at E8.5 because of their overall reduced size, a failure of the embryo proper to undergo its characteristic turning and blebbing of the yolk sac. In the placenta, the ectoplacental cone is smaller than normal (Riley *et al.*, 1998). The outer layer of trophoblast cells, which should contain trophoblast giant cells, has several defects in the *Hand1* mutants (Figure 2). First, the outer layer has many fewer cells than normal and, as a result, the overall conceptus is within a much smaller sac (Riley *et al.*, 1998; Scott *et al.*, 2000). The number of cells present is not significantly different than the number of trophectoderm cells present at the blastocyst stage. Normally, this number increases as new giant cells differentiate at the edge of the ectoplacental cone. Differences in

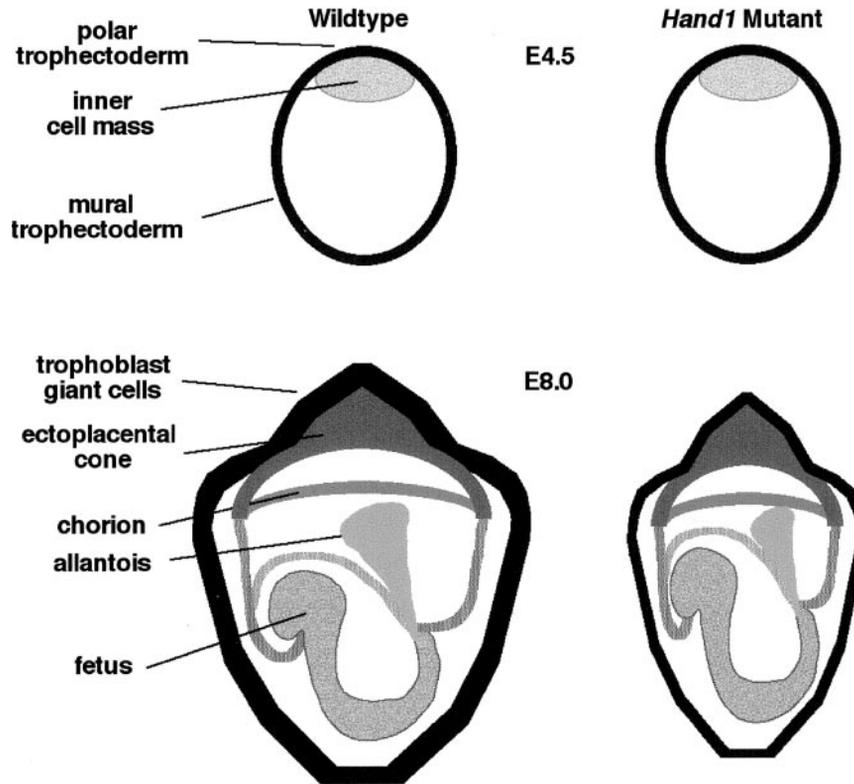


FIG. 2. Summary of the development of *Hand1* null mutant conceptuses at embryonic day (E) 4.5 and 8.0.

gene-expression patterns are consistent with the conclusion that this secondary differentiation does not occur (Riley *et al.*, 1998). The second major difference is that the trophoblast cells in the outer layer of the placenta, which are present in the *Hand1* mutants, failed to undergo the characteristic morphological giant transformation (Riley *et al.*, 1998; Scott *et al.*, 2000). Therefore, *Hand1* is essential for trophoblast giant cell differentiation.

While *Hand1* promotes differentiation of trophoblast giant cells, another bHLH gene has the opposite effect. *Mash2* is required for the maintenance of giant cell precursors (Guillemot *et al.*, 1994) and its overexpression in Rcho-1 cells prevents giant cell differentiation (Cross *et al.*, 1995; Kraut *et al.*, 1998; Scott *et al.*, 2000). *Hand1* and *Mash2* mRNA expression overlaps in the ectoplacental cone and spongiotrophoblast, layers of the placenta that contain giant cell precursors (Scott *et al.*, 2000). This observation implies that the

opposing activities of *Hand1* and *Mash2* must be coordinated. *Hand1* function is required to turn off *Mash2* mRNA expression coincident with trophoblast giant cell differentiation (Riley *et al.*, 1998). In addition, the Hand1 protein can compete with Mash2 for binding to E proteins (Scott *et al.*, 2000). Because Mash2 must dimerize with an E protein in order to bind DNA and therefore regulate transcription, this competition can titrate away Mash2 function. However, the *Hand1* null mutant phenotype is not solely explained by ectopic activity of Mash2, as the *Hand1* mutant phenotype is not altered by further mutation of *Mash2* (Scott *et al.*, 2000). Although the Hand1 protein dimerizes with E proteins *in vitro*, the latter do not appear to be expressed in trophoblast giant cells (Scott *et al.*, 2000). This suggests that Hand1 likely dimerizes with some other factor(s) in these cells. Candidates include Hand1 itself, since Hand1 can homodimerize at least *in vitro* (Firulli *et al.*, 2000; Scott *et al.*, 2000). Another bHLH protein encoded by the *Stra13* gene is expressed in trophoblast giant cells (Boudjelal *et al.*, 1997) but its ability to interact with Hand1 has not been tested. The complexity of the multiple potential bHLH protein interactions involving Hand1 has been one factor that has slowed the identification of Hand1 DNA recognition site(s) and target genes. One likely scenario is that Hand1 complexed with different partner proteins has different DNA binding specificities.

B. PLACENTAL VILLI AND SYNCYTIOTROPHOBLAST

While bHLH genes regulate the formation of trophoblast giant cells, no bHLH genes have been discovered that directly regulate the differentiation of syncytiotrophoblast cells or the villous structures of the labyrinth. The *Mash2* and *Hand1* genes are expressed focally within the labyrinth layer in mice but neither pattern of expression is obviously localized to syncytiotrophoblast. While we have found other bHLH factors that are expressed in the labyrinth (I.C. Scott, K. Dawson, J.C. Cross, unpublished observations), none have shown syncytiotrophoblast-specific expression.

In looking for other transcription factors that control cell-fate decisions, we became interested in the *Gcm* (for glial cells missing) gene family. In *Drosophila*, *Gcm* mutants show a cell fate change in the nervous system such that precursor cells that normally divide to give rise to neural and glial derivatives instead only give rise to neurons (Wegner and Riethmacher, 2001). The gene name is slightly misleading because the mutants also show defects in hemocyte development, implying that *Gcm* regulates cell fate in general rather than specifies glial cell fate. The first reports describing attempts to identify mammalian homologues of *Gcm* revealed two genes in both mice and humans that were called *Gcm1/Gcm-a* and *Gcm2/Gcm-b* (Akiyama *et al.*, 1996; Altshuller *et al.*, 1996; Kim *et al.*, 1998). The initial published report indicating that *Gcm1* mRNA was only detected in the placenta, based on northern blotting (Altshuller *et al.*, 1996), prompted further investigation. It soon was discov-

ered that expression of *Gcm1* in the mouse placenta is confined to the labyrinth layer (Kim *et al.*, 1998; Basyuk *et al.*, 1999), including syncytiotrophoblast cells (Basyuk *et al.*, 1999). Expression initiates at the time that the labyrinth first begins to form at E8.5 and persists as long as the labyrinth grows through repeated branching morphogenesis (Basyuk *et al.*, 1999).

Detailed mRNA expression analysis has shown that *Gcm1* gene expression is initiated around E8.0–8.5 in small clusters of cells within the chorion layer that otherwise contains trophoblast stem cells (Anson-Cartwright *et al.*, 2000). These *Gcm1* expressing cells are found at sites where the chorionic plate folds to form branches, regions where syncytiotrophoblast differentiation also begins. Both the initiation of chorioallantoic branching (Figure 3) and the formation of syncytiotrophoblast are blocked completely when *Gcm1* function is ablated (Anson-Cartwright *et al.*, 2000). As a result, the labyrinth layer of the placenta does not form in *Gcm1* null mutants and the chorion layer persists (Anson-Cartwright *et al.*, 2000; Schreiber *et al.*, 2000). Without the labyrinth, the embryos die around E10. Given that *Gcm* in the *Drosophila* nervous system

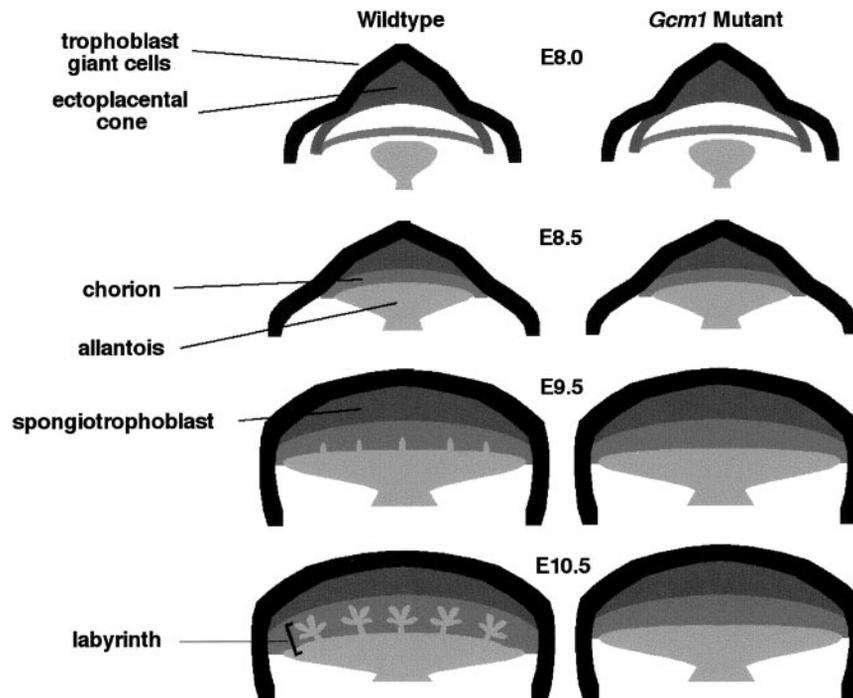


FIG. 3. Summary of the development of *Gcm1* null mutant conceptuses between embryonic day (E) 8.0 and 10.5.

regulates a cell-fate change, it is interesting to consider what happens in the *Gcm1*-deficient mice and whether the phenotype can be interpreted in a similar way. Because the chorionic trophoblast cell lineage has not been described in detail, this is a matter for speculation only. However, a reasonable hypothesis is that *Gcm1* is required for trophoblast cells to “choose” a syncytiotrophoblast fate rather than remain as stem cells.

III. Transcription Factors Controlling Development Also Regulate Placental Hormone Production

Both *Hand1* and *Gcm1* were discovered as orphan transcription factors in the sense that, although their DNA-binding specificities were learned early on, their biologically important target genes remain largely unknown. It is safe to say that this is true for the vast majority of transcription factors that have been described. The criteria that prove that a transcription factor is an important and direct regulator of a gene are 1) the transcription regulatory regions of the gene should contain binding sites for the factor, 2) selective mutation of the binding site should alter gene expression, and 3) gene expression should be similarly reduced in mutants lacking the transcription factor. Trophoblast subtype-specific hormone genes fulfill some of these criteria and suggest them as target genes of *Hand1* and *Gcm1*.

The mouse *Pll* gene (encoding the prolactin-like hormone, placental lactogen I) is specifically expressed in trophoblast giant cells (Faria *et al.*, 1991). The expression of *Pll* mRNA is dramatically reduced in *Hand1* mutants, indicating that *Pll* is genetically downstream of *Hand1* (Firulli *et al.*, 1998; Riley *et al.*, 1998). Because *Hand1* mutants show other abnormalities in giant cell differentiation, the reduced expression in the mutants could be indirect. However, the mouse *Pll* promoter contains a variant E-box-like element (Figure 4) that conforms to the consensus site to which *Hand1*/E47 dimers can bind (Hollenberg *et al.*, 1995). Although a point mutation of this site has not been tested, deletion of a 86-bp region of the promoter (between -274 and -188 relative to the transcription start site) that encompasses this element results in a reduction in overall promoter activity in transfected Rcho-1 trophoblast giant cells (Figure 4) (Shida *et al.*, 1993). Co-transfection of *Hand1* expression vector with a *Pll* promoter/reporter gene construct results in dose-dependent transactivation (Figure 4) (Cross *et al.*, 1995; Scott *et al.*, 2000). Deletion of the promoter between -274 and -188 prevents transactivation by *Hand1* (Cross *et al.*, 1995). These data suggest that *Hand1* likely regulates *Pll* gene promoter activity directly. It is important to note, however, that *Hand1* must not provide cell-type specificity to *Pll* transcription on its own because the *Hand1* gene is expressed in other cells and tissues where *Pll* is not. For example, in the trophoblast lineage, *Hand1* is expressed in the ectoplacental cone/syncytiotrophoblast (Cross *et al.*, 1995; Scott *et al.*, 2000). It is likely that *Hand1* interactions with other trophoblast subtype-

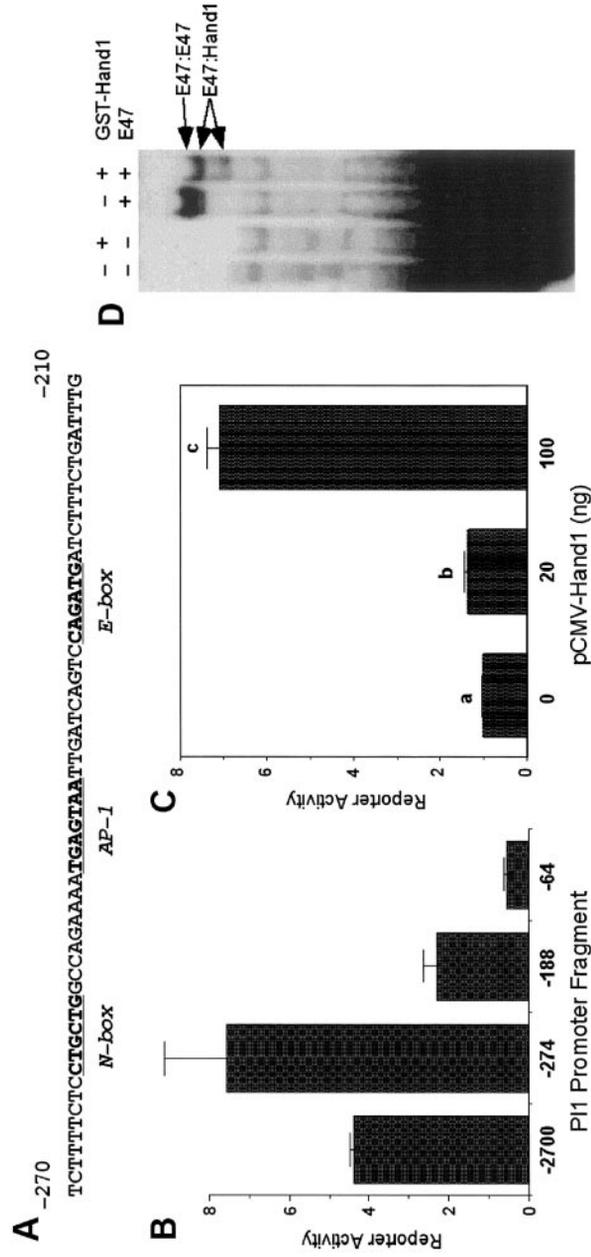


FIG. 4. HandI regulates *PII* gene expression. (A) Partial sequence of the *PII* gene promoter showing a variant E-box element. (B) Effect of 5' deletions on the activity of the *PII* promoter in transfected Rcho-1 trophoblast giant cells. [Based on work in Cross *et al.*, 1995.] (C) HandI activates the *PII* promoter. Rcho-1 cells were co-transfected with a *PII* promoter/luciferase reporter gene and a HandI expression vector. [Based on work in Scott *et al.*, 2000.] (D) HandI binds to a variant E-box element. Recombinant E47 and HandI were mixed and subjected to electrophoretic mobility shift analysis using an oligonucleotide sequence similar to that present in the *PII* promoter. [Based on work in Scott *et al.*, 2000.]

specific transcription factors together provide the specificity to *Pll* gene expression. These other factors could include Gata factors (Ng *et al.*, 1994) and/or the bHLH factor Stra13 (Boudjelal *et al.*, 1997).

In contrast to the directed studies aimed at making a connection between Hand1 and *Pll* transcription, the Gcm1 transcription factor has been associated with regulation of the *Cyp19* gene but by a more indirect route. In characterizing the *Cyp19* promoter by conventional molecular and biochemical means, Yamada and colleagues localized a critical regulatory element to a short region of DNA designated TSE2 (Yamada *et al.*, 1999). The TSE2 element was used in a one-hybrid screen in which DNA sequences are employed as the bait to identify transcription factors able to bind it. This screen identified a single cDNA clone from a human placental cDNA library that turned out to encode GCM1. In retrospect, the critical regulatory element fit the known consensus for a Gcm1/GCM1-binding element (Schreiber *et al.*, 1997,1998). Yamada also showed that a similar DNA sequence was present in the placental enhancer of the leptin gene (Yamada *et al.*, 1999). What is obviously missing from the analysis is proof that Gcm1/GCM1 is critical for transcriptional regulation of *Cyp19* and *Leptin* but the data thus far appear quite compelling.

IV. Conclusions

How do we interpret the observations that, while the Hand1 and Gcm1 transcription factors are critical for development of distinct trophoblast cell subtypes, they also appear to regulate cell subtype-specific genes? In the case of the hormone genes implicated to date, it seems unlikely that their function underlies the developmental roles of their transcriptional regulators. A more likely explanation is simply that what has been observed in other systems holds true in trophoblast cells as well: transcription factors controlling cellular development have ongoing effects in the same cells to regulate the transcription of genes whose function is peculiar to those cells. Not so many years ago, it was argued that studying the promoters of cell type-specific genes, as a means of getting insights into transcription factors that control cell fate, would be an endless road. As regulators would be identified, the search would turn to “the regulators of the regulators,” and so on. If the *Pll* and *Cyp19* genes are any indication, however, the procession from key transcription factor to cell type-specific gene may be much less complex and, indeed, can occur in a single step without need for a cascade of gene-induction events. With the discovery that the human genome may contain many fewer genes than originally thought, in retrospect, it may make sense that regulatory networks would involve fewer steps.

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