The topographical regulation of embryonic stem cell differentiation

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The potential use of pluripotent stem cells for tissue repair or replacement is now well recognized. While the ability of embryonic stem (ES) cells to differentiate into all cells of the body is undisputed, their use is currently restricted by our limited knowledge of the mechanisms controlling their differentiation. This review discusses recent work by ourselves and others investigating the intercellular signalling events that occur within aggregates of mouse ES cells. The work illustrates that the processes of ES cell differentiation, epithelialization and programmed cell death are dependent upon their location within the aggregates and coordinated by the extracellular matrix. Establishment of the mechanisms involved in these events is not only of use for the manipulation of ES cells themselves, but it also throws light on the ways in which differentiation is coordinated during embryogenesis.

Keywords: embryonic stem cells; basement membrane; epithelia; apoptosis; cavitation; primitive endoderm

1. INTRODUCTION

Mouse embryonic stem (ES) cells are isolated from cultured blastocysts at a stage when the blastocoelic surface of the inner cell mass (ICM) is lined by a layer of primitive extraembryonic endoderm cells (figure 1; Nadijcka & Hillman 1974; Robertson 1987; Brook & Gardner 1997). After culture for several days on feeder cell layers and/or in the presence of the cytokine leukaemia inhibitory factor (LIF), the cells are disaggregated and cultured further, during which time colonies of ES cells may be identified and selected. Cultured in the presence of LIF, mouse ES cells appear to maintain their pluripotent state indefinitely. However, a proportion of cells in ES cell cultures can always be seen to 'spontaneously' differentiate even in the presence of LIF (Smith 2001). The reason for the development of this heterogeneity is unclear. Furthermore, species- and strain-specific differences determine the success of attempts to derive ES cells, but even those strains of mice routinely used to produce ES cells (e.g. 129) yield low and variable results (Robertson 1987; Brook & Gardner 1997). Although considerable progress has been made by identifying a number of transcription factors that are necessary for the derivation and maintenance of mouse ES cells (Buehr *et al.* 2003), the reasons for variable yields and the lack of response of some ES cells (e.g. human) to LIF (Thomson *et al.* 1998) are also unknown.

Fortunately, two complementary sets of observations provide clues that may help explain this behaviour: first, the removal of extraembryonic endoderm cells from the conceptus of a LIF-unresponsive mouse strain has led to the successful derivation of ES cell lines (McWhir *et al.* 1996; Brook & Gardner 1997). Second, ES cell populations

can be maintained and expanded in the absence of LIF by selectively removing primitive endoderm cells as they differentiate (Mountford *et al.* 1998). Taken together, these observations indicate that intercellular interactions regulate the differentiation of ES cells and thereby can compromise their self-renewal. Thus, analysis of the interactions between ES cells is necessary to understand and control their potentially problematic tendency to differentiate. Furthermore, this analysis is likely to provide valuable information about the mechanisms that regulate the development of early embryonic cell lineages *in vivo*. Although much remains to be done, it is now becoming clear that not only do intercellular interactions regulate the self-renewal of ES cells, but they also specify their divergent directions of differentiation, together with the sequence, location and survival of nascent cell lineages.

2. THE RELATIONSHIP OF EMBRYONIC STEM CELLS TO EMBRYONIC CELLS *IN VIVO*

(a) *The derivation of embryonic stem cells*

Although ES cells may be isolated from the ICM, the identity of the cells from which they are derived is somewhat ambiguous. The ICM initially comprises a population of pluripotent cells that arise from the asymmetric cleavage of polarized blastomeres of the compacted morula. Thus, blastomeres inheriting an apical cell membrane enter the trophectodermal lineage, whereas the basal daughter cells lacking the apical membrane become part of the ICM of the blastocyst. Almost immediately after blastocyst formation, a layer of primitive endoderm arises from the cells of the ICM that line the blastocoel cavity (Nadijcka & Hillman 1974). The primitive endoderm cells give rise to two extraembryonic cell lineages: the parietal endoderm that migrates under the trophectoderm, and the visceral endoderm that differentiates from the primitive

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Figure 1. Schematic diagram showing the relationship between development of the conceptus *in vivo* (a) and the differentiation of ES cells in embryoid bodies *in vitro* (b). The broken line indicates the inhibition of differentiation of parietal and visceral endodermal cells by LIF. The term 'epiblast epithelial cells' refers to ES cells that have polarized to form a columnar epithelium and although morphologically they resemble primitive ectoderm, they do not express primitive ectoderm-specific genes. 'Presumptive primitive ectoderm' refers to ES cells that resemble primitive ectoderm in terms of their expression profile but have not yet polarized to form an epithelium. BM, basement membrane; BMP, bone morphogenetic protein; FGF, fibroblast growth factor.

endoderm cells that remain in contact with the ICM following implantation. The visceral endoderm is necessary for the further differentiation and molecular patterning of the primitive ectoderm (Spyropoulos & Capecchi 1994; Beddington & Robertson 1998; Gu *et al.* 1998; Sirard *et al.* 1998). Once the primitive endoderm (or hypoblast) has formed, the remaining cells of the ICM are often referred to as the *epiblast*, from which ES cells are derived. However, it is unclear if these non-endodermal cells of the ICM differ from ICM cells prior to differentiation of the primitive endoderm.

The confusion between the terms *ICM* and *epiblast* arises because the term *epiblast* is also applied to the cells underlying the visceral endoderm in the early post-implantation period, prior to their polarization to form the primitive ectoderm. However, these cells clearly differ from the preimplantation epiblast cells in having distinct gene expression profiles. Thus, only the former express *CRTR-1* and *Rex1*, and only the latter express *Fgf5* and *PRCE* (Haub & Goldfarb 1991; Pelton *et al.* 2002), although both cell types express *Oct-4*, a marker of pluripotent cells (Rosner *et al.* 1990). Most importantly, these two cell populations also display differences in developmental potential, for ES cells can only be derived from the pre-implantation ICM (Rossant 1977; Beddington 1983). Given that the available evidence points to the identity of ICM and 'preimplantation epiblast' cells as the source of ES cells, then the term *epiblast* will be reserved for later

stages of ICM cell differentiation, which we will show to be dependent upon the initial differentiation of primitive endodermal cells. Thus, we argue that ICM cell differentiation is a sequential process, intercellular interactions between primitive endodermal cells and remaining undifferentiated ICM cells being responsible for the subsequent differentiation of other cell lineages (figure 1).

(b) Intracellular signalling pathways regulating embryonic stem cell pluripotency

Detailed studies have been performed to analyse the mechanisms that regulate ES cell pluripotency (for reviews see Niwa (2001); Pesce & Scholer (2001)). It has been shown that LIF signals via a heterodimer of the low-affinity LIF receptor (LIFR) and gp130 receptor to activate the JAK kinase-mediated phosphorylation of latent STAT3 to support ES cell self-renewal (Niwa *et al.* 1998; Matsuda *et al.* 1999). In addition, LIF also activates the mitogen-activated protein kinase (MAPK) pathway, inhibition of which enhances ES cell self-renewal (Burdon *et al.* 1999a). Thus, LIF can activate antagonistic cell-signalling pathways, the balance of which determines whether the ES cells remain pluripotent or differentiate (Burdon *et al.* 1999b).

In addition to the requirement for LIF, maintenance of the steady-state levels of the transcription factor Oct-4 has been shown to be required for the self-renewal of pluripotent ES cells (Niwa *et al.* 2000) and is also necessary

Figure 2. Immunofluorescence showing activated STAT3 (pSer727-STAT3) (green) and Oct-4 (red) in ES cell culture and embryos at embryonic day 4.0 (E4.0). (a) ES cell colony cultured on gelatinized tissue-culture plastic in the presence of LIF. (c) Confocal image of E4.0 embryo. (b,d) Bright-field images of (a) and (c), respectively. e, primitive endoderm; i, inner cell mass; t, trophoblast.

for the initial development of pluripotency in ICM cells (Nichols *et al.* 1998). Experimentally induced increases and decreases of Oct-4 levels result in the loss of pluripotency of ES cells and their differentiation towards extra-embryonic endodermal and trophoblast cell lineages, respectively. However, maintenance of Oct-4 does not obviate the requirement for LIF to prevent differentiation (Niwa *et al.* 2000), indicating that both maintenance of Oct-4 expression and LIF-induced activation of STAT3 are necessary to sustain the pluripotent state of ES cells. While it remains unknown how the LIF/STAT3 and Oct-4 pathways interact, evidence has been presented that they are together required to maintain the level of expression of an as yet unidentified inhibitor that suppresses expression of transcription factors such as *GATA-6*, which in turn regulate expression of endoderm-specific genes (Fujikura *et al.* 2002). Recently, a novel homeobox-containing protein, *Nanog*, has also been shown to be required for maintenance of mouse ES cell pluripotency (Chambers *et al.* 2003; Mitsui *et al.* 2003). Although over-expression of *Nanog* does not abrogate the requirement for Oct-4, it does by-pass the need for activated STAT3. During pre-implantation development, *Nanog* is first expressed in the inner cells of the morula and, following blastocyst formation, is downregulated in the nascent primitive endoderm (Chambers *et al.* 2003; Mitsui *et al.* 2003). It will be interesting to see if *Nanog* is involved in the suppression of *GATA-6*.

(c) *Intercellular interactions that promote embryonic stem cell differentiation*

While ES cells can be maintained in a pluripotent state by monolayer culture on fibroblast feeders in the presence of LIF, they can also be cultured in suspension, under which conditions the ES cells form aggregates called embryoid bodies (Robertson 1987). The initial stage of embryoid body development occurs on the first day following growth in suspension culture, and involves ES cells clumping together to form cellular aggregates. By day 2, the cells on the periphery of the embryoid bodies have begun to differentiate to become primitive endoderm (Murray & Edgar 2001b). In embryoid bodies, LIF does not inhibit the topologically restricted differentiation of primitive endodermal cells at the surface of the cell aggregate (Shen & Leder 1992), consistent with the apparent lack of effect of LIF on the normal development of ICM cells that are not experiencing implantation delay (Nichols *et al.* 2001). Thus, the stimulus for the loss of pluripotency and differentiation of ES and ICM cells when located at the surface of aggregates is dominant over any inhibitory effect exerted by LIF.

It should be noted that the subsequent differentiation of primitive endodermal cells to form parietal and visceral endoderm derivatives is effectively blocked by LIF (figure 1; Murray & Edgar 2001a). The absence of visceral endoderm cells in the presence of LIF is therefore likely to account for the indirect suppression of epiblast cell

differentiation by LIF in embryoid bodies (Murray & Edgar 2001a). These observations raise the intriguing possibility that when used to maintain pluripotent ES cells in culture, LIF may act by blocking the further differentiation of primitive endodermal cells which spontaneously arise in ES cultures even in the presence of LIF (see Smith 2001). Experiments performed using embryoid bodies derived from ES cells transfected with the *Oct-4* promoter upstream of a neomycin resistance gene (OKO cells) strongly support this hypothesis (Mountford *et al.* 1998); in the presence of antibiotic, nascent primitive endoderm cells on the surface of OKO embryoid bodies are killed as soon as they begin to downregulate *Oct-4*. Under such conditions, the core cells of the embryoid body maintain pluripotency even in the absence of LIF (Mountford *et al.* 1998), indicating that the requirement for LIF can be bypassed if primitive endoderm is ablated.

In vivo, although STAT3 expression was not detected in the ICM prior to implantation (Antczak & Van Blerkom 1997), we have recently been able to demonstrate the presence of activated STAT3 in the nuclei of primitive endoderm cells differentiating from both ES cells *in vitro* and in blastocysts *in vivo* (figure 2): however, activated STAT3 was absent from the cells within the ICM. Thus, LIF/STAT3 signalling during the pre-implantation period may act to prevent the precocious development of visceral endoderm. This would be of particular importance during diapause (delayed implantation) where any development of visceral endoderm would be expected to result in the premature differentiation of the pluripotent cells within the ICM, thus causing the demise of the embryo. In support of this hypothesis, it has been shown that gp130-null mice (defective in LIF/STAT3 signalling) do not survive implantation delay (Nichols *et al.* 2001). While this result clearly points to a role for LIF signalling in pluripotent cell maintenance during delayed implantation, in the light of the previous observations it remains to be established if LIF acts directly on ES cells or alternatively if it exerts its effects via STAT3 signalling in the primitive endoderm during delayed implantation.

3. PRIMITIVE ENDODERM DIFFERENTIATION

(a) *Is primitive endoderm differentiation determined by lineage or location?*

Normally, the first cell lineage to 'spontaneously' differentiate from cultured ES cells or cells of the ICM *in vivo* is that of the extraembryonic endoderm. There are two possibilities that explain why the differentiation of primitive endoderm cells is restricted to the surface of the ICM and embryoid bodies. First, the cells within the aggregates could comprise a heterogeneous population, some of which are already committed to become either ES cells or primitive endoderm. Those cells committed to the primitive endoderm lineage would then migrate to the surface of the aggregate. Consistent with this lineage-dependent model of primitive endoderm differentiation, it is known that prior to compaction, individual blastomeres inherit different gene products from the oocyte (Antczak & Van Blerkom 1997). However, there is presently no evidence that these have any influence on the fate of ICM cells. The second possibility for primitive endoderm differentiation is based on the premise that if ICM and ES cells prior to

aggregation comprise a homogeneous population, then their fate must be determined by intercellular interactions dependent upon their position within the aggregates, the innermost cells thereby remaining undifferentiated while the surface cells differentiate to become primitive endoderm.

Experiments performed with F9 embryonal carcinoma cells and ES cell-derived embryoid bodies strongly support the position-dependent model for primitive endoderm differentiation. In F9 cell aggregates, the peripheral cells become extraembryonic endoderm, expressing high levels of endoderm-specific genes such as laminin in response to retinoic acid (Becker *et al.* 1992). Importantly, it was established that endodermal cells were not already present within the aggregates, ruling out the possibility that endodermal cells migrated to the periphery in response to retinoic acid (Becker *et al.* 1992). Furthermore, if the F9 cells were cultured in a monolayer, retinoic acid induced the whole population to become endoderm (Li & Gudas 1996). These observations show that the way in which F9 cells respond to retinoic acid depends upon their interaction with other cells; being centrally located within an aggregate renders F9 cells incapable of responding to endoderm-inducing cues.

In contrast to F9 carcinoma cell aggregates, the peripheral cells of ES cell-derived embryoid bodies spontaneously differentiate to become primitive endoderm in the absence of added retinoic acid (Robertson 1987). As with F9 cells, primitive endoderm cells are not detectable during the early stages of ES cell-derived embryoid body formation, as cells expressing endoderm-specific genes only become apparent at the periphery after 2 days of aggregate culture (Murray & Edgar 2001b). Importantly, experiments performed using a pure ES cell population generated by prior selection of cells expressing the *Oct-4* ES cell marker showed that following 8 days growth under antibiotic selection, embryoid bodies were produced that consisted entirely of undifferentiated ES cells (Mountford *et al.* 1998). These cells were demonstrated to exhibit normal behaviour by showing that a layer of primitive endoderm-like cells with characteristic absence of *Oct-4* expression differentiated at the embryoid body periphery following removal of antibiotic and addition of LIF (Mountford *et al.* 1998). Moreover, when injected into host blastocysts, ES cells can give rise to extraembryonic endoderm *in vivo*, albeit with a lower frequency than that with which they enter embryonic lineages (Beddington & Robertson 1989). Taken together, these observations indicate that ES cells are competent to differentiate along the primitive endodermal cell lineage, and that this differentiation is stimulated by their location at the surface of cell aggregates.

(b) *Signalling pathways required for primitive endoderm differentiation*

(i) *The role of FGF signalling*

There are numerous reports showing that FGF4 is expressed by ES and ICM cells (Niswander & Martin 1992; Rappolee *et al.* 1994), where it is regulated by the *Oct-4* and *Sox2* transcription factors involved in the maintenance of ES cell pluripotency (Yuan *et al.* 1995). While the proliferation of *FGF-4*^{-/-} ES cells is apparently normal (Rappolee *et al.* 1994; Wilder *et al.* 1997), ablation of

the *FGF4* gene demonstrated that it is necessary for the development of extraembryonic endoderm *in vivo*. Analysis of FGF signalling after blockade with dominant-negative FGF receptor constructs has provided evidence that the phosphoinositide 3'-kinase (PI3-kinase) and Akt/protein kinase B pathway downstream of the FGF receptor is necessary for endodermal cell differentiation (C. S. Chen *et al.* 2000; Li *et al.* 2001). Additionally, it has been demonstrated that Grb-2, acting upstream of the Ras-MAPK pathway is required for the appearance of endodermal cells (Cheng *et al.* 1998). Interestingly, while involved in regulation of differentiation, the MAPK pathway does not appear to be involved in regulation of ES cell proliferation (Burdon *et al.* 1999b) owing to the absence of the cyclin D checkpoint in G1 of the ES cell cycle (Savatier *et al.* 1996). This observation may explain the observation that FGF4 signalling is apparently unnecessary for ES cell replication (Rappolee *et al.* 1994; Wilder *et al.* 1997). Taken together, these observations demonstrate that multiple FGF signalling pathways are required for endodermal cell differentiation, but they do not explain why this signalling is restricted to the surface cells of embryoid bodies.

(ii) *The role of transcription factors*

In addition to the importance of FGF signalling pathways, gene targeting studies have shown that the transcription factors GATA-4 and -6 are essential for extraembryonic endodermal cell development *in vivo* (Kuo *et al.* 1997; Molkentin *et al.* 1997; Morrissey *et al.* 1998; Koutsourakis *et al.* 1999). Although there appears to be some redundancy between these two factors (Fujikura *et al.* 2002), the phenotype of mutant embryos suggests that GATA-6 is required at an earlier stage of development than GATA-4 (Kuo *et al.* 1997; Molkentin *et al.* 1997; Morrissey *et al.* 1998; Koutsourakis *et al.* 1999). It has been shown that GATA-6 is expressed in some of the ICM cells of the early blastocyst and is subsequently upregulated and restricted to the extraembryonic endoderm (Koutsourakis *et al.* 1999; Rossant *et al.* 2003), although the mechanisms responsible for this transition in expression profile are currently unknown.

In vitro experiments have confirmed that GATA-6 regulates extraembryonic endoderm differentiation, for forced expression of *GATA-6* in ES cell monolayers induces the entire population to adopt a parietal endoderm fate even in the presence of LIF (Fujikura *et al.* 2002). However, when ES cells overexpressing *GATA-6* are cultured in suspension to form embryoid bodies, it seems that only the peripheral cells differentiate to become visceral extraembryonic endoderm (Fujikura *et al.* 2002), indicating that while GATA-6 may be required for extraembryonic endoderm differentiation, it is not sufficient. This notion is supported by the observation that while *GATA-6* is expressed in cells dispersed throughout the ICM of the early blastocyst, only the peripheral cells become primitive endoderm (Rossant *et al.* 2003). It appears, therefore, that we have a situation that is analogous to the differentiation of F9 cells, in that ES cells centrally positioned within an aggregate are incapable of responding to endoderm-inducing cues.

How does GATA-6 induce endoderm differentiation? It has been shown that GATA-6, in addition to upregulating

its own expression and that of *GATA-4*, also upregulates expression of *COUP-TFs* (Fujikura *et al.* 2002). *COUP-TFI* is expressed in the primitive endoderm cells of both the mouse blastocyst at embryonic day 3.5 (E3.5) and in embryoid bodies at day 2 (Murray & Edgar 2001b). Forced expression of *COUP-TFI* in ES cells grown in monolayer induces the whole population to adopt an epithelial-like morphology and upregulate laminin genes (Fujikura *et al.* 2002). Thus, GATA-6 induces endoderm-specific gene expression via its upregulation of *COUP-TFs*. Additionally, the variant hepatocyte nuclear factor-1 (vHNF1), first expressed in the primitive endoderm, is also required for the further differentiation of these cells (Barbacci *et al.* 1999). It is interesting to note that *COUP-TFs* are responsible for the induction of vHNF1 expression in liver cells (Power & Cereghini 1996), and importantly, this requires the presence of an octamer-binding site in the proximal promoter region (Power & Cereghini 1996). This observation might explain the seemingly contradictory results concerning the role of Oct-4 in primitive endoderm differentiation: while overexpression of *Oct-4* in ES cell monolayers induces primitive endoderm differentiation (Niwa *et al.* 2000), the developmental relevance of this is not clear because it is known that *Oct-4* is downregulated in the primitive endoderm cells of the blastocyst (Rosner *et al.* 1990). However, in contrast to the expression profile of *Oct-4* mRNA, it has been reported that Oct-4 protein levels are elevated in primitive endoderm cells *in vivo* (Palmieri *et al.* 1994). These apparently contradictory observations prompted us to investigate the expression of Oct-4 protein in day 2 embryoid bodies and E4.0 blastocysts. Consistent with a recent report (Rossant *et al.* 2003), our results show that Oct-4 is not expressed in the primitive endoderm cells (figure 2), although from our data we cannot rule out the possibility that downregulation of Oct-4 is preceded by a transient increase in expression. It is likely that the suppression of Oct-4 is mediated by *COUP-TFs*, for it has been shown that *COUP-TFs* repress Oct-4 in both embryonal carcinoma cells (Ben-Shushan *et al.* 1995) and ES cells (Fujikura *et al.* 2002). Thus, taking all these observations into account, we suggest that the role of Oct-4 in the initial stages of extraembryonic endoderm differentiation is to enable *de novo* expressed *COUP-TFs* to transactivate vHNF1. Accumulation of *COUP-TFs* in the primitive endoderm cells would then result in downregulation of Oct-4, an event that is likely to be necessary for further extraembryonic endoderm development.

(iii) *The role of intercellular interactions*

Given that the initial differentiation of ES cells is restricted to those at the surface of embryoid bodies, then any hypothesis aimed at explaining the mechanisms controlling differentiation must be derived from the differences in cellular environment of inner and outer cells. Clearly, the surface cells are unique in having a free surface that is not involved in the E-cadherin-dependent adhesion responsible for the clustering of ES cells into developing embryoid bodies (Larue *et al.* 1996). It has been reported that E-cadherin-mediated cell adhesion can stimulate phosphorylation of the docking protein Gab-1 involved in activation of Ras/MAPK and PI3-kinase/Akt cascades (Shinohara *et al.* 2001), both of which have been

implicated in endodermal cell differentiation (Cheng *et al.* 1998; Li *et al.* 2001). Thus, the surface location of ES cells may regulate the function of the FGF-dependent signalling pathways, shown above to be necessary for endodermal cell differentiation.

Any cadherin-dependent activation of FGF signalling pathways in surface cells may be regulated by changes in the availability of Gab-1 or other scaffold proteins necessary for FGFR signalling owing to the polarization of surface ES cells. Alternatively, FGF signalling could be activated by morphological changes, for the ES cells at the surface of embryoid bodies become attenuated (Murray & Edgar 2001b), exhibiting changes in cytoskeletal organization as demonstrated by immunohistochemistry (Cheng *et al.* 1998). These morphological changes occur as the cells begin to express the early endodermal markers including *COUP-TFs I* and *II* (Murray & Edgar 2001b). Significantly, *Grb2*^{-/-} ES cells also show the morphological and cytoskeletal changes typical of primitive endoderm, yet fail to upregulate laminin genes (Cheng *et al.* 1998). These observations are consistent with the hypothesis that endodermal cell differentiation is stimulated by changes in ES cell morphology.

It has been known for many years that cell morphology can have a profound effect on many cellular processes including proliferation and differentiation (Folkman & Moscona 1978), although more recently evidence has been presented that the generation of cytoskeletal tension rather than a shape change *per se* is responsible for driving these processes (for a review see Huang & Ingber (2000)). Indeed, tension within the actin cytoskeleton has been shown to influence several of the signalling components involved in endodermal cell differentiation, for example by promoting the translocation of MAPK from the cytoplasm to the nucleus (Aplin & Juliano 2001) or influencing the cytoskeletal binding of Akt (Sawada & Sheetz 2002). It remains to be seen if any of these potential mechanisms is responsible for the surface-dependent differentiation of surface ES cells along the endodermal pathway.

4. DEVELOPMENT OF PRIMITIVE ECTODERM EPITHELIAL CELLS

Despite our lack of detailed knowledge of the mechanisms responsible for endodermal cell differentiation, the consequences of this differentiation are clear: the surface endodermal cells express large amounts of basement membrane components, resulting in the deposition of a basement membrane which induces epithelium formation in the subjacent ES cells (Murray & Edgar 2000; Li *et al.* 2001). Additionally, further differentiation of the surface cells along the extraembryonic endodermal lineage is necessary for the expression of factors that promote the differentiation of ES cells along the ectodermal lineage (see figure 1). Thus, this initial differentiation of surface ES cells is responsible for the location and sequence of subsequent events by which ES cells differentiate along the ectodermal lineage (Li *et al.* 2003).

(a) *Morphological differentiation of the ectodermal epithelium*

While ES cells positioned at the periphery of embryoid bodies initially differentiate into the endodermal lineage,

application of the basement membrane component laminin to the apical surface of these cells drives differentiation towards an ectodermal-like fate, the ES cells undergoing a reversal of polarity in that their outer surface membrane now becomes the basal membrane of a columnar ectodermal epithelium rather than the apical surface of primitive endodermal cells (Li *et al.* 2001). Cadherin-mediated cell-cell interactions are promoted in differentiating embryoid bodies so that junctional complexes develop in the ectodermal epithelium after basement membrane contact (Larue *et al.* 1994; Li *et al.* 2002). Thus, the initiation of ectodermal epithelium formation by ES cells, its location and the polarity of ectodermal cells are all determined by the juxtaposition of cells relative to the basement membrane. It is important to note, however, that despite the primitive ectoderm-like morphology of these cells, they do not display the correct gene expression profile in the absence of extraembryonic endoderm and are restricted in their ability to undergo further differentiation, indicating that the basement membrane alone is not sufficient for the differentiation of epiblast epithelial cells (Li *et al.* 2001).

(i) *The role of laminin*

We have developed a system to analyse laminin deposition and the function of basement membranes by targeting the *LAMC1* genes of mouse ES cells (Smyth *et al.* 1999). The consequent absence of the laminin $\gamma 1$ subunit results in loss of the trimeric type-1 laminin molecule, failure of basement membrane formation and early embryonic lethality (Smyth *et al.* 1999). Rescue of basement membranes by addition of exogenous laminin to cultured embryoid bodies allowed us to demonstrate that laminin is necessary for basement membrane deposition and subsequently for ectodermal epithelium formation (Murray & Edgar 2000).

In an attempt to determine the ES cell receptors used by the laminin to drive epithelium formation, we made use of ES cells lacking either the $\beta 1$ integrin subunit or α -dystroglycan (Li *et al.* 2002). Although both of these putative laminin receptors had been shown previously to play a role in the deposition of laminin on ES cell membranes (Henry *et al.* 2001), surprisingly neither $\beta 1$ integrins nor α -dystroglycan were necessary for ectodermal epithelium formation (Li *et al.* 2002). This indicates that either there is functional redundancy between the two receptors, or alternatively the ES cells interact with laminin via a receptor other than a $\beta 1$ -integrin or α -dystroglycan. Consistent with the latter possibility, proteolytic fragments of laminin and modified recombinant subunits identified the laminin LG $\alpha 4$ sub-domain as essential for epithelium differentiation (Li *et al.* 2002). This sub-domain binds heparin, consistent with the hypothesis that the ES cells can use a heparan sulphate proteoglycan (HSPG) other than α -dystroglycan for epithelialization in response to laminin (Li *et al.* 2002).

Cell-extra-cellular matrix interactions mediated by members of the syndecan cell membrane HSPG family have been reported to be involved in epithelial cell maintenance (Kato *et al.* 1995), and are able to stimulate intracellular signalling pathways common to both integrins and α -dystroglycan in addition to their synergistic action with growth factor signalling (Hoffman *et al.* 1998; Bernfield *et al.* 1999; Rapraeger 2001). Furthermore, it has been

shown that syndecan and E-cadherin expression are mutually dependent (Kato *et al.* 1995; Leppa *et al.* 1996). It will therefore be interesting to see if a syndecan mediates the effects of laminin to stimulate ectoderm epithelium formation.

(ii) *Extracellular matrix-induced signalling*

While it is clear that the initial trigger for ectodermal epithelial formation is the interaction between laminin and receptors on the ES cell membrane (Li *et al.* 2001), how does this promote the key events involved in epithelialization; namely, cell polarization and maturation of intercellular junctions? From the work done on various model systems to analyse the development of cell polarity, many candidate regulatory components may well be involved. However, recent observations have implicated members of the Rho family of small GTPases in ectodermal epithelium formation. Thus, knockout of the Cdc42 member of the family results in a specific failure of ectodermal epithelium formation, correlated with aberrant actin cytoskeletal organization indicating defective cell polarization (Chen Y. *et al.* 2000). Cdc42 has been shown to be activated by laminin–cell interactions in other systems (Weston *et al.* 2000), and therefore has the potential to effect epithelial morphogenesis by regulating the organization of the actin cytoskeleton (Johansson *et al.* 2000; Prehoda *et al.* 2000; Yamanaka *et al.* 2001; Edlund *et al.* 2002). In addition, Rac1 (another member of the Rho-GTPase family) has been shown to promote the laminin-dependent reversal of polarity of aggregated MDCK epithelial cells (O'Brien *et al.* 2001). Details of the mechanisms by which members of the Rho family regulate ectodermal epithelium formation await further investigation.

(b) Biochemical differentiation of the primitive ectoderm

Although a columnar ectodermal epithelium fails to develop in basement-membrane-deficient *LAMC1*^{−/−} embryoid bodies, nevertheless the primitive ectoderm-specific marker *FGF-5* is upregulated subsequent to endoderm formation and at later time-points the differentiation of endothelial, neuronal and muscle cells is observed (Smyth *et al.* 1999; Murray & Edgar 2000). Conversely, in the presence of basement membrane components, although ES cells adopt the characteristic morphology of primitive ectoderm cells, they retain a gene expression profile that more closely resembles that of ES and ICM cells and are restricted in their developmental potential (Sirard *et al.* 1998; Yang *et al.* 1998; Li *et al.* 2001; Murray & Edgar 2001a; Pelton *et al.* 2002). Thus, induction of the epithelial morphology and gene expression of ectodermal epithelial cells are distinct and apparently independent processes, both of which are dependent upon the initial differentiation of extraembryonic endodermal cells at the surface of the embryoid body. While we have been able to make some progress with the analysis of the role of the primitive endodermal basement membrane in the induction of ectodermal epithelialization, the means by which cells of the visceral endodermal lineage are able to induce the ectodermal gene expression profile are not so clear.

However, some progress has been made in determining the mechanisms that induce the differentiation of visceral

endoderm from primitive endoderm. Shortly after implantation, the primitive endoderm cells directly overlying the ICM differentiate to become visceral endoderm, while those positioned at the ICM-trophoblast contact zone become parietal endoderm. It has been suggested previously that parietal endoderm cells differentiate from primitive endoderm cells that lose contact with the ICM as a result of overcrowding caused by their continuing proliferation (Gardner 1982); the inference being that ICM-derived factors are required for the initial differentiation of visceral endoderm. In support of this hypothesis, it has recently been shown that in embryoid bodies derived from epiblast-like cells rather than ES cells, no visceral endoderm cells are formed, and all primitive endoderm precursors become instead parietal endoderm (Lake *et al.* 2000).

There is good evidence to suggest that signalling from bone morphogenetic proteins (BMPs) is required for visceral endoderm differentiation, for inhibition of BMP signalling was found to block the development of visceral endoderm *in vitro* (Coucouvanis & Martin 1999). Furthermore, in embryoid bodies derived from S2 embryonal carcinoma cells where all primitive endoderm cells differentiate to parietal endoderm, BMPs were found to promote trans-differentiation of parietal endoderm to visceral endoderm (Coucouvanis & Martin 1999). In support of a role for *BMP4* in visceral endoderm differentiation, it has been shown that this factor is expressed specifically in the ICM of the blastocyst but is downregulated in the epiblast following implantation (Coucouvanis & Martin 1999). Moreover, targeted deletion of *SMAD4*, an essential intracellular component of the BMP signalling pathway, produces defects in the differentiation of visceral endoderm (Sirard *et al.* 1998; Yang *et al.* 1998). Thus, the entry of primitive endodermal cells into the visceral endodermal lineage is likely to be dependent upon the continued presence of undifferentiated ES or ICM cells expressing *BMP4*.

In a neat twist of ontogeny, the visceral endodermal cells are responsible for the loss of the very cells that induced them by stimulating the differentiation of ES cells into the ectodermal lineage. This essential role of visceral endoderm in primitive ectoderm development *in vivo* has been amply demonstrated by a variety of gene knockout studies. *GATA6*^{−/−} and *evx1*^{−/−} embryos have a very early lethality, characterized by the demise of the primitive ectoderm. In both cases it is apparent that the death of the primitive ectoderm cells resulted from a failure of visceral endoderm differentiation (Spyropoulos & Capecchi 1994; Koutsourakis *et al.* 1999).

In vitro analysis has shown that LIF blocks primitive ectoderm differentiation in embryoid bodies, as assayed by the absence of expression of the primitive ectoderm marker *FGF-5* and lack of mesodermal derivatives. However, the differentiation of primitive endoderm and basement membrane deposition is unaffected by LIF (Shen & Leder 1992; Murray & Edgar 2001a). Despite the absence of primitive ectoderm cell differentiation, ES cells aligned against the primitive endodermal basement membrane formed a columnar epithelium and cavitation ensued (Murray & Edgar 2001a). Similarly, addition of laminin to embryoid bodies derived from ES cells expressing a dominant-negative FGF receptor induced columnar epithelial formation and cavitation, even though primitive

ectodermal cells failed to differentiate (Li *et al.* 2001). Significantly, it has been shown that endoderm-conditioned media can promote primitive ectoderm differentiation in ES cells cultured in the presence of LIF (Rathjen *et al.* 1999). Thus, a plausible explanation for the absence of primitive ectoderm differentiation in the presence of either LIF or defective FGF signalling is that in both cases the development of visceral endoderm cells is inhibited, thereby indirectly preventing ectodermal cell differentiation (Li *et al.* 2001; Murray & Edgar 2001a).

5. CAVITATION

The already mentioned sequence of extraembryonic endoderm formation by peripheral cells of embryoid bodies followed by the differentiation of ectodermal epithelial cells from subjacent ES cells recapitulates the initial stages of cell differentiation of the ICM. Furthermore, the subsequent development of embryoid bodies also mimics ICM development in that a cavity forms within the cell aggregates, analogous to the proamniotic cavity that develops *in vivo*.

(a) Mechanisms of cavitation

There are several potential mechanisms of cavity formation; for instance, the development of the blastocoel and exocoelomic cavities that form during the early stages of embryogenesis is dependent upon a process of fluid infiltration (Bonnevie 1950). Alternatively, the programmed cell death (PCD) of cells centrally positioned within solid cellular aggregates is also commonly used during development to create lumens and it is likely to be required during post-natal life for maintenance of lumen patency (Crosby & Hill 1962; Debnath *et al.* 2002).

In embryoid bodies, cavitation results from PCD (Coucouvanis & Martin 1995) and is dependent upon the basement membrane deposited by primitive endodermal cells (Murray & Edgar 2000). Thus, there is no cavitation in the absence of basement membranes, and cavitation is restored or even accelerated by restitution of the basement membrane (Murray & Edgar 2000). More detailed investigation suggested that the lack of PCD results from the absence of polarized primitive ectodermal epithelial cells, the initial stages of cavitation being caused by the selective PCD of cells positioned at the apical surfaces of the newly formed epithelial cells (Murray & Edgar 2000, 2001a).

It has been suggested that factors derived from visceral endoderm cells induce PCD (Coucouvanis & Martin 1995), but it has subsequently been demonstrated that visceral endoderm cells are not required for this process (Murray & Edgar 2001a). More recently, the redundancy of the extraembryonic endoderm has been clearly demonstrated by the addition of high concentrations of laminin-1 or Matrigel (a basement membrane substitute) to newly formed embryoid bodies, which results in complete blockade of endoderm differentiation without affecting columnar epithelial formation or cavitation (Li *et al.* 2001). However, there is evidence that the basement membrane is not sufficient for this PCD as there is a failure of epithelialization and PCD in embryoid bodies derived from S2 embryonal carcinoma cells or from ES cells expressing a dominant-negative BMP receptor construct, despite the presence of a basement membrane (Coucouvanis &

Figure 3. Toluidine blue-stained section of day 5 embryoid body. In the early stages of cavitation, cell death is restricted to the apical surfaces of the newly formed columnar epithelium (black arrow). Note that the epithelial cells are juxtaposed to non-polarized cells (white arrow). Black arrow, columnar epithelium; white arrow, non-polarized cells; C, cavity containing cell debris.

Martin 1999). These observations support the hypothesis that the role of the basement membrane in PCD is indirect, its main function being to induce the formation of an epithelium, which in turn directly effects cavity formation.

In striking resemblance to proamniotic cavity formation, it was found that PCD occurred in an *in vitro* model of mammary gland development subsequent to the formation of polarized mammary epithelial cells, this polarization in turn being dependent upon the presence of a basement membrane (Debnath *et al.* 2002). Following on from this, it was demonstrated that forced expression of the cell adhesion molecule CEACAM1-4S induces polarization of mammary carcinoma cells in contact with the basement membrane, and PCD of centrally located cells to form a cavity (Kirshner *et al.* 2003). Thus, the sequence of events leading to cavitation in embryoid bodies not only recapitulates proamniotic cavity formation but also reflects processes necessary for the maintenance of normal tissue structure that are compromised in cancer (Debnath *et al.* 2002; Kirshner *et al.* 2003).

(b) The role of epithelial cells in programmed cell death

Localized areas of PCD are already observed in embryoid bodies subjacent to small groups of newly polarized epithelial cells before the primitive ectodermal epithelium is complete (figure 3). Thus, it is unlikely that the PCD is due to limited access of essential nutrients or growth factors to the inner cells. Furthermore, in the mammary gland model of cavitation-linked PCD, it was found that fluorescein-labelled dextrans as large as 40 kDa

were able to access the cavity (Debnath *et al.* 2002). These observations are consistent with the hypothesis that polarized epithelial cells may actively induce the PCD of cells positioned at their apical surfaces.

Histological analysis of cavitating embryoid bodies has shown that while a large proportion of dying cells display characteristics of apoptosis, others undergo necrotic cell death (P. Murray and D. Edgar, personal observations; Boyd *et al.* 1984). Furthermore, the inner cells of mammary acini normally undergo apoptotic cell death, but overexpression of the anti-apoptotic protein Bcl2 results in the dying cells displaying the characteristics of vacuolating autophagy (Debnath *et al.* 2002). Taken together, these observations suggest that PCD can be induced by more than one intracellular pathway. To date, there has been scant analysis of the molecular pathways operating to induce death of inner cells during cavitation. In embryoid bodies it has been shown recently that caspase-3 is activated, cytochrome *c* is released from the mitochondria and the apoptosis-inducing factor (AIF) translocates from the mitochondria to the nucleus (Joza *et al.* 2001). There are data suggesting that AIF is essential for PCD based on the observation that embryoid bodies derived from AIF-deficient ES cells do not cavitate. However, it is important to note that the ability of such embryoid bodies to deposit a basement membrane or generate a columnar epiblast epithelium has not yet been demonstrated. Consequently, the defect in cavitation observed in AIF-null embryoid bodies may be an indirect effect similar to that seen in *LAMC1*^{-/-}, *integrin β 1*^{-/-} and dominant-negative FGF receptor embryoid bodies where epithelialization does not occur (Murray & Edgar 2000; Joza *et al.* 2001; Li *et al.* 2001, 2002).

6. CONCLUDING REMARKS

Mammalian embryogenesis comprises a sequence of events that results in the generation of differentiated cells and in the organization of those cells within the body. One of the fundamental processes involved is the generation of cellular asymmetries both between cells within groups and at the single cell level with the generation of cell polarity. This review on the behaviour of ES cells shows that such asymmetries result from intercellular interactions, involving either direct contacts between cells or indirect interactions via the extracellular matrix. The spatial cues arising from such interactions are determined by what is initially the very simple inside–outside topography that clumps of identical cells possess. However, even the initial few steps of ES cell differentiation make it clear that topographically determined interactions sequentially build up complex structures in which distinct differentiated cells are correctly located as necessary for subsequent development (figure 1).

As the principles of regulation of ES cell differentiation are now becoming clear, it is possible to delineate some direct questions that need to be addressed to understand and direct their maintenance and differentiation.

- (i) What are the mechanisms responsible for the divergent differentiation of ES cells? We have proposed that ES cell differentiation is regulated by changes in cell–cell interactions that occur when they are

cultured as embryoid bodies. Specifically, we hypothesize that primitive endoderm cell differentiation is induced by the polarization and/or shape changes that occur in the cells located at the periphery of the embryoid body, and suppressed in the cells located interiorly. Future investigations therefore need to be directed towards (1) the mechanisms that restrict the consequences of FGF signalling to the outer cells of embryoid bodies necessary for endoderm formation and (2) how the basement membrane induces polarization of ES cells to divert their differentiation into the primitive ectoderm lineage.

- (ii) How do intercellular interactions induce the PCD necessary for cavitation? Many organs contain epithelium-lined lumens, the integrity of which is essential for normal function, but very little is known about the mechanisms involved. From our work with cavitation in embryoid bodies we have hypothesized that the ectodermal epithelial cells play an active part in inducing the PCD of ES cells positioned at their apical surfaces. The molecular mechanisms involved in this intercellular interaction are currently being investigated, but the general applicability of this hypothesis to lumen formation in other systems also needs to be evaluated.

The authors thank Dr D. Spiller for help with confocal imaging. P.M. is the recipient of a Royal Society Dorothy Hodgkin Research Fellowship. The work described in this review that was carried out in the authors' laboratory was supported by grants from the Wellcome Trust, the Medical Research Council, the European Union and the British Federation of Women Graduates.

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