Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines

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SUMMARY

Embryonic stem cells are established directly from the pluripotent epiblast of the preimplantation mouse embryo. Their derivation and propagation are dependent upon cytokine-stimulated activation of gp130 signal transduction. Embryonic stem cells maintain a close resemblance to epiblast in developmental potency and gene expression profile. The presumption of equivalence between embryonic stem cells and epiblast is challenged, however, by the finding that early embryogenesis can proceed in the absence of gp130. To explore this issue further, we have examined the capacity of gp130 mutant embryos to accommodate perturbation of normal developmental progression. Mouse embryos arrest at the late blastocyst stage when implantation is prevented. This process of diapause occurs naturally in lactating females or can be induced experimentally by removal of the ovaries. We report that gp130−/− embryos survive unimplanted in the uterus after ovariectomy but, in contrast to wild-type or heterozygous embryos, are subsequently unable to resume development. Inner cell masses explanted from gp130−/− delayed blastocysts produce only parietal endoderm, a derivative of the hypoblast. Intact mutant embryos show an absence of epiblast cells, and Hoechst staining and TUNEL analysis reveal a preceding increased incidence of cell death. These findings establish that gp130 signalling is essential for the prolonged maintenance of epiblast in vivo, which is commonly required of mouse embryos in the wild. We propose that the responsiveness of embryonic stem cells to gp130 signalling has its origin in this adaptive physiological function.

Key words: Pluripotency, Blastocyst, Diapause, Epiblast, Self-renewal, Stem cell, Mouse

INTRODUCTION

The epiblast is a transient population of pluripotent cells from which the entire mammalian foetus is derived (Gardner and Beddington, 1988; Hogan et al., 1994). The pluripotent capacity of the epiblast normally persists for only 4-5 days in early mouse embryogenesis. However, a potential for prolonged propagation of epiblast can be revealed by culturing embryos in the presence of the cytokine leukaemia inhibitory factor (LIF), which results in the establishment of embryonic stem (ES) cell lines (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981; Nichols et al., 1990). The continued propagation of pluripotent ES cells in culture is sustained by LIF provided either by co-culture with a feeder layer of embryonic fibroblasts (Rathjen et al., 1990), or as a recombinant protein added to the culture medium (Rathjen et al., 1990; Smith et al., 1988; Williams et al., 1988). These cells can be expanded indefinitely in culture whilst retaining the full differentiation capacity of the epiblast. They also express various antigenic and gene markers characteristic of the epiblast. Most impressively, ES cells have the ability to respond appropriately to developmental cues in the blastocyst and re-enter embryonic development (Beddington and Robertson, 1989; Bradley et al., 1984). ES cells thus appear to be in vitro counterparts of the early epiblast (Gardner and Brook, 1997; Smith, 2001).

In the absence of LIF, ES cells differentiate and pluripotency cannot be maintained. LIF acts by engaging a heterodimeric cell surface receptor complex comprising the LIF receptor subunit (LIFR) (Gearing et al., 1991) and glycoprotein 130 (gp130; also known as Il6st) (Davis et al., 1993). A family of related cytokines, including cardiotoxin 1, oncostatin M and ciliary neurotrophic factor, that interact with the LIFR/gp130 complex can substitute for LIF and support ES cell self-renewal (Conover et al., 1993; Pennica et al., 1995; Rose et al., 1994). Alternatively, the combination of interleukin 6 and soluble interleukin 6 receptor activates gp130 homodimers and can be used to derive and maintain ES cells without involvement of LIFR (Nichols et al., 1994; Yoshida et al., 1994).

LIF, LIFR and gp130 mRNAs are all expressed in the mouse blastocyst, and moreover in a reciprocal pattern between the trophectoderm and the inner cell mass (ICM) (Nichols et al.,
1996). This is suggestive of a paracrine interaction whereby cytokine production by the trophectoderm would act to sustain embryonic pluripotency. However, embryos lacking LIF, LIFR or gp130 develop normally, at least until mid-gestation. Lif mutants are viable into adulthood, although homozygous females are sterile due to a failure of the uterus to support implantation (Stewart et al., 1992). Liffr−/− embryos die around the time of birth with severe deficits in motor neuron and glial cell populations (Li et al., 1995; Ware et al., 1995). Gp130 mutants exhibit defects in placental, cardiac and haematopoietic development in addition to the nervous system and die between 12 and 18 days post coitum (d.p.c.), depending on genetic background (Nakashima et al., 1999; Yoshida et al., 1996). No evidence for early embryo loss has been reported for any of these mutations. In contrast to the dependency of ES cells on gp130 signalling, therefore, the epiblast in vivo does not appear to rely on this pathway in the course of unperturbed development.

Embryogenesis in mice can be arrested temporarily at the blastocyst stage. This phenomenon of diapause has evolved in certain mammals to overcome sub-optimal conditions for reproduction associated with climate or demands on maternal nutrients due to the presence of a suckling litter (Mantalenakis and Ketchel, 1966; Yoshinaga and Adams, 1966). Under such conditions embryos develop to the hatched blastocyst stage, but then cease development and remain unimplanted in the uterus. In the mouse this state of diapause may persist for several weeks throughout which the blastocysts remain capable of resuming development once restored to an oestrogen-rich environment. Diapause can be induced experimentally by removal of the ovaries after fertilisation. Following ovariectomy embryos progress through blastocyst formation, hatch from the zona pellucida, and then arrest. When transferred to a primed recipient such embryos can implant and develop normally. This alteration of the normal schedule of development necessitates extended maintenance of the epiblast. Interestingly, ES cells were first established from embryos in diapause (Evans and Kaufman, 1981) and this generally appears to facilitate their derivation (Brook and Gardner, 1997).

We have examined Lifr and gp130 mutant embryos for their ability to support maintenance of epiblast cells during embryonic diapause. Whereas normal embryos can produce foetuses after several weeks in diapause, we find that this capacity is completely lost in the mutants. These findings establish a cryptic but critical role for gp130 receptor signalling in the mouse epiblast in vivo and thus provide an explanation for the responsiveness of ES cells to this pathway.

MATERIALS AND METHODS

Embryos
Mice carrying targeted deletions of the gp130 (Yoshida et al., 1996) or Lifr (Li et al., 1995) genes were maintained on both 129 Ola (inbred) and CD-1 (outbred) backgrounds. As no difference was observed in the phenotypes on either background, results have been pooled. Compound heterozygous lines were established by crossing the single heterozygotes on a CD-1 background. Experimental embryos were generated by natural intercross matings of heterozygotes. The morning of detection of a vaginal plug was designated 0.5 d.p.c.

Delaying implantation of embryos and analysis of developmental potential
Embryonic diapause was induced by surgical removal of the ovaries of pregnant female mice at 2.5 d.p.c. and subcutaneous administration of 0.5 mg of Depo provera. Diapause commences 2 days later, just before the normal time for implantation (4.5 d.p.c.). The ability of delayed blastocysts to resume development was investigated by flushing embryos from the uterus at the designated time and transferring them to pseudopregnant recipients at 2.5 d.p.c. Dissections were performed 7 days later (9.5 d.p.c. recipient age). Intact embryos were examined by light microscopy and genotyped by Southern blotting, as described previously (Li et al., 1995; Yoshida et al., 1996). All subsequent genotyping on embryos, ICMs and trophectoderm lysates was performed using polymerase chain reaction (PCR).

PCR genotyping
Gp130 genotypes were determined by PCR using an oligonucleotide located at the gp130 initiation codon 5′-AGATGTCACACCA-AGGATTTGCTA-3′, in combination with oligonucleotides located in the second intron of gp130 5′-CCCCAACCCTTTCATATA-TGGAGGTAG-3′ or in the tk promoter of tk neo 5′-CCG-ACCTGCACTCTGCGTGTTCAAG-3′. This generated products of 300 base pairs (bp) for wild-type and 450 bp for mutant alleles respectively. Embryos or trophectoderm debris were lysed as previously described (Nichols et al., 1998). Amplification was carried out on 1-7 μl of DNA for 35 cycles (following 95°C hot start for 10 minutes) of 94°C, 15 seconds; 60°C, 12 seconds; 72°C, 60 seconds, with a final extension at 72°C for 10 minutes. Reaction products were resolved by agarose gel electrophoresis.

Immunosurgery
Immunosurgical isolation of internal cell populations (Solter and Knowles, 1975) was carried out on freshly flushed delayed blastocysts. Whole anti-mouse antisera was used at 20% (v/v) in phosphate-buffered medium 1 (PB1; Whittingham and Wales, 1969). Embryos were incubated in this at 37°C for 2-3 hours, rinsed in PB1 containing 10% foetal calf serum (FCS), then exposed to 20% rat serum (non-heat inactivated as a source of complement) in PB1 at 37°C for 15 minutes. Embryos were then transferred individually to drops of Glasgow modified Eagle’s medium (GMEM) with 10% FCS under mineral oil for a further hour. Trophoectodermal debris was then separated and transferred into individual tubes containing 10 μl of lysis buffer for PCR genotyping.

SSEA-1 immunofluorescence
Freshly isolated ICMs were rinsed in PB1 with 0.15% BSA and incubated in anti-SSEA-1 (stage-specific embryonic antigen-1) supernatant (1/1000 dilution) at 4°C for 45 minutes. After rinsing in PB1 with BSA they were transferred to FITC-conjugated rabbit anti-mouse IgM (1/10 dilution) for 30 minutes at 4°C. They were then rinsed and observed using a fluorescence microscope. ICMs were then processed separately for genotyping by PCR. The anti-SSEA-1 monoclonal antibody developed by Solter and Knowles was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242 (Solter and Knowles, 1978).

Outgrowth of isolated ICMs in culture
Following immunosurgery residual inside cell clumps were transferred to individual gelatinised wells in 4-well plates containing GMEM + 20% FCS. Outgrowths were monitored daily and cultures were fixed for in situ hybridisation analysis after 5 days in culture.

In situ hybridisation
In situ hybridisation was carried out according to the protocol
described by Rosen and Beddington (Rosen and Beddington, 1993) adapted for use on cell cultures. The digoxigenin-labelled antisense riboprobe corresponds to nucleotides 7-1013 of SPARC (secreted acidic cysteine rich glycoprotein; GenBank accession number 040177).

ICM cell counts

Cell counts were facilitated by disaggregating freshly isolated ICMs into single cells or doublets. Five minutes' incubation in 0.5% pronase in PBS was followed by at least 20 minutes in Ca2+ and Mg2+-free KSOM (Bhatnagar et al., 1995) until the cells could be separated easily.

Detection of dead cells

Hoechst staining was used to identify condensed or fragmented nuclei, indicative of dead cells. Blastocysts undergoing diapause were flushed from uteri, rinsed in PBS, then incubated in 0.05 mM Hoechst in absolute ethanol at 4°C for at least an hour. Before observation they were rinsed in absolute ethanol for 30 minutes, then placed individually into drops of glycerol on a glass slide, each covered with a fragment of siliconised coverslip for fluorescence microscopy. After observation and scoring for dead cells each delayed blastocyst was rinsed in PBS and placed in 10 μl of PCR buffer for genotyping. In subsequent experiments fragmented DNA was detected by TdT-mediated dUTP nick end labelling (TUNEL) according to the manufacturer’s protocol (Roche Molecular Biochemicals).

RESULTS

Gp130/LIFR are required for recovery from embryonic diapause

The capacity of embryos lacking LIFR and/or gp130 to maintain developmental competence when the preimplantation period is extended was investigated by experimental induction of diapause (delayed implantation). Compound heterozygotes for gp130 and Lifr were intercrossed and pregnant females ovariecctomised at 2.5 d.p.c. Thirty two blastocysts were recovered after 6 days in delay and transferred directly to the uter of pseudopregnant females. Twenty-eight implantation sites were dissected 7 days after transfer (equivalent to 9.5 d.p.c. of normal embryo development) and foetuses examined and genotyped. Of 17 conceptuses recovered, 4 were Lifr/−− (Fig. 1). Two of these were normal and 2 abnormal. The remaining 13 embryos carried at least one wild-type allele for both gp130 and Lifr. No gp130 null embryos were recovered (see below). In addition there were 11 empty implantation sites.

Using intercross matings between Lifr heterozygotes the period in delay was extended to 12 days. Of 26 implantation sites produced after transfer of 31 blastocysts, 19 contained normal foetuses, but on genotyping none of these was found to be Lifr/−−. The remaining 7 implantation sites were empty and presumably attributable to null embryos that had implanted in the uterus, but failed to resume development. We conclude that gp130 is required for recovery from even a relatively brief period of diapause and that a requirement for LIFR becomes evident during prolonged diapause.

Gp130−− embryos are unable to resume embryogenesis after diapause

The observation that mutant foetuses could not be recovered from recipient females after uterine transfer of delayed blastocysts could be due to embryo loss during diapause or to inability to reinitiate development. The incidence of empty decidua after transfer suggested that mutant embryos had implanted in the host uterus but failed to develop subsequently (Fig. 1). To confirm this, embryos were flushed from pregnant females of gp130 heterozygous intercross matings after 6 days in diapause and genotyped by PCR. The predicted Mendelian ratio for the three genotypes was obtained (15 +/+, 24 +/−, 13 −−/−−), establishing that gp130−− blastocysts do persist during diapause. Gp130 signalling therefore plays a specific role in maintaining the developmental potential of the embryo during implantation delay.

Pluripotent cells are absent in gp130−− blastocysts after diapause

Internal cell populations were isolated immunosurgically (Solter and Knowles, 1975) from blastocysts generated by heterozygous intercross matings after 6 days in diapause. Expression of the epiblast-specific marker SSEA-1 (Solter and Knowles, 1978) was examined by whole-mount immunofluorescence staining, followed by PCR genotyping. Whereas ICMs from wild-type or heterozygous embryos exhibited a distinct region of fluorescence representing the epiblast component of the embryo (Fig. 2A,B), gp130−− ICMs showed no immunoreactivity (5/5; Table 1). This implies that although gp130−− embryos can survive diapause, the epiblast compartment is not maintained.

Table 1. SSEA-1 immunostaining of ICMs isolated from gp130 heterozygous intercross blastocysts delayed for 6 days

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<th>Genotype</th>
<th>Wt/het</th>
<th>null</th>
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<tr>
<td>Positive</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>5</td>
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Wt/het, ICMs wild type or heterozygous for gp130; null, ICMs homozygous null for gp130.
To examine directly the differentiation potential of the internal cells in \textit{gp130}\(^{-/-}\) blastocysts after diapause, ICMs isolated as described above were cultured under conditions that allow attachment and growth (Nichols et al., 1998). Wild-type and heterozygous embryos generally maintained a prominent mass of cells derived from the ICM with a distinct rind and core structure (62/68). They also gave rise to outgrowing parietal and visceral endoderm cells (hypoblast derivatives; Fig. 2C; Table 2). In contrast, inner cells from homozygous mutant embryos did not exhibit a central mass and produced only dispersed refractile cells of stellate morphology (10/10 of \textit{gp130}\(^{-/-}\) outgrowths; Fig. 2D; Table 2). Subsequent whole-mount in situ hybridisation analysis for Sparc mRNA (Murray et al., 1990; Rothstein et al., 1992) substantiated the morphological identification of these dispersed cells as parietal endoderm, the primary derivative of the hypoblast. Thus, the pluripotent epiblast cells are not maintained during diapause, and the residual ICM is composed entirely of hypoblast or its derivatives.

**Gp130\(^{-/-}\) embryos have reduced inside cell numbers after 6 days of diapause**

ICMs from \textit{gp130}\(^{-/-}\) delayed blastocysts appear smaller than their wild-type or heterozygous littermates (Fig. 2A). This is likely to reflect a reduced number of cells in mutant embryos. To confirm this quantitatively, cells were counted from isolated ICMs from intercross embryos delayed for 2 days and 6 days (Fig. 3). After 2 days in delay there was no significant difference between the number of inside cells in wild type or heterozygous versus homozygous null embryos (\(P>0.1\) by Student’s \(t\)-test). During the following 4 days the inside cell number in wild-type and heterozygous embryos showed an

<table>
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<th>Table 2. Outgrowth of ICMs isolated from \textit{gp130} intercross blastocysts delayed for 6 days</th>
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<tr>
<td>Wt/het</td>
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<tr>
<td>Persistent ICM</td>
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<td>Parietal endoderm only</td>
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Wt/het, ICMs wild type or heterozygous for \textit{gp130}; null, ICMs homozygous null for \textit{gp130}.
apparent modest increase. Conversely, in homozygotes the number of inside cells declined significantly (P<0.002 by Student’s t-test), such that the average inside cell number of homozygous mutant delayed blastocysts is less than half that of wild-type or heterozygous embryos (P<0.001). This suggests that the epiblast cells of gp130−/− embryos may be dying during diapause.

Gp130−/− embryos are subject to increased cell death during delayed implantation

Fluorescent DNA stains can be used to detect dead cells, since their nuclei may appear condensed or fragmented. We therefore applied Hoechst staining to gp130 intercross embryos during delayed implantation. After 3 days of diapause blastocysts were flushed from uteri, stained with Hoechst, scored for the presence of condensed or fragmented nuclei, and subsequently genotyped by PCR. As shown in Fig. 5, significantly more dead cells were detected in embryos lacking gp130 than in wild-type or heterozygous embryos (P<0.002 by Student’s t-test).

These findings were extended using an alternative detection method. Blastocysts from intercross matings were flushed from uteri after 3 days in delay and examined in whole-mount for DNA strand breaks by means of TUNEL. DNA fragmentation is characteristic of cells undergoing apoptosis. Fluorescently labelled cells were counted (Fig. 4), and each blastocyst was subsequently genotyped by PCR. Significantly more labelled cells were detected in mutant embryos than in those of their wild-type or heterozygous littermates (P<0.01 by Student’s t-test; Fig. 5). It is therefore likely that the dead cells detected using Hoechst staining are arising as a result of apoptosis. The greater window for detection of DNA fragmentation compared with the overt morphological appearance of condensed or fragmented nuclei probably accounts for the greater number of TUNEL-positive cells.

**DISCUSSION**

The ability of embryos bearing mutations in the components of the LIF pathway to undergo apparently normal epiblast expansion leading to egg cylinder formation and gastrulation (Li et al., 1995; Stewart et al., 1992; Yoshida et al., 1996) appears in conflict with the central role of gp130 in maintaining embryonic stem cells. This paradox can be resolved by the finding that blastocysts lacking gp130 are unable to resume development after diapause specifically because of a loss of the epiblast component. Absence of epiblast was demonstrated by consistent failure to detect SSEA-1 immunofluorescence in gp130−/− delayed blastocysts, and by the corresponding restricted in vitro differentiation potential in this group of embryos. The loss of the epiblast component was found to occur by cell death with evidence of DNA fragmentation, a diagnostic hallmark of apoptosis. These observations define a function for gp130 in vivo that is consistent with its role in the maintenance of pluripotent ES cells in vitro.

The loss of epiblast identity in gp130−/− embryos after 6 days in delay was indicated by the lack of SSEA-1 immunostaining on freshly isolated ICMs (Fig. 2A,B). These ICMs produced only parietal endoderm in culture (Fig. 2D), whereas ICMs from wild-type or heterozygous blastocysts exhibited a prominent mass of epiblast and outgrew both visceral and parietal endoderm (Fig. 2C). Formation of visceral endoderm...
requires sustained contact with the epiblast (Hogan and Tilly, 1981). Therefore the absence of visceral endoderm in outgrowths from gp130−/− ICMs provides further evidence for the lack of a functional epiblast.

The reduction in the number of ICM cells in gp130−/− embryos during delayed implantation (Fig. 3) implicates cell death rather than inappropriate differentiation as the likely mechanism for loss of the epiblast in gp130−/− embryos during diapause. The elevated numbers of dead cells detected in the gp130−/− embryos compared with their wild-type and heterozygous littermates substantiates this interpretation (Fig. 5). The detection of a number of dead cells in wild-type and heterozygous delayed blastocysts indicates that the cell death observed during normal blastocyst development (Copp, 1978; El-Shershaby and Hinchliffe, 1974) is also ongoing during diapause, albeit at a lower level. Overall there is a modest increase in ICM cell numbers in normal embryos during diapause, as previously suggested by Evans and Kaufman (Evans and Kaufman, 1981). It is striking that we have never observed mutant embryos with more than four dead or dying cells at any one time. Thus the loss of epiblast does not appear to occur simultaneously, but rather to be progressive. Trophoderm cells in the blastocyst have been shown to exhibit phagocytic activity (Rassoulzadegan et al., 2000). It is possible that dead cell debris is rapidly removed by the trophoderm and degraded so that only nascent dead cells are detectable, and a cumulative increase in the number of dead cells therefore is not observed. TUNEL-positive cells appearing in the trophoderm region of delayed blastocysts may also be attributable to trophoderm phagocytic activity.

There are two possible reasons why epiblast cells might enter into apoptosis in the absence of gp130 signalling. One possibility is that gp130 provides a direct cell survival signal. STAT3, which is activated downstream of gp130, has been shown to have anti-apoptotic activity in some circumstances (Hirano et al., 2000). However, although gp130 signalling can enhance ES cell viability under sub-optimal conditions, its main function in these cells in vitro is to suppress differentiation (Smith, 2001). Therefore an alternative explanation for the diapause phenotype could be that, in the absence of gp130, epiblast cells may begin to differentiate inappropriately and that this subsequently results in apoptosis.

During unperturbed development gp130 and Lifr are expressed in the ICM of the blastocyst, and this expression is maintained during diapause (Nichols et al., 1996, and unpublished data). As shown in Fig. 1, some Lifr−/− embryos are able to resume development after 6 days in delay, although embryogenesis may be compromised. After 12 days of diapause, however, no Lifr−/− embryos were found following transfer, although wild-type or heterozygous embryos were still viable. These data indicate that epiblast maintenance during delayed implantation shows a more acute dependence on gp130 than LIFR. This implies that the action of LIF or related cytokines that signal through the LIFR/gp130 heterodimeric complex, are augmented, at least for a limited period, by a cytokine acting through a separate gp130 receptor complex that does not include LIFR. Interleukin 6 (IL6), which acts via gp130 homodimers is a candidate, since IL6 and IL6Rα mRNAs have been detected in blastocysts (Murray et al., 1990; Rothstein et al., 1992). We have previously shown that IL6/sIL6Rα can support both the derivation and propagation of ES cells with similar efficiency to LIF (Nichols et al., 1994; Yoshida et al., 1994).

Diapause is observed in many different mammals. The hormonal mechanisms, growth characteristics, stimuli (lactational, seasonal or nutritional) and potential duration are sufficiently diverse to imply that this phenomenon has been adopted independently during evolution (Renfree and Shaw, 2000). A speculative possibility is that there may be a relationship between the persistence of gp130/LIFR expression and the distinct duration of diapause seen in different species. The finding that gp130 signalling serves a highly evolved adaptive function is consistent with the proposal that an alternative pathway plays the primary role in supporting normal transient propagation of the epiblast (Dani et al., 1998; Lake et al., 2000; Rathjen et al., 1999). It is noteworthy that mouse epiblast cells appear more amenable to conversion into ES cells if they have been subjected to diapause (Brook and Gardner, 1997; Robertson, 1987). This may be related to the activation of dependency on gp130 signalling that could prime the cells for continued self-renewal. However, ES cell derivation is a multifactorial process (Smith, 2001) and imposition of diapause alone is not sufficient in non-permissive mouse strains or other rodents such as the rat.

The results presented here may have implications for attempts to derive and propagate ES cell equivalents from mammals that do not undergo diapause. Primate blastocyst-derived stem cells have been reported not to respond to gp130 cytokines and to be more difficult to propagate than mouse ES cells (Reubinoff et al., 2000; Thomson et al., 1998). However, the generality of these observations is not certain (Schuldiner et al., 2000). It is worth noting that LIFR and gp130 mRNAs have been detected in blastocysts of various species including humans (Geisterler and Gauldie, 1996; Nichols et al., 1996; Sharkey et al., 1995). Therefore, the expression of these components has been conserved, but whether this extends to complete or only partial functionality remains to be determined.

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