Constitutive PtdIns(3,4,5)P₃ synthesis promotes the development and survival of early mammalian embryos

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Mammalian preimplantation embryos develop in the oviduct as individual entities, and can develop and survive in vitro, in defined culture media lacking exogenous growth factors or serum. Therefore, early embryos must generate intrinsic signals that promote their development and survival. In other cells, activation of class I phosphoinositide 3-kinase (PI3K) is a universal mechanism to promote cell proliferation and survival. Here, we examined whether PI3K is intrinsically activated during preimplantation development. Using GFP-tagged pleckstrin homology domains to monitor PtdIns(3,4,5)P₃ synthesis, we show that PI3K is constitutively activated in mouse preimplantation embryos. E-cadherin ligation promotes PtdIns(3,4,5)P₃ synthesis at sites of blastomere adhesion at all cleavage stages. In addition, in culture conditions that promote autocrine signalling, a second pool of PtdIns(3,4,5)P₃ is generated in the apical membrane of early stage blastomeres. We show that constitutive PtdIns(3,4,5)P₃ synthesis is necessary for optimal development to blastocyst and to prevent large-scale apoptosis at the time of cavitation.

KEY WORDS: Preimplantation development, PI 3-kinase, PIP3, E-cadherin, Apoptosis

INTRODUCTION

Early mammalian development consists of a complex series of events by which the fertilized egg develops into a blastocyst capable of implanting in the uterus. Initially, the single-cell zygote develops into a morula through a series of cleavage divisions and compaction. This is followed by the formation of a fluid-filled cavity to produce a blastocyst that contains the first committed cell lineages: the inner cell mass which is the precursor of the embryo proper, and the outer trophectoderm, which will form most of the extra-embryonic tissues (Johnson and McConnell, 2004). Interestingly, preimplantation development can be recapitulated in vitro, in defined culture media lacking growth factors or serum. How early mammalian embryos maintain active proliferation and little cell death in the absence of exogenous growth factors has been a longstanding mystery.

In other cells, a universal mechanism to promote cell proliferation and survival is the activation of phosphoinositide 3-kinase class I (PI3K) by growth factors, hormones or cytokines (Vanhaesebroeck et al., 2001; Hawkins et al., 2006). The PI3K lipid product PtdIns(3,4,5)P₃ binds to pleckstrin homology (PH) domain-containing proteins, such as the master kinase Akt, resulting in the activation or inhibition of a number of downstream effectors (Manning and Cantley, 2007). PtdIns(3,4,5)P₃ is virtually absent in quiescent cells and its synthesis upon receptor stimulation is balanced by its degradation by lipid phosphatases. Thus, PtdIns(3,4,5)P₃ is considered to be a genuine lipid second messenger.

The PI3K/Akt pathway has been suggested to be functional during preimplantation development, although PI3K activation was not formally demonstrated (Bi et al., 2002; Lu et al., 2004; Riley et al., 2005). The mechanisms and spatiotemporal dynamics of PI3K activation in early development are also unknown. In this study, we used GFP-tagged PH domains to monitor PtdIns(3,4,5)P₃ synthesis in early mouse embryos. We show that PtdIns(3,4,5)P₃ production is constitutive, and is necessary for optimal development and survival during the preimplantation period.

MATERIALS AND METHODS

Embryo collection and culture

Female MF1 mice (3- to 4-weeks old) were superovulated by intraperitoneal injection of 7.5 IU pregnant mare’s serum gonadotrophin (PMSG, Intervet) followed, 48 hours later, by 5 IU human chorionic gonadotrophin (hCG, Intervet). Female mice were mated with F1 males at the time of hCG administration, and one-cell embryos were collected 24-26 hours later, in Hepes-buffered KSOM supplemented with amino acids. Embryos were cultured in KSOM (Specialty Media) supplemented with amino acids, in a 5% CO₂ incubator.

For high-density culture, embryos were placed into small drops of KSOM under mineral oil, at a density of one embryo per µl (typically, 10 embryos in 10-µl drops). For low-density culture, embryos were cultured singly in 100-µl drops of KSOM, under oil. For culture without mineral oil, 20-30 embryos were placed into 3 ml of KSOM.

For embryo aggregation, one-cell zygotes were freed of their zona pellucidae by incubation in acidic Tyrode solution, and cultured in groups of 2 or 3. Culture conditions and growth factors were as described (Rogers et al., 2006).

Preparation of cRNA encoding GFP-tagged PH domains

GFP-PHGRP1 in pEGFP-C1 was described previously (Gray et al., 1999; Viard et al., 2004). This construct incorporates a nuclear export signal resulting in the exclusion of GFP-PHGRP1 from the nucleus. GFP-PHGRP1 was subcloned into pcDNA3.1 for cRNA synthesis from the T7 promoter. GFP-PHAkt in pHiro was a kind gift from Tobias Meyer (Stanford, CA, USA). GFP-PHAkt was replaced with GFP to generate GFP-PHAkt and cRNA was prepared from the SP6 promoter of pHiro. cRNAs were prepared and polyadenylated using the mMessage mMachine kit (Ambion), purified using RNeasy columns (Qiagen), and stored at –80°C.
Image acquisition and analysis
Embryos were placed on glass-bottom dishes (MatTek) and confocal images (3.5 μm thick) were acquired with an LSM510meta confocal microscope (Carl Zeiss MicroImaging) using a 40× (1.3NA) oil-immersion lens. The microscope was fitted with an incubator to maintain the temperature at 37°C during image acquisition. Excitation was provided by a 488-nm Argon laser, and GFP and TUNEL fluorescence were collected through a 505-530 band-pass emission filter. Confocal images were analyzed with MetaMorph (Molecular Devices).

Statistical analysis
The distribution of PtdIns(3,4,5)P3 according to culture conditions and the developmental rates of the different populations of embryos were analysed using Fisher’s exact test (two-tailed), using the free online calculator GraphPad QuickCals.

RESULTS AND DISCUSSION
PtdIns(3,4,5)P3 is constitutively produced in early embryos
To detect PtdIns(3,4,5)P3 in living mouse embryos, we expressed a PtdIns(3,4,5)P3-specific GFP-tagged PH domain, GFP-PHGRP1 (Gray et al., 1999; Viard et al., 2004) by injecting the corresponding cRNA (0.18 μg/μl in the pipette) into one-cell zygotes. Embryos were cultured at high density (1 embryo/μl), in a defined medium (KSOM) without exogenous growth factors or serum. Surprisingly, we observed a constitutive production of PtdIns(3,4,5)P3 at all preimplantation stages (Fig. 1).

PtdIns(3,4,5)P3 accumulated at sites of blastomere apposition at all cleavage stages [referred to as junctional PtdIns(3,4,5)P3]. PtdIns(3,4,5)P3 also accumulated at the contact zone between blastomeres and the second polar body (see Fig. 2). In addition, PtdIns(3,4,5)P3 was detected in non-apposing membranes in one-, two- and four-cell embryos [apical PtdIns(3,4,5)P3]. In blastocysts, PtdIns(3,4,5)P3 was detected at cell-cell contacts in both the trophectoderm layer and the inner cell mass, but was absent from the membranes facing the extracellular milieu or the blastocoel cavity.

A consistent feature of PtdIns(3,4,5)P3 dynamics during preimplantation development was the disappearance of apical PtdIns(3,4,5)P3 from the 8-cell stage onwards (Fig. 1). This time frame coincides with blastomeres acquiring apico-basal polarity in preparation for the differentiative cell divisions (Johnson and McConnell, 2004). Recent data suggest that PtdIns(3,4,5)P3 segregation to the basolateral membrane is required for proper differentiation and maintenance of the basolateral surface in epithelial cells (Gassama-Diagne et al., 2006). We are currently investigating whether a similar mechanism operates during blastomere polarization at the 8-cell stage.

To confirm that GFP-PHGRP1 accumulation at the membrane was due to PtdIns(3,4,5)P3 synthesis, we tested the effects of the PI3K inhibitors wortmannin and LY294002. In preliminary experiments, we found that the mineral oil universally used to cover drops of embryo culture medium acts as a sink for these inhibitors, preventing their action (see Fig. S1 in the supplementary material). By contrast, in embryos cultured without mineral oil, PtdIns(3,4,5)P3 production was inhibited at all stages by 100 nM wortmannin and 10 μM LY294002 (see Fig. S1 in the supplementary material; data not shown).

Apical PtdIns(3,4,5)P3 is promoted by high-density culture
Culture of mammalian embryos at high density is known to improve proliferation and survival rates, and overall blastocyst quality. There is substantial evidence that high-density culture promotes the action of autocrine trophic factors at the two-cell stage (Paria and Dey, 1990; Lane and Gardner, 1992; Stoddart et al., 1996; Brison and Schultz, 1997; O’Neill, 1998). The mechanisms of autocrine signalling are still poorly understood. However, it is noteworthy that most of the proposed autocrine factors, such as TGFB, PDGF, IGF/II and PAF (reviewed by Kane et al., 1997), are coupled to PI3K activation in other cells.

Consistent with previous studies, we found that one-cell embryos cultured at low density (one embryo/100 μl) had a poor rate of development to blastocyst (~35% reaching blastocyst after 4 days of culture; n=41) compared with embryos cultured at high density (10 embryos/100 μl; ~75% blastocysts at 4 days; n=46). To test the hypothesis that PI3K activity is promoted by high-density culture, we examined PtdIns(3,4,5)P3 synthesis in two-cell embryos from high- or low-density cultures, using GFP-PHGRP1 (Fig. 2A,B). In high-density culture, the majority of embryos exhibited PtdIns(3,4,5)P3 accumulation in junctional and apical membranes, while a minority displayed only junctional PtdIns(3,4,5)P3. By contrast, in low-density culture, the majority of embryos showed only junctional PtdIns(3,4,5)P3 synthesis (Fig. 2A,B).

Similar results were obtained using another PtdIns(3,4,5)P3 probe, GFP-PHAkt (Fig. 2A,B), which binds both PtdIns(3,4,5)P3 and its metabolite PtdIns(3,4)P2 (Franke et al., 1997; Gray et al., 1999). Therefore, the lack of apical PtdIns(3,4,5)P3 labeling in low-density culture is not due to its conversion to PtdIns(3,4)P2. These data suggest that apical PtdIns(3,4,5)P3 synthesis is promoted by high-density culture, suggesting it originates from autocrine signalling.

 Junctional PtdIns(3,4,5)P3 is generated through E-cadherin signalling
The distribution of junctional PtdIns(3,4,5)P3 suggests that PI3K activation may result from cell-cell adhesion, which in mammalian embryos is mediated by E-cadherin (Ogou et al., 1982; Vestweber et al., 1987; de Vries et al., 2004). To test this hypothesis, we monitored PtdIns(3,4,5)P3 production during manipulation of blastomere adhesion. First, we found that inhibition of E-cadherin ligation, by incubation in a Ca2+-free medium, resulted in the loss of junctional PtdIns(3,4,5)P3 (Fig. 3A). This effect was reversible as Ca2+ readmission restored PtdIns(3,4,5)P3 at cell-cell contacts (Fig. 3A). Secondly, the function-inhibitory anti-E-cadherin antibody...
ECCD-1 (Yoshida-Noro et al., 1984) prevented junctional PtdIns(3,4,5)\(_3\) accumulation (Fig. 3B). Blastomere adhesion and embryo compaction were also inhibited by ECCD-1, demonstrating that E-cadherin function was abolished. Finally, we performed embryo aggregation, which is an E-cadherin-mediated process (Neganova et al., 2000). Aggregation of two-cell embryos resulted in the accumulation of PI3K lipid products at newly formed contact region between two aggregated two-cell embryos. PI3K may become activated following its recruitment to the E-cadherin adhesion complex, as suggested in epithelial cells (Pece et al., 1999). Alternatively, E-cadherin ligation could promote ligand-independent stimulation of receptor signalling, followed by PI3K activation, as shown in ovarian cancer cells (Reddy et al., 2005).

**PtdIns(3,4,5)\(_3\) is necessary for development beyond the two-cell stage**

To examine the effects of chronic PI3K inhibition, one-cell embryos were cultured continuously in the presence of 10 \(\mu\)M LY294002 (or the equivalent amount of DMSO for controls) in 3 ml of medium, in the absence of mineral oil. In these low-density culture conditions, 40% of the control embryos reached the eight-cell stage, but only \(~10\%\) reached the blastocyst stage (Fig. 4A). By contrast, the great majority of embryos treated with LY294002 did not develop beyond the two-cell stage (Fig. 4A). These embryos were negative for TUNEL staining (\(n=25\); data not shown), suggesting that this early developmental arrest was not due to apoptosis. To confirm this early requirement for PtdIns(3,4,5)\(_3\), we injected embryos with a 10-fold higher GFP-PHGRP1 cRNA concentration (1.8 \(\mu\)g/\(\mu\)l; \(10\times\)). Overexpressed GFP-PHGRP1 is expected to compete with endogenous PtdIns(3,4,5)\(_3\) targets and thereby to interfere with PtdIns(3,4,5)\(_3\) signalling (Várnai et al., 2005). Strikingly, most of these embryos failed to develop beyond the two-cell stage during high-density culture (Fig. 4B). By contrast, embryos injected with the low GFP-PHGRP1 cRNA concentration (0.18 \(\mu\)g/\(\mu\)l; \(1\times\)), and embryos overexpressing GFP only (GFP cRNA: 2.0 \(\mu\)g/\(\mu\)l), cleaved and reached the blastocyst stage with a success rate similar to control non-injected embryos (Fig. 4B). These data suggest that PtdIns(3,4,5)\(_3\) synthesis in early embryos is essential for development beyond the two-cell stage. As the two-cell stage is also characterized by a major transcriptional activation of the embryonic genome (Schultz, 2002), we suggest that PtdIns(3,4,5)\(_3\) may control the pattern of gene expression during the maternal-to-zygotic transition, ultimately resulting in improved developmental rate and blastocyst quality.
Fig. 4. PI3K activity promotes early embryo development and survival.
(A) Chronic PI3K inhibition with LY294002 prevents development beyond the two-cell stage. One-cell embryos were cultured continuously in the presence of LY294002 (10 μM, grey) or the equivalent amount of DMSO (black), in 3 ml of medium. Over 100 embryos were scored in each experimental condition. *P<0.0001; #P<0.01. (B) Developmental competence of embryos injected with 0.18 μg/μl (grey bar, 1×) or 1.8 μg/μl (dark green, 10×) of GFP-PHGRP1 cRNA, or GFP cRNA (2.0 μg/μl, light green). Control, non-injected embryos are shown in black. Culture was performed in high-density conditions. The number of embryos examined is indicated on the graph. *P<0.0001. (C) PI3K inhibition with LY294002 prevents the morula-blastocyst transition. Embryos were cultured in high-density conditions until they reached a given stage, before being exposed to LY294002 (without mineral oil). Embryos were checked the next day to examine their progression to the next developmental stage (indicated on the x-axis). Thirty to 50 embryos were scored for each developmental transition. *P<0.0001. (D) 16-32 cell morula recovered from a high-density culture and exposed to 10 μM LY294002 for 20 hours, without mineral oil. TUNEL staining reveals apoptosis in virtually all blastomeres (right panel). The arrowhead indicates a small blastocoel-like cavity. (E) Expanded blastocyst obtained after culturing a 16-32 cell morula in the presence of DMSO for 20 hours, without mineral oil. Little apoptosis was detectable (TUNEL staining, right). (F) Eight-cell embryo cultured in the presence of ECCD-1 from the one-cell stage, in high-density conditions. All blastomeres were negative for TUNEL staining (right). (G) 16-32 cell embryo cultured in the presence of ECCD-1 from the one-cell stage, in high-density conditions, exhibiting extensive TUNEL staining (right). All TUNEL images (D-G) are projections of multiple confocal sections across the whole embryo, and are representative of 12-26 similar observations.

Junctional PtdIns(3,4,5)P_3 prevents apoptosis during the morula-blastocyst transition
To identify other developmental transitions sensitive to PI3K inhibition, embryos were cultured in high-density conditions before being exposed to 10 μM LY294002 for 15-20 hours, at which time transition to the next developmental stage was scored. In these conditions, PI3K inhibition did not affect embryo cleavage and compaction, up to the morula (16-32 cells) stage (Fig. 4C), and did not trigger apoptosis (data not shown). However, 16- to 32-cell morulae exposed to LY294002 experienced a dramatic wave of apoptosis resulting in embryo death before blastocyst formation (Fig. 4C.D). The extent of cell death was such that cell counts were impossible in these embryos. Small blastocoel-like cavities could be observed in a third (n=9/26) of these embryos (see Fig. 4D), suggesting that the apoptotic program was induced around the onset of cavitation. By contrast, control morulae treated with DMSO formed expanded blastocysts (n=26/31) that contained few, if any, apoptotic cells (Fig. 4C.E). To provide further evidence for the anti-apoptotic role of PtdIns(3,4,5)P_3, we inhibited E-cadherin-induced PtdIns(3,4,5)P_3 synthesis from the one-cell stage, using ECCD-1. Interestingly, these embryos underwent successive cleavage divisions (but no compaction) up to the 16-cell stage (day 3), without noticeable lethality (Fig. 4F; data not shown). However, after 3.5 days of culture, at the time when control embryos underwent cavitation, a wave of apoptosis triggered the death of virtually all blastomeres in embryos exposed to ECCD-1 (Fig. 4G). These results suggest that E-cadherin-mediated junctional PtdIns(3,4,5)P_3 synthesis is critically required at the morula-blastocyst transition to prevent widespread apoptosis and embryonic death. This finding could explain why mouse embryos deficient in zygotic E-cadherin die before cavitation (Larue et al., 1994). The apoptotic program in this case appears to be developmentally regulated because, in our experiments, cell death occurred specifically at the morula-blastocyst transition, even though PtdIns(3,4,5)P_3 synthesis was inhibited at earlier stages (using LY294002 or ECCD-1). Blastocyst formation is a critical metabolic transition, as the embryo switches to glucose as the main energetic substrate, and dramatically increases its oxygen consumption (Houghton et al., 1996). Therefore, junctional PtdIns(3,4,5)P_3 synthesis may be primarily required to promote cell survival during this period of high metabolic and oxidative stress.
Conclusion

Sustained PtdIns(3,4,5)P_3 production is a rare phenomenon in normal cells. However, constitutively elevated PtdIns(3,4,5)P_3 levels are frequently observed in transformed cells, providing them with the ability to grow and survive under anchorage-independent conditions (Vivanco and Sawyers, 2002). In this study, we have shown that preimplantation embryos represent a remarkable example of constitutive PtdIns(3,4,5)P_3 synthesis in a physiological context. Using a combination of autocrine and adhesion signals, early embryos generate two pools of PtdIns(3,4,5)P_3 that are vital for optimal development to blastocyst. In this regard, it is noteworthy that mouse embryos lacking PI3Kbeta die before implantation, suggesting a specific requirement for this PI3K isoform during early development (Bi et al., 2002). Further studies of the mechanisms of PtdIns(3,4,5)P_3 signalling in early embryos should provide important advances into our understanding of early mammalian development, which is a major concern for embryo-based technologies in man and domestic animals.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/3/425/DC1

References


