Trophic signals acting via phosphatidylinositol-3 kinase are required for normal pre-implantation mouse embryo development

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Summary

The growth and survival of the preimplantation mammalian embryo may be regulated by several autocrine trophic factors that have redundant or overlapping actions. One of the earliest trophic factors to be produced is embryo-derived platelet-activating factor (1-*O*-alky-2-acetyl-*sn*-glyceryl-3-phosphocholine). The addition of platelet-activating factor to embryo culture media exerted a trophic effect, but structurally related lipids (3-*O*-alky-2-acetyl-*sn*-glyceryl-1-phosphocholine, 1-*O*-alky-*sn*-glyceryl-3-phosphocholine, octadecyl-phosphocholine) had no effect. Platelet-activating factor induced a pertussis toxin-sensitive $[Ca^{2+}]_i$ transient in two-cell embryos that did not occur in platelet-activating factor-receptor null (*Pafr*–/–) genotype embryos. Fewer *Pafr*–/– mouse zygotes developed to the blastocyst stage in vitro compared with *Pafr*+/+ zygotes ($P<0.02$), those that developed to blastocysts had fewer cells ($P<0.001$) and more cells with fragmented nuclei ($P<0.001$). The inhibition of 1-*O*-phosphatidylinositol 3-kinase (LY294002 (3 μM and 15 μM) and wortmannin (10 nM and 50 nM)) caused a dose-dependent inhibition of platelet-activating factor-induced $[Ca^{2+}]_i$ transients ($P<0.001$). The two-cell embryo expressed 1-*O*-phosphatidylinositol 3-kinase catalytic subunits p110α, β, γ and δ, and regulatory subunits p85α and β. LY294002 and wortmannin each caused a significant reduction in the proportion of embryos developing to the morula and blastocyst stages in vitro, reduced the number of cells within each blastocyst, and significantly increased the proportion of cells in blastocysts with fragmented nuclei. The results indicate that embryo-derived platelet-activating factor (and other embryotrophic factors) act through its membrane receptor to enhance embryo survival through a 1-*O*-phosphatidylinositol 3-kinase-dependent survival pathway.

Key words: PAF, PAF-receptor, Phosphatidylinositol 3-kinase, Calcium signalling, Preimplantation embryo

Introduction

There are many aspects of the regulation of the growth, survival and development of the preimplantation mammalian embryo that remain enigmatic. The increasing production of such embryos by assisted reproductive technologies in human medicine, animal production and conservation biology has highlighted the relatively poor viability of embryos produced by such methods, with many embryos failing to survive past the normal time of implantation, making the preimplantation stage one of the most developmentally fragile periods.

The embryo produces its own array of trophic factors and receptors and it has been widely proposed that early embryo development is subject to autocrine, paracrine and endocrine stimulation of cell growth and survival (for reviews see Hardy and Spanos, 2002; Kane et al., 1997; Kaye, 1997). 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (platelet-activating factor, PAF) was the first characterized factor released by the preimplantation embryo to be identified (O'Neill, 1985a; O'Neill, 1985b). It was subsequently shown that PAF also stimulated embryo metabolism (Ryan et al., 1989), cell cycle progression (Roberts et al., 1993) and embryo viability (Ryan et al., 1990; Spinks and O'Neill, 1988; Spinks et al., 1990), thereby providing evidence for an autocrine loop in the early embryo.

Despite the wide experimental support in vitro for a role for trophic factors in the support of preimplantation embryo development, it has been surprisingly difficult to provide conclusive evidence for a physiologically relevant role for these factors. To date there have been no reports of preimplantation embryo lethality resulting from the genetic deletion of putative autocrine trophic factors or their receptors. One possible explanation is that, given the panoply of trophic factors reported to act on the embryo, there is considerable overlap or redundancy in the actions of these autocrine trophic factors such that deletion of any given factor has relatively little impact on development. Any test of this hypothesis requires a detailed understanding of the mode of action of candidate embryotrophic factors.

Because PAF was the first described and, to date, is the best defined of the embryonic trophic factors, we have chosen to

investigate its mechanism of action in detail. There are several reports of an apparent trophic action for PAF on embryos (O'Neill et al., 1989; Roudebush et al., 1996; Stoddart et al., 1996). Although PAF seems to act to enhance cell-cycle progression (Roberts et al., 1993) it did not act as a classical growth factor (one that triggers progression of cells through specific cell-cycle checkpoints) but rather acted to enhance the survival of cells within the embryo (O'Neill, 1998).

A functional PAF-receptor was described that was a single polypeptide composed of 342 (human) or 341 (mouse) amino acids with the structure of a heptahelical transmembrane protein that links to G-proteins (for a review, see Honda et al., 2002). The receptor has wide tissue distribution (Ishii and Shimizu, 2000) and is expressed in the early mouse embryo (Roudebush et al., 1997; Stojanov and O'Neill, 1999). A single receptor subtype is currently thought to mediate all the actions of PAF described (Ishii and Shimizu, 2000). The PAF-receptor in various tissues has been shown to couple with multiple G-proteins apparently activating calcium, cyclic AMP (cAMP) and multiple kinase signalling pathways. Mice with the PAF-receptor gene deleted (*Pafr*–/–) (Ishii et al., 1998) had an absence of surface PAF-receptors in a variety of tissues, had attenuated PAF-induced calcium signal transduction in neutrophils and displayed defects in sperm function in vitro (Wu et al., 2001).

Embryo-derived PAF acts to induce a characteristic transient increase in intracellular calcium ($[Ca^{2+}]_i$) (Roudebush et al., 1997; Emerson et al., 2000). As $[Ca^{2+}]_i$ transients are universal pleiotypic signal transducers, it seems likely that this event is a mechanism for inducing the trophic actions of PAF within the embryo. The calcium transient requires the co-dependent influx of external calcium through a $Ca_v1.2$ ($\alpha_{1\text{-}C}$ L-type) channel (Lu et al., 2003) and release of internal inositol trisphosphate-sensitive calcium stores (Emerson et al., 2000).

This study identifies the known G-protein-coupled receptor as being responsible for the PAF-induced calcium signal transduction in the two-cell mouse embryo and shows that the signal is transduced via the activation of phosphatidylinositol 3-kinase. The actions of the PAF-receptor and the transduction element phosphatidylinositol 3-kinase were necessary for normal development and survival of the preimplantation embryo. The study also showed that the actions of PAF were at least partially redundant to the actions of other autocrine factors. Our results provide direct support for the hypothesis that early embryo survival requires the action of a range of autocrine trophic factors that have overlapping modes of action.

Materials and Methods

Animals

The use of animals was in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and was approved by the Institutional Animal Care and Ethics Committee. The four strains of mice used in particular experiments are indicated in the report for each experiment: Quackenbush outbred strain (QS), F1 (C57BL/6J X CBA/J), PAF-receptor knock-out (*Pafr*–/–) and corresponding wild-type (*Pafr*+/+) (Department of Biochemistry and Molecular Biology, University of Tokyo) (Ishii et al., 1998). The *Pafr*–/– strain were backcrossed to wild-type C57BL/6J mice more than 12 times. All animals were housed and bred in the Gore Hill Research Laboratory, St Leonards, NSW.

Embryo collection and culture

All components of media were tissue culture grade from Sigma Chemical Co. (St Louis, MO). Unless otherwise stated all media were supplemented with 3 mg bovine serum albumin (BSA)/ml (Fraction V, CSL, Melbourne, Victoria, Australia).

Female mice, 6-9 weeks old, were superovulated by intraperitoneal injection of 5 units of equine chorionic gonadotrophin (Folligon, Intervet International, Boxmeer, The Netherlands) followed 48 hours later by 5 units of human chorionic gonadotrophin (hCG) (Chorulon, Intervet). Females were then paired with males of proven fertility. Day 1 of pregnancy was confirmed by the presence of a copulation plug. Mice were killed by cervical dislocation. Embryos were flushed from the reproductive tract using Hepes-buffered modified human tubal fluid medium (Hepes-modHTF: 102 mM NaCl, 4.6 mM KCl, 0.20 mM $MgSO_4$, 0.4 mM KH_2PO_4, 21.4 mM Na lactate, 1 mM glutamine, 0.33 mM Na pyruvate, 2.78 mM glucose, 2.0 mM $CaCl_2$, 4 mM $NaHCO_3$, 21 mM Hepes buffer, pH 7.35; 285 mOsm/l). Embryos were collected at either the zygote or two-cell stage by flushing from the oviducts 20-21 hours or 40-42 hours after hCG. Zygotes were freed of any remaining cumulus cells by brief exposure to 300 i.u. hyaluronidase (Sigma Chemical Co.). Embryos were thoroughly washed in three changes of Hepes-modHTF, recovered in a minimal volume and assigned to various treatments as required in modHTF (same as Hepes-modHTF except that Hepes was replaced with $NaHCO_3$). Embryos were cultured in 10 μl volumes in 60-well HLA plates (LUX 5260, Nunc, Naperville, IL) overlaid by approximately 2 mm of heavy paraffin oil (BDH Laboratory Supplies, Poole). Plates were allowed to equilibrate in the culture incubator for at least 4 hours before addition of the embryos. Embryos were cultured either individually or in groups of ten in each drop.

Calcium imaging

Embryos from QS mice were used for imaging studies except where otherwise indicated. Embryos were washed three times in Hepes-modHTF and incubated with Fura-2 AM (1 μM; Molecular Probes, Eugene, OR) in BSA-free perfusion medium for 30 minutes and then washed three times in BSA-free perfusion medium. Perfusion medium was the same composition as Hepes-modHTF with 3 mg BSA/ml. Embryos were incubated in recombinant plasma-type PAF acetylhydrolase (rPAF acetylhydrolase, 175 μg/ml; ICOS, Bothell, WA) in Hepes-mod HTF for 15 minutes and washed in Hepes-modHTF medium before calcium imaging.

Embryos in BSA-free perfusion medium were placed onto a Cell-Tak® (Collaborative Biomedical Products, Bedford, MA) treated glass coverslip that was attached to the perfusion chamber. The chamber contained ~0.5 ml of medium and was perfused with medium at 37°C at a rate of 1 ml/minute. Treatment drugs were added to the perfusion medium unless otherwise stated.

Because zona-pellucida-free embryos adhered more effectively to the Cell-Tak the zona pellucida was removed by brief treatment with 0.5% pronase, followed by extensive washing in Hepes-modHTF with BSA followed by washing in the same medium lacking BSA. After establishing baseline readings and subtracting background, perfusion was initiated. Relative changes in $[Ca^{2+}]_i$ were measured using fluorescence ratiometric imaging of Fura-2 at excitation wavelengths of 340 and 380 nm. One frame (0.04 seconds) was captured at each wavelength every 5 seconds. The $[Ca^{2+}]_i$ was averaged over an entire two-cell embryo and thus does not reflect the peak calcium concentrations achieved within regions of a cell. Results were recorded with a Panasonic video camera (model WV-BP 310/A) linked to a Macintosh computer via a Pixelpipeline framegrabber. Images were captured and analysed with Ionvision™ software (Improvision, Coventry, UK). All imaging was performed on a Nikon Diaphot microscope using 100 W Xenon illumination, and a ×20 Olympus DPlan Apo UV lens. Up to ten embryos were within the field of view of this objective and were imaged simultaneously.

Intracellular calcium concentrations were calculated using the equations of Grynkiewicz et al. (Grynkiewicz et al., 1985). R_{max} (340/380 nm ratio value at high calcium concentrations) was determined in the presence of 2 mM calcium and ionomycin (1 μM, Calbiochem, Alexandria, NSW, Australia). R_{min} (340/380 nm ratio value in calcium-free conditions) was determined by perfusing with Ca^{2+}-free perfusion medium until a stable baseline was achieved. Calcium-free media had $CaCl_2$ replaced by NaCl and contained 50 μM 1,2-bis(2-aminophenoxyl)-ethane-N,N,N′,N′-tetra-acetic acid tetrakis (BAPTA) (Emerson et al., 2000).

Manganese quench analysis

Analysis of calcium influx channel activity was performed by measuring the quenching of Fura-2 fluorescence at 360 nm by Mn^{2+} as previously described (Lu et al., 2003). Replacement of calcium in extracellular media with Mn^{2+} provides a means of analysing the activation of cation influx channels. Preparation of embryos and imaging of Mn^{2+} quenching was performed in a similar manner to calcium imaging, except that the perfusion medium had $CaCl_2$ replaced with 0.1 mM Mn^{2+} and osmotically adjusted with NaCl. Imaging was performed at excitation wavelengths of 360 nm and the epifluorescence recorded separately as arbitrary units of fluorescence. The imaging chamber containing embryos was initially perfused with calcium and protein-free perfusion media for 60 seconds. The media was then changed to the Mn^{2+} containing media with BSA and perfusion was performed for 50 seconds before the application of PAF (37 nM) in the same media.

Treatments

PAF (Sigma, equal mixture of 1-*o*-octadecyl/hexadecyl-2-acetyl-2-*sn*-glyceryl-3-phosphocholine), enantiomeric-PAF, lysoPAF, octadecyl-phosphocholine (each from NovaBiochem AG, Darmstadt, Germany) was prepared as a 1 mg/ml stock solution in chloroform. Aliquots were removed to a siliconized glass test tube, reduced to dryness under a stream of N_2 and dissolved in perfusion medium to the desired concentration. Glycerol, hexadecanol and acetate (Sigma) were prepared directly in culture media.

Some inhibitors and antagonists were also used (all from Calbiochem). They were initially prepared as 2000-fold concentrated stocks in either perfusion medium or dimethylsulfoxide (Me_2SO) and then diluted to working concentrations in perfusion medium. In all experiments in which Me_2SO was used as a solvent, control medium contained the same concentration of Me_2SO. Stocks of the following agents were prepared in media: cholera toxin; dibutyryl cAMP; genistein; pertussis toxin; while the following AG82; Chelerythrine chloride; forskolin; Lavendustin A; LY294002; LY303511; PP2; staurosporine; and Wortmannin were prepared as stocks in Me_2SO. In calcium imaging studies embryos were pretreated with these agents for 20 minutes (except for pertussis toxin, which was pretreated for 4 hours because of its low membrane permeability) and during PAF challenge. Other drug treatments are as described in the text.

Detection of mRNA for phosphatidylinositol 3-kinase

Evidence for the expression of mRNA for phosphatidylinositol 3-kinase subunits was sought using reverse transcriptase polymerase chain reaction (RT-PCR). Primers were designed and purchased from Sigma-Genosys (Sydney, NSW, Australia). The primers (accession numbers) and the product size were as follows: p110α (accession number NM008839) 5′-ATCTCCACAGCCACACCCTAC, 3′-CATAATTCAGCCATTCATTCCACC, 238bp; p110β (AJ297560) 5′-GCCTTCAACAAAGATGCCC, 3′-CACTATGTCTGTCACCAATCC, 146bp; p110γ (MMU249413) 5′-CACCCCTCAAAAATTTCC, 3′-CTACTCCACTCCTTCCTTC, 111bp; p110δ (AJ292990) 5′-TGAACAAAAGCAACATGGCGG, 3′-CGTAGGTGAGAATGAAGGGGAC, 294bp; p85α (BC002168) 5′-GAGAAAGACAAGAGAACCAATAC, 3′-ATCCTCATCATCTTCTACC, 128bp; p85β (Y13569) 5′-ATGAAGAATACACACGGAC, 3′-CCTTCTCATTTCCCTCTC, 159bp; and β-actin (MMACTBR) 5′-CGTGGGCCGCCCTAGGCACCA-3′ and 5′-GGGGGACTTGGGATTCCGGTT-3.

For all RT-PCR assays the following controls were always undertaken: (1) mouse β-actin was used as a positive control for the effectiveness of RNA extraction and RT-PCR reactions (the β-actin primer pair was designed to span the first intron (87 bp in length) of the rodent β-actin gene, thus contaminating genomic DNA could be detected using these primers); (2) to control for false positive PCR amplification of contaminating genomic DNA, some samples did not include reverse transcriptase; (3) water was added instead of template to test for contamination with extraneous DNA; (4) some samples were randomly treated with RNase I (Promega Corp., Madison, WI) before RT, confirming the RNA origin of positive RT-PCR reactions.

Two-cell F1 embryos were thoroughly washed in five changes of HEPES-modHTF and three changes of PBS. Then they were transferred in minimal volume of six embryos/10 μl into diethylpyrocarbonate (DEPC) treated autoclaved tissue culture grade milliQ water with 1× gold PCR buffer and 1 unit of RNAase inhibitor. Then the cells were lysed by three times repeats of freezing in liquid nitrogen and thawing while vortexing. Isolated RNA was treated with DNase (RQ1 DNase; Promega Corp., Madison WI, USA) to eliminate contamination with genomic DNA, by resuspending the RNA pellet in 20 μl of resuspension solution (Tris-HCl 40 mM, NaCl 10 mM, and $MgCl_2$ 6 mM, pH 7.9) containing 2 units of RQ1 DNase (Promega) and incubated at 37°C for 30 minutes. Following the addition of a second equal volume of resuspension solution, RNA was phenol-chloroform re-extracted. The RNA pellet was dissolved in double-autoclaved Milli-Q water in the presence of RNase Inhibitor (Promega) (final concentration 1 U/ml).

RNA was reverse transcribed at 42°C for 30 minutes with 1.5 units of murine leukaemia virus reverse transcriptase primed with 0.25 μM oligo (dT) in 20 μl of reaction mix containing 3 mM $MgCl_2$, 60 mM KCl, 50 mM Tris-HCl, pH 8.3, 1 mM each dNTP and 1 unit of RNase Inhibitor (all reagents supplied by Perkin-Elmer Life Sciences). The RT reaction was then terminated by heating at 99°C for 5 minutes and cooling to 5°C.

Twenty microlitres of RT reaction volume were used for test sample in a final PCR reaction volume of 50 μl containing 2 mM $MgCl_2$, 10 mM KCl, 50 mM Tris-HCl, pH 8.3, 0.2 mmol each dNTP, 2.5 units of AmpliTaq DNA polymerase and 0.4 μM each of a specific primer pair were subjected to 35 rounds of amplification in a Corbett Thermal Reactor. PCR reaction products were analysed by electrophoresis on 2% agarose gel stained with ethidium bromide to visualize PCR product on a UV transilluminator. Fragments were verified by size and the product extracted and the sequence analyzed to confirm they were from the target gene (ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit from Perkin-Elmer, Foster City, CA, performed by SUPAMAC, Redfern, NSW, Australia).

Cell counts and embryo morphology

The developmental stage and morphology of embryos was assessed by visualizing the embryos with an inverted phase contrast microscope (Nikon Diaphot, Japan) at 24 hour intervals after zygote collection. Cell counts and integrity of nuclei was assessed by visualization of cell nuclei following staining with 4 μg/ml Hoechst dye 33342 (bisbenzimide, Sigma Chemical Co.). Embryos were left in this solution for 40 minutes and then prepared as wet mounts on a glass microscope slide under a coverslip. Nuclei were visualized using mercury lamp ultraviolet illumination and epiflourescence on a Nikon Optiphot microscope with an Olympus DPlanApo 40 UV objective.

Statistical analysis

Statistical analyses were performed on SPSS statistical package (version 11.5, SPSS, Chicago, IL). Comparisons of peak amplitude of $[Ca^{2+}]_i$ responses were by *t*-test or one-way analysis of variance where several drug concentrations were tested. The proportion of embryos developing to the given developmental landmarks following culture in vitro in the presence of drugs was assessed by binary logistic regression analysis, treating the proportion developing to the given landmark as the dichotomous dependent variable and the drug treatment as the categorical covariate and replicate as covariate in the model. Where the combined effects of embryo culture density and genotype were assessed, both density and genotype were treated as covariates and interaction effects were tested in the model. The effect of treatments on the number of apparently normal or fragmented nuclei in the resulting blastocysts were assessed using univariate regression analysis within the General Linear Model. In the model, the effect of treatment on the number of normal nuclei within blastocysts was tested by treating the number of cells with normal nuclei as the dependent variable, drug dose (or genotype) and (where appropriate) embryo density were treated as fixed factor main effects, and the number of cells with fragmented nuclei was incorporated in the model as a covariate. Full factorial analysis was performed with significant factor contrasts performed to assess difference between individual treatments. Effects of treatments on the number of fragmented nuclei was assessed by treating this parameter as the dependent variable and the number of normal nuclei as a covariate.

Results

Supplementing media with PAF enhanced the cell number of two-cell embryos culture in vitro to the blastocyst stage; however, a range of structurally related compounds had no such effect (Table 1). Two-cell QS mouse embryos were cultured for 72 hours in modHTF supplemented with 1 μM of the chemicals shown in Table 1. There was no difference ($P>0.05$) between treatments in the proportion of embryos that formed blastocysts (all greater than 90%); however, PAF caused a marked and consistent increase in the number of cells present in blastocysts. The related phospholipids, enantiomeric PAF (3-*O*-alky-2-acetyl-*sn*-glyceryl-1-phosphocholine) and lysoPAF (1-*O*-alky-*sn*-glyceryl-3-phosphocholine) and the related molecule octadecyl-phosphocholine had no significant ($P>0.05$) effect on the number of cells in the resulting blastocysts. Compounds that form part of the PAF structure (glycerol, hexadecanol, acetate) also had no effect on the number of cells in blastocysts ($P>0.05$). PAF is an ether phospholipid that is capable of modifying membrane structure and function in a receptor-independent manner (Sawyer and Anderson, 1989); it is also a lipid that could conceivably provide a source of nutritional benefit to the embryo (phospholipids do not form a normal part of embryo culture media). However, the strict structural requirements for PAF's observed embryotrophic action argue that PAF's actions were unlikely to be due either to receptor-independent effects on the membrane lipid phase or a nutritional effect.

Table 1. The trophic actions of PAF and structurally related compounds on the number of cells present within blastocysts that were cultured from the 2-cell stage in media which was supplemented with the listed compounds

	Cells per blastocyst (mean±s.e.m.)	
Treatment	Control	Treatment (1μM)
PAF	76.3±8.1	118.0±9.3**
LysoPAF	84.9±15.1	90.6±12.1
Enantiomeric PAF	82.6±10.7	86.8±13.1
Glycerol	80.2±9.6	75.0±5.5
Hexadecanol	80.2±7.3	81.5±7.0
Octadecyl-phosphocholine	86.8±10.1	91.0±6.9
Sodium acetate	75.1±12.1	81.1±8.8

Results are the means and s.e.m. of three replicates with at least 15 embryos in each replicate. **$P<0.01$ compared with corresponding control; other treatments did not differ from their respective controls.

The addition of exogenous PAF (37 nM) caused a transient increase in $[Ca^{2+}]_i$ in two-cell mouse embryos (Fig. 1A), confirming earlier reports (Emerson et al., 2000; Roudebush et al., 1997). It is shown for the first time that these transients were blocked by previous exposure of embryos to pertussis toxin (Fig. 1A), suggesting the requirement of a pertussis-sensitive G-protein-coupled receptor. Two-cell *Pafr*–/– embryos showed no $[Ca^{2+}]_i$ response to PAF challenge, whereas *Pafr*+/+ embryos displayed a characteristic $[Ca^{2+}]_i$ transient (Fig. 1B). This result shows that the G-protein-coupled PAF-receptor that is present in the early embryo was

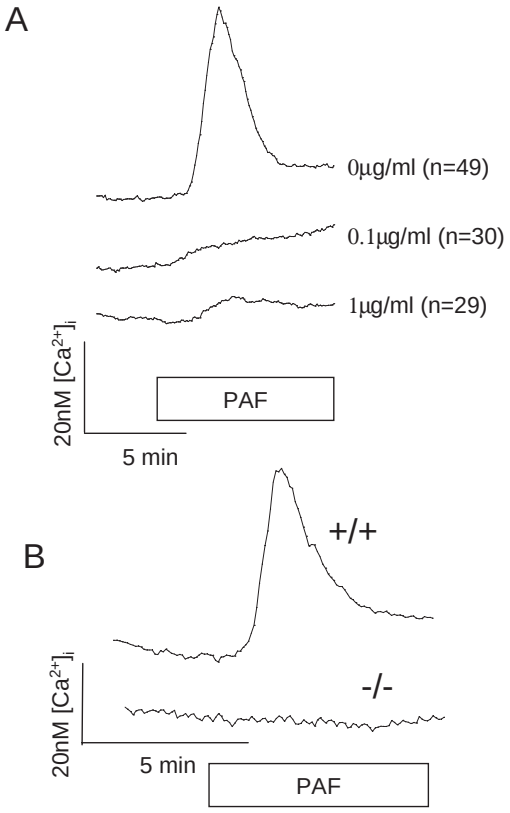

Fig. 1. (A) The effect of pertussis toxin on PAF-induced Ca^{2+} transients in two-cell embryos. Traces show the mean $[Ca^{2+}]_i$ from the number of embryos shown in brackets. The experiments were repeated three times. Embryos were pretreated with the concentration of pertussis indicated and then challenged with 37 nM PAF/ml. (B) The role of the PAF-receptor in PAF-induced calcium transients in two-cell embryos and expression of receptor in human embryos. Two-cell embryos collected from either Pafr+/+ or Pafr–/– parents were exposed to rPAF acetylhydrolase and then challenged with 20 ng PAF/ml. Traces are representative of over 30 embryos from three replicates.

required for signal transduction responses to PAF in the two-cell embryo. *Pafr*–/– zygotes were cultured in vitro and their development compared with *Pafr*+/+ controls (Fig. 2). There was no apparent effect of genotype or density on the development of embryos over the first 48 hours of culture, but by 72 hours development was retarded (P<0.001) as a consequence of both the *Pafr*–/– genotype and low embryo culture density. This effect was more pronounced by 96 hours culture. Fewer *Pafr*–/– embryos developed to the blastocyst stage (P<0.02) (Fig. 2A); those that did develop to blastocysts had fewer normal cells (P<0.001), and more cells with fragmented nuclei (P<0.001) (Fig. 2B). This adverse effect of the *Pafr*–/– genotype was still present, but was less apparent (P<0.001), when the embryos were cultured in groups of ten rather than individually. Despite the additive effects of culture density and genotype, there was no significant interaction effect (P>0.05) between the two factors for either development rate or the number of normal cells present in each embryo. There was, however, an interaction effect (P<0.005) between the genotype and density and the number of cells with fragmented nuclei in each embryo.

A range of inhibitors (Fig. 3) were used to investigate the mode of PAF's induction of $[Ca^{2+}]_i$ transients in the two-cell embryo. The tyrosine kinase inhibitor genistein failed to inhibit (P>0.05) PAF-induced $[Ca^{2+}]_i$ transients at a concentration (25 μM) at which it acts as a selective tyrosine kinase inhibitor, but

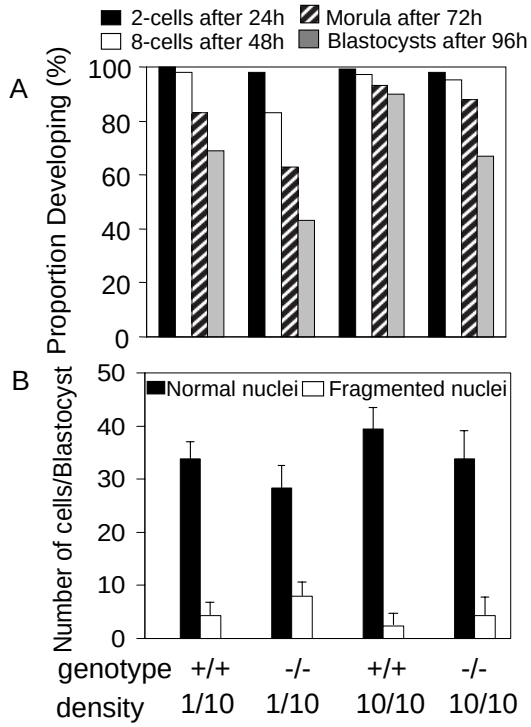

Fig. 2. The effect of Pafr expression and embryo density on the development of zygotes in vitro. Pafr+/+ or Pafr–/– embryos were cultured either individually (1/10) or in groups of 10 (10/10) in 10 μl drops of media for 96 hours. (A) The proportion of embryos achieving expected developmental landmarks at each 24 hour interval was assessed, and (B) for those zygotes that developed to morphologically normal blastocysts after 96 hours culture the number of cells per embryo (mean ± s.d.) with apparently normal or obviously fragmented nuclei was assessed.

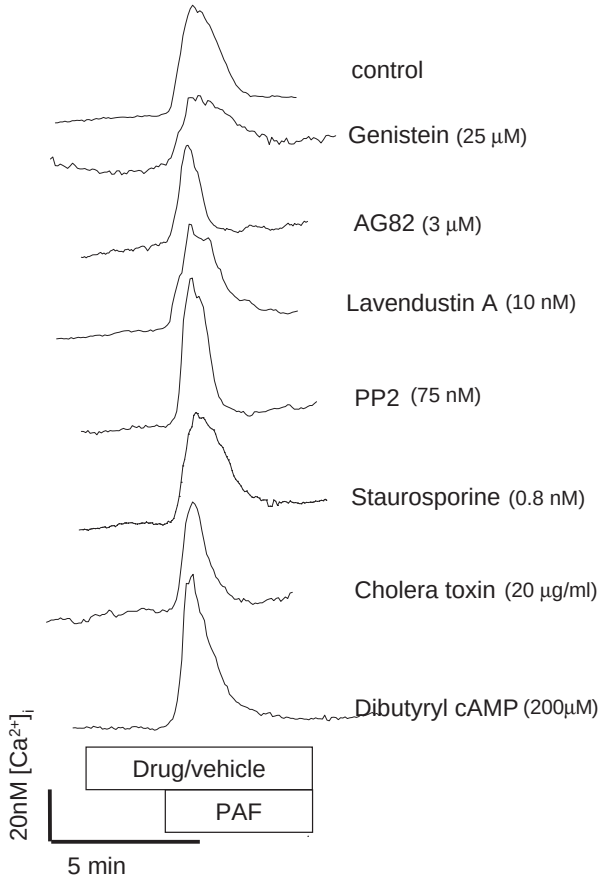

Fig. 3. The effect of inhibitors of various signal transduction agents on PAF-induced Ca^{2+} transients in two-cell embryos. Traces show the mean intracellular calcium of at least 30 embryos from three replicates. Embryos were treated with PAF:acetylhydrolase, then with inhibitors shown for 30 minutes and then challenged with 37 nM PAF/ml in the presence of inhibitor.

did inhibit signalling at higher nonselective concentrations (50-100 μM, not shown). Other tyrosine kinase inhibitors (AG82, 3-75 μM; Lavendustin A, 10-50 nM; and PP2, 0.5 μM) were without effect (P>0.05) on PAF-induced calcium signalling. The protein kinase C inhibitors staurosporine (0.8 nM) (Fig. 3) and chelerythrine chloride (1 μM) (not shown) were also without effect (P>0.05), excluding a role for protein kinase C in calcium signal transduction. However, at a higher (nonselective) concentration (80 nM) staurosporine did inhibit (P<0.001) calcium transients (not shown). Cholera toxin (5-20 μg/ml, not shown) did not prevent PAF-induced $[Ca^{2+}]_i$ transients (P<0.05), whereas the addition of dibutyryl cAMP (200 μM) or forskolin (10 μM) had no effect (P<0.05) on unstimulated cells and no effect on the PAF-induced $[Ca^{2+}]_i$ transients (Fig. 3).

1-*O*-phosphatidylinositol 3-kinase inhibitors (LY294002 and wortmannin) (Fig. 4A) selectively inhibited PAF-induced calcium transients in a dose-dependent manner (P<0.001) but LY 303511 (an inactive analogue of LY 294002) had no effect (P>0.05). Neither drug had any effect on the baseline calcium concentration before PAF challenge. The results shown are for two-cell embryos from F1 strain mice; equivalent results were also obtained from C57BL/6J strain mice (not shown).

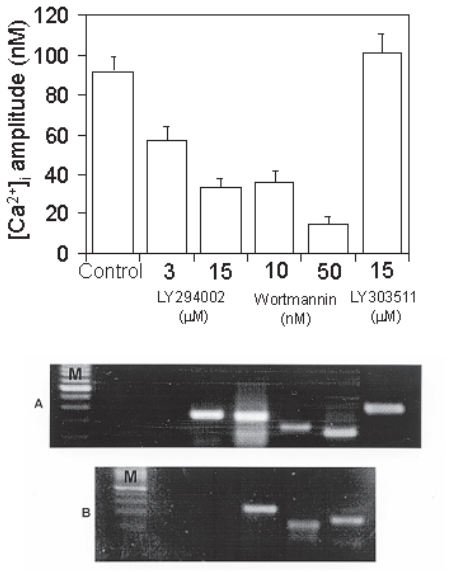

Fig. 4. (A) The effect of inhibitors of phosphoinositide 3-kinase on PAF-induced Ca^{2+} transients in two-cell embryos. The results are the mean and s.e.m. of at least 30 embryos from three replicates. Embryos were treated with PAF:acetylhydrolase, then with the inhibitors and concentrations shown and then challenged with 37 nM PAF/ml in the presence of inhibitors. (B) The expression of phosphoinositide 3-kinase subunits in the two-cell embryo. A: mRNA for P110 catalytic subunits in two-cell embryos and brain tissue. M is molecular weight size markers (GeneRuler DNA Ladder Mix). Lane 1 lacked reverse transcriptase; Lane 2, no template acted as a negative controls; Lane 3, β-actin (243 bp; positive control); Lane 4, p110α (238 bp); Lane 5, p 110β (146 bp); Lane 6, p110γ (111 bp); and Lane 7, p110δ (bp 294). B: mRNA for P85 regulatory subunits in two-cell embryos. M is molecular weight size markers. Lane 1, no reverse transcriptase; Lane 2, no template; Lane 3, two-cell β-actin; Lane 4, P85 α-subunit (128bp), Lane 8 P85, β-subunit (159 bp).

RT-PCR was performed to determine if the F1 two-cell embryo expressed mRNA coding for phosphatidylinositol 3-kinase. Primers for the catalytic subunits p110α, β, γ and δ, and regulatory subunits p85α and β were used and transcripts corresponding to all these gene products were detected in two-cell embryos (Fig. 4B). Transcript identity was confirmed in all cases by DNA sequencing. The results show for the first time that the early mouse embryo expresses multiple phosphatidylinositol 3-kinase isoforms.

It was previously shown that PAF-induced $[Ca^{2+}]_i$ transients required the influx of calcium into two-cell embryos via an L-type calcium channel (Lu et al., 2003). This influx could be measured by replacing calcium in media with manganese and observing the consequent quenching by manganese of Fura-2 fluorescence at 360 nm. LY294002 (5 μM) significantly reduced (P<0.01) and 10 μM completely blocked PAF-induced manganese influx in two-cell embryos (Fig. 5), indicating that activation of this enzyme was required for L-type calcium channel opening.

To assess the role of phosphatidylinositol 3-kinase activation in normal embryo development, F1 zygotes were cultured in the presence of LY294002 (3 μM and 15 μM) (Fig.

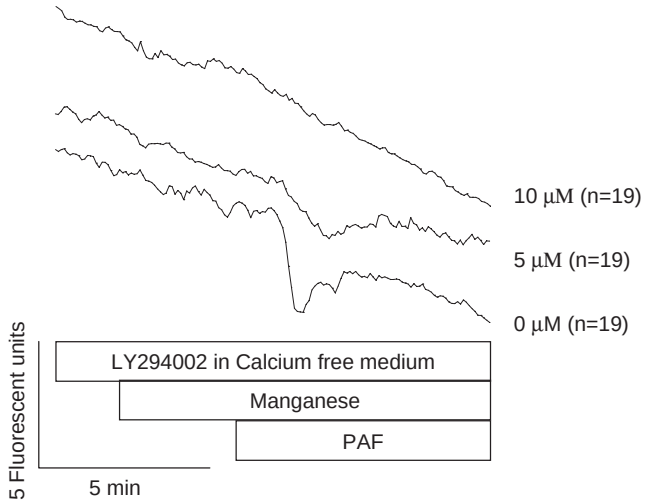

Fig. 5. The role of phosphoinositide 3-kinase in PAF-induced Ca^{2+}-influx as assessed by Mn^{2+}-quenching of Fura-2. The relative fluorescence (arbitrary units) of Fura-2 loaded two-cell embryos measured at 360 nm in perfusion media in which calcium has been replaced with 0.1 mM Mn^{2+}. The lines in each graph show the mean traces from the number of embryos shown in brackets. The boxed areas on the x-axis represent the time interval that the treatments indicated were applied.

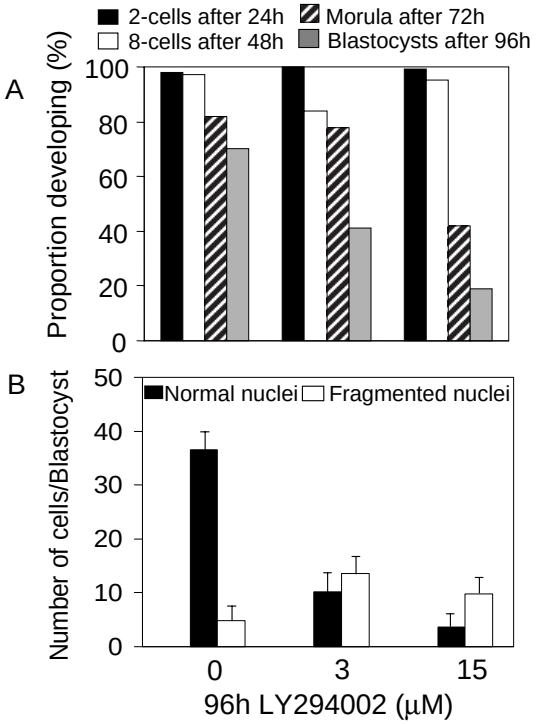

Fig. 6. The effect of the phosphoinositide 3-kinase inhibitor LY294002 on the development of zygotes in vitro. Zygotes were cultured individually in 10 μl drops of media for 96 hours. The three groups of bars in A and B show the results for incubation with 0 μl, 3 μl and 15 μl LY294002, respectively. (A) The proportion of embryos achieving expected developmental landmarks at each 24 hour interval was assessed, and (B) for those zygotes that developed to morphologically normal blastocysts after 96 hours culture the number of cells per embryo (mean ± s.d.) with apparently normal or obviously fragmented nuclei was assessed.

6). There was no effect of LY294002 on the proportion of embryos that developed to morphologically normal two-cell embryos after 24 hours culture. After 48 hours culture 15 μM (P<0.01) but not 3 μM LY294002 (P>0.05) caused a significant reduction in the proportion of embryos that developed to the four-cell stage, and by 72 hours both 3 and 15μM caused a significant reduction in the proportion of embryos developing to the morula stage. The effect of 15 μM was significantly (P<0.05) greater than 3 μM and a similar effect was found for the proportion developing to the blastocyst stage after 96 hours culture in LY294002 (Fig. 6A). There was also a significant effect of drug dose (P<0.001) on the number of cells with normal nuclei present in the embryos that developed to the blastocyst stage (Fig. 6B), and drug dose caused a significant increase in the number of cells within each blastocyst that had fragmented nuclei (P<0.001). The culture of embryos in wortmannin (10 nM and 50 nM) caused similar effects, with a significant dose-dependent inhibition of development of zygotes to the morula and blastocyst stages after 72 hours and 96 hours culture, respectively (Fig. 7A). After 96 hours culture, zygotes that developed to the blastocyst stage had fewer cells with normal nuclei (P<0.001) and more cells with fragmented nuclei (P<0.001) than controls (Fig. 7B). The effect of both the phosphatidylinositol 3-kinase inhibitors on embryo development and survival was more profound than the phenotype observed for the *Pafr*–/– zygotes cultured in vitro. In this regard it is noteworthy that PAF-induced calcium signalling is restricted to the late zygote and two-cell stage (Emerson et al., 2000). Thus, exposure of embryos to the signalling inhibitors throughout the entire preimplantation phase may not be analogous to the situation in *Pafr*–/– embryos. A further experiment was therefore performed whereby zygotes were exposed to LY294002 (a reversible inhibitor) for only 30 hours (throughout the zygote and two-cell stage) and then cultured in the absence of the inhibitor for a further 66 hours (Fig. 8). Such treatment had no effect (P>0.05) on the rate of embryo development for the first 48 hours (Fig. 8A) after treatment but by 72 hours there was a significant (P<0.005) reduction in the proportion of embryos reaching the blastocyst stage. Of those that reached the blastocyst stage (Fig. 8B) there was also a significant decrease in the number of normal cells (P<0.05) and a corresponding increase (P<0.05) in cells with fragmented nuclei. The impact of restricting LY294002 treatment to the first two cell cycles was not as profound as the effect of culture in the drug throughout the entire preimplantation period, but did account for a significant proportion of the developmental retardation induced by inhibition of this survival factor signalling pathway. Inhibition of phosphatidylinositol 3-kinase over this restricted time frame caused a loss of developmental potential that was similar to that observed in *Pafr*–/– embryos.

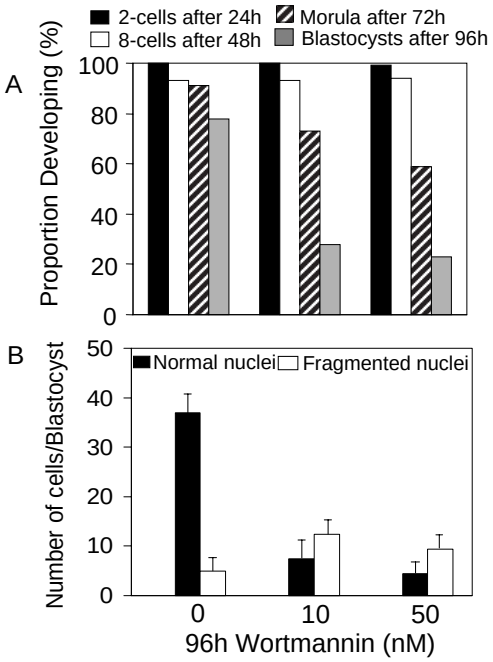

Fig. 7. The effect of the phosphoinositide 3-kinase inhibitor Wortmannin on the development of zygotes in vitro. The zygotes were cultured individually in 10 μl drops of media for 96 hours. (A) The proportion of embryos achieving expected developmental landmarks at each 24 hour interval was assessed, and (B) for those zygotes that developed to morphologically normal blastocysts after 96 hours culture the number of cells per embryo (mean ± s.d.) with apparently normal or obviously fragmented nuclei was assessed.

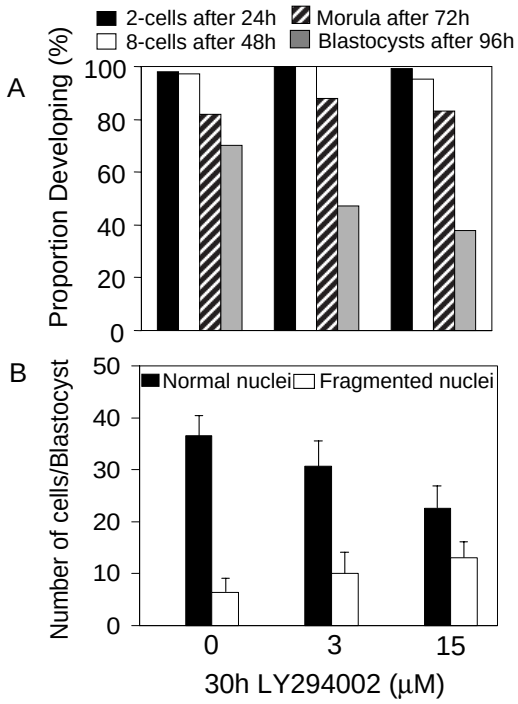

Fig. 8. The effect on zygotes treated with the reversible phosphoinositide 3-kinase inhibitor LY294002 inhibitor (LY294002) for 30 hours, followed by culturing without LY294002 for a further 66 hours. Zygotes were cultured either individually in 10 μl drops of media for 96 hours. The three groups of bars in A and B show the results for incubation with 0 μl, 3 μl and 15 μl LY294002, respectively. (A) The proportion of embryos achieving expected developmental landmarks at each 24 hour interval was assessed. (B) For those zygotes that developed to morphologically normal blastocysts after a total culturing time of 96 hours, the number of cells with apparently normal and also obviously fragmented nuclei was assessed per embryo (mean ± s.d.).

Discussion

Cell survival requires the action of a repertoire of tissue-specific survival factors and it is argued that in the absence of such survival signals cells undergo apoptosis by default (Raff et al., 1994). There is ample evidence that the preimplantation embryo constitutively expresses the machinery for apoptosis (Exley et al., 1999; Jurisicova et al., 1998), and that a range of stresses to the early embryo induce apoptosis (Brison and Schultz, 1997; Hardy, 1999; Jurisicova et al., 1996; Moley et al., 1998). Yet the preimplantation embryo displays autonomous growth and survival in vitro, showing apparently normal development without the need for exogenous proteins or hormones (Whitten and Biggers, 1968). The preimplantation embryo is more resistant to pharmacological activation of the default death programme than somatic cells (and cells from more advanced stages of embryo development) (Weil et al., 1996). It was concluded that the early embryo was able to keep the death programme suppressed in the absence of survival signals (Weil et al., 1996). However, an alternative explanation may be that in the absence of exogenous factors autocrine signalling is responsible for suppressing the death programme.

Many survival factors act via membrane receptors to activate phosphatidylinositol 3-kinase (for a review, see Marte and Downward, 1997), which in turn stimulates cell survival signalling pathways (for a review, see Lawlor and Alessi, 2001). PAF-induced calcium transients were dependent on the expression of the known G-protein-coupled PAF-receptor and were inhibited by pertussis toxin, which phosphatidylinositol 3-kinase was expressed within the two-cell embryo and its action were an essential component of the PAF-induced signal transduction pathway in the two-cell embryo. The absence of the PAF-receptor resulted in a reduced rate of embryo development in vitro, a decrease in the number of cells per embryo and an increase in the proportion of cells in each blastocyst with fragmented nuclei. This phenotype was similar to the effect on embryo development of treatment with phosphatidylinositol 3-kinase inhibitors or by buffering intracellular calcium in vitro (Emerson et al., 2000). These results support a role for embryo-derived PAF acting via its G-protein-coupled receptor to activate phosphatidylinositol 3-kinase-dependent pathways for cell survival.

To our knowledge, this is the first report of the expression of multiple forms of phosphatidylinositol 3-kinase in the preimplantation embryo, of the enzyme's activity within the early embryo and of its role in normal preimplantation stage embryo survival. Deletion of some phosphatidylinositol 3-kinase subunits genes does not result in the death of the preimplantation embryo (Bi et al., 2002), but given the multiple expression of phosphatidylinositol 3-kinase isoforms in the embryo it is unlikely that deletion of just one type would be sufficient to cause lethality within the preimplantation embryo. The effectiveness of LY294002 and wortmannin at low concentrations (where they have high selectivity for phosphatidylinositol 3-kinase (Powis et al., 1994)), the failure of LY303511 (an inactive structural analogue of LY 294002) to inhibit PAF-induced calcium transients, and the failure of inhibitors of several other common cellular kinases to influence PAF-induced signal transduction, argues that the effects of LY294002 and wortmannin were selective for phosphatidylinositol 3-kinase inhibition. The report does not define phosphatidylinositol 3-kinase's targets in the embryo, yet the enzyme is often involved with the activation of L-type calcium channels (Quignard et al., 2001) and phospholipase Cγ (Hiller and Sundler, 2002), and it is shown here that phosphatidylinositol 3-kinase inhibitors blocked PAF-induced calcium influx into the embryo

There is a positive correlation between the amount of PAF released by embryos in vitro and their pregnancy potential following embryo transfer (O'Neill et al., 1987; Vereecken et al., 1990). Furthermore, the production of mouse embryos by in vitro fertilization caused a marked reduction in the release of PAF by those embryos compared with those fertilized in the reproductive tract (O'Neill, 1997). In vitro fertilization embryos also had reduced viability, with an increased death rate of cells within the embryo (O'Neill, 1998). Treatment of embryos with PAF-receptor antagonists (Emerson et al., 2000) or an anti-PAF antibody (Roudebush et al., 1994) caused a similar loss of embryo viability. The similarities of those results with the phenotype of *Pafr*–/– embryos cultured in vitro argues that the *Pafr*–/– phenotype was indeed a consequence of defective PAF signalling. We further show that there was an additive detrimental effect on embryo viability of low embryo culture density and the absence of the PAF-receptor (culture in groups partially compensated for the absence of PAF-signalling). It is argued that this cooperativity may reflect the actions of alternative autocrine trophic factors (Lane and Gardner, 1992; O'Neill, 1997; Paria and Dey, 1990; Salahuddin et al., 1995).

The absence of a functional *Pafr* gene from embryos caused a reduction in the proportion of individual zygotes developing to the blastocyst stage by around 35%. Simplistically, it might be inferred from this result that a single trophic factor such as PAF contributes approximately one third of the trophic drive required for normal early embryo development in vitro. If it is assumed that each embryotrophic factor has a similar contribution to embryo viability as PAF, then the actions of perhaps as few as three of these factors may be sufficient to support normal early embryo development. Given that there are many more than three trophic factors reported to act on the early embryo (for reviews, see Hardy and Spanos, 2002; Kane et al., 1997; Kaye, 1997), it is probable that loss-of-function of several of these factors may be well tolerated by the embryo if sufficient alternative trophic support is present. PAF-induced $[Ca^{2+}]_i$ signal transduction is apparently restricted to the late zygote and two-cell stage. Given this ontogeny, it was of interest to observe that restricting phosphatidylinositol 3-kinase inhibition to the zygote-two-cell stage resulted in less embryopathy then was the case for exposure throughout the preimplantation phase. Indeed, treatment with phosphatidylinositol 3-kinase inhibitors over the first two cell-cycles caused a degree of embryopathy that was similar to that observed in untreated *Pafr*–/– embryos cultured in vitro. The greater embryopathy caused by treatment with phosphatidylinositol 3-kinase inhibitors throughout the entire preimplantation stage raises the not very surprising possibility that the stimulation of phosphatidylinositol 3-kinase activity by PAF-receptor-independent mechanisms (possibly by other trophic/survival factors) during the remaining stages of preimplantation embryo development is also required for normal embryo development.

The study of the growth regulation and survival of the

preimplantation embryo has been a difficult area of study. The current work shows that the combined use of genetically modified mice, in vitro embryo culture, cell biological and pharmacological analysis allows aspects of this problem to be defined. The results show that the preimplantation mouse embryo requires the action of trophic/survival factors, including PAF, that act via receptors to stimulate a phosphatidylinositol 3-kinase-dependent signalling pathway. The attenuation of this pathway leads to a high incidence of embryopathy early in development. Given the increasing importance of the early embryo in a range of medical therapies, from assisted reproductive technologies to the generation of embryonic stem cells for use in cell therapy, such detailed studies of the factors and pathways involved in promoting early embryo growth and survival is warranted. As our results support the conclusion that a considerable degree of overlap in the actions and functions of the trophic factors exists in the early embryo, our report provides a basis for the definition of the mechanism of action of PAF on the embryo, and this should provide valuable insights into the potential common actions of the class of compounds that exert trophic actions on the early embryo.

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