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# Insulin-like growth factor 1 acts as an autocrine factor to improve early embryogenesis *in vitro*

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**ABSTRACT Consideration of embryo-derived growth factors, such as IGF1, is important when culturing an embryo in an *in vitro* fertilization (IVF) setting, or when studying the effect of growth factors on embryo development *in vitro*. Addition of IGF1 to the culture medium has been reported to cause a range of developmental responses in preimplantation mouse embryos. This variability may be due to culture of embryos in suboptimal culture media and at different culture densities/volumes. This study examined the role of exogenous and autocrine IGF1 on mouse preimplantation development *in vitro*, by treatment of embryos with an IGF1R neutralising antibody (IGF1R nAb) under low density (1 embryo/100 μl) or high density (1 embryo/1 μl) culture conditions. At low density, IGF1R nAb reduced development to the blastocyst stage, hatching, and total cell numbers in blastocysts and increased the number of apoptotic cells in blastocysts, suggesting that autocrine IGF1 signalling is occurring, even at low density. This signalling is independent of IGF1 present in the zona pellucida, since culturing embryos in the absence of their zona pellucida had no effect on blastocyst development. Addition of 10 ng/ml IGF1 increased blastocyst development at low density, but decreased hatching at high density. Similarly, high levels of exogenous IGF1 at low density decreased hatching. IGF1 appears to play a role in cell survival and treatment of blastocysts with IGF1 increased Akt phosphorylation. The IGF1R antagonist picropodophyllin was also used in this study, but was found to have non-specific effects on the mitotic spindle. In conclusion, IGF1 is an important growth factor for the improvement of preimplantation development; however, for optimal development the total amount of IGF1 present must be tightly controlled.**

KEY WORDS: *IGF1, embryogenesis, autocrine growth factor*

## Introduction

The preimplantation embryo develops in luminal fluid secreted by the maternal reproductive tract. This fluid contains a large number of maternal and embryo-derived growth factors (reviewed in (Hardy and Spanos, 2002)). Interestingly, preimplantation embryo development can occur in the absence of such factors, in a simple chemically defined medium (Whitten, 1956). However, embryos that develop in this way exhibit a slower rate of cleavage and a higher amount of developmental arrest compared to embryos that develop *in vivo* (reviewed in Summers and Biggers, 2003). Although there has been substantial improvement in media for mammalian preimplantation embryo culture, even the best media currently used may not be optimal and inevitably cause imbalances and stress to the embryos (reviewed in Summers and Biggers, 2003). Growth factors present *in vivo* may enable embryos to attain optimum development not possible in simple, chemically defined medium. Thus the inclusion of one or more growth factors in culture media and their concentration must be carefully considered. The benefit of the presence of growth factors in culture media is highlighted by the culture of embryos in high density compared to low density. Under high density embryo culture conditions, where the embryo is cultured in a small volume of medium or at high embryo concentrations, embryo-derived

*Abbreviations used in this paper:* BSA, Bovine serum albumin; Hepes mod-HTF, Hepes buffered modified synthetic human tubal fluid; hCG, Human chorionic gonadotropin; IGFBP, IGF binding protein; IGF1, insulin-like growth factor 1; IGF1R, Insulin-like growth factor 1 receptor; IGF1R nAb, Insulin-like growth factor 1 receptor neutralising antibody; IVF, *in vitro* fertilisation; KSOM, Potassium simplex optimized medium; KSOM/AA, Potassium simplex optimized medium/amino acids; MAPK, Mitogen activated protein kinase; M II, Mitosis phase 2; NS, No significant difference; PAF, Platelet activating factor; PBS, Phosphate buffered saline; PDGF α, Platelet-derived growth factor- α; PI3K, Phosphoinositide 3-kinase; PMS, Pregnant mares' serum gonadotropin; PPP, Picropodophyllin; TGF-α, Transforming growth factor- α.

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growth factors become concentrated in the medium and then act on the preimplantation embryo to improve development (Lane and Gardner, 1992, Melin *et al.*, 2009, Paria and Dey, 1990). While under low density culture conditions, where the embryo is cultured in a large volume of medium, embryo-derived growth factors are diluted resulting in poor development (O'Neill, 1997, O'Neill, 1998).

IGF1 is one such growth factor which is secreted by the preimplantation embryo and IGF1 and its receptor are present from the oocyte stage and throughout the preimplantation period (Inzunza *et al.*, 2010). In the mouse and rabbit, IGF1 binds to the zona pellucida, the extracellular matrix which surrounds the preimplantation embryo (Herrler *et al.*, 1997, Smith *et al.*, 1993). Removal of the zona pellucida decreases blastocyst development (Suzuki *et al.*, 1995), suggesting that IGF1 found in the zona pellucida may contribute to the available autocrine IGF1.

Although there was a great deal of work on the action of IGF1 in the early embryo in the late 80s and early 90s, this work occurred before the culture density dependence of embryos was realised and when only suboptimal culture media was available. As a result, a range of developmental responses by preimplantation mouse embryos to the addition of IGF1 to the culture medium has been reported. These include increased blastocyst formation, protein synthesis and the number of cells in the inner cell mass or a decrease in apoptosis (Brison, 2000, Harvey and Kaye, 1992, Markham and Kaye, 2003). While others have reported no accelerated rate of development (Heyner *et al.*, 1993, Paria and Dey, 1990), or only an increased rate of hatching (Heyner *et al.*, 1993). Firstly, the interpretation of a negative result needs to be considered carefully as suboptimal culture media has been directly linked to decreases in *Igf1*, *Igf1r*, *Igf2* and *Igf2r* expression (Ho *et al.*, 1995). Secondly, some studies reported that IGF1 improved development at high embryo concentrations (Harvey and Kaye, 1992, Markham and Kaye, 2003), in which activation of autocrine signalling pathways would be expected. Thus the question of whether this positive effect of IGF1 was a result of synergy between growth factors remains. None the less, at least some of the variability reported, in response to IGF1, may be due to the presence of endogenous IGF1 and other embryo-derived growth factors.

This discrepancy in the literature of the effect of exogenous IGF1 warrants new investigation into IGF1 action. In the present study, experiments have been carefully conducted in order to elucidate the role of IGF1 in mouse preimplantation development. The current study utilised potassium simplex optimised medium (KSOM), an optimal culture medium that provides excellent support for mouse embryo development (Erbach *et al.*, 1994). KSOM supports normal expression of some genes involved in metabolism, transcription and cell proliferation including *Igf1*, *Igf1r*, *Igf2* and *Igf2r* (Ho *et al.*, 1995). In the present study the density of culture was varied to determine the role of a physiological concentration of IGF1 on preimplantation embryo development from the zygote stage. Furthermore, the involvement of autocrine IGF1R signalling in preimplantation development was investigated using inhibitors of the IGF1R, namely picropodophyllin (PPP) and an IGF1R neutralising antibody (IGF1R nAB). Since activation of the IGF1R has been shown to decrease apoptosis in preimplantation embryos (Spanos *et al.*, 2000) the effect of IGF1 on apoptosis and pAkt activation were examined.

## Results

### *Effect of IGF1 on development of preimplantation embryos cultured at different densities*

Since the discrepancies in previously reported studies of IGF1 on development may be due to the lack of consideration for culture density, the effect of IGF1 on preimplantation embryos was assessed

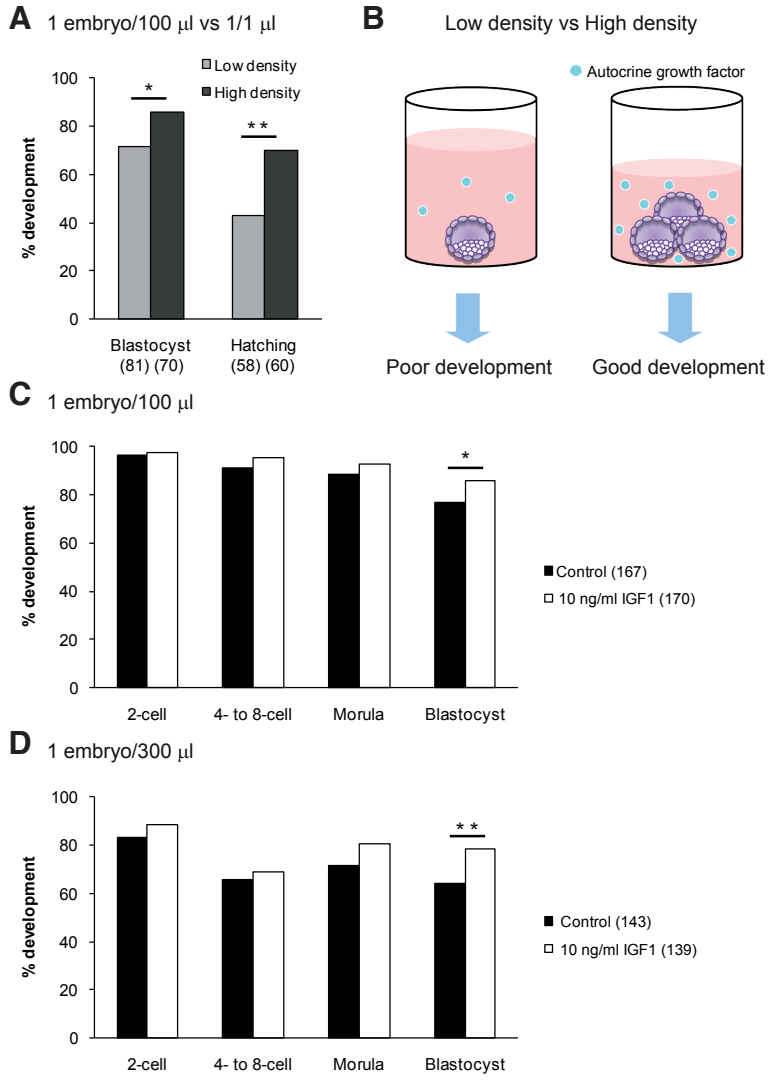



**Fig. 1. Exogenous IGF1 improves development to the blastocyst stage at low density, but not at high density. (A)** *Development of zygotes to the blastocyst stage and proportion of blastocysts hatching after culture at low density (1 embryo/100 µl) or in groups at high density (1 embryo/1 µl).* **(B)** *Schematic showing the two culture densities: low density (1 embryo/100 µl), individual culture and high density (1 embryo/1 µl), group culture. Development of zygotes to the 2, 4-8, morula and blastocyst stage after culture at low density at* **(C)** *1 embryo/100 µl or* **(D)** *1 embryo/300 µl, in the absence or presence of 10 ng/ml IGF1. The results are displayed as the percentage of embryos developed to each stage or hatched from the zona pellucida, pooled from at least three experiments (n values in parentheses). Chi-square analysis was used to compare the control to the treatment groups. * indicates* $P<0.05$ *** indicates* $P<0.01$.

at high density, when autocrine growth factors, including IGF1 are concentrated, and at low density, when autocrine growth factors are diluted. In the present study embryo viability was assessed in terms of blastocyst development, hatching from the zona pellucida, cell numbers and apoptosis. Firstly, we confirmed that culture of embryos at high density significantly increased the percentage of embryos that developed to the blastocyst stage ($P<0.05$) and that hatched from their zonae pellucidae ($P<0.01$), compared to the paired low density control (Fig. 1A).

Culture of zygotes individually in 100 μl or 300 μl of medium in the presence of 10 ng/ml IGF1 caused a significant increase in development to the blastocyst stage (Fig. 1C; $P<0.05$, Fig. 1D; $P<0.01$). No improvement in development was observed prior to the blastocyst stage. Furthermore no effect on hatching was observed in either 100 μl ($P=0.487$, data not shown) or 300 μl ($P=0.466$, data not shown). The number of cells in blastocysts cultured in 100 μl of medium was also determined. IGF1 had no effect on total cell numbers ($P=0.685$; n≥101, data not shown) or cell numbers in the ICM ($P=0.065$; n≥39, data not shown). Furthermore, IGF1 had no effect on the number of apoptotic cells in blastocysts cultured at low density ($P=0.478$; n≥88, data not shown).

### *Effect of high concentrations of IGF1 on blastocyst development*

Treatment of zygotes cultured at high density with 10 ng/ml IGF1 had no effect on development to the blastocyst stage (Fig. 2B; $P=0.820$), or total cell numbers in blastocysts ($P=0.127$, n≥42, data not shown). Embryos treated with IGF1 (10 ng/ml) at high density however, had a decreased level of hatching compared to the control (Fig. 2A; $P<0.05$) but this was not due to an increased number of apoptotic cells in blastocysts ($P=0.796$; n≥42, data not shown).

Previously, treatment of mouse blastocysts with 100 ng/ml IGF1 was shown to down regulate the IGF1R (Chi *et al.*, 2000, Smith *et al.*, 1993) and induce apoptosis (Chi *et al.*, 2000). The effect of 30 and 100 ng/ml IGF1 on development to the blastocyst stage, hatching, cell numbers and apoptosis at low density was investigated. Neither 30 nor 100 ng/ml IGF1 improved blastocyst development (Fig. 2B; $P=0.721$; and $P=0.616$). Instead, both concentrations decreased hatching compared to the control (Fig. 2B; $P<0.01$). 30 and 100 ng/ml IGF1 had no effect on total cell numbers ($P=0.804$; n=19, data not shown), cell numbers in the ICM ($P=0.753$; n≥9, data not shown), or on the number of apoptotic cells in blastocysts ($P=0.669$; n=19, data not shown).

### *Role of autocrine IGF on preimplantation embryo development in vitro*

The role of autocrine IGF1 signalling during early embryo development *in vitro* was examined using an IGF1R nAb to specifically inhibit IGF1 binding to its receptor. Embryos were cultured in the presence or absence of IGF1R nAb (0.1-2 ng/ml IGF1R nAb) at low and high density to specifically inhibit IGF1R signalling. At low density (1 embryo/100 μl) 2 ng/ml IGF1R nAb reduced development to the blastocyst stage (Fig. 3A) and hatching (Fig. 3B) compared to the control ($P<0.05$). This loss of viability occurred from compaction onwards (data not shown). 0.1-1 ng/ml IGF1R nAb had no effect on blastocyst development (Fig 3A) or hatching (Fig. 3B). Total cell numbers in blastocysts cultured at low density were decreased by treatment with 1 and 2 ng/ml IGF1R nAb (Fig. 3C; $P<0.05$). 0.5 ng/ml and 0.1 ng/ml IGF1R nAb had no effect on cell numbers (Fig. 3C; $P>0.05$). 2 ng/ml IGFR nAb increased the number of apoptotic cells in blastocysts cultured at low density (Fig. 4B; $P<0.001$).

Culture of embryos at low density (1 embryo/100 μl) with 2 ng/ml goat IgG had no effect on development to the blastocyst stage ($P=0.369$; n≥51, data not shown), hatching ($P=0.175$; n≥33, data not shown) or cell numbers ($P=0.101$; n≥31, data not shown). Treatment of embryos cultured at high density with 2 ng/ml IGF1R nAb had no effect on blastocyst development, hatching, cell numbers, or apoptosis (data not shown).

### *Effect of IGF1 on Akt phosphorylation in blastocysts*

The best defined pathways by which IGF1R signalling can prevent apoptosis are mediated by phosphoinositide 3-kinase (PI3K) and Akt. Since treatment of embryos cultured at low density with 2 ng/ml IGF1R nAb increased apoptosis in blastocysts, the effect of IGF1 on Akt phosphorylation was investigated by western blotting. Treatment of mouse blastocysts with 100 ng/ml IGF1 increased phosphorylation of Akt after 10 minutes (Fig. 4C).

### *Effect of IGF1R neutralising antibody on blastocyst development in the absence of the zona pellucida*

The role of the zona pellucida in the autocrine action of IGF1 on development was investigated as IGF1 has been shown to bind to the zona pellucida of mouse embryos (Smith *et al.*, 1993). Culturing

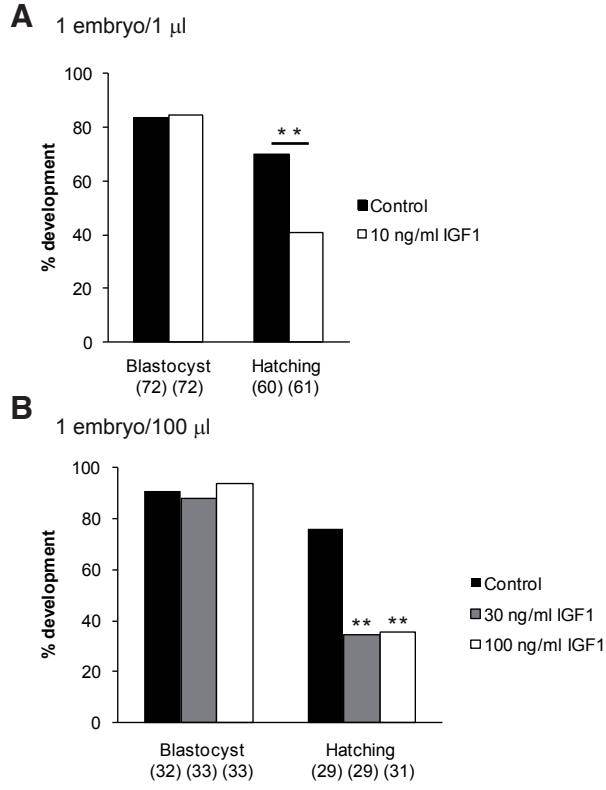
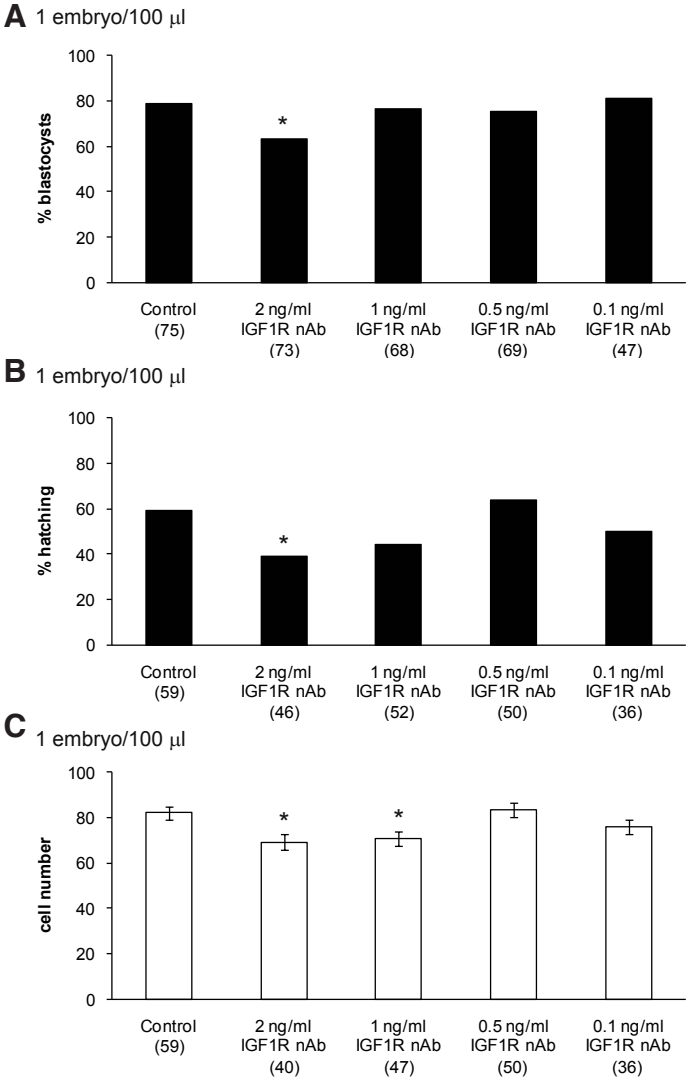



**Fig. 2. High concentrations of IGF1 decrease blastocyst hatching.** *Development of zygotes to the blastocyst stage and proportion of blastocysts hatching after culture* **(A)** *in the absence or presence of 10 ng/ml IGF1 in groups at high density (1 embryo/1 μl) and* **(B)** *individually at low density (1 embryo/100 μl) in the absence or presence of 30 or 100 ng/ml IGF1. The results are displayed as the percentage of embryos developed to blastocyst stage or hatching, pooled from at least three experiments (n values in parentheses). Chi-square analysis was used to compare the control to the treatment groups. ** indicates P<0.01.*

embryos at low density (1 embryo/100 μl) in the absence of their zonae pellucidae had no effect on development to the blastocyst stage (Fig. 5; $P$=0.456). Furthermore, the presence of 2 ng/ml IGF1R nAb still decreased blastocyst development in zona free embryos cultured at low density (Fig. 5; $P$<0.05).

### *Effect of picropodophyllin on blastocyst development in vitro*

The effect of the IGF1R antagonist picropodophyllin (PPP) was investigated as it has been shown to completely inhibit mouse embryo development (Inzunza *et al.*, 2010). The culture of embryos at both low and high density in the presence of varying concentrations of PPP inhibited development and reduced cell numbers (Fig. S1). Since blocking the IGF1R with the neutralising antibody and PPP produced differing results, we investigated whether the greater potency of PPP was due to its non-specific action on the mitotic spindle as reported in other cell types (Kelleher, 1977, Loike *et al.*, 1978). 26 out of 28 zygotes arrested during mitosis when cultured for 24 hours in the presence of 0.25 μM PPP (Fig. 6A).



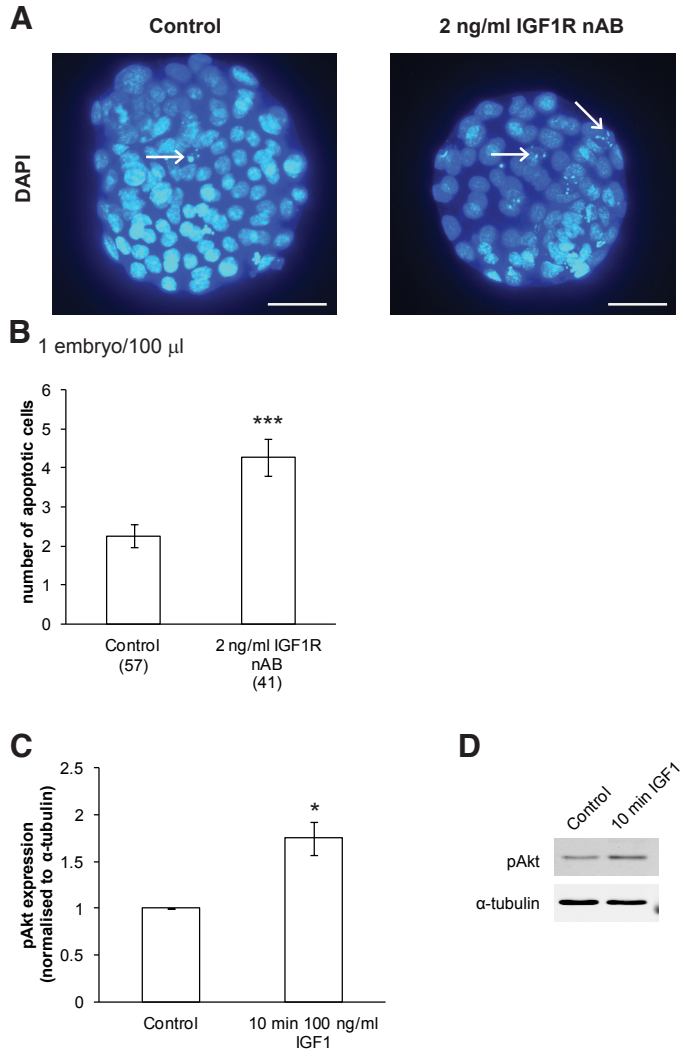




**Fig. 3 (left). IGF1R neutralising antibody decreases development to the blastocyst stage and blastocyst cell numbers.** *Zygotes were cultured at low density (1embryo/100 μl) in the presence or absence of 0.1-2 ng/ml IGF1R nAb. The results are displayed as* **(A)** *the percentage of embryos developed to blastocyst stage,* **(B)** *the percentage of blastocysts hatching and* **(C)** *the average total number of cells per blastocyst ± SEM, pooled from at least three experiments (n values in parentheses). Chi-square analysis was used to compare the development and hatching rate of the control to the treatment groups. One-way ANOVA followed by Dunnett's multiple comparisons test was used to compare cell numbers of the control to the treatment groups. * indicates* $P \leq 0.05$.

**Fig. 4 (right). IGF1 decreases apoptosis by activation of Akt. (A)** *Representative images of blastocysts cultured from the zygote stage in the presence or absence of 2 ng/ml IGF1R nAb. Arrows show examples of apoptotic cells. Scale bars represent 100 μm.* **(B)** *The average number of apoptotic cells per blastocyst ± SEM, pooled from at least three experiments (n values in parentheses).* **(C)** *Effect of 100 ng/ml IGF1 on phospho-Akt levels in blastocysts, detected by western blotting. Data are average p-Akt band intensities (±SEM, n=3) normalised to α-tubulin and are expressed relative to the control.* **(D)** *Representative western blot of blastocysts exposed to 100 ng/ml IGF1 for 10 minutes. Western blots were probed for anti-p-Akt (upper panel) and anti-α-tubulin (lower panel). T-test analysis was used to compare the control to the treatment groups. * indicates* $P<0.05$ **** indicates* $P<0.001$.

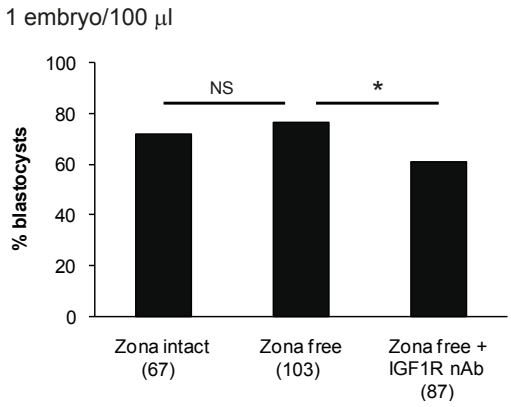



**Fig. 5. Culturing embryos without their zona pellucida has no effect on development to the blastocyst stage.** *Zygotes were treated with pronase to remove their zonae pellucidae and then cultured at low density (1 embryo/100 ml) with or without the addition of 2 ng/ml IGF1R nAb. The results are displayed as the percentage of embryos developed to blastocyst stage, pooled from at least three experiments (n values in parentheses). The development rates between zona free and zona intact or zona free and zona free with 2 ng/ml IGF1R nAb were compared by chi-square analysis. NS indicates not significant, * indicates P<0.05.*

This compares to 29 out of 30 control embryos that had reached the 2-cell stage. Incubation of metaphase II oocytes in medium containing 0.5 μM PPP over a period of 6 hours caused disruption of spindles and dispersal of the chromosomes throughout 100% of oocytes (n=28, Fig. 6B, 6C and Supplementary video S1). All of the control oocytes (n=27), not exposed to PPP, maintained normal spindle structure and had chromosomes that were aligned along the metaphase plate (Fig. 6B).

## Discussion

In the present study the culture of embryos at high density significantly improved the proportion of blastocysts developing and hatching from their zonae pellucidae, compared to those cultured at low density. It is well established that increasing embryo culture density improves embryo viability due to the presence of embryo-derived growth factors that are beneficial to development (reviewed in (O'Neill, 2008)). Under low density culture conditions, embryo-derived growth factors are diluted resulting in poor embryo development (O'Neill, 1997, O'Neill, 1998). Increasing embryo culture density, by culturing embryos in groups (Lane and Gardner, 1992, O'Neill, 1997, Paria and Dey, 1990), or by reducing culture volume (Melin *et al.*, 2009) results in improved embryo development due to the concentration of embryo-derived growth factors. IGF1 is one growth factor that may contribute to the improved development seen at high density, as IGF1 and its receptor are present from the oocyte stage and throughout the preimplantation period (Inzunza *et al.*, 2010). However, various effects of IGF1 on the preimplantation embryo have been reported, possibly due to the use of suboptimal culture media which is known to impact on expression of mRNA encoding the IGFs and IGFRs as well as other trophic factors and their receptors, such as the platelet activating factor receptor (Ho *et al.*, 1995, Stojanov *et al.*, 1999, Stojanov and O'Neill, 2001).

In the present study embryos were cultured in potassium simplex optimized medium (KSOM), a culture medium optimised

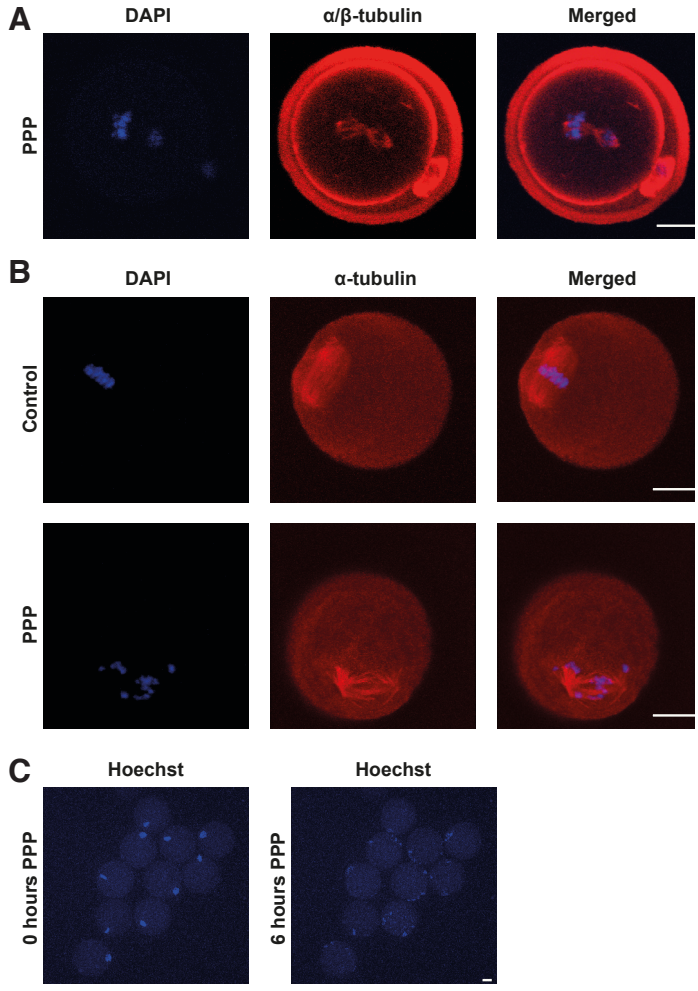



**Fig. 6. Picropodophyllin disrupts the spindle in zygotes and oocytes.** **(A)** *Representative confocal image of a zygote arrested at the 1-cell stage after treatment with 0.25 μM PPP for 24 hours. Anti-α/β-tubulin staining in red and DAPI staining in blue.* **(B)** *Representative 3-D projected confocal image of oocytes cultured in either the absence or presence of 0.5 μM PPP for six hours. Anti-β-tubulin staining in red and DAPI staining in blue.* **(C)** *Oocytes cultured in the presence of 0.5 μM PPP at zero and six hours. Hoechst staining in blue. Scale bars represent 20 μm.*

for mouse zygote development. At low density (1 embryo/100 or 300 μl) the presence of IGF1 significantly increased the percentage of embryos that developed to the blastocyst stage, with IGF1 having its affect after the morula stage. This finding correlates with previous studies by Harvey and Kaye (1992) that show increased blastocyst development from compaction onwards. In low density culture (1 embryo/ 100 μl or 300μl), it was assumed that the level of autocrine growth factors was low. However, our results suggest that there was still sufficient embryo-derived IGF1 to impact on development, since the IGF1R nAb, decreased development to the blastocyst stage, hatching and total cell numbers in blastocysts.

High concentrations of IGF1 are known to cause down-regulation of the IGF1R in mouse blastocysts and can be detrimental to development (Chi *et al.*, 2000). This was confirmed by our study, since high concentrations of IGF1 (30 or 100 ng/ml) in low density culture and addition of exogenous IGF1 to high density culture reduced blastocyst hatching. The concentrations used in this study are

similar to the concentration of embryo-derived IGF1 that has been measured in medium after culture of mouse embryos in groups of 30 for 4.5 days (Inzunza *et al.*, 2010). These results suggest that low concentrations of IGF1 improve embryo development in vitro, while high concentrations are detrimental.

A change in blastocyst hatching was one of the predominant responses to IGF1 observed in this study. Inhibition of IGF1R signalling, with the neutralising antibody and down-regulation of the IGF1R by culture in high concentrations of IGF1, both reduced blastocyst hatching. A variety of mechanisms have been proposed to mediate embryo hatching. It is thought that when a crucial cell number is reached thinning of the zona pellucida occurs (Montag *et al.*, 2000). The presence of the IGF1R nAb increased the number of apoptotic cells in blastocysts suggesting that IGF1 stimulates cell survival pathways in the mouse embryo. This was confirmed by our study as treatment of mouse blastocysts with IGF1 increased phosphorylation of Akt. Akt is also implicated in the hatching process since inhibition of Akt in the blastocyst, decreases hatching (Riley *et al.*, 2005). Thus, the decrease in hatching seen when the IGF1R was inhibited may not be due solely to the reduced number of cells in the blastocyst but also due to reduced Akt activity.

Treatment of embryos cultured at high density with IGF1R nAb had no effect on development to the blastocyst stage, hatching, total cell numbers or apoptosis. It is therefore possible that other growth factors present at high density can overcome the loss of IGF1R signalling, possibly by activating the PI3K/Akt pathway. Most of the proposed autocrine factors, such as TGF$\alpha$, PDGF$\alpha$ and PAF (reviewed in (Hardy and Spanos, 2002)) are known to be coupled to PI3K activation and PI3K activation, is promoted by high-density culture, compared to low density (Halet *et al.*, 2008).

IGF1 has been found to bind to the zona pellucida in the mouse and rabbit (Herrler *et al.*, 1997, Smith *et al.*, 1993). Throughout development the rabbit zona pellucida undergoes a structural transformation (Herrler *et al.*, 1997). IGFBP-3 is incorporated into rabbit zona pellucida during this transformation and has been suggested to act here by regulating the transport and availability of IGF to its receptor on the embryo's plasma membrane (Herrler *et al.*, 1997, Herrler *et al.*, 2002). Although evidence suggesting morphological transformation of the mouse zona pellucida is lacking, removal of the zona pellucida in the mouse has been shown to decrease blastocyst development (at 1 embryo/20 $\mu$l; Suzuki *et al.*, 1995), or have no effect (at 1 embryo/1 $\mu$l) (Ribas *et al.*, 2006), suggesting a possible role of autocrine signalling by the zona pellucida. In the present study no adverse effect on blastocyst development was seen by zona pellucida removal followed by culture at low density (1 embryo/100 $\mu$l). The IGF1R nAb decreased development to a similar level in both zona free and zona intact embryos, suggesting that IGF1 found in the zona pellucida of the mouse does not have a significant role in autocrine growth factor signalling *in vitro*.

In addition to the IGF1R nAb the cyclolignan PPP was also used in this study to inhibit IGF1 signalling. PPP inhibits IGF1R autophosphorylation (Girnita *et al.*, 2004) and has been shown to inhibit mouse embryo development at 0.5 and 0.1 $\mu$M (Inzunza *et al.*, 2010). In the present study PPP caused embryo arrest in a dose dependent manner at both low and high density. These results differ from our IGF1R nAb studies which show that a loss of IGF1R signalling could be overcome by culture at high density. Furthermore, mice carrying null mutations of the IGF1R gene complete gestation (Liu *et al.*, 1993), suggesting that although IGF1R activation improves development, it is not essential. These discrepancies led us to investigate potential non-specific actions of PPP. Podophyllotoxin (PPT), a cyclolignan related to PPP, displays cytotoxicity due to its interaction with $\beta$-tubulin which prevents microtubule assembly ($ID_{50}$= 0.6 $\mu$M), causing mitotic arrest in metaphase (Kelleher, 1977, Loike *et al.*, 1978). PPP has a 50-fold lower inhibitory effect on microtubule assembly ($ID_{50}$= 30 $\mu$M) (Kelleher, 1977; Loike *et al.*, 1978). Addition of PPP to MII oocytes over a period of 6 hours disrupted the spindle causing the chromosomes to disperse throughout the oocytes. Additionally, zygotes treated with 0.25 $\mu$M PPP arrested in metaphase and displayed an abnormal spindle. Therefore, we suggest that the detrimental effect of PPP on embryo development is due to non-specific actions, on the mitotic spindle, rather than its ability to prevent IGF1R signalling.

Although it is known that IGF1 improves embryo survival, the signalling pathways activated in the mouse embryo by IGF1 have not been directly determined. In the bovine pre-implantation embryo IGF1 is reported to have a dual role, as a mitogen, promoting cell proliferation through mitogen-activated protein kinase (MAPK) and as a survival factor, inhibiting apoptosis through activation of the PI3K/Akt pathway (reviewed in (Velazquez *et al.*, 2009)). In the mouse embryo, recent evidence suggests that full activation of the IGF1R requires interaction with E-cadherin (Bedzhov *et al.*, 2012). The present study demonstrates for the first time that IGF1 activates the PI3K/Akt pathway in the preimplantation mouse embryo and thereby improves development by inhibiting apoptosis.

In conclusion, by carefully controlling for the effect of embryo culture density and by using a specific IGF1R blocker (IGF1R nAb) this study explains the discrepancies in embryonic responses to IGF1 that have previously been reported and demonstrates an important role for IGF1 in the preimplantation mouse embryo. More importantly, to ensure optimal development, the total amount of IGF1 in the culture media is critical and the presence of other growth factors also needs to be considered.

## Materials and Methods

### *Media and chemical preparation for embryo collection and culture*

The medium used for embryo collection was Hepes (N-2- Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered modified synthetic human tubal fluid medium (Hepes mod-HTF (mM); NaCl (102), KCl (4.6), $KH_2PO_4$ (0.4), $MgSO_4$ (0.2), $NaHCO_3$ (4), Na-lactate (21.4), Na pyruvate (0.4), glucose (2.8), $CaCl_2$ (2.04), Hepes (21), 0.06 mg/ml penicillin, 0.04% (w/v) phenol red, pH 7.4, ~300 mosM/L) containing 3 mg/ml bovine serum albumin (BSA; Sigma-Aldrich; St Louis, MO, USA). The medium used for embryo culture was potassium simplex optimized medium (KSOM (mM); NaCl (95), KCl (2.5), $KH_2PO_4$ (0.35), $MgSO_4$ (0.2), $NaHCO_3$ (25), $Na_2EDTA$ (0.01), Na-lactate (10), Na-pyruvate (0.2), L-glutamine (1), glucose (0.2), $CaCl_2$ (2.04), 0.06 mg/ml penicillin, 0.04% (w/v) phenol red, pH 7.4; ~260 mosM/L) containing 1 mg/ml BSA. Media was made up from stock solutions prepared from reagents of tissue culture grade (Sigma-Aldrich).

Recombinant mouse IGF1 (R&D Systems; Minneapolis, MN USA) was reconstituted at 100 $\mu$g/ml in sterile phosphate buffered saline (PBS; AMRESCO; Solon, OH, USA). 2 $\mu$l aliquots were prepared and stored at -80ºC. Prior to use, aliquots were diluted to 10 $\mu$g/ml in sterile PBS and then diluted in KSOM to give the desired final concentration.

Human/mouse IGF1R neutralising antibody (IGF1R nAb; AF-305-NA; R&D Systems) was reconstituted at 0.2 mg/ml in PBS. 10 $\mu$l aliquots were prepared and stored at -80ºC. IGF1R nAb was diluted in KSOM to a final concentration of 0.1 to 2 $\mu$g/ml.

Picropodophyllin (PPP; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to a concentration of 5 mM and stored at 4ºC. A 1:10 dilution of this stock in DMSO was made prior to its addition to KSOM. Concentrations of PPP ranging from 0.05 to 0.5 μM were prepared by serial dilution in KSOM.

***Zygote collection***

All procedures involving the use of animals were conducted in accordance with the Australian Code of Practice for Use of Animals in Research and approved by the University of Sydney Animal Care and Ethics Committee. Quackenbush Swiss (QS) mice (Animal Resource Centre, Perth, Australia) were used in this study and housed under a 12-hour light: 12-hour dark cycle. Female mice (4-10 weeks old) were superovulated by intraperitoneal injection of 10 International Units (I.U.) of pregnant mare serum gonadotrophin (PMSG; Intervet, Sydney, Australia) followed by intraperitoneal injection of 10 I.U. of human chorionic gonadotrophin (hCG; Intervet) 48 hours later. They were then paired with a stud male QS mouse (10-30 weeks old) overnight. The presence of a vaginal plug the following day indicated successful mating.

Female mice were euthanized by cervical dislocation 20-22 hours after hCG administration in order to recover zygote stage embryos. Zygotes were isolated from the oviducts into Hepes mod-HTF (37ºC) and then isolated from their surrounding cumulus cells by addition of approximately 100 μl of 1 mg/ml hyaluronidase (Sigma-Aldrich) to the Hepes mod-HTF (3 ml). Zygotes were then collected and washed in at least three changes of KSOM. In some instances embryos were cultured in the absence of their zonae pellucidae. In these cases zygotes, were treated for approximately 3 minutes with pronase (10 mg/ml) in Hepes mod-HTF until their zonae thinned, upon which they were washed in KSOM to completely dissociate the zygote from its zona.

***Embryo culture***

Embryos were cultured from the zygote stage in round bottom 96-well plates (Corning, NY, USA) containing KSOM, pre-warmed to 37ºC and equilibrated at 5% $CO_2$ for a minimum of two hours. High density culture conditions were achieved by culturing embryos in groups of 10-15 at a concentration of 1 embryo per 1 μl medium. Embryos cultured at high density were in contact with one another. Low density culture conditions were achieved by culturing embryos individually in 100 μl or 300 μl medium. Embryos were incubated at 37ºC and 5% $CO_2$. Embryos were scored according to their developmental stage (2-cell, 4-cell, 8-cell, compacted, morula, cavitated and blastocyst) each day at 24 hour intervals. Blastocyst development and hatching from the zona pellucida was noted on day six of development (144 hours post-hCG). The medium was not changed over the six day period.

***Cell number and apoptotic cell determination***

In order to differentially stain inner cell mass and trophectoderm cells, cultured blastocysts were transferred to a 1:10 dilution of anti-mouse lymphocyte antibody (Sigma-Aldrich) in Hepes mod-HTF for 8 minutes at 37ºC allowing the antibody to bind to the trophectoderm cells. Blastocysts were then washed five times in Hepes mod-HTF before being transferred to a 1:10 dilution of guinea pig complement containing propidium iodide (25 μg/ml; Sigma-Aldrich) in Hepes mod-HTF for 4 minutes. Blastocysts were then washed 5 times in Hepes mod-HTF before being fixed in 4% PFA for 15 minutes. Blastocysts were then quickly rinsed in PBS + 1 mg/ml PVA and mounted in 5 μl Vectashield containing 1.5 μg/ml 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

Nuclei were visualised using confocal microscopy (LSM 510 Meta, Carl Zeiss, Germany) using a 405 nm laser and 561 nm laser at 40 X objective. A Z-stack was taken through the embryo, at 2.5 μm intervals, enabling nuclei at all depths to be visualised. Cells were counted manually using LSM Image Browser software (Carl Zeiss). Nuclei stained only with DAPI and not propidium iodide were counted as Inner cell mass (ICM) cells (Fig. S2). For total cell counts and counts of apoptotic nuclei blastocysts were fixed in 4% PFA for 15 minutes, rinsed in PBS + PVA and mounted in 5 μl Vectashield + DAPI. Nuclei were visualised using confocal microscopy (for total cell counts) or epifluorescence (for apoptotic nuclei, Olympus IX51).

***Western blotting***

Blastocysts and morula were isolated from the reproductive tract 94 hours after hCG administration and cultured in KSOM for 24 hours until fully expanded. Blastocysts were then treated with 100 ng/ml IGF1 for 10 minutes, washed in cold PBS + 1 mg/ml PVA and transferred to lysis buffer (Cell Signaling, Beverly, MA, USA) + 1 mM PMSF (Sigma) before being snap frozen in liquid nitrogen. Protein samples were diluted with 6x Laemmli buffer (35 mM Tris-HCl, pH 6.8, 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.05% (w/v) bromophenol blue; Sigma) and heated for 5 minutes at 100ºC. Proteins were electrophoresed on an 8% SDS–polyacrylamide gel, transferred to a nitrocellulose transfer membrane (Hy-BLOT, Australia) and then incubated in blocking buffer (Odyssey; Li-cor Biosciences, Lincoln, NE, USA) overnight at 4ºC with gentle shaking. Membranes were probed overnight at 4ºC in blocking buffer + 0.1% Tween-20 with 1:500 rabbit anti-phospho-Akt (Ser473) (Cell signaling, #4058) or 1:1000 mouse anti-α-tubulin (Sigma, #T9026). Membranes were washed in Tris-buffered saline + Tween 20 (TBST; 10 mM Tris–HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20) and subsequently incubated for 2 hours with either 1:4000 Donkey anti-Rabbit IRDye 800CW (Odyssey) or 1:4000 Donkey anti-Mouse IRDye 680LT (Odyssey). Proteins were visualised using the Odyssey infrared imager.

***Time-lapse microscopy to determine effects of PPP on spindle structure***

Oocytes were isolated from the oviducts of unmated female mice 14-17 hours after hCG administration into Hepes mod-HTF (37ºC). Oocytes were treated with 0.4 nM α-Chymotrypsin (Sigma-Aldrich) in Hepes mod-HTF at 37ºC until their surrounding cumulus cells and zonae pellucidae were removed. Oocytes were incubated with 10 μg/ml Hoechst 33258 (Roche, Indianapolis, IN, USA) in Hepes mod-HTF at 37ºC for 15 minutes. Oocytes were then treated with or without 0.5 μM PPP for 6 hours. Oocytes were imaged over the 6 hour period using the CellVoyager™ CV1000 Confocal Scanner System (Yokogawa, Tokyo, Japan) using 405 nm laser at 5% laser power. A Z-stack was taken through the embryo, at 1.7 μm intervals, every 5 minutes. Oocytes were processed for immunofluorescence at the 6 hour end-point. Additionally a set of control and treatment oocytes were kept in the incubator at 37ºC where they were processed for immunofluorescence at the 6 hour end-point.

***Immunofluorescence of oocytes and embryos***

Oocytes and embryos were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then washed in PBS + poly-vinyl alcohol (PVA). They were then permeabilised with PBS + PVA + 0.3% Triton-X-100 for 15-30 minutes and then washed three times, with the final wash lasting 10 minutes. They were then blocked in PBS + PVA + 0.1% Tween-20 + 0.7% BSA for 15-30 minutes. Primary antibodies were diluted in PBS + PVA + 0.1% Tween-20 + 0.7% BSA. Oocytes were incubated 1:200 mouse anti-β-tubulin (MAB3408; Millipore; Billerica, MA, USA) overnight at 4ºC. Embryos were incubated in 1:100 mouse anti-β-tubulin and 1:100 mouse anti-α-tubulin (T9026; Sigma-Aldrich) overnight at 4ºC. Following primary antibody incubation oocytes/embryos were washed in PBS + PVA + Tween-20 + BSA three times, with the final wash lasting 10 minutes. They were then incubated in secondary antibody (1:200 Alexa Fluor 594 goat anti-mouse IgG; A-11005; Molecular Probes® Invitrogen, Carlsbad, CA, USA) for two hours at room temperature. They were then washed in PBS + PVA + Tween-20 + BSA three times, with the final wash lasting 10 minutes. Then they were transferred to 5 μl Vectashield + DAPI. Nuclei and secondary antibody fluorescence were visualised using confocal microscopy using a 405 nm and Argon laser (458, 477, 488 and 514 nm lines) at 40 X objective.

***Statistical analysis***

Embryos/oocytes were randomly allocated into control and treatment groups. Chi-squared analysis of the effects of culture conditions on embryo development were performed on at least three pooled experiments unless

stated otherwise and compared the control group to the treatment group. Cell counts from at least three pooled experiments were compared using unpaired t-tests for single comparisons or One-way ANOVA followed by Dunnett's multiple comparisons test (performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com), for multiple comparisons to the control. Cell counts were expressed as the mean ± standard error of the mean (SEM). Western blot analysis was performed on groups of 150 embryos (n=3). Densitometry was performed using Image-J software. The optical density of pAkt bands was normalised to $\alpha$-tubulin and expressed relative to the control, followed by statistical analysis using unpaired t-tests.

*Declaration of interest*

*The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.*

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