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Research article

IGF-1/IGFBP-1 increases blastocyst formation and total blastocyst cell number in mouse embryo culture and facilitates the establishment of a stem-cell line

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Abstract

Background: Apoptosis occurs frequently for blastocysts cultured *in vitro*, where conditions are suboptimal to those found in the natural environment. Insulin-like growth factor-1 (IGF-1) plays an important role in preventing apoptosis in the early development of the embryo, as well as in the progressive regulation of organ development. We hypothesize that IGF-1 and its dephosphorylated binding protein (IGFBP-1) may be able to improve embryo culture with an associated reduced cell death, and that the resultant increase in the total cell number of the embryo could increase the chances of establishing an embryonic stem-cell line.

Results: *In vivo* fertilized zygotes were cultured in medium containing supplementary IGF-1, or IGFBP-1/IGF-1. The stages of the resultant embryos were evaluated at noon on day five post-hCG injection. The extent of apoptosis and necrosis was evaluated using Annexin V and propidium iodine staining under fluorescent microscopy. The establishment of embryonic stem-cell lines was performed using the hatching blastocysts that were cultured in the presence of IGF-1 or IGFBP-1/IGF-1. The results show that the rate of blastocyst formation in a tissue-culture system in the presence of IGF-1 was 88.7% and IGFBP-1/IGF-1 it was 94.6%, respectively, and that it was significantly greater than the figure for the control group (81.9%). IGFBP-1/IGF-1 also resulted in a higher hatching rate than was the case for the control group (68.8% vs. 48.6% respectively). IGF-1 also increased the number of Annexin V-free and propidium iodine-free blastocysts in culture (86.8% vs. 75.9% respectively). Total cell number of blastocyst in culture was increased by 18.9% for those examples cultured with dephosphorylated IGFBP-1/IGF-1. For subsequent stem-cell culture, the chances of the successful establishment of a stem-cell line was increased for the IGF-1 and IGFBP-1/IGF-1 groups (IGF-1 vs. IGFBP-1/IGF-1 vs. control: 45.8% vs. 59.6% vs. 27.3% respectively).

Conclusion: IGF-1 or dephosphorylated IGFBP-1/IGF-1 supplement does result in an anti-apoptotic effect for early embryo development in culture, with a subsequent increased total cell

number resulting from cell culture. The effect is beneficial for the later establishment of a stem-cell line.

Background

For the new generation of assisted reproductive technology, it has been previously suggested that in order to decrease the frequency of ovarian hyperstimulation syndrome and resultant multiple pregnancies, the dosage of gonadotropin used needs to be reduced to lessen the follicle stimulation [1,2]. Accordingly, the number of oocytes retrieved and the number of embryos transferred via IVF (*in vitro* fertilization) get decreased. Under such a condition, strategies such as attempting to elicit an increase in the number of viable embryos *in vitro* and a reduction in apoptosis in order to attempt to improve the overall quality of embryos may contribute to this challenge.

Apoptosis occurs as natural cell death during embryonic development.[3]. An increased rate of apoptosis has been observed for mouse and human blastocysts cultured *in vitro* in response to specific culture conditions which are deemed to be suboptimal to those arising in the natural environment featuring complete and stable autocrine and paracrine systems [4–7]. In order to improve the results of *in vitro* embryo culture, practitioners need concern themselves with three major factors: 1) the recognition of appropriate apoptotic regulators [3,6,8], 2) the potential for reduced cell death [3,7,8], and 3) the recognition of appropriate maturation regulators [9,10]. Apoptotic regulators include the ICE (interleukin-1b converting enzyme) family, the family of caspases, and bcl-2 and related genes, eg Bax [7,11,12]. In a number of previous studies, the potential beneficial factors for embryo development have been reported to be IGF-1, IGF-2, EGF, β-fibroblast growth factor (β-FGF), transforming growth factor-α (TGF-α), TGF-β, leukemia inhibitory factor (LIF), and interleukin-6 (IL-6).[3,7,8,11–14]. The potential candidates for a role as anti-apoptotic regulators in embryo culture and selected to reduce the rate of embryonic cell death include TGF-α, IGF-1, IGF-2, insulin, and EGF [3,8,12,14]. The objective of this study is to evaluate the effect of IGF-1 and its cofactor, dephosphorylated IGFBP-1, upon the preimplantation development of mouse embryos and on the subsequent influence in the establishment of embryonic stem-cell lines.

IGF-1 is a single-chain polypeptide of 70 amino-acid residues cross-linked by three disulfide bridges [15]. This entity IGF-1, also known as somatomedin C, is secreted from the liver into the general circulation in a process regulated by pituitary growth hormone, it therefore mediates the growth-promoting activity of growth hormone (GH) [16]. Further, IGF-1 may act as a mitogenic factor for a variety of cells and is able to elicit a control over cell proliferation and differentiation [8,12,13], and IGF-1, as a member of the IGF family, also elicits a very special and complex autocrine and paracrine effect upon normal ovarian function.[4,5,10]. The ovarian regulation mediated by IGF-1, IGF-1R and IGFBP-1, the so-called "intraovarian IGF-IGFBP system" plays a significant role in the processes of follicular development, oocyte maturation and ovulation, and, ultimately, even the development of the embryo [17–19]. Furthermore, the regulation of IGF-1 through IGFBP-1 makes a comprehension of the effect of IGF-1 more complicated than would appear to be the case at first glance; IGFBP-1, as a transport vehicle for IGF-1, controls the distribution of IGF-1 and the action of IGF-1 at a cellular level [20,21]. Further, IGFBP-1 may elicit both restricting and augmenting effects upon the action of IGF-1, depending upon the degree of phosphorylation of IGFBP-1 [20]. Dephosphorylated IGFBP-1, reflecting a decreased affinity for IGF-1, acts as reservoir for IGF-1 and stimulates the activity of IGF-1-induced DNA synthesis [20].

The activity of the IGF system plays a role in embryo development [7,19,22–25]. Kleffens et al. (1999) reported that the expression of IGF system components preceded the occurrence of apoptosis during mouse embryo development and organ-specific development [26]. Further, IGF-1 also prevents the likelihood of induced apoptosis due to the inhibition of caspase-3-like activation and Bcl-2 and Bax expression [13]. Therefore, IGF-1 does play an important role in the prevention of apoptosis in early embryo development and the onward regulation of organ development. We hypothesize that the anti-apoptotic effect of IGF-I and/or its cofactor, IGFBP-1 upon blastomeres, and the reduced likelihood of cell death during blastocyst formation could contribute to an improvement in the results of *in vitro* embryo culture with reduced cell death rather than the proliferation of cells. Therefore, the possible increased total cell number of embryos in culture with IGF-1 or IGFBP-1/IGF-1 complex could benefit the subsequent embryo culture for the establishment of embryonic stem-cell lines (Figure 1).

Table 1: The effect of IGF-1 upon the developmental competence of *in vivo* fertilized mouse zygotes.

Culture medium	Total n	Zygotes arrested in one-cell to morula stage (%)	Zygotes developed to Blastocyst (%)	
			Blastocyst (%)	Hatched blastocyst (%)
kSOM/AA with IGF-1	106	12(11.3)	94(88.7) *	
			43(40.6) **	51(48.1) **
kSOM/AA without IGF-1	83	15(18.1)	68(81.9) *	
			31(37.3) **	37(44.6) **

IGF-1: 100 ng/ml. The results were pooled from 5 repeats of experiments. * P = 0.043 ** P > 0.05

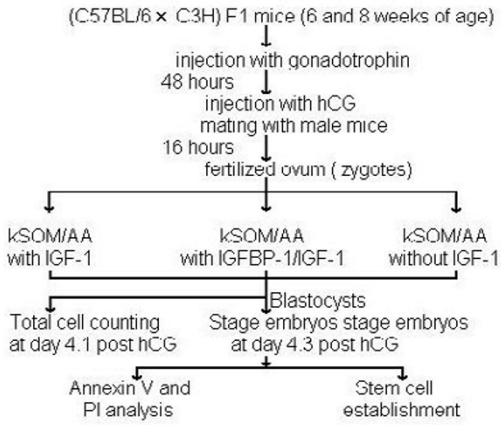

Figure 1
The scheme of conducted experiments

Results

The effect of IGF-1 upon in vivo fertilized mouse zygotes (Experiment-1)

The development of in vivo *fertilized zygotes collected from six-to-eight-week-old mice in different culture media*

The results indicated below are a composite pooled from five repeats of our tissue culture experiments involving the in vivo fertilized mouse zygotes. The cleavage and blastocyst formation rates for the embryos were evaluated daily under an inverted microscope until day 4.3 post-fertilization. The results show that the proportion of embryos that arrested from the one-cell stage to the morula stage for the experimental group (11.3 %) was lower than was the case for the control group (18.1 %). A significant increase in the blastocyst formation rate for the IGF-1-supplemented group as compared to the control group (88.7% vs. 81.9% respectively; p = 0.043) was also noted, although the hatching rate for blastocysts did not appear to differ significantly between the two groups (Table 1).

The evaluation of the level of necrosis and apoptosis for those embryos cultured in different media

A total of 106 and 83 *in vivo* zygotes were subjected to IGF-1-supplemented medium (IGF-1 group) and control medium (control group), respectively. All embryos, including those arrested at different stages of cleavage and those that had developed into the blastocyst stage were subjected to propidium iodide (PI) and Annexin V staining, and then examined under epifluorescence microscopy.

The results are illustrated in Table 2, from which it can be seen that a significant increase in the level of Annexin V- and PI-free embryos arose for the IGF-1 group as compared to the control group (86.8% vs. 75.9%; $p < 0.05$). Twelve (11.3%) embryos arrested prior to blastocyst formation for the IGF-1-supplemented group, and five (4.7%) of these demonstrated PI-positive cells. All of the Annexin V-positive and PI-negative embryos (n = 5) also arrested prior to the blastocyst stage (Tables 1 &2). The arrested stages for these embryos varied widely, ranging from a two-cell stage to the morula stage. Two cleaved embryos arrested at the eight-cell and morula stages, respectively, and none of the blastocysts from the IGF-1 group exhibited positivity for either Annexin V or PI stains.

Amongst the control group, there were 15 embryos which arrested prior to the blastocyst stage, of which about one half (7/15) were PI-positive (Figure 2B) whilst the other half (8/15) were Annexin V-positive and PI-negative (Figure 2A). Beside these arrested embryos, five blastocysts from the control group stained positively with Annexin V but did not express PI fluorescence (Figure 2C).

Table 2: The effect of IGF-1 upon the *in vivo* fertilized embryos examined for apoptosis and necrosis with fluorescent microscopic examination of PI and Annexin V staining.

Study methods			Propidium iodide and Annexin V --FITC studies		
Culture medium	Total n	With PI-positive cells *	With Annexin V-positive and PI-negative cells **	With Annexin V-positive or PI-positive cells ***	Without Annexin V- and PI-positive cells ****
kSOM/AA with IGF-1	106	5(4.7%) All are arrested embryos	5(8.5%) All are arrested embryos	10(9.4%)#	96(86.8%)# All blastocysts and 2 arrested embryos
kSOM/AA without IGF-1	83	7(8.4%) All are arrested embryos	13(15.6%) Arrested embryos and five blastocysts	20(24.1%)#	63(75.9%)# All are blastocysts

IGF-1: 100 ng/ml. The results were pooled from 5 repeats of experiments. # $P < 0.05$ * With necrotic cells; at least one PI-positive cell in the embryo, no matter if Annexin V-stained cells are present or not. ** With apoptotic cells; at least one Annexin V-positive cell in the embryo, but no PI-positive cells observed. *** With apoptotic cells or necrotic cells. **** Normal without apoptotic cells or necrotic cells.

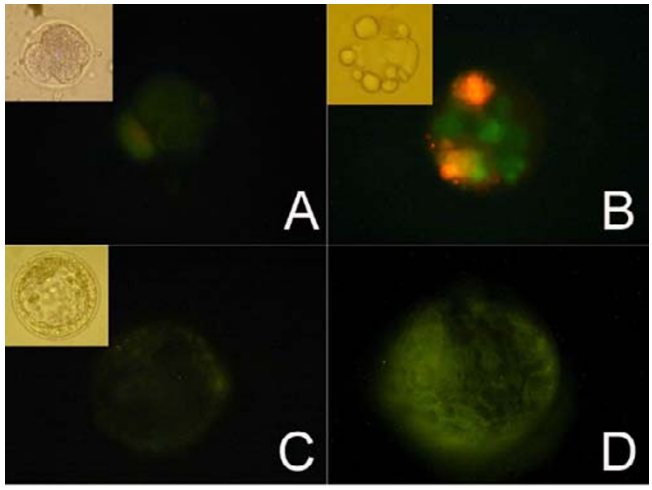

Figure 2
The *in vivo* fertilized *in vitro* grown embryos examined for apoptosis and necrosis. (A) A day five *in vitro* cultured embryo arrested at six-cell stage from the control group is observed before and after epifluorescent technique. Green fluorescence by Annexin V is observed over the outer cell membrane and means that apoptosis is underway. There is negative PI fluorescence at the time of harvest. (B) An arrested embryo at the morula stage becomes severely fragmented. Green fluorescence can be noted following Annexin V staining with simultaneous red fluroscence by PI staining. (C) A blastocyst cultured from the control group appears to be normal looking prior to epifluorescent observation. The same blastocyst under epifluorescent microscope shows apoptotic changes with Annexin V fluorescence and absence of PI fluorescence. (D) An apoptotic blastocyst, following Annexin V and PI staining, revealed green fluorescence. Under epifluoresvent microscope, Annexin V staining revealed a well fluorescent membrance with central clearing.

The effect of dephosphorylated IGFBP-1/IGF-1 complex on in vivo *fertilized mouse zygotes (Experiment-2)*

The results depicted below derive from the pooled results from four repetitive experiments. The cleavage and blastocyst rates of the embryos were investigated daily until day 4.3 post-fertilization using an inverted microscope. More embryos developed into the blastocyst stage for the IGFBP-1/IGF-1 group than was the case for the control group (94.6% vs. 81.9% respectively; $p = 0.05$). The hatching rate for the IGFBP-1/IGF-1 group was significantly higher than the corresponding figure for the control group (68.8% vs. 48.6%; $p = 0.026$; Table 3).

The effect of dephosphorylated IGFBP-1/IGF-1 complex upon total blastocyst cell count (Experiment-3)

The results shown below are pooled from three repeats of the culture experiments. The developmental stages for the cultured embryo were determined daily until day 4.1 post-fertilization using an inverted microscope. They were evaluated before hatching on the morning of day five, that is day 4.1 post-fertilization, instead of day 4.3 post-fertilization, because the hatching of the cultured embryos may distort the integrity of their figures and therefore interfere with the counting of the total cell number. In order to enumerate the total cell number of cultured embryos, the embryos that progressed to the blastocyst stage for both groups were cultured in Hoechst H33342 medium (5 μg / ml) for three to five minutes, and then mounted onto a slide and examined under a fluorescence microscope. The results of cell enumeration are shown in Table 4. There appeared to be a significantly-increased total cell count for the blastocysts deriving from the IGFBP-1/IGF-1-supplemented group than was the case for the control group (87.6 ± 5.3 vs. 73.7 ± 7.1 respectively, $p < 0.01$; Figure 3).

IGF-1 or IGFBP-1/IGF-1-supplemented embryo culture and stem-cell establishment (Experiment-4)

Zygotes recovered from female mice (C57BL/6 J x C3H), naturally mated with C3H males, were cultured in kSOM + amino acids (kSOM/AA) medium alone, and served as

Table 3: The effect of dephosphorylated IGFBP-1/IGF-1 complex upon the developmental competence of *in vivo* fertilized mouse zygotes.

Culture medium	Total n	Zygotes arrested in one-cell to morula stage (%)	Zygotes developed to blastocyst (%)	
			Blastocyst (%)	Hatched blastocyst (%)
kSOM/AA with IGFBP-1/IGF-1	93	5(15.4)	88(94.6) **	
			24(25.5)	64(68.8) *
kSOM/AA without IGFBP-1/IGF-1	72	13(18.1)	59(81.9) **	
			22(33.3)	37(48.6) *

The results were pooled from 4 repeats of experiments. * $P < 0.05$, ** $P = 0.050$

Table 4: Dephosphorylated IGFBP-1/IGF-1 complex increases the total cell number of cultured blastocysts.

Culture Medium	No of zygotes	No. of blastocysts tested	Total cell number of blastocysts
kSOM/AA with IGFBP-1/IGF-1	38	15	87.6 ± 5.3 *
kSOM/AA without IGFBP-1 /IGF-1	45	18	73.7 ± 7.1 *

The results were pooled from 3 repeats of experiments. * $P < 0.01$

the control group. The culture medium used for one experimental group was supplemented with IGF-1 (100 ng/ml), whilst the medium for the other experimental group was supplemented with an IGFBP-1/IGF-1 complex (100 ng/ml). The randomly-selected hatching blastocysts deriving from these experimental groups were placed into feeder wells for subsequent establishment of the stem-cell lines used herein. The embryonic stem-cell lines were established from the inner-cell mass (ICM) of the hatched blastocyts following 23-25 days of co-culture with an STO feeder-cell layer, such a procedure resulting in an increased proportion (percentage) of successfully-established stem-cell lines (45% vs. 59.6% vs. 27.3% respectively, $p < 0.05$; Figure 4).

Discussion

The effects of IGF-1 upon the developmental competence of in vivo *fertilized mouse zygotes*

Autocrine and paracrine secretion of growth factors, and the timely expression of relevant specific receptors would likely affect the success of *in vitro* culture of the embryos [7,17,27]. The deprivation of necessary growth factors for such embryos has been shown to trigger apoptosis for a wide variety of *in vitro* cultured cells [4]. In recent studies pertaining to apoptosis, it has been suggested that TGF-α plays a critical role in the regulation of apoptosis as a "survival factor" for mouse blastocysts *in vitro*.[3]. Other putative survival factors for *in vitro* cultured embryos include IGF-1 and PAF (platelet activating factor) [4,19,28]. It has been previously demonstrated that culture-medium supplementation with IGF-1 promoted the development of bovine embryos to the morula stage at day five, and that the addition of 10 μg/ml insulin to the medium increased the cell number of bovine blastocysts cultured *in vitro* [28]. Significantly-enhanced effects were shown for LIF, IL-6, TGF-α, EGF, IGF-1, IGF-2, and TGF-βsupplement as regards improving embryonic hatching of post-thawed mouse morulae, in particular, the hatching rate, the level of which ranged from 61% for the control group to 82% for the IGF-1-supplemented group [14].

It has previously been suggested that the core anti-apoptotic effect of IGF-1 was mediated via IGF-1R [12,29,30]. Further, the IGF-1 receptor can be activated by either IGF-1 or insulin, however, the relative dimension of the anti-apoptotic effect elicited by IGF-1 upon the IGF-1 receptor would appear to be more efficient than is the case for insulin [31]. Although the IGF-1 receptor and the insulin receptor utilize a common pathway through IRS-1 (Insulin receptor substrate-1) and PI3-kinase (phosphatidyli-

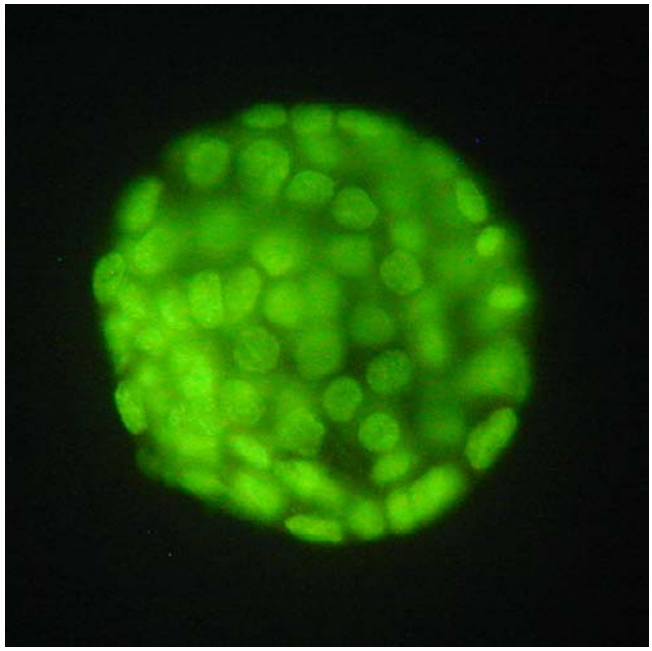

Figure 3
The total cell count of the H33342-stained blastocyst. An H33342-stained blastocyst from the control group revealed a total of 77 visible nuclei observed from different planes.

nositol 3-kinase), the IGF-1 receptor can act through an alternative anti-apoptotic pathway which appears to be IRS-1 and PI3-kinase-independent and is not shared with the insulin receptor [32]. This suggests that the mitogenic activity of IGF-1 through the IGF-1 receptor, which correlates with the tyrosine kinase domain, may be able to elicit a more-powerful anti-apoptotic effect than expected anti-apoptotic effect through pathway of the IRS-1. Therefore, further studies are needed.

In our study, the benefit of supplementation of culture medium with IGF-1 for the development of *in vivo* fertilized embryos has clearly been demonstrated (Table 1). The blastocyst formation rate was significantly increased for the IGF-1-supplemented group as compared to the control group (88.7% vs. 81.9% respectively), although the significance did not appear to be particularly prominent or dramatic ($p = 0.043$). This may be due to the moderately high blastocyst-formation rate (81.9%) for our control group, in which the apoptotic factors might be less than those in other *in vitro* studies with low blast-formation rate.[3,8,22,25,33]. In the studies of Lighten et al. (1998), treating cultured human embryos with 1.7 nM IGF-1 increased the rate of blastocyst formation by 25%, (60% versus 35% for the control; $p= 0.0018$).[25]. There were low blastocyst-formation rate in their control group (35%), in which suboptimal conditions with apoptotic factors may be encountered. The relative increase in the level of blastocyst formation was greater and more dramatic in their study as compared to ours.[25]. The anti-apoptotic effect of IGF-1 noted here would appear to be mainly due to its role as a protector of embryonic cells from possible injury [11,20,34]. Therefore, as regards elevating blastocyst formation rate, the more-prominent effect of IGF-1 would likely be determined under the more suboptimal culture conditions. Herrler and his colleagues (1998) have demonstrated that IGF-1 might act as a "survival factor" during preimplantation development, in which situation the apoptosis was induced by UV irradiation (254 nm wave length; 11.8 W/m^2).[22]. Indeed, our studies have demonstrated that the supplementation of culture medium by IGF-1 would likely improve the blastocyst-formation rate of the *in vivo* fertilized embryo cultured *in vitro*. This significant but not prominent effect of IGF-1, when compared to the results obtained in other studies with imposed apoptotic factors [14,22], might have resulted from the absence of the induction of a significant level of apoptosis in our experimental study.

Anti-apoptotic effect

To study the survival effect of IGF-1 alone over the kSOM cultured embryos, we observed the necrotic and apoptotic changes as revealed by Annexin V and PI staining using an epifluorescent microscope. As indicated in Table 2, a significantly-lower number of apoptotic and necrotic embryos were observed for the IGF-1-supplemented group than was the case for the control group (9.4% vs. 24.1% respectively, $p < 0.05$). Further, we also noted that there were fewer Annexin V-revealed apoptotic cells amongst blastocysts for the IGF-1 group as compared to the control group (n= 0 vs. n = 5 respectively). Such results reveal a significant anti-apoptotic and, subsequently, an anti-necrotic effect elicited by IGF-1 supplementation of embryo culture medium.

The proportion (percentage) of apoptotic blastocysts, revealed by only Annexin V fluorescence staining at the termination of embryo culture in kSOM/AA medium either with or without IGF-1 supplementation was 3.1% (5/162), a result that appeared to be quite different from that of Kamjoo and his colleagues in 2002 [6], from whose studies, the apoptosis index (dead cells/total cells) revealed with TUNEL staining for mouse embryos cultured in kSOM was 14.4 % [1]. We were unable to determine the dead-cell count for each blastocyst, because the quality of the Annexin V fluorescence in the blastocysts appeared to be quite scant in our study, although the total cell number of the blastocysts examined in our study was higher than that reported by Kamjoo and his colleagues in 2002 [6].

In our study, the embryos were collected from F1 female C57BL/6 mice mated with C3H males and were cultured in kSOM/AA culture medium. Such a model appears to demonstrate a better potential to promote blastocyst formation in culture and to result in an increased blastocyst total cell number than the models with other culture media and strain of mouse [6,35]. The blastocyst formation rate for the control group in our study was 81.9%. By using a MF1 x MF1 strain and C57BL6/CBA x C57BL6/CBA mice, Kamjoo and his colleagues (2002) were able to report a blastocyst formation rate of 62% (total cell number = 78.0, apoptotic index = 20.3%) and 98% (total cell number = 55.0, apoptotic index = 14.4%), respectively for these two strains [6]. Therefore, blastocysts deriving from MF1x MF1 revealed a higher apoptotic index than did those deriving from C57BL6/CBA x C57BL6/CBA (20.3% vs. 14.4% respectively) cultured in kSOM medium [6]. Such difference in results may be attributable to the different types of mouse strain used for the different experiments [6].

Quite independently of the type of mouse strain used for experimentation, different methods for the determination of apoptosis, such as Annexin V, TUNEL, 4'-6-diamidino-2-phenylindole, *in situ* nick translation, and Bcl-2 and Bax protein levels, may also make for different results of detection of apoptosis.[6,8,13,22,36]. In the studies of Berlerkom and Patrick in 1998 pertaining to apoptosis with TUNEL and Annexin V staining over the oocytes, most Annexin V fluorescence appears subsequent to the documented fragmentation of the oocytes, although such an event appeared to occur coincidentally with PI staining.[36]. Annexin V fluorescence, however, was reported by Berlerkom and Patrick to have only appeared to be associated with positive PI fluorescence, thus there were no oocytes that were stained with only Annexin V and not featuring PI fluorescence in their studies.[36]. In these workers' studies, no temporal or spatial relationship between the appearance of TUNEL- and annexin V-stained fragments in the same oocyte was reported.[36]. In our embryo studies, the process of apoptosis for embryos did not proceed by way of the oocytes; the plasma membrane alternations with the associated translocation of phosphatidyserine from the inner layer to the outer layer, which was revealed by Annexin-V FITC staining, occurred prior to (Figure 2A) or coincidentally with the appearance of PI-stained necrotic changes (Figure 2B). Further, we noted that 48.1% (13/27) of arrested embryos exhibited the Annexin V stain only (Figure 2A), although 44.4% (12/27) of arrested embryos did exhibit PI-stained neuclei, for which the Annexin V stain was frequently observed coincidentally (Figure 2B). This arose because Annexin V was also able to stain the inner plasma membrane of ruptured necrotic cells.

Early in our studies, we had tried some preliminary investigation with TUNEL evaluation for apoptosis at the completion of embryo culture. The frequency of observed TUNEL fluorescence for blastocysts *in vitro* appeared to be higher than that of the Annexin V fluorescence, the latter appearing to be extremely rare for the well-developed blastocysts. Therefore, the observed apoptotic changes involving the DNA fragmentation of blastomeres would appear to have resulted from the activation of an endogenous nuclear endonuclease, which was labeled by the fluorescein-dUTP in the TUNEL staining technique.[37]. Such a change appeared to occur more frequently and earlier for blastocysts than did the phosphatidylserine translocation in the plasma membrane during the apoptotic changes of blastocysts. Therefore, in the study of Kamjoo et al. (2002), apoptotic blastomeres were frequently seen, this study revealing an apoptotic index of 14.4% for the embryos collected from C57BL6/CBA x C57BL6/CBA mice [6]. In our study, 8.5–15.6% of the cultured embryos exhibited only Annexin V stain positivity, in which cases the necrotic embryos were excluded by the PI stain. If this coincidental PI-revealed necrosis in Annexin V stained embryo was not excluded from the statistic counting of apoptotic changes of the cultured embryos, there would be a total of 9.4–24.1% of Annexin V-stained embryos exhibiting ongoing apoptotic changes. Since there is no temporal or spatial relationship between the occurrence of TUNEL- and annexin V-staining positivity for the degenerating oocytes, these relationships during embryo development still need to be investigated further.[36].

The effects of IGFBP-1/IGF-1 upon the in vitro *developmental competence of the* in vivo *zygotes*

As shown, we had demonstrated that IGF-1 decreased the level of apoptosis of mouse embryos *in vitro*. The question thus arises, how is IGF-1 regulated and transported in the microenvironment during the preimplantation period, and, further, will IGFBP-1 decrease or increase the influence of IGF-1? Following on, a further question arises will IGF-1 be transported into the ICM of an embryo? The variation in apoptosis with different microenvironments and different strains of test animals is likely to be the result of variable embryonic expression of intracellular and extracellular regulators. Herrler et al. (1998) used UV radiation as an apoptotic factor to demonstrate that IGF-1 acted principally and significantly as a " survival factor".[22].

IGFBP-1 is a 25 kD protein produced predominantly by hepatocytes and decidualized ovarian endometrium [16,20]. IGFBP-1 provides a transport vehicle for IGF-1 and IGF-II, and controls the distribution of IGF-1 and possibly directs it to a specific receptor site [16,20]. IGFBP-1 also controls IGF action at the cellular level by either restricting or augmenting the access of IGF-1 to an

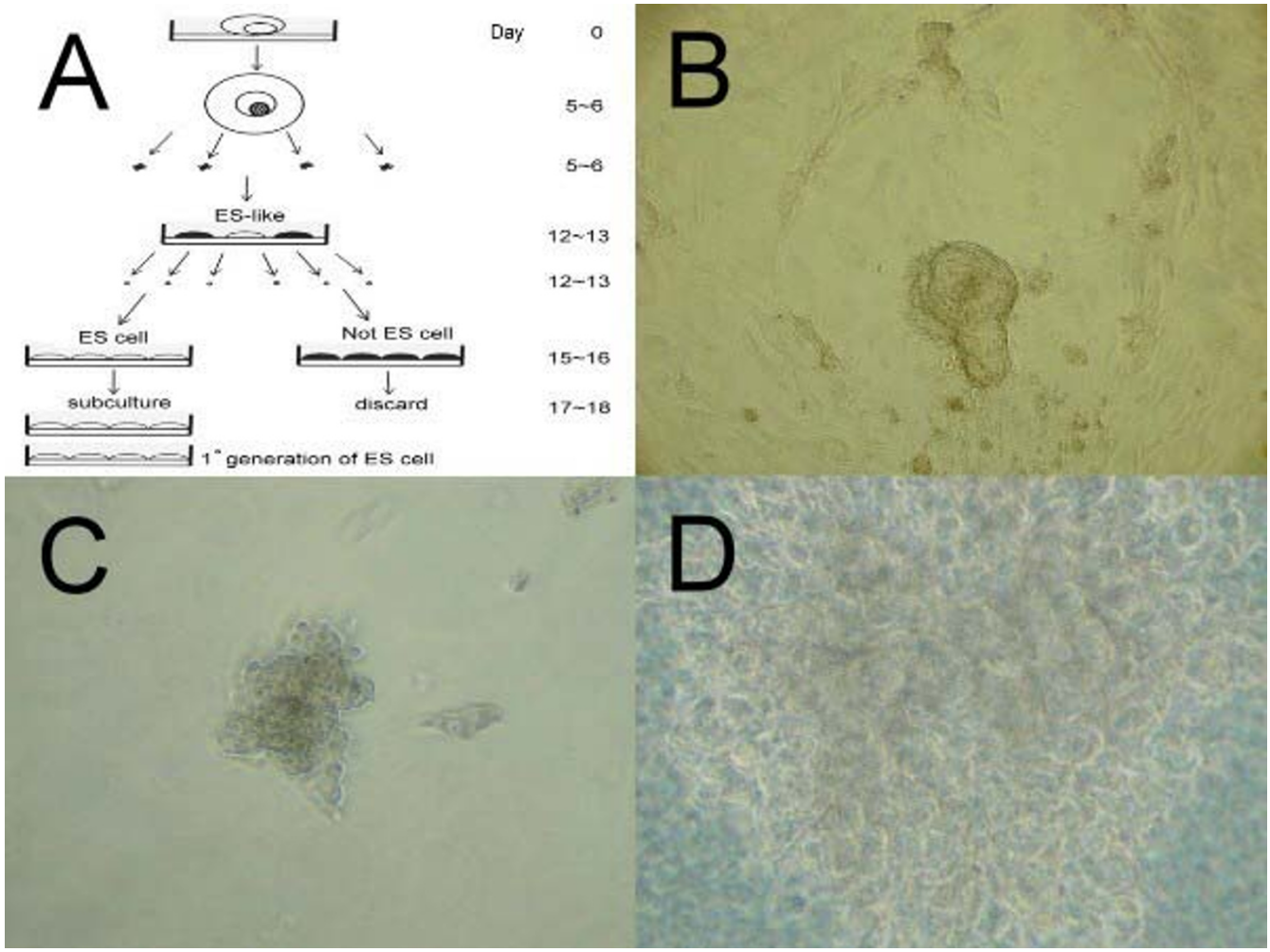

Figure 4
The establishment of ES cell line deriving from the hatching blastocysts. (A) The procedure for establishing ES cell line. (B) When the ICM-derived clump has reached the stage illustrated in figure by day 5–6th after explantation into culture, the ICM-derived component is clearly identified from the trophoblast cell. The cell clump is ready to be disloged and transfered for stem cell culture. (C) Cultured embryonic stem cells following dispersion have been transferred to another culture dish for further growth of the ES cells. (D) Well-established ES cells line, derived from the hatching blastocysts, will aggregate to form a burst-like ES cell colony.

appropriate receptor [16,20]. Further, IGFBP-1 appears to reveal a very high affinity for IGF-I and IGF-II [16,20], therefore, it usually sequesters IGF-I, acting as a reservoir and inhibitor of IGF-1 by preventing its binding to the IGF-I receptor site [20].

IGFBP-1 may demonstrate both stimulatory and inhibitory effects upon IGF-1-induced DNA synthesis [20]. These effects relate, predominantly, to the degree of phosphorylation and de-phosphorylation of the IGFBP-1 molecular species, and three mechanisms, including phosphorylation, proteolysis, and the association with the extracellular matrix (ECM)/integrin, influence the dissociation of IGF-1 from IGFBP-1 [16,20]. Phosphorylation of the molecule increases the affinity of IGFBP-1 for IGF-1 and inhibits IGF-1 binding to its receptor, such that de-phosphorylation of IGFBP-1 reduces its affinity for IGF-1 resulting in increased IGF-1 bioavailability [16,20]. Therefore, the ratio of non-phosphorylated or a lesser phosphorylated form to the phosphorylated form of IGFBP-1 determines the level of IGF-1 bioactivity *in vivo* [20,38]. IGFBP-1 can be effectively proteolysed into fragments, which display a reduced affinity for IGF-1, such that following phosphorylation of IGFBP-1, IGF-1 will be

released in a free form to bind with the IGF-1 receptors subsequent to the proteolysis step [21,39]. The association between IGFBP-1 and ECM/integrin results in a decreased affinity for IGF-1, thus the IGFBP-1/IGF-1 complex is modulated by ECM/integrin interaction [16,20,21,39,40]. As for the extracellular regulators, the role of IGFBP-1 deserves mention in this regard here, as does further study of that role since little free IGF is detectable in the human serum *in vivo*. Most IGFs are bound to larger proteins and only as little as 0.4% of total IGF-1 is unbound and free in the serum [41].

As a coordinating factor, we chose the dephosphorylated IGFBP-1 molecular complex to evaluate the influence of IGFBP-1/IGF-1 upon embryo development, IGFBP-1 being clearly vital to the expression of IGF-1. In the results of our experiments as depicted in Table 3, the synergistic effect of the IGFBP-1/IGF1 complex embryo development was significant. The rates of blastocyst formation and hatching were both significantly elevated for the experimental groups of IGFBP-1/IGF-1 as compared to the control group (blastocyst formation: 94.6% vs. 81.9%, $p = 0.05$; hatching: 68.8% vs. 48.6%, $p < 0.05$). The IGFBP-1 component of the IGFBP-1/IGF-1 complex used in our studies was initially isolated from the human hepatoma cell line Hep G2, and was found to exist primarily in its phosphorylated form. To obtain the IGFBP-1/IGF-1 complex, the IGFBP-1 moiety was dephosphorylated with alkaline phosphatase and reacted with IGF-1 in a 1:1 ratio (as per product information in Sigma No 12024; Sigma catalogue). Therefore, it seems likely that the "used" IGFBP-1 in our studies should be all dephosphorylated and demonstrate a low affinity for IGF-1, such that the resultant sequestration of IGF-1 due to the binding of IGF-1 to IGFBP-1 should be minimal. Further, the IGF-1 molecule was able to be deactivated in the culture medium in the presence of fetal calf serum (FCS), and the dephosphorylated IGFBP-1, in the form of an IGF-1/IGFBP-1 complex, was able to play a role in the preservation and carriage of IGF-1. This effect appears to be more important during the peri-implantation period and later embryo development than at earlier stages of embryo development [42].

IGFBP-1/IGF-1 complex decreases the level of apoptosis revealing an increased total cell number (TCN) for blastocysts

Several growth factors have been reported to be anti-apoptotic; LIF, IL-6, TGF-α, EGF, IGF-1, IGF-II, and TGF-β etc., all appearing to be beneficial to post-thawed mouse blastocysts as regards expansion in culture and subsequent hatching [3]. LIF has been proposed to play a role in maintaining the proliferative ability of ICM cells, yet it failed to increase the total cell number of cultured blastocysts, whilst TGF-α has been reported to demonstrate a marginal potential to increase the cell number of blastocysts [3]. There would appear to be several different pathways for these growth factors to benefit the embryos to which they are exposed in culture, and also, there appear to be several parameters that can be assessed in order to reveal the relative positive effects of such growth factors, these including cell number and accelerated development, embryo grading, hatching ratio, and even *zona pellucida* thickness variation [43]. The ultimate outcome for the blastocyst is to become a viable embryo featuring an abundance of pluripotential stem cells before and shortly after implantation [44]. Therefore we choose cell number per blastocyst and the passage of established stem-cell culture as the landmarks of the beneficial effect of growth factors upon embryo development for such potent mitogenic agents as IGF-1 [14].

For the evaluation of the effects of the IGF-1/IGFBP-1 complex upon the total cell count of the blastocysts, we randomly choose a selection of embryos cultured in medium supplemented with IGF-1/IGFBP-1 complex and a non-supplemented analogue for Hoechst 33342 staining. Fifteen embryos deriving from the IGFBP-1/IGF-1 group and 18 embryos from the control group were able to be scored after having been successfully stained with Hoechst 33342. This BisBenzimide fluorescent DNA stain is plasma membrane permeable, and intercalates in the A-T regions of the DNA molecule, it thus being useful for staining DNA, chromosomes, and nuclei [45]. Thus, Hoechst 33342 is able to stain the nuclei of both viable and non-viable cells. Therefore, the total cell count of the blastocysts which were enumerated for cell number was determined by counting the total number of stained nuclei at the end of culture using fluorescent microscopy. Use of the Hoechst 33342 stain revealed that there was a significant increase in the total cell number of blastocysts in the IGFBP-1/IGF-1 group than was the case for the control group (87.6 ± 5.3 vs. 73.7 ± 7.1 respectively).

The total cell number of the blastocyst is dependent upon the developmental environment and the particular stage of the embryo at which it is assayed [6,44]. Over a similar time course, blastocysts that developed *in vivo* revealed a higher cell number than those cultured *in vitro* [6]. From the results of Kamjoo's group (2002), the total cell number of the murine blastocyst flushed from the uterus on day four was, respectively, 87.4 ± 2 and 61.3 ± 4.4 cells for mice of the C57BL6/CBA x C57BL6/CBA and MF1 x MF1 strains of mice [6]. In the same study, the average day-four TCN of blastocysts developed from *in vivo* fertilized embryos cultured *in vitro* with kSOM was 55.0 ± 2.3 and 66.9 ± 1.6 cells for, respectively, C57BL6/CBA x C57BL6/CBA and 57BL6/CBA x MF1 mouse strains [6]. In our study, the embryos were flushed out from the oviducts on day zero, after the confirmation of a vaginal plug hav-

ing developed. The *in vivo* fertilized embryos were then cultured in kSOM/AA-supplemented medium either in the presence or absence of IGFBP-1/IGF-1 until day 4.1 post-fertilization (day five morning). For our control group (with no IGFBP-1/IGF-1 supplementation), the mean TCN of the blastocysts was 73.7 ± 7.1 (Table 4), such a figure being similar to that for the MF1 x MF1 embryo in kSOM-supplemented culture on the morning of day five following pregnancy, as has been reported previously [6].

Certain apoptotic factors have been noted to be present in the *in vitro* culture of mouse embryos, as evidenced by the observation of the occurrence of developmental retardation and the level of apoptosis having both increased amongst mouse embryos following *in vitro* culture [6,46]. The embryos cultured *in vitro* which is clearly a suboptimal condition as compared to the natural environment in the uterus, have not only been deprived of a whole family of necessary growth factors but have also faced exposure to a variety of apoptotic factors in the *in vitro* environment, suggesting that a decrease in total cell number of the blastocyst would likely be expected.

IGF-1 and embryonic stem-cell establishment

Early differentiation of the embryo leads the development of two distinct lineages-the inner cell mass and the trophectoderm [44,47]. For the mouse, a number of immortal stem-cell lines can be obtained from both cell types [48]. The pluripotent internal cell component of the blastocyst, the ICM, is segregated into a subcompartment, the epiblast, prior to blastocyst implantation [4,44]. It is evident that ES cells originate from the epiblast, and isolation of epiblast cells has been reported to enhance the efficiency of ES cell derivation [49]. Prior to the induction of diaphase (implantation delay), an increase in epiblast cell numbers appears to enhance the efficiency of ES cell generation [50–52]. In our study, the significantly-increased success in the establishment of a stem-cell line as compared to control group was the indirect evidence suggesting an increased level of viable cells in culture and a decreased level of apoptosis for the blastocysts cultured in medium supplemented with IGF-1 and IGFBP-1/IGF-1.

Conclusion

The supplementation of culture medium with IGF-1 100 ng/ml significantly increased blastocyst formation from *in vivo* fertilized/*in vitro* cultured embryos, the increase being 8.3%. Although such a result was statistically-significant, the increase, however, appeared mediocre. IGFBP-1 controls IGF's action at the cellular level by regulating the access of IGFs to appropriate receptors as well as at a microenvironment level where IGFBP-1 acts as a carrier and reservoir for IGF-1 [16,20,21,40]. Dephosphorylated IGFBP-1 with a low affinity for IGF-1 encourages the IGFBP-1/IGF-1 complex to elicit a similar beneficial effect to IGF-1 upon the cultured embryo, the effect being, principally, to enhance blastocyst formation. A synergistic effect of dephosphorylated IGFBP-1/IGF-1 appears possible, since from our study, the hatching rate also appeared to have increased for embryos cultured in medium supplemented with this complex when compared to those cultures supplemented with IGF-1 only.

Arrested embryos may be free from the presence of Annexin V staining positivity in the cell membrane and also free from PI staining positivity in the nucleus, such an outcome suggesting that Annexin V (positive) staining of the arrested embryos reveals that apoptosis is a late event in the development of the compromised embryos. Those arrested embryos, which did not normally demonstrate any mitogenic activity but still maintained normal biological activity therefore must have maintained a normal Annexin V stain-free plasma membrane. Necrotic changes in the blastomere of the embryo, as revealed by PI staining, appear to be a much later event for the compromised embryos. PI-stained cells were only found in the arrested embryos for either the IGF-1 group or the control group. Annexin V revealed apoptosis of embryo cells was able to be detected, although seldom, in the well-developed blastocysts of the control group. IGF-1 decreased the level of apoptosis for cells in blastocysts cultured *in vitro*. Therefore, IGF-1 has truly elicited a significant anti-apoptotic and an anti-necrotic effect upon the preimplantation development of experimental *in vivo* fertilized/*in vitro* cultured embryos.

IGFBP-1/IGF-1 increased the total cell number of *in vitro* cultured blastocysts, we noting an 18.9% increase in the total cell number of the blastocysts for the IGFBP-1/IGF-1-supplemented group as compared to the control group. The anti-apoptotic effect provided by IGF-1 decreased the triggering of cell death in culture, and enhanced the chances of the proliferation of viable cells. Therefore, in this study, IGF-1 contributed to a net increase in the total cell number of those blastocysts cultured *in vitro*.

Blastocysts cultured in IGF-1- and IGFBP-1/IGF-1-supplemented medium revealed a significantly-increased number of viable cells for subsequent embryos. The greater the number of viable cells in the hatching blastocysts the greater will be the subsequent ICM and the number of epiblast cells [53,54]. The greater the total ICM and the greater the number of epiblast cells that were placed in culture, the greater the number of subsequent colonies that could be derived and the greater the chances for subsequent successful stem-cell line establishment. The facilitated enhanced efficiency of ICM-derived colony formation and stem-cell line establishment in this study implies that IGF-1 or IGFBP-1/IGF-1 supplementation of

culture medium increases the viable cell numbers for the involved cultured blastocyst.

Materials and methods

Collection and in vitro *culture of* in vivo *zygotes*

All embryos used in this study were collected from F1 female mice (C57BL/6 J x C3H, six to eight weeks old) mated with C3H males. Mice were maintained under a daily light regime of 14 hours L: 10 hours D (Light: Dark). Female mice were superovulated by an injection of pregnant mare serum gonadotropin (5IU; PMSG, Sigma, St Louis, MO, USA) at 5 p.m., followed by an injection of human chorionic gonadotrophin (5IU; hCG, Sigma, St Louis, MO, USA) 48 hours later. Females were placed in cages with males subsequent to the administration of hCG to the former. On the following morning, the females were examined for the presence of a vaginal plug. Females exhibiting a vaginal plug were sacrificed 16 hours post-hCG injection by means of cervical dislocation and their oviducts were excised. The *in vivo* fertilized zygotes were then flushed out from the oviducts of these mice [47]. The embryos so collected were randomly allocated to control and treatment groups with different *in vitro* culture conditions. In each repeat experiment, three to four pairs of successfully mated mice were used and the results of each experiment were pooled from several repeats.

Preparation of culture media for each experiment

The culture medium used in our study was kSOM/AA medium [23,24,35,55] and all other chemicals used in this study were purchased from Sigma Chemicals unless otherwise specified.

Experiment 1

The *in vivo* fertilized zygotes were randomly allocated into control and treatment groups. There were totally 83 zygotes in the control group and 106 zygotes in the experiment group in the five repeats of experiments. For the control group, the embryos were cultured in kSOM/AA medium. Further supplementation of the culture medium used to culture the embryos in the treatment group was made with insulin-like growth factor-1 (IGF-1; 100 ng/ml). The developmental stages of the various cultured embryos were evaluated daily until day 4.3 post-fertilization (24 hr × 5 – 5 hr post-hCG injection - 12(11–13.5)hr ovulation time after hCG injection [44]: day five noon). Apoptosis was evaluated using Annexin V and PI staining under fluorescent microscopy.

Experiment 2

Seventy-two *in vivo* fertilized zygotes in the control group were cultured in kSOM/AA medium as indicated above. In the culture medium for the 93 *in vivo* fertilized zygotes in the treatment group, insulin-like growth factor binding protein-1/IGF-1 complex (IGFBP-1/IGF-1) was supplemented (100 ng/ml). The evaluation of the developmental competence of cultured embryos was performed as described above for experiment one.

Experiment 3

Forty-five *in vivo* fertilized zygotes in the control group were cultured in kSOM/AA medium. For the culture medium for 38 *in vivo* fertilized zygotes in the treatment group, further supplementation of IGFBP-1/IGF-1 was made (100 ng/ml). Blastocyst-stage embryos obtained both from the control and treatment groups on day 4.1, measured from time of fertilization, i.e. 12 hr(11–13.5 hr) after hCG induced ovulation (24 hr × 4 + 16 hr post-hCG injection - 12(11–13.5)hr ovulation time after hCG injection [44]: day five morning) were fixed for cell-number evaluation.

Experiment 4

The medium used for the *in vitro* culture of embryos corresponding to the control group was kSOM/AA. The medium for one experimental group was kSOM/AA supplemented with IGF-1 (100 ng/ml); the medium for the other experimental group was kSOM/AA supplemented with IGFBP-1/IGF-1 complex (100 ng/ml). The experiment was pooled from 3 repeats and there were totally 11, 12, and 13 hatched blastocysts in control, IGF-1, and IGFBP-1/IGF-1 groups respectively. The methods used for the establishment of embryonic stem cells in this study was that described in 1983 by Kaufman at al. [51] using STO cells here as the necessary feeder cells.

Culture of embryos

The in vivo fertilized zygotes were cultured in three types of culture medium, kSOM/AA, kSOM/AA with 100 ng/ml IGF-1, and kSOM/AA with 100 ng/ml IGFBP-1/IGF-1 complex, respectively, at 37°C in an atmosphere of 5% CO_2-in-air. For experiments 1 and 2, the final stages of development of the cultured embryos were determined on day 4.3 post fertilization under an inverted microscope. For experiment 3, the cultured blastocyst-stage embryos obtained on day 4.1 post-fertilization were cultured in Hoechst H33342 (5 μg/ml; Sigma, St Louis, MO, USA) staining medium in DPBS at 37°C with 5% CO_2 for a period of three to five minutes. The cell numbers of these cultured blastocyst-stage embryos were then enumerated under a fluorescence microscope following fixation. For experiment 4, the hatched blastocysts obtained from in vitro culture on day 4.3 post-fertilization were placed into feeder cell-containing wells with ES medium.[51] for establishment of embryonic stem-cell lines.

Table 5: Subsequent stem-cell establishment from the hatched blastocysts derived from IGF-1- or IGFBP-1/IGF-1-supplemented embryo culture.

Culture Medium	No. of hatched blastocysts tested	No. of blastocysts up to three passages (%) to establish ES cell lines	No of wells * (%) of established ES cell lines
kSOM/AA with IGF-1	12	6 (50)	22(45.8) **
kSOM/AA with IGFBP-1/IGF-1	13	8(61.5)	31(59.6) **
kSOM/AA	11	4(36.3)	12(27.3)

The results were pooled from 3 repeats of experiments. * The cellular clump from ICM of each blastocyst was dissociated and implanted into four separate culture wells, which were labeled and traced back after the establishment of ES cell line. ** $P < 0.05$

Fluorescent staining with Annexin V-FITC, propidium iodide (PI) and Hoechst 33342 stain

The embryos subjected to Annexin V and PI staining were dual stained in phosphate-buffed saline (PBS) containing fluorescein isothiocyanate (FITC) conjugate of Annexin V (1 μg/mL) and propidium iodide (500 ng/mL) at 37°C for 45 minutes. The embryos were then examined under epifluorescent fluorescence microscopy using a Leitz 4867709 Cube microscope (BP450-490/FT510/LP520) for Annexin V - FITC and a Chroma 31005 cube microscope (propidium iodide cube) for PI. As for TCN counting, the individual blastocysts were incubated in a droplet of Hoechst 33342 for three to five minutes at 37°C and then fixed for fluorescence examination under epifluorescent fluorescence microscopy using a Leitz 4867709 Cube microscope [56].

Preparation of feeder dishes

The procedure for the preparation of feeder dishes was modified from a technique suggested by Hogan et al. in 1994 [44,47]. Briefly, confluent STO cells (National Health Research Institutes, Taiwan) in tissue culture dishes were treated with freshly-prepared DMEM plus 10% fetal calf serum (FCS) and mitomycin C (10 ug/ml), following which, the dishes were returned to a humidified incubator at 37°C with a 5% CO_2-in-air atmosphere for a period of two-to-three hours. Following this, the dishes were washed extensively with several changes of Dulbecco's Phosphate Buffered Saline (DPBS) and the cells subsequently collected by trypsinization. Following this, the cells were sedimented by low-speed centrifugation (1,000 rpm for a period of five minutes), and then the pellet was resuspended with fresh DMEM plus 10% FCS, and the cells counted, and then diluted DMEM plus 10% FCS to yield a final cell density of 3×10^5/ml. Next, the cells so prepared were placed into tissue culture dishes (Nunclon 153066) which had been pretreated with collagen. The collagen-pretreating procedure for the dish was performed by flooding the dish with a 0.1% solution of collagen for a period of two hours, following which, the collagen was removed.

Culture of embryonic stem (ES) cells (Figure 4)

The hatched blastocysts recovered from the 3 different in vitro culture media, as shown in experiment 4, were implanted into feeder wells, as day zero of de novo isolation of embryonic stem cells. By day five to six, the ICM transformed into a distinct cellular mass (Figure 4B) within the trophoblast outgrowth, which were extracted and washed initially with Ca^{++}/Mg^{++}- free PBS, then incubated in PBS with 0.25% trypsin/0.2%EDTA as an additive. resulting in a clump of cells. A pasteur pipette was used to gently separate the clump of cells into smaller aggregates (Figure 4C), which were implanted into 4 fresh feeder wells allowing them to grow until the 12th or the 13th day. (The ES-like cell clump from each blastocyst was implanted into 4 labeled wells. The establishment of ES cell line was traced back to these original labeled wells). Growths resembling stem cells were drawn out from the culture media and subjected to disaggregating process during day 5–6, only this time the cells were separated into single cells. These were collected and reseeded into new culture feeder wells and allowed to grow for another 4–5 days before stem cell-like growths were again drawn out and segregated for another repeat culture (day 17–18). At this subculture, the successful growth of stem cells was labeled as 1st generation of embryonic stem cells. Successful and unsuccessful culture of stem cells arising from a common blastocyst were followed for 3 more generations at an interval of four-to-five days in order to establish permanent ES cell lines (Figure 4D). Together with the unsuccessful ones, the permanent ES cell lines were traced to the initial common blastocyst and labeled wells of cellular aggregates separated from ICM-derived cellular clump and accounted accordingly. (modified from Hogan et al.1994) [44,47].

List of abbreviations used

Bax: Bcl-2 associated X protein

Bcl-2: a human proto-oncogene discovered as the translocated locus in a B-cell

DMEM: Dulbecco's Modified Eagle's Medium.

DNA: deoxyribonucleic acid

DPBS: Dulbecco's Phosphate Buffered Saline.

ECM: Extracellular Matrix

EGF: epidermal growth factor

FCS: newborn calf serum, fetal calf serum

β-FGF: β-fibroblast growth factor

ES cell: embryonic stem cell

FITC: fluorescein isothiocynate

GH: growth hormone

H33342: Hoechst BisBenzimide 33342

hCG: human chorionic gonadotrophin

ICE: interleukin-1b converting enzyme

ICM: inner cell mass

IGF-1: insulin like growth factor-1

IGF-2: insulin like growth factor-2

IGFBP-1: insulin like growth factor-1 binding protein

IGF-1R: IGF-1 receptor

IL-6: interleukin-6

IRS-1: insulin receptor substrate-1

IVF: *in vitro* fertilization

kSOM/AA: kSOM with amino acids

LIF: leukemia inhibitory factor

PAF: platelet activating factor

PBS: phosphate-buffed saline

PI: Propidium Iodide

PI3-kinase: phosphatidylinositol 3-kinase

STO: A permanent cell line derived from mouse fetal fibroblasts.

TCN: total cell number

TGF-α: transforming growth factor-α

TGF-β: transforming growth factor-β

TUNEL: terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling

UV radiation: ultraviolet light radiation

Authors' contributions

TCL conceived of the study, participated in its design, and drafted the manuscript. JMY performed the embryo and stem cell culture. KBG carried out the epifluorescent microscopic observation and performed the statistical analysis. TTH helped to plan and coordinate the studies. LRC supervised and interpreted the studies and helped manuscript preparation.

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References

1. Navot D, Bergh PA and Laufer N: **Ovarian hyperstimulation syndrome in novel reproductive technologies: prevention and treatment.** *Fertil Steril* 1992, **58:**249-261.
2. Homburg R and Howles CM: **Low-dose FSH therapy for anovulatory infertility associated with polycystic ovary syndrome: rationale, results, reflections and refinements.** *Hum Reprod Update* 1999, **5:**493-499.
3. Brison DR and Schultz RM: **Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha.** *Biol Reprod* 1997, **56:**1088-1096.
4. O'Neill C: **Role of autocrine mediators in the regulation of embyo viability; lesions from animal models.** *J. Sassist. Reprod. Genet* 1998, **15:**460-465.
5. Pantaleon M and Kaye PL: **IGF-I and insulin regulate glucose transport in mouse blastocysts via IGF-I receptor.** *Mol Reprod Dev* 1996, **44:**71-76.
6. Kamjoo M, Brison DR and Kimer SJ: **Apoptosis in the preimplantation mouse embryo: effect of strain difference and in vitro culture.** *Mol Reprod Dev* 2002, **61:**67-77.
7. Jurisicova A, Latham KE, Casper RF and Varmuza SL: **Expression and regulation of genes associated with cell death during murine preimplantation embryo development.** *Mol Reprod Dev* 1998, **51:**243-253.
8. Spanos S, Becker DL, Winston RM and Hardy K: **Anti-apoptotic action of insulin-like growth factor-I during human preimplantation embryo development.** *Biol Reprod* 2000, **63:**1413-1420.
9. Harper KM and Brackett BG: **Bovine blastocyst development after follicle-stimulating hormone and platelet-derived growth factor treatment for oocyte maturation in vitro.** *Zygote* 1993, **1:**27-34.
10. Pawshe CH, Rao KB and Totey SM: **Effect of insulin-like growth factor I and its interaction with gonadotropins on in vitro maturation and embryonic development, cell proliferation, and biosynthetic activity of cumulus-oocyte complexes and granulosa cells in buffalo.** *Mol Reprod Dev* 1998, **49:**277-285.
11. Tamatani M, Ogawa S and Tohyama M: **Roles of Bcl-2 and caspases in hypoxia-induced neuronal cell death: a possible neu-**

roprotective mechanism of peptide growth factors. *Brain Res Mol Brain Res* 1998, **58:**27-39.
12. Prisco M, Romano G, Peruzzi F, Valentinis B and Baserga R: **Insulin and IGF-I receptors signaling in protection from apoptosis.** *Horm Metab Res* 1999, **31:**80-89.
13. Tamatani M, Ogawa S, Nunez G and Tohyama M: **Growth factors prevent changes in Bcl-2 and Bax expression and neuronal apoptosis induced by nitric oxide.** *Cell Death Differ* 1998, **5:**911-919.
14. Desai N, Lawson J and Goldfarb J: **Assessment of growth factor effects on post-thaw development of cryopreserved mouse morulae to the blastocyst stage.** *Hum Reprod* 2000, **15:**410-418.
15. Li CH, Yamashiro D, Gospodarowicz D, Kaplan SL and Vliet GV: **Total synthesis of insulin-like growth factor I (somatomedin C).** *Proc Natl Acad Sci U S A* 1983, **80:**2216-2220.
16. Ranke MB and Elmlinger M: **Functional role of insulin-like growth factor binding proteins.** *Horm Res* 1997, **48 Suppl 4:**9-15.
17. Yoshimura Y: **Insulin-like growth factors and ovarian physiology.** *J Obstet Gynaecol Res* 1998, **24:**305-323.
18. Herrler A, Einspanier R, Schams D and Niemann H: **Effect of recombinant bovine somatotropin (rBST) on follicular IGF-I content and the ovarian response following superovulatory treatment in dairy cows: a preliminary study.** *Theriogenology* 1994, **41:**601-611.
19. Herrler A, Lucas-Hahn A and Niemann H: **Effects of insulin-like growth factor-I on in-vitro production of bovine embryos.** *Theriogenology* 1992, **37:**1213-1224.
20. Clemmons DR: **Insulin-like growth factor binding proteins and their role in controlling IGF actions.** *Cytokine Growth Factor Rev* 1997, **8:**45-62.
21. Baxter RC: **Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities.** *Am J Physiol Endocrinol Metab* 2000, **278:**E967-76.
22. Herrler A, Krusche CA and Beier HM: **Insulin and insulin-like growth factor-I promote rabbit blastocyst development and prevent apoptosis.** *Biol Reprod* 1998, **59:**1302-1310.
23. Lawitts JA and Biggers JD: **Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium.** *Biol Reprod* 1991, **45:**245-251.
24. Lawitts JA and Biggers JD: **Optimization of mouse embryo culture media using simplex methods.** *J Reprod Fertil* 1991, **91:**543-556.
25. Lighten AD, Moore GE, Winston RM and Hardy K: **Routine addition of human insulin-like growth factor-I ligand could benefit clinical in-vitro fertilization culture.** *Hum Reprod* 1998, **13:**3144-3150.
26. Kleffens M, Groffen C, Neck JW, Vermeij-Keers C and Drop SL: **mRNA and protein localization of the IGF system during mouse embryonic development in areas with apoptosis.** *Growth Horm IGF Res* 1999, **9:**195-204.
27. Lonergan P, Gutierrez-Adan A, Pintado B, Fair T, Ward F, Fuente JD and Boland M: **Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro.** *Mol Reprod Dev* 2000, **57:**146-152.
28. Matsui M, Takahashi Y, Hishinuma M and Kanagawa H: **Insulin and insulin-like growth factor-I (IGF-I) stimulate the development of bovine embryos fertilized in vitro.** *J Vet Med Sci* 1995, **57:**1109-1111.
29. O'Connor R, Kauffmann-Zeh A, Liu Y, Lehar S, Evan GI, Baserga R and Blattler WA: **Identification of domains of the insulin-like growth factor I receptor that are required for protection from apoptosis.** *Mol Cell Biol* 1997, **17:**427-435.
30. Allan GJ, Flint DJ, Darling SM, Geh J and Patel K: **Altered expression of insulin-like growth factor-I and insulin like growth factor binding proteins-2 and 5 in the mouse mutant Hypodactyly (Hd) correlates with sites of apoptotic activity.** *Anat Embryol (Berl)* 2000, **202:**1-11.
31. Harvey MB and Kaye PL: **Mediation of the actions of insulin and insulin-like growth factor-I on preimplantation mouse embryos in vitro.** *Mol Reprod Dev* 1992, **33:**270-275.
32. Damario MA, Liu HC, Mele CA, Horenstein MG and Rosenwaks Z: **Immunohistochemical analysis of insulin-like growth factor-binding proteins -1, -2, and -3 in implantation sites of the mouse.** *J Assist Reprod Genet* 1998, **15:**513-520.
33. Makarevich AV and Markkula M: **Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during in vitro maturation and culture.** *Biol Reprod* 2002, **66:**386-392.
34. Smith S, Francis R, Guilbert L and Baker PN: **Growth factor rescue of cytokine mediated trophoblast apoptosis.** *Placenta* 2002, **23:**322-330.
35. Ho Y, Wigglesworth K, Eppig JJ and Schultz RM: **Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression.** *Mol Reprod Dev* 1995, **41:**232-238.
36. Berlerkom JV. , Patrick WD: **DNA strand breaks and phosphatidylserine redistribution in newly ovulated and cultured mouse and human oocytes: occurrence and relationship to apoptosis.** *Human Reproduction* 1998, **13:**1317-1324.
37. Gorczyca W, Gong J and Darzynkiewicz Z: **Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays.** *Cancer Res* 1993, **53:**1945-1951.
38. Keogh R and jakeman PM: **Excerase-induced changes in insulin-like growth factor bioavailablility in man: evidence of reduced affinity of IGFBP-I for insulin-like growth factor.** *The journal of physiology* 2000:523.
39. Firth SM and Baxter RC: **Cellular actions of the insulin-like growth factor binding proteins.** *Endocr Rev* 2002, **23:**824-854.
40. Galiano RD, Zhao LL, Clemmons DR, Roth SI, Lin X and Mustoe TA: **Interaction between the insulin-like growth factor family and the integrin receptor family in tissue repair processes. Evidence in a rabbit ear dermal ulcer model.** *J Clin Invest* 1996, **98:**2462-2468.
41. Frystyk J, Skjaerbaek C, Dinesen B and Orskov H: **Free insulin-like growth factors (IGF-I and IGF-II) in human serum.** *FEBS Lett* 1994, **348:**185-191.
42. Sakaguchi M, Dominko T, Leibfried-Rutledge ML, Nagai T and First NL: **A combination of EGF and IGF-I accelerates the progression of meiosis in bovine follicular oocytes in vitro and fetal calf serum neutralizes the acceleration effect.** *Theriogenology* 2000, **54:**1327-1342.
43. Gabrielsen A, Bhatnager PR, Petersen K and Lindenberg S: **Influence of zona pellucida thickness of human embryos on clinical pregnancy outcome following in vitro fertilization treatment.** *J Assist Reprod Genet* 2000, **17:**323-328.
44. Hogan B, Beddington R, Costantini F and Lacy E: **Manipulating the mouse embryo: A laboratory manual.** *Cold Spring Harbor Laboratory Press* 2thth edition. *New York, Cold Spring Harbor Laboratory*; 1994.
45. Handyside AH and Hunter S: **A rapid procedure for visualising the inner cell mass and trophectoderm nuclei of mouse blastocysts in situ using polynucleotide-specific fluorochromes.** *J Exp Zool* 1984, **231:**429-434.
46. Byrne AT, Southgate J, Brison DR and Leese HJ: **Effects of insulin-like growth factors I and II on tumour-necrosis-factor-alpha-induced apoptosis in early murine embryos.** *Reprod Fertil Dev* 2002, **14:**79-83.
47. Labosky PA, Barlow DP and Hogan BL: **Embryonic germ cell lines and their derivation from mouse primordial germ cells.** *Ciba Found Symp* 1994, **182:**157-68; discussion 168-78.
48. Rossant J: **Stem cells from the Mammalian blastocyst.** *Stem Cells* 2001, **19:**477-482.
49. Brook FA and Gardner RL: **The origin and efficient derivation of embryonic stem cells in the mouse.** *Proc Natl Acad Sci U S A* 1997, **94:**5709-5712.
50. Bradley A, Evans M, Kaufman MH and Robertson E: **Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines.** *Nature* 1984, **309:**255-256.
51. Kaufman MH, Robertson EJ, Handyside AH and Evans MJ: **Establishment of pluripotential cell lines from haploid mouse embryos.** *J Embryol Exp Morphol* 1983, **73:**249-261.
52. Kaufman MH, Evans MJ, Robertson EJ and Bradley A: **Influence of injected pluripotential (EK) cells on haploid and diploid parthenogenetic development.** *J Embryol Exp Morphol* 1984, **80:**75-86.
53. Gardner RL: **The origin and efficient derivation of embryonic stem (ES) cells in the mouse.** *Pathol Biol (Paris)* 1998, **46:**676-677.
54. Gardner RL: **Contributions of blastocyst micromanipulation to the study of mammalian development.** *Bioessays* 1998, **20:**168-180.

55. Lawitts JA and Biggers JD: **Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media.** *Mol Reprod Dev* 1992, **31:**189-194.
56. Pursel VG, Wall RJ, Rexroad CE, Hammer Jr.R.E. and Brinster RL: **A rapid whole-mount staining procedure for nuclei of mammalian embryos.** *Theriogenology* 1985, **6:**687-691.