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Effects of Glucose and IGF-I on Expression of Glucose Transporter 1 (Glut1) and Development of Preimplantation Mouse Embryo

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생쥐의 착상전 배아의 발생과 Glucose Transporter 1 (Glut1) 발현에 대한 포도당과 IGF-I의 영향

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요 약 : Na^+이온 비의존적으로 작동하는 포도당 수송체 (glucose transporter 1, Glut1)는 생쥐 배아의 세포막을 경계로 포도당을 수송하는 주요통로이다. 성장인자 가운데 insulin-like growth factor-I (IGF-I)은 생쥐배아에서 포도당의 유입을 증가시키는 것으로 알려져있으나 이러한 효과가 IGF-I에 의한 Glut1의 전사조절 효과에 기인한 것인지는 알려져 있지 않다. 본 연구는 포도당과 IGF-I이 생쥐의 착상전 배아 발생과 Glut1 발현에 미치는 영향을 조사함으로써 이들에 의한 배발생 조절기작을 이해하고자 시행하였다. 2-세포기 배아는 배양액내 pyruvate 존재하에 포도당의 유무와 관계없이 포배로 발생하였다. IGF-I은 2-세포기에서 체외 발생한 중기포배내 할구수를 유의하게 증가시켰다. 2-세포기부터 체외발생한 상실배의 Glut1 전사체의 양에는 배양액내 포도당의 유무에 따른 차이가 없었으며, IGF-I은 포도당과 무관하게 Glut1의 발현을 증가시켰다. 이러한 결과에서 상실기 생쥐배아의 경우 단순히 포도당의 결핍에 의해 Glut1의 발현이 전사수준에서 촉진되지 않으며, Glut1 발현의 증가는 IGF-I에 의한 배발생 촉진효과와 관련이 있는 것으로 사료된다.

ABSTRACT : A sodium-independent facilitative glucose transporter 1 (Glut1) is a major route by which glucose can be transported across the plasma membrane of mouse embryo. Although it has been known that insulin-like growth factor-I (IGF-I) promotes glucose transport into the mouse embryo, whether IGF-I directly regulates transcription of Glut1 has been uncovered in mouse preimplantation embryo. This study was aimed to elucidate the role of glucose and IGF-I in development and Glut1 expression in preimplantation mouse embryo. Two-cell embryos developed in blastocyst regardless of the glucose in the presence of pyruvate. IGF-I significantly increased the number of blastomeres in the mid-blastula. Deprivation of glucose did not affect the amount of Glut1 transcripts in molula cultured from 2-cell embryo. IGF-I potentiated Glut1 expression in molula cultured from 2-cell embryo even in the absence of glucose. Taken together, it is concluded that depletion of glucose does not promote Glut1 expression the in morula cultured form 2-cell embryo, and that increment of Glut1 expression possibly mediates embryotropic effect of IGF-I on preimplantation mouse embryo.

Key words: Glucose transporter 1, IGF-I, Embryo, Mouse.

INTRODUCTION

Mouse preimplantation embryo in culture was known to require pyruvate for the first cleavage division (Biggers et al., 1967) and glucose as the sole energy substrate was unable to support development until the four- to eight-cell stage (Brinster, 1965a: Brinster & Thomson, 1966). It was reported that accumulation of glucose in fertilized mouse egg affected embryonic metabolism and thus evoked developmental block in vitro (Chatot et al., 1989; Menezo & Khatchadourian, 1990). However, after the switching of energy source from pyruvate or lactate to glucose in 8-cell embryo (Leese & Barton, 1984), there is an obligatory requirement for glucose before the morula stage (Brown & Whittingham, 1991; 1992). Moreover Chatot et al. (1994) reported that brief exposure of embryo to high concen-

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tration of glucose (27 mM), at any time between 50~80 h post-hCG was sufficient to overcome the two cell block and promoted development to blastocyst in blocking strain. It suggests obligate role of glucose in development of preimplantation mouse embryo.

Glucose can be transported across the plasma membrane by two different mechanisms: via a sodium-coupled active carrier system that is expressed in epithelial cells (Esposito, 1984) or by a sodium-independent facilitative glucose transporter system (Wheeler & Hinkle, 1985). Five facilitative glucose transporter proteins (Glut1, Glut2, Glut3, Glut4, and Glut5) have been described to date and their cDNA have been cloned (Bell et al., 1990; Kasanicki and Pilch, 1990; Thorens et al., 1990). The expression of Glut1 was reported in oocytes and preimplantation mouse embryos, and Glut2 was in 8-cell stage mouse embryo onwards (Hogan et al., 1991; Schultz et al., 1992). Glut1 and Glut2 display different cellular distribution in blastocyst. Glut1 has wide spread distribution in both trophectoderm and inner cell mass, indicative of its engagement in intracytoplasmic glucose uptake, while Glut2 is located on plasma membrane of trophectoderm facing the blstocyst cavity (Aghayan et al., 1992). It suggested a different functional significance of Glut isoforms during mouse embryonic development.

Preimplantation mouse embryos expressed some growth factors and homologous receptors (Schultz & Heyner, 1993). Autocrine and paracrine action of growth factors has been known to promote cell proliferation of mammalian embryo (Paria & Dey, 1990; Gardner & Kaye, 1991; Harvey & Kaye, 1992; Smith et al., 1993). Some growth factors promote synthesis of DNA, RNA, and protein (Heyner et al., 1989; Harvey & Kaye, 1991; Rappolee et al., 1992) as well as morphological development (Dardik & Schultz, 1992; Smith et al., 1993) of preimplantation embryos.

Insulin-like growth factor-I (IGF-I) is a highly conserved 70 amino acid peptide with diverse biological functions. In mammals, IGF-I receptor mediates short term metabolic effect and long term mitogenic effect of IGF-I (Harvey & Kaye, 1991). Biological responses to IGF-I was known to be dependent on the numbers of cognate receptors on the cell surface. Expression of IGF-I is influenced by hormonal, nutritional, tissue-specific, and developmental factors (Cohick & Clemmons, 1993). Expressions of IGF-I receptor (Heyner et al., 1989; Rappolee et al., 1990) and insulin receptor (Harvey & Kaye, 1988; 1991; Mattson et al., 1988) were found in 8-cell mouse embryo onwards. Recently it was found that IGF-I and insulin increased glucose uptake in 8-cell mouse embryos and promoted development to blastocysts *in vitro* (Pantaleon & Kaye, 1996). It implicates relationship between glucose transport systems and switching of metabolic reliance of embryo from pyruvate to glucose (Leese & Barton, 1984). Previousely, it was reported that some growth factors and calf serum were implicated in up-regulation of Gluts expression (Hiraki et al., 1988; Baldwin, 1993) and that Glut1 activity was regulated by glucose concentration in murine fibroblasts (Haspel et al., 1986). However, the molecular evidences supporting the idea that up-regulation of expression of Glut1 by IGF-I and sensitization of Glut1 expresseion by glucose deprivation were poorly understood in preimplantation embryos.

Present study was aimed to uncover the regulation of embryonic expression of Glut1 by IGF-I and glucose at transcription level. Development of mouse preimplantation embryos and expression of Glut1 were examined in the presence or absence of glucose in combination with IGF-I in different developmental stages of mouse embryos.

MATERIALS AND METHODS

1. Oocyte and embryo retrieval and culture

Six weeks-old mice (ICR strain) were injected with 5 IU of pregnant mare serum gonadotropin (PMSG) and followed by injection of 5 IU of human chorionic gonadotropin (hCG) 48 h later. Hormone injected mice were mated with 3 months-old males. Two-cell embryos were collected by flushing the oviduct with M2 medium (0.4% BSA) 48 h after hCG injection, placed in M16 medium under mineral oil, and cultured at 37℃, 5% CO_2, 95% air and 100% humidity (Biggers et al., 1971). Immature oocytes with intact germinal vesicle (GV-oocyte) were collected by puncture of follicles and cumulus cells were detached by repeated flushing with narrow bore pipette.

Oocytes were directly used for RT-PCR analysis. More than 100 embryos were subjected to culture and χ^2-independence test was done for statistical analysis of developmental rates of embryos.

2. Glucose and IGF-I treatment

To verify whether glucose can influence IGF-I regulation of development of 2-cell embryo to blastocyst and Glut1 expression, culture of late 2-cell embryos to blastocyst stage was conducted in two different ways. In group I, 2-cell embryos were cultured to blastocysts in the presence of glucose. In group Ⅰ-Ⅰ, 2-cell embryos were cultured in glucose medium untill their development to 8-cell stage and then IGF-I was introduced to culture. Group Ⅱ embryos were cultured in glucose-free medium throughout the culture. Group Ⅱ-Ⅰ embryos were cultured in glucose-free medium to their development to 8-cell stage and then IGF-I was introduced to culture. In Gruop Ⅲ, 2-cell embryos were cultured in glucose-free medium, transferred to glucose medium at 8-cell stage and cultured to blastocysts. In Group Ⅲ-Ⅰ, 2-cell embryos were cultured in glucose-free medium, transferred to glucose medium supplemented with IGF-I at 8-cell stage, and cultured to blastocysts. Concentrations of glucose and IGF-I were 5 mM and 4 μg/ml, respectively (Pantaleon et al., 1996).

3. Nuclear staining of blastocyst

Zona pellucida was removed by brief incubation in acid Tyrode solution (Hogan et al., 1994). After rinse in M2/BSA, zona-free blastocysts were fixed in 1% glutaraldehyde in phosphate buffered saline (PBS), washed in PBS, and stained with bisbenzamide (10 μg/ml in PBS) for 10 min. After several washing in PBS, embryos were mounted in glycerol and observation was done under epifluorescence microscope (Leitz, Dialux). More than 29 embryos per group were evaluated for their cell numbers. Mean cell numbers per embryo were statistically analyzed by Student's *t*-test.

4. RNA preparation

All solutions were prepared with water treated with 0.1% dithyolpyrocarbonate (DEPC). Oocytes and embryos were washed with Ca^{2+}, Mg^{2+}-free PBS and transferred to a chilled Trizol (GIBCO BRL). The sample was vortexed vigorously, and stroed at −70℃ until use. Prior to isolation of the RNA, the tubes were thawed on ice and rabbit α-globin mRNA (0.1 pg per embryo) was added. This mRNA served as an internal control for RNA recovery and efficiency of the reverse transcription-PCR reaction.

5. Reverse transcription

Reverse transcription (RT) was conducted on total RNA from 30 oocytes or 30 embryos. The reaction was carried out in 50 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 5 mM $MgCl_2$ containing 1 mM each of dATP, dGTP, dCTP, dTTP, 20 units of RNase inhibitor, and 50 pmol oligo(dT)20-M4 adaptor primer (Takara). The reaction mixture was incubated at 37℃ for 2 min, 5 units of AMV reverse transcriptase XL (Takara) were added and then transferred to a PCR thermal cycler (Takara, Model 480). Reverse transcription was conducted for 1 hr at 42℃. The samples were heated for 5 min at 99 ℃ and then placed on ice. At this point the samples were either used directly for amplification or stored at −20℃.

6. Polymerase chain reaction (PCR)

PCR amplification of Glut1 cDNA was performed with 5 embryos equivalent of cDNA reverse transcribed from 0.1 μg total RNA in 100 μl of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of the 4 dNTPs, 1 unit *Taq* polymerase (Takara), 25 pmol each of the appropriate 3′ and 5′ pimers, and 5 μl of the reverse transcription reaction. The primers for amplification of RT product of Glut1 were 5′-CTGCAGGAGCAGCTGCCTTGGATG-3′ and 5′-GATCTC ATCGAAGGTTCGGCCTTT-3′ (Birnbaum et al., 1986). The primers for α-globin were 5′-GCAGCCACGGTGGCGAGTAT-3′ and 5′-GTGGGACAGGAGCTTGAAAT-3′, respectively (rabbit, Cheng et al., 1986). The primers for hypoxantine phosphoribosyltransferase (Hprt) were 5′-CCTGCTGGATTACATTAAAGCACT-3′ and 5′-GTCAAGGGCATATCCAACAACAAA-3′ (Jeske et al., 1996).

The Glut1, α-globin, and Hprt primers gave rise to diagnostic fragments of 361 bp, 257 bp, and 354 bp, respecti-

ively. The basic PCR program used was incubation at 95℃ for 3 min, followed by a cycle program of 94℃ for 30 sec, 60℃ for 1 min, and 72℃ for 10 min. The last cycle was conducted with a 10 min extension at 72℃. The numbers of cycles were 40, 35, and 35 for Glut1, α-globin, and Hprt respectively. Following PCR, the amplification products (20 μ l) were run on 2.5% agarose gels containing 0.5 μg/ml ethidium bromide and photographed under UV light. Band intensity was analyzed using image analysis system (Bioprofill version 96, France). Semiquantitative analysis of Glut1 transcript was done according to the method by Manejwala et al (1991).

RESULTS

1. Effects of glucose and IGF-I on preimplantation development of mouse embryos

Effects of glucose and IGF-I on development of 2-cell embryos were summarized in Table 1. In group I embryos cultured in glucose medium, 59.2 % (74/125) of embryos developed to blastocysts. Inclusion of glucose throughout the culture and addition of IGF-I during their development from 8-cell onwards did not increased development to blastocysts (58.3 %, 74/127, group Ⅰ-Ⅰ). In glucose-free medium 60.6 % of embryos developed to blastocysts (group Ⅱ). Addition of IGF-I to 8-cell embryo slightly but not significantly increased development to blastocysts in glucose-free medium (67.7 %, 84/124, group Ⅱ-Ⅰ). In group Ⅲ, addition of glucose to 8-cell embryos which developed in glucose-free medium, 60.4 % (81/134) of embryos developed to blastocysts. Inclusion of glucose and IGF-I in culture of 8-cell embryos which developed in glucose-free medium slightly increased development to blastocyst but not significantly (65.5 % (93/142)) (group Ⅲ-Ⅰ).

Table 1. Development of late 2-cell mouse embryos cultured in M16 with or without glucose and IGF-I

Groups	Post-hCG			No. of Embryo Cultured	No. of Embryos Developed			
	48~66 hrs	67~96 hrs			72 hrs post-hCG		96 hrs post-hCG	
	Glu	Glu	IGF-I		≤8-cell	Mo (%)	≤Mo	Bla (%)
Group Ⅰ	+	+	−	125	64	61(48.4)	51	74(59.2)
Group Ⅰ-Ⅰ	+	+	+	127	63	64(50.4)	53	74(58.3)
Group Ⅱ	−	−	−	130	71	69(53.1)	51	79(60.8)
Group Ⅱ-Ⅰ	−	−	+	124	61	63(50.8)	40	84(67.7)
Group Ⅲ	−	+	−	134	61	73(54.5)	53	81(60.4)
Group Ⅲ-Ⅰ	−	+	+	142	68	74(52.1)	49	93(65.5)

The late 2-cell mouse embryos were collected 48 h post-hCG and cultured for 48 h in the presence or absence of glucose (5mM) and IGF-I (4μg/ml). Glu, glucose; Mo, morula; Bla, blastocyst. Degenerated embryos are not shown in this table.

Table 2. Total cell number of early- and mid-blastocysts cultured in M16 with or without glucose and IGF-I

Groups	Post-hCG			No. of Embryo Examined	96 hrs post-hCG			
	48~66 hrs	67~96 hrs			Early-lastocyst*		Mid-blastocyst**	
	Glu	Glu	IGF-I		No. of Embryo	Cells / Embryo	No. of Embryo	Cells / Embryo
Group Ⅰ	+	+	−	66	31	25.3±4.7	35	34.9±7.3^{a}
Group Ⅰ-Ⅰ	+	+	+	62	29	28.9±5.2	33	45.9±7.7$^{a'}$
Group Ⅱ	−	−	−	76	35	25.8±5.4	41	30.6±7.5^{bc}
Group Ⅱ-Ⅰ	−	−	+	77	38	28.0±6.2	39	40.1±6.9$^{b'}$
Group Ⅲ	−	+	−	67	35	29.7±6.2	32	39.3±6.6$^{c'}$
Group Ⅲ-Ⅰ	−	+	+	66	36	31.9±6.8	30	45.0±7.1

The late 2-cell mouse embryos were collected 48 h post-hCG and cultured for 48 h in the presence or absence of glucose (5mM) and IGF-Ⅰ (4μg/ml). Glu; Glucose.
*, blastocysts which start to develop blastoceal; **, blastocysts which have well developed blastoceal.
a, a'; b, b'; c, c' : significantly different from each other ($p<0.05$). Cells per embryo are Mean±SD.

2. Effects of glucose and IGF-I on cell numbers of mouse blastocyst

Effects of glucose and IGF-I on the mean cell numbers of the early and the mid-blastocysts were summarized in Table 2. In the presence of glucose, numbers of blastomeres of the early and the mid-blastocysts were 25.3±4.7 and 34.9±7.3, respectively (group I embryos). Under the same regime, addtion of IGF-I on culture of 8-cell embryos significantly ($p<0.05$) increased mean cell numbers of the early and the mid-blastocysts (28.9±5.2 and 45.9±7.7, respectively) compared to group I.

In the absence of glucose throughout the culture, mean cell numbers of the early and the mid-blastocysts were 25.8±5.4 and 30.6±7.3 (group Ⅱ), and which was not different from group Ⅰ. Addtion of IGF-I on culture of 8-cell embryos in glucose-free medium (group Ⅱ-Ⅰ) significantly ($p<0.05$) increased cell numbers of the early and the mid-blastocysts (28.0±6.2 and 40.1±6.9, respectively).

Mean cell numbers of the early and the mid-blastocysts which developed from 2-cell in the absence of glucose and then transferred to glucose (group Ⅲ) were 29.7±6.7 and 39.3±6.6, respectively. This result was significantly different from gruop Ⅱ ($p<0.05$). Under the same regime, addtion of IGF-I on culture of 8-cell embryos onwards did not increased cell numbers of the early and the mid-blastocyst (group Ⅲ-Ⅰ).

3. Expression of Glut1 in preimplantation mouse embryos

Glut1 transcript was detected after RT-PCR in GV-oocytes and all stages of preimplantation mouse embryos examined (Fig. 1A). RT-PCR of α-globin mRNA as a marker for recovery of total RNA resulted in consistent amplification in all stage of embryos examined (Fig. 1B). Relative amount of Glut1 mRNA to α-globin mRNA was the highest in GV-oocyte and gradually decreased during development to 4-cell embryo. In 8-cell embryos onwards, gradual increase of Glut1 transcript was found (Fig. 1C).

4. Changes in Glut1 expression in morula in the presence or absence of glucose and IGF-I

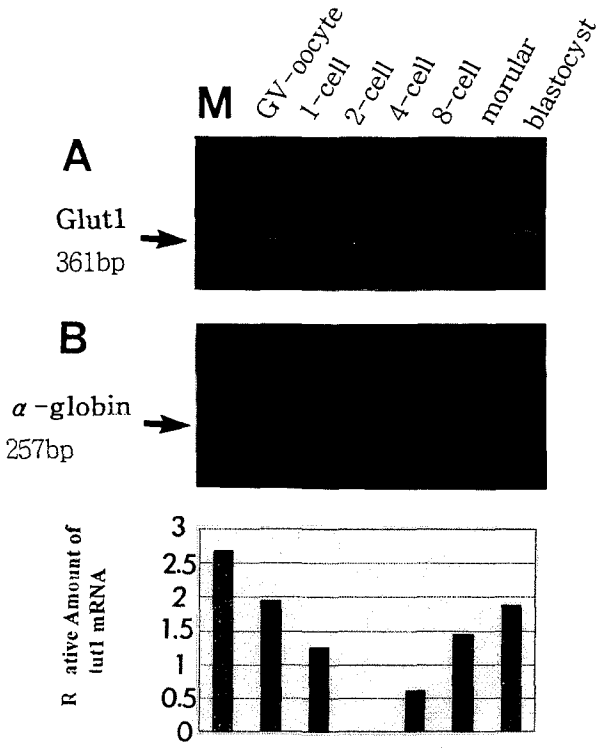

Fig. 1. The temporal expression of glucose transporter 1 (Glut1) in mouse oocytes and preimplantation embryos. RNA isolated from oocytes and preimplantation embryo was subjected RT-PCR, and was analyzed for Glut1 expression (A). RT-PCR of α-globin mRNA which served as an internal marker to monitor RNA recovery (B). (C) Relative amount of Glut1 mRNA to α-globin mRNA. M, φX174/Hae Ⅲ ladder; 1, GV oocyte; 2, 1-cell; 3, 2-cell; 4, 4-cell; 5, 8-cell; 6, morula; 7, blastocyst.

In all groups of embryos, RT-PCR product of Glut1 mRNA in morula (Fig. 2A) and relative amount of Glut1 mRNA to Hprt transcript increased in the presence of IGF-I regardless of glucose in the medium (Fig. 2C). RT-PCR of Hprt mRNA which serves as a internal maker for embryonic transcription resulted in consistent amplification in all stage of embryos examined (Fig. 2B).

DISCUSSION

Temporal pattern of Glut1 expression in oocyte and early embryos (Fig. 1) coincided with Hogan et al. (1991). It has been known that most of the maternally derived mRNAs decreased after ovulation (Bachvarova1 & De Leon, 1980) and embryonic transcrption is under control of zygotic gene

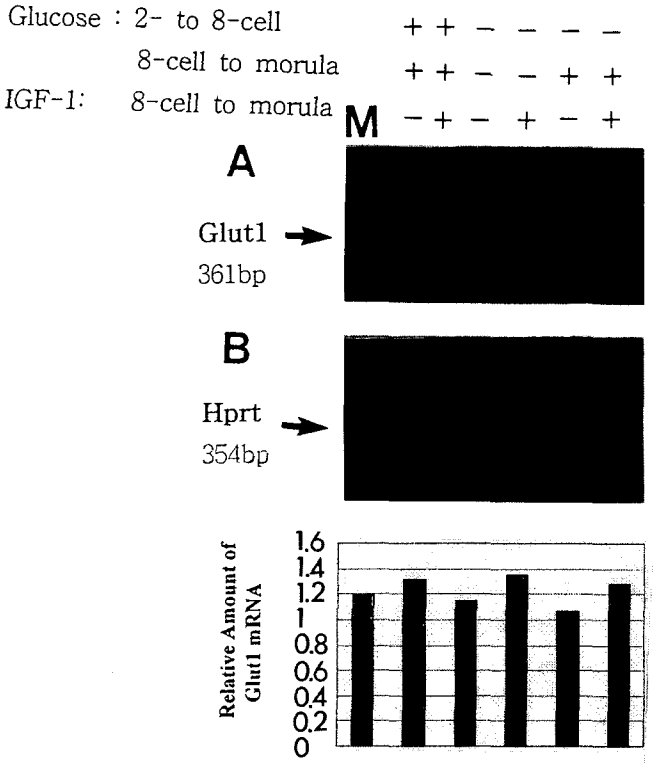

Fig. 2. The regulation of expression of glucose transporter1 (Glut1) in morula by glucose and IGF-I.
RNA isolated from morula which were culture in the presence or absence of glucose and IGF-I was subjected RT-PCR, and was analyzed for Glut1 expression (A). RT-PCR of Hprt mRNA which served an internal marker to monitor relative amount of Glut1 RNA (B). (C) Relative amount of Glut1 mRNA to Hprt mRNA. M, ϕX174/Hae Ⅲ ladder.

activation at 2-cell embryo in mouse (Flach et al., 1982). The gradual decrease in RT-PCR product of Glut1 transcript to 4-cell embryo following the highest expression in GV-oocyte suggested that transcrption of Glut1 in the mouse embryo is under zygotic control. Constitutive pattern of Glut1 expression in 4-cell embryo onwards indicates engagement of Glut1 in glucose uptake in mouse ealry embryos. The development of 2-cell embryos to blastocysts *in vitro* was not different according to experimental regimes (Table 1). However, the development of 8-cell embryos which were cultured from 2-cell in the absence of glucose showed slight increase in development to blastocysts (group Ⅰ vs group Ⅱ and group Ⅲ). In this regard, it was reported that mouse embryos do not use glucose as an energy substrate until early 8-cell stage (Brinster, 1965; Brinster & Thomson, 1966) and that inclusion of glucose in culture of early embryos retarded development (Menezo & Khatchadourian, 1990). Regardless of the glucose, mean cell numbers of the mid-blastocysts was increased by IGF-I as reported previousely (Rappolee et al., 1992; Smith et al., 1993). It was also reported that short term effect of IGF-I was metabolic (Harvey & Kaye, 1988; Gardner & Kaye, 1991) and mitogenic in long term (Harvey & Kaye, 1992) in mouse embryos. Therefore it can be suggested that glucose and IGF-I is not essential for embryonic viability and development to blastocyst but promotive to developmental potency of the mouse embryos in long term as shown by by increment in cell numbers per embryo (Table 2). Under the same regime of embryo culture, the RT-PCR product of Glut1 transcript in morula was slightly increased in response to IGF-I, and it was not dependent on the presence of glucose (Fig 2C). It suggested that up-regulation of Glut1 expression at transcription level was possiblly responsible for short term effect of IGF-I on glucose uptake in mouse embryo (Pantaleon & Kaye, 1996). It was reported that embryonic expression of IGF-I receptor which mediated biological effect of IGF-I initiated at 8-cell stage in mouse embryos (Heyner et al., 1989; Rappolee et al., 1990) and that IGF-I and insulin increased glucose uptake in mouse embryos (Pantaleon & Kaye, 1996). Therefore, it can be suggested that embryotropic effect of IGF-I was temporally coincident with the onset of embryonic expression of IGF-I receptor as well as the switching of energy substrate from pyruvate or lactate to glucose at 8-cell stage.

Under glucose deprivation, there was no detectable increase in embryonic expression of Glut1 at RNA level (Fig. 2). Similarly, Haspel et al. (1986) reported that chronic glucose deprivation produced 10-40-fold increase in total amount of Glut protein but not the level of *in vitro* translatable Glut mRNA in murine fibroblast. Therefore it can be suggested that embryonic adaptation to increase glucose uptake under glucose deprivation occurs downstream of Glut transcription. However, possibilities that switching of Glut expression from Glut1 to other isoforms and post-translational modification of Glut1 protein in preimplantation embryo can not be excluded.

In summary, it was found that up-regulation of Glut1 by IGF-I at transcription level independent from glucose and it might mediate short term metabolic effect and long term mitogenic effect of IGF-I on preimplantation development of 8-cell mouse embryo onwards.

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