

## Delay of ZGA initiation occurred in 2-cell blocked mouse embryos

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### ABSTRACT

One-cell mouse embryos from KM strain and B6C3F1 strain were cultured in M16 medium, in which 2-cell block generally occurs. Embryos of KM strain exhibited 2-cell block, whereas B6C3F1 embryos, which are regarded as a nonblocking strain, proceeded to the 4-cell stage in our culture condition. It is often assumed that the block of early development is due to the failure of zygotic gene activation (ZGA) in cultured embryos. In this study we examined protein synthesis patterns by two-dimensional gel electrophoresis of [<sup>35</sup>S] methionine radiolabeled 2-cell embryos. Embryos from the blocking strain and the nonblocking strain were compared in their development both in vitro and in vivo. The detection of TRC expression, a marker of ZGA, at 42 h post hCG in KM embryos developed in vitro suggested that ZGA was also initiated even in the 2-cell arrested embryos. Nevertheless, a significant delay of ZGA was observed in KM strain as compared with normally developed B6C3F1 embryos. At the very beginning of major ZGA as early as 36 h post hCG, TRC has already been expressed in B6C3F1 embryos developed in vitro and KM embryos developed in vivo. But for 2-cell blocked KM embryos, TRC was still not detectable even at 38 h post hCG. These evidences suggest that 2-cell-blocked embryos do initiate ZGA, and that 2-cell block phenomenon is due not to the disability in initiating ZGA, but to a delay of ZGA.

**Key words:** mouse embryo, two-cell block, zygotic gene activation (ZGA).

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### INTRODUCTION

During mouse development, the formation of a 2-cell embryo marks the transition from maternal to zygotic gene dependence[1-3], a phenomenon characteristic of most, if not all, metazoa[4]. Before maturation, oocytes are transcriptionally active[5]. Transcription stops when oocytes undergo meiosis to form eggs. Oocyte maturation initiates the degradation of maternal mRNA, and this destruction is essentially complete by the end of the 2-cell stage.

Following fertilization the early embryo is essentially transcriptionally silent and the early development is directed by the complement of maternally inherited mRNAs and proteins. The onset of zygotic gene activation (ZGA), which is a time dependent event delayed for about 24 h, does not occur until the 1-cell embryo undergoes cleavage to form a 2-cell embryo. The maternal to embryonic transition then occurs, in which further development is directed by the zygotic transcripts. Thus, ZGA is absolutely essential for continued development[6].

Transcription of TRC (transcription requiring complex) gene(s) constitutes one of the earliest indicators of ZGA during the 2-cell stage. It is a family of three related proteins of ~ 70 kD. Each of these proteins is heterogeneous with respect to pI (6.2 ~

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6.8), nuclear-association, and insolubility in 2% Triton X-100/0.3 M KCl. The synthesis of these proteins, which can constitute 5-10% of total protein synthesis in the 2-cell stage, is restricted to the 2-cell stage and is a-amanitin-sensitive[7].

When cultured in vitro, most mammalian embryos cannot accomplish their preimplantation development[8]. In mice, with the exception of some inbred and F1 hybrid strains, 1-cell embryos undergo a single cleavage division in vitro and are arrested at 2-cell stage, which has been termed a "2-cell block". On the other hand, the culture of 2-cell embryos to the blastocyst stage can be performed easily[9-11]. Reciprocal crosses between blocking and nonblocking strains indicated that this in vitro 2-cell block is a maternally regulated occurrence [12]. The block can be rescued by the transfer of cytoplasm of embryos from nonblocking strain. This indicated that the block is mediated by a cytoplasmic component, which may be lacking in embryos from blocking strains[13]. The ability of 1-cell mouse embryos to develop in vitro has been shown to be a function of mouse strain, medium components, and culture conditions[10].

In the past decades intensive efforts have been made to elucidate the cause of 2-cell block. However there is still little information on the mechanism of this event. It has often been assumed that the block of early development is due to the failure of zygotic gene activation (ZGA) in cultured embryos. This assumption was based on the finding that both the start of the appreciable expression of the zygotic genome and the developmental arrest occurs at 2-cell stage. If the transcription is blocked in the embryos from the nonblocking strain by a-amanitin, the arrest occurs at G2 phase in 2-cell stage[11]. Although the temporal coherence of the initial phase of ZGA with the critical period of 2-cell arrest is not likely to be explained just as coincidence, confirmation is still required. We therefore, in this study, compared protein synthesis patterns of 2-cell stage embryos of blocking strain and nonblocking strain mice developed either in vitro or in vivo by two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis (2-D IEF/SDS-PAGE). Data demonstrated at this point indicated that ZGA was also initiated even in 2-cell arrested embryos, but ZGA was delayed for

some reason in 2-cell blocked embryo as compared with those that were developed normally.

## MATERIALS AND METHODS

### *Embryo collection and culture*

Female KM and B6C3F1 mice, 5-6 weeks of age, were used to obtain the blocked and unblocked embryos at the 2-cell stage respectively. KM mice were obtained from Shanghai Experimental Animal Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. B6C3F1 mice were F1 hybrids of C57BL/6J females and C3H males which are both non-blocking strains[11, 14], purchased from the Jackson Laboratory, Bar Harbor, Maine, USA and raised in the animal facility of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.

One-cell embryos were collected from the oviducts of superovulated KM females mated with male KM mice or from B6C3F1 females mated with male B6C3F1 mice 21 h following hCG injection as previously described[15]. The collection medium was bicarbonate free minimal essential medium (Earle's salts) supplemented with pyruvate (100  $\mu$ g/ml), polyvinylpyrrolidone (3  $\mu$ g/ml), and 25 mM HEPES, pH 7.2 (MEM/PVP). Cumulus cell were removed with 100 IU/ml hyaluronidase (Sigma) in MEM/PVP. Following four washes through 200  $\mu$ l drops of MEM/PVP and 2 additional drops of M16 medium, the embryos were cultured until the indicated stage in M16 medium with or without 24  $\mu$ g/ml a-amanitin (Sigma) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air[16].

### *[<sup>35</sup>S]Methionine radiolabeling of embryos*

Typically groups of 50-100 embryos were radiolabeled for 2 h at 37°C in 50 ml microdrops of M16 medium supplemented with 1 mCi/ml [<sup>35</sup>S]Methionine (specific activity > 1,100 Ci/mM; Amersham), overlaid with medium-washed paraffin oil (Sigma). Based on a 20 h cell cycle for one-cell embryo, embryos at the initial phase of ZGA were radiolabeled 36 h or 38 h post hCG. Mid-2-cell-stage embryos were radiolabeled 42 h post hCG. At the end of the radiolabeling period, only healthy looking embryos that had developed properly were washed free of extracellular [<sup>35</sup>S]Methionine through six 200 ml drops of PBS/PVP, embryos were transferred into 0.5  $\mu$ l Eppendorf tubes, centrifuged and stored with a minimum amount of medium in 0.5 ml Eppendorf tubes at -70°C.

The radiolabeled embryos were then either lysed directly or extracted first in a solution of 50 mM Tris-HCl (pH 7.4), 2% Triton X-100, and 0.3 M KCl for 10 min at room temperature. The TRC remained insoluble under these conditions, whereas most of the radiolabeled proteins became soluble. The zona pellucidas that retained the insoluble proteins (zona pellucida ghosts) were then washed in 50 mM Tris-HCl, pH 7.4, and the insoluble proteins associated with them were then analyzed by gel electrophoresis[7].

### *2-dimensional gel electrophoresis*

Radio labeled samples, prepared as above, were dissolved in

O'Farrell lysis buffer[17] and that of  $\times 10^5$  c.p.m. were applied to the gel. Two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis (2-D IEF/SDS-PAGE) was carried out over a pH range of 4.5 to 7 (with ampholytes of 1.6% pH 5-7, 0.4% pH 3-10) on 4% polyacrylamide tube gels for the first dimension and on 12% polyacrylamide slab gels for the second-dimensional separation. Mini-Protean II Cell (Bio-Rad) was used according to the manufacturer's instruction manual. Bio-Rad's 2-D SDS-PAGE Standards were added to determine pIs and molecular weights of sample proteins or served as a marker for 2-D gel matching. The resulting gels were fixed, dried and exposed to Fuji X-ray film between 4-6 weeks at  $-70^\circ\text{C}$ . The 2-D gel electrophoresis for each individual sample was repeated at least three times.

## RESULTS

KM is regarded as one of the mouse strains sensitive to the 2-cell block[18], whereas B6C3F1 is well known as a nonblocking strain[11]. To confirm this in our culture condition, we tested the rates of development beyond 2-cell stage. Fertilized eggs of two strains were cultured in a widely used and chemically defined media M16, in which 2-cell block usually occurs[19]. As shown in Tab 1, nearly all the embryos from both B6C3F1 and KM developed to the 2-cell stage. About 80% of embryos from B6C3F1 further developed to the 4-cell stage. In contrast, only 6% of embryos from KM reached the 4-cell stage, as most of them were blocked at the 2-cell stage. Thus, we confirmed the definitely different abilities between the two strains to overcome two-cell block in the same culture condition.

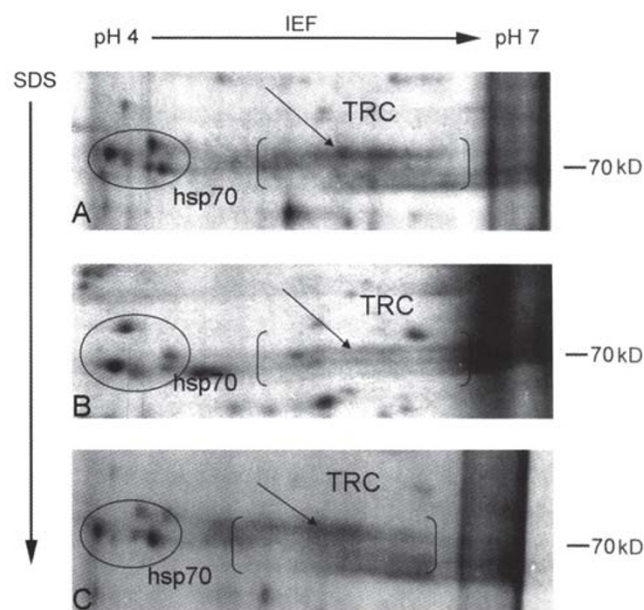
**Tab 1.** Development beyond the two-cell stage of embryos from different mouse strains

Embryos	No. of 1-cell embryos cultured	No. (%) of embryos developed to:	
		2-cell	$\geq 4$ -cell
KM	120	109	8(6)
B6C3F1	159	27	126(80)

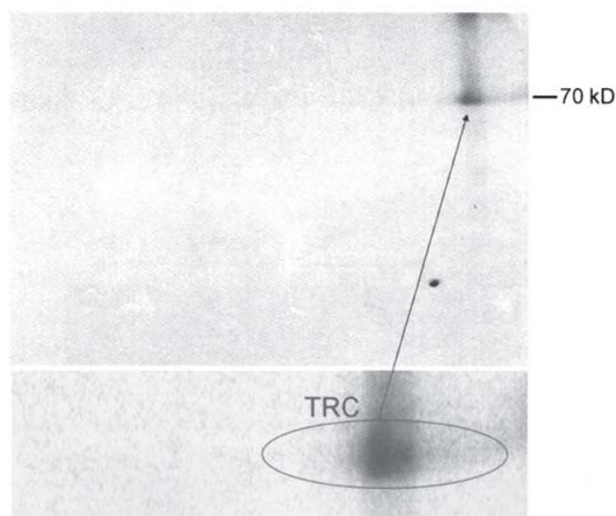
The 70 kD TRC complex has been generally used as a sensitive marker of zygotic transcription activation due to its stage specificity and high level of synthesis. The protein is encoded by an mRNA that is specifically transcribed at the two-cell stage. In this study, we also used TRC as a sign of ZGA to examine the transcription ability of embryos in the early stages.

Embryos were radiolabeled from 42 h to 44 h post-hCG, corresponding to mid-two-cell stage, at

which point the synthesis of the TRC is maximal[20]. After that, samples were subjected to two-dimensional gel electrophoresis. Surprisingly, a group of



**Fig 1.** Two-dimensional gel analysis of the incorporation of [ $^{35}\text{S}$ ] Methionine into embryos (A) 2-cell KM embryos in vitro, 42 h post hCG. (B) 2-cell KM embryos in vivo, 42 h post hCG. (C) 2-cell B6C3F1 embryos in vitro, 42 h post hCG. Arrow: TRC, circle: hsp70.

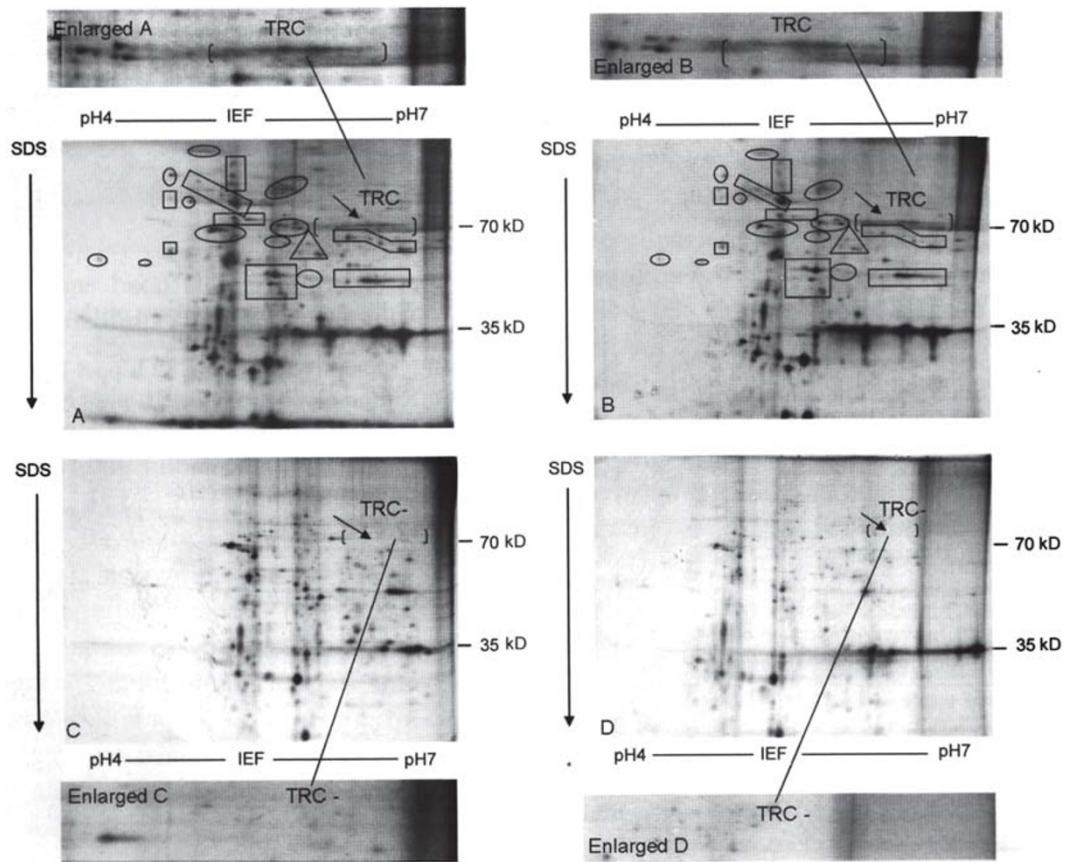


**Fig 2.** Two-dimensional gel analysis of the incorporation of [ $^{35}\text{S}$ ] Methionine into B6C3F1 2-cell embryos extracted with 2% Triton X-100/0.3 M KCl. Nearly all of the soluble proteins disappeared from the gel, but the insoluble TRC retained.

three ~70 kD proteins with pI 6.2-6.8, referred as TRC, were strongly detected in 2-cell blocked KM embryos, as well as that developed in vivo and the nonblocking B6C3F1 embryos developed in vitro (Fig 1).

To confirm that the TRC like complex identified on the gel is really TRC, we examined its solubility property. TRC was reported to be nuclear-associated proteins and exhibited similar properties to those of nuclear lamins with low solubility in a solution of 2% Triton X-100/0.3 M KCl. The radiolaabeled embryos were extracted first in the solution of

2% Triton X-100/0.3 M KCl before applying to the gel. Most proteins were desolved away, but TRC remained insoluble following the extraction. As expected, the consequent gel was nearly blank, except a cluster of three proteins of 70 kD with pI 6.2~6.8 (Fig 2). The exact molecular weight, pI, and solubility properties proved it to be TRC. Since the appearance of TRC marks the initiation of ZGA, the detection of it even in 2-cell blocked embryo appeared to be in disagreement with the previous consideration that the arrest of development at two-cell stage was due to an inability to undergo ZGA.



**Fig 3.** Two-dimensional gel analysis of the incorporation of [<sup>35</sup>S] Methionine into embryos

(A) 2-cell KM embryo of 42 h post hCG developed in vitro.

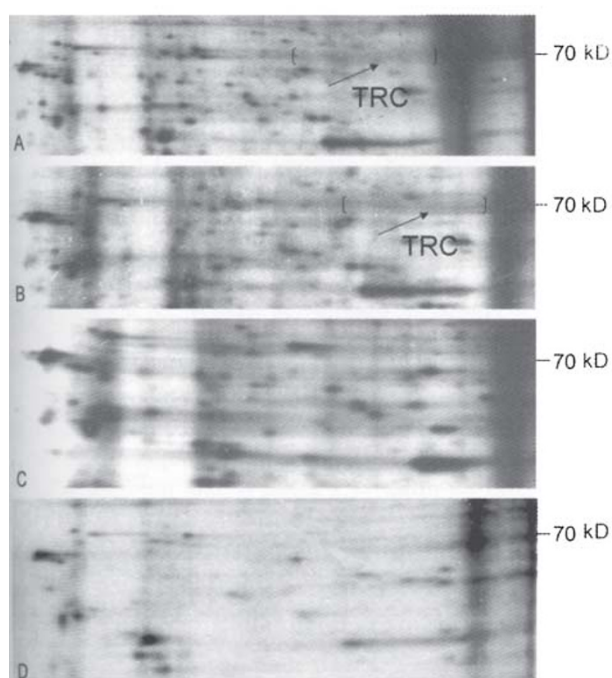
(B) 2-cell B6C3F1 embryo of 42 h post hCG developed in vitro.

(C) 1-cell KM embryo of 27 h post hCG developed in vivo.

(D) 2-cell B6C3F1 embryo of 42 h post hCG developed with  $\alpha$ -amanitin.

Spots or clusters of proteins encircled in (A) and (B) showed great similarity with each other, but when compared with (A) and (C), many different spots or clusters were found.

The rectangles on the top of (A) and (B), and those below (C) and (D) were their replicative enlarged view of proteins around 70 K TRC complex.



**Fig 4.** Two-dimensional gel analysis of the incorporation of [<sup>35</sup>S] Methionine into 2-cell embryos at 36 h post hCG.

- (A) KM 2-cell embryo 36 h post hCG developed in vivo.  
 (B) B6C3F1 2-cell embryo 36 h post hCG developed in vitro.  
 (C) KM 2-cell embryo 36 h post hCG developed in vitro.  
 (D) KM 2-cell embryo 38 h post hCG developed in vitro.

At the period as early as 36 h or 38 h post hCG, TRC was clearly observed in 2-cell embryos from KM developed in vivo and B6C3F1 embryos developed in vitro. But for KM developed in vitro, no TRC complex was detectable at this early stage.

Onset of ZGA is a time dependent event that does not begin until 24 h after fertilization, approximately 36 h post-hCG on the basis of fertilization occurred at about 12 h post-hCG. We then examined the expression of TRC at 36 h or 38 h post-hCG, the time equal to or shortly after ZGA initiation in the normal condition. The results showed that both B6C3F1 embryos cultured in vitro (Fig 4B) and KM embryos developed in vivo (Fig 4A) expressed TRC and other zygotic proteins on time. While in the case of KM embryo developed in vitro, however, TRC was barely detectable at either 36 h post hCG or 38 h post hCG (Fig 4). These results taken together with the above suggest that the initiation of ZGA was delayed some hours in 2-cell blocked embryos, though not absolutely disabled.

Additionally, the potent inhibitor of RNA

polymerase,  $\alpha$ -amanitin was used to distinguish zygotically encoded transcripts from maternally encoded ones. The expression of TRC, which is one of the main products of ZGA, is absent as reported in the case that ZGA was inhibited by 24 mg/ml  $\alpha$ -amanitin. Particular dots or clusters on the 2-D gel that were absent in the  $\alpha$ -amanitin treated embryos but present in the intact ones were identified as zygotic proteins. Our results demonstrated that other zygotic proteins besides TRC were also expressed in the 2-cell blocked KM embryos. The 2-D patterns of blocked embryos showed great similarity with those of the non-blocked embryo (Fig 3, compare A and B), while comparing with those of the 1-cell stage embryos many different spots or clusters were identified (Fig 3, A and C). Therefore, it can be concluded that even embryos blocked at the 2-cell stage underwent ZGA. Such an observation raises a further question about whether ZGA initiates on time in blocking strain embryos cultured in vitro.

## DISCUSSION

The data presented here contradict with a previous report, in which the authors concluded that a suppression of initiation of ZGA is involved in the 2-cell block. This was proposed by the authors as an explanation of a decrease in mRNA levels observed corresponding to the critical period for 2-cell block induced by phosphate exposure at the time of ZGA [21]. Our finding of this work, which strongly suggested that ZGA do initiate even in 2-cell arrested embryo, however, minimize the likelihood of the above proposal.

Effect of different culture media on protein synthesis was examined in an earlier study[15]. An enhanced rate of total protein synthesis and higher level of synthesis of proteins were observed in embryos cultured in CZB medium, which supports the developments beyond 2-cell stage also for blocking strain embryos. It was also reported in that paper that protein synthesis is markedly retarded in embryos cultured in Whitten's medium without EDTA that arrest the embryos at two-cell stage. This finding is in parallel with the results obtained in our study that aimed at the comparison of two different strains of embryo with different ability to progress beyond 2-cell stage in the same M16 medium. It's probably not only the retardation of the rate of total

protein synthesis as mentioned by those authors, but also a delay in transcription as evidenced by the mRNA decrease observed in 2-cell block embryos [21] that may account together for the inability of the second round of embryonic cleavage.

Zygotic genome expression is governed by a time dependent "zygotic clock" of unknown mechanism that delays the onset of transcription for about 24 h postfertilization (i.e. post-hCG 36 h), regardless of whether the 1-cell embryo has completed DNA replication or undergone mitosis. Acquisition of a transcriptionally permissive state occurs during the late 1-cell stage of mouse embryogenesis, but the major transcription-dependent expression begins after the formation of a 2-cell embryo[22-24]. This delay in zygotic gene expression provides a window of opportunity for remodeling parental chromosomes without accidentally and prematurely expressing their genes[25, 26]. The timing of ZGA follows the rule "go when ready". Changes in chromatin structure may underlie the timing and basis for ZGA[6]. And post-translational modification of maternally derived transcription factors is also likely involved in this time dependent event[27]. Recently, a 2-cell-stage-specific increase in polyribosomal abundance of maternal mRNA encoding some transcription factors (TFs) was observed. It was hypothesized that the regulated recruitment of maternal TFs mRNA may control the time of ZGA by controlling the time of appearance of certain TFs[28]. From this point of view, we can suppose that 2-cell block must involve delayed expression or activation of one or more transacting factors required for transcription or translation, or both of those genes that will trigger the zygotic clock[4]. Additionally, the DNA-binding activity of a number of transcription factors depends on the cellular redox potential; the oxidized forms lose their DNA-binding activity. Oxidative stress during embryo culture can thus detrimentally affect development[29]. Therefore, intrinsic causes, arising within the embryo, or extrinsic causes, present in the environment provided by the medium[16], could interfere with the precise and complicated regulation of zygotic clock, the slowing down of which may thus lead to a delay in triggering of ZGA. Autocrine diffusible factors have been reported to be necessary during 2-cell-stage for subsequent survival and normal development to the blastocyst stage

[30]. Thus, one of the possible explanations of 2-cell block would be that the delay of ZGA sequentially leads to a retardance or deficiency in producing autocrine growth/survival factors that generally initiated at late 2-cell-stage.

In conclusion, protein synthesis patterns by two-dimensional gel electrophoresis of [<sup>35</sup>S] Methionine radiolabeled 2-cell embryos were examined, comparing blocking strain with nonblocking strain embryos either developed *in vitro* or *in vivo*. Our results suggest that it is not the disability in initiating ZGA but the delay of the initiation that is involved in the 2-cell block of mouse embryos *in vitro*.

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