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EIF1A Expression in Sertoli Cell Nuclear Transfer Embryos during Preimplantation Development

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Abstract: Global transcriptome studies have provided important information advancing our understanding of nuclear reprogramming in somatic cell nuclear transfer (SCNT) embryos. The expression of the *Eif1a* gene is down-regulated in mouse Sertoli cell nuclear transfer (SeCNT) embryos during the preimplantation stages, however its protein expression level remains unclear. Here we observed the protein expression level of EIF1A by immunofluorescence and western blot analysis during the preimplantation stages in mouse SeCNT embryos. The results reveal that EIF1A protein is constantly expressed in germinal-vesicle and metaphase II oocytes and preimplantation stage embryos. Importantly, the localization and expression level of EIF1A were similar in control *in vitro* fertilized embryos and SeCNT embryos. Thus, since maternally derived EIF1A persisted throughout preimplantation development, repression of *Eif1a* is not likely to be involved in the developmental potency of mouse SeCNT embryos.

Key words: EIF1A, Mouse clone embryos, Reprogramming, Immunofluorescence imaging, Western blotting

Introduction

Somatic cell nuclear transfer (SCNT) is an excellent approach for understanding the reprogramming of mammalian differentiated cell nuclei, because through this artificial technique, terminally differentiated cells can reverse to the totipotent state and are able to differentiate into all kinds of body cells [1]. Although the technique has improved in the last decade, the survival rate of cloned embryos is still not as good as expected [2], and the mechanism underlying nuclear reprogramming remains to be clarified.

Comparison of gene expression profiles between SCNT and control embryos (generated in *in vitro* fertilization, IVF, or intracytoplasmic sperm injection) provide a good way of improving our understanting of the reprogramming mechanism and determining abnormal nuclear reprogramming. In a previous study, we conducted comprehensive transcriptome analysis in Sertoli cell nuclear transferred (SeCNT) embryos [3]. The results revealed that the gene expression profile differs significantly depending on the type of donor cell in mice. Furthermore, a number of abnormalities have been identified in murine [4], bovine [5], and porcine [6] SCNT embryos by transcriptome analysis using a microarray technique. In a *Xist* knock-down experiment, Inoue *et al.,* showed that the over-expression of *Xist* in SeCNT embryos was responsible for a number of abnormal gene expressions, and the survival rate of SeCNT embryos was significantly improved [7].

We recently performed transcriptome analysis of SeCNT embryos throughout preimplantation development [8]. A set of inappropriately expressed genes was identified at every developmental stage, and it might be responsible for the embryonic disorders. Among these genes, *Eif1a*, a eukaryotic translation initiation factor, was expressed at a low level in SeCNT embryos at the 2-cell, 4-cell and 8-cell stages. It is known that EIF1A catalyzes the 40S and 80S ribosomal subunit-mediated formation of initiation complexes, and facilitates the transfer of Met-tRNA to 40S subunits [9, 10]. Furthermore, the up-regulation of *Eif1a* has also been used as a marker for detecting zygotic gene activation in bovine embryos [11, 12]. Importantly, a lack of this protein may cause a dysfunction in gene translation and protein production of the genes important for early embryo development and may affect zygotic gene activation. Thus, the repression of *Eif1a* may cause developmental delay or failure of early embryos. However, although the transcriptome analysis

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data revealed that the gene expression of *Eif1a* is down-regulated in SeCNT embryos during the preimplantation stages, its protein expression level was not clear. Here, we observed the protein expression level of EIF1A by immunofluorescence and western blot analysis in SeCNT embryos.

Materials and Methods

Oocytes and embryos

Germinal vesicle (GV) oocytes were collected from female B6D2F1 mice 46 h after injection of pregnant mare's gonadotropin, and metaphase II (MII) oocytes were collected 14 h after administration of human chorionic gonadotropin (hCG). Embryos were incubated in potassium simplex optimization medium (KSOM) at 37 °C, under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 until use. *In vitro* fertilization (IVF) embryos were used as control embryos, and SeCNT embryos were produced as described previously [13].

Immunofluorescence observation

At each preimplantation stage, 5 embryos were used for each experiment, which was repeated 3 times. In total 120 embryos were used for immunofluorescence observation. After the removal of the zona pellucida by 0.5% protease treatment, embryos were fixed in 4% paraformaldehyde for 1 h, permeabilized in 0.25% TritonX-100/PBS for 1 h and washed for 10 min, 3 times in wash solution (0.1% TritonX-100, 0.3%BSA/PBS). Next, blocking was performed with blocking solution (PBS including 0.1% TritonX-100, 1% skim milk, 5% BSA) for 1 h. Rabbit polyclonal anti-EIF1A antibody (Abcam, MA, USA), and Alexa Fluor® 488 F (ab')$_2$ fragment of goat anti-rabbit IgG (H+L) (Invitrogen, NY, USA) were used for detection of EIF1A. Anti-EIF1A antibody at a concentration of 1 μg/ml was added and incubated at 4 °C overnight. After 3 washes with wash solution, secondary antibody at 1/5000 dilution was added and reacted for 50 min. 4', 6-Diamidino-2-phenylindole (DAPI: 2 μg/ml) was used for cell nuclear staining. Fluorescence images were acquired with an Olympus IX71 microscope controlled by MetaMorph software (Molecular Device, CA, USA).

RT-PCR

Single 8-cell IVF (n=5) and SeCNT (n=5) embryos were used for RT-PCR of *Eif1a*. After total RNA extraction (Qiagen) and cDNA synthesis (Ambion), *Eif1a* was amplified by an ABI 7500 Real time PCR system (Applied Biosystem). *β*-Actin, a housekeeping gene that shows constant expression in our microarray data, was used as an internal control to normalize the relative expression levels between IVF and SeCNT embryos. *Eif1a* forward primer: GCGTTTTGGTCACTACTCAGGA; reverse primer: GAAGATCCACAGGCAGCAAA.

β-actin forward primer: GAAATCGTGCGTGACATCAAAG; reverse primer: CTGTGGCATCCATGAAACTACA.

Western blotting

Forty embryos pooled at each stage (GV, MII, 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stage) were used for western blotting following the standard protocol. Western blotting was performed twice for verification. Briefly, samples were lysed in 12 μl EZapply® SDS-PAGE loading buffer (ATTO, Tokyo, Japan), protein was separated on 12.5% polyacrylamide gel by SDS–PAGE (PAGE) and transferred onto a PVDF membrane and blocked and hybridized with anti-EIF1A antibody using Can Get Signal® (Toyobo, Osaka, Japan) reagent. Rabbit polyclonal anti-EIF1A antibody was purchased from Abcam (MA, USA), and used at a final concentration of 1 μg/ml. ECL anti-rabbit IgG (GE, NA, UK) at 1:5000 dilutions was used as a secondary antibody. Luminol/Enhancers (ECL prime reagent, GE, NA, UK) were used for detection. Anti-tubulin antibody (Abcam) was used to detect tubulin as a positive control. Images were captured using a LAS-1000plus (Fujifilm, Tokyo, Japan).

Results

Following our previous microarray report [3], we focused on the *Eif1a* gene expression. We found that the *Eif1a* transcripts in IVF embryos first appeared at the 1-cell stage, and then, the level increased remarkably at the 2-cell stage (Fig. 1A). The expression lasted till the 8-cell stage; however, it ceased at the morula and blastocyst stages. In contrast, in SeCNT embryos, the *Eif1a* expression was restricted at the 2-cell stage and to one-fourth the level of that in IVF embryos. To validate these results, *Eif1a* mRNA was amplified by q-PCR in 8-cell embryos, and the results show that the expression of *Eif1a* in IVF 8-cell embryos was 3.7 fold higher than that in SeCNT embryos (Fig. 1B). Thus, *Eif1a* mRNA expression is significantly repressed in SeCNT embryos.

Next, we conducted immunofluorescence imaging analysis and western blotting to detect EIF1A protein. The results of EIF1A immunofluorescence imaging of IVF and SeCNT embryos are shown in Fig. 2. EIF1A was detected with distinct signals in GV and MII oocytes in both the nucleus and cytoplasm. Protein signals were constantly observed throughout the early embryonic de-

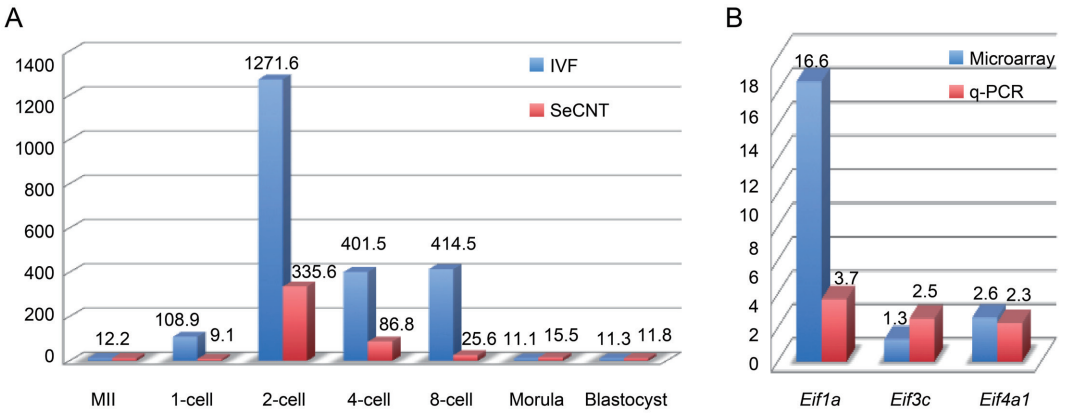

Fig. 1. Gene expression level of *Eif1a* in SeCNT embryos detected by microarray and q-PCR.
A. Gene expression data of *Eif1a* from the MII to blastocyst stages by microarray. Y-axis, RAW value; X-axis, embryo stage. B. Fold change (IVF / SeCNT) of *Eif1a*, *Eif3c* and *Eif4a1* at the 8-cell stage by q-PCR (n=5). Bar in blue is the fold change of IVF/SeCNT by microarray data. Bar in red is fold change of IVF/SeCNT by q-PCR. Y-axis, fold change.

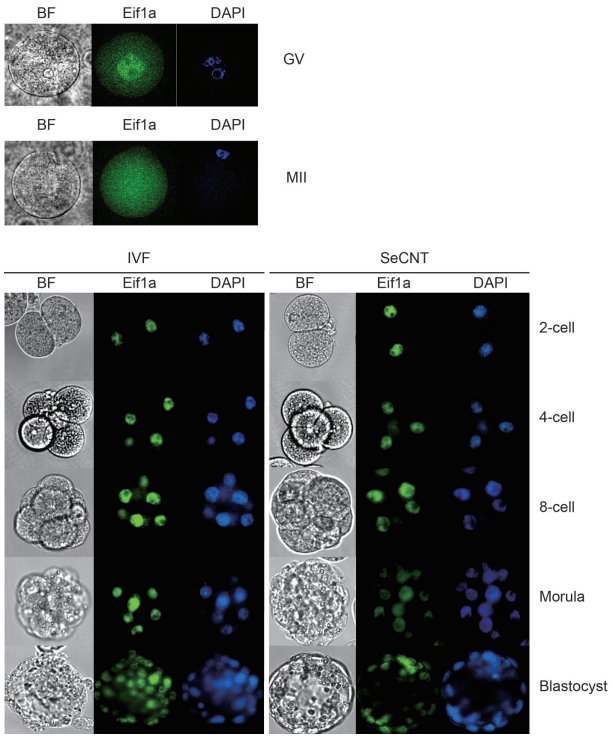

Fig. 2. Immunofluorescence results of EIF1A from the GV to blastocyst stages in IVF and SeCNT embryos.
BF, bright field; EIF1A, green; DAPI, blue. Left panel: IVF embryos; right panel: SeCNT embryos.

velopment from the MII oocyte to the blastocyst stages. At the GV and MII stages, the EIF1A was localized in both the nucleus and cytoplasm; however, EIF1A was sequestered into the pronuclear after fertilization. The nucleus-specific localization of EIF1A was observed throughout development to the blastocyst stage. Importantly, EIF1A showed similar patterns of localization and expression in IVF and SeCNT embryos. In IVF embryos, *de novo Eif1a* transcription was activated around the 4-cell stage, but its mRNA expression was much lower in SeCNT embryos. At the blastocyst stage, EIF1A was detected in the cell nuclei of both the inner cell mass and trophectoderm cells. This suggests that some of the EIF1A protein was translated from the embryonic mRNA in IVF embryos, but the *de novo* translation was limited in SeCNT embryos. We confirmed the EIF1A levels by western blotting (Fig. 3). The results reveal that the protein level of EIF1A was constant in the GV and MII oocytes and IVF derived preimplantation stage embryos.

Discussion

EIF1A is a member of the eIF family, the main function of which is the initialization of protein synthesis [14]. Using microarray and qPCR data, we confirmed that *Eif1a* together with *eif3c* and *eif4a1* are down-regulated in SeCNT embryos (Fig. 1B). These results suggested that the dysfunction of the eIF family is one of the reasons for embryonic development failure. However, the present immunofluorescence and western blot analysis findings contradict this hypothesis, because the results clearly show that maternally derived EIF1A existed abundantly in GV and MII stage oocytes, and seemed to be stable throughout preimplantation development. Therefore, the repression of *Eif1a* transcription in SeCNT embryos did

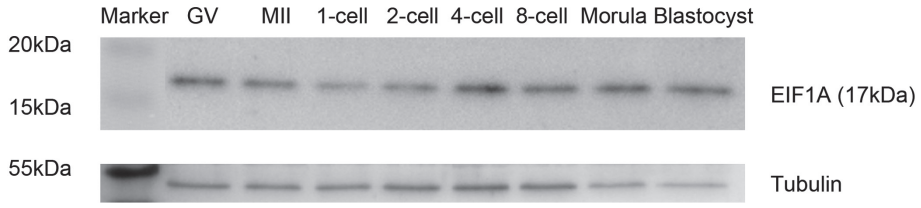

Fig. 3. Western blot analysis of EIF1A from GV oocytes to blastocysts in IVF embryos. GV-germinal vesicle oocyte; MII- metaphase II oocyte. Tubulin was used as a positive control.

not reflect the EIF1A levels directly, discrediting the possibility that the repression of *Eif1a* is involved in the developmental failure of SeCNT embryos.

To date, many notable reports have shown that a number of genes are inappropriately reprogrammed in SCNT embryos, inhibiting gene activation and repression at the correct stages [3, 15, 16]. For example, the expression of *Sox2* and *Fgf4*, is repressed in cumulus cell clones, Sertoli cell clones and ES cell clones, and this is known to cause embryonic lethality after implantation [3, 17, 18]. However, only a few studies at the protein level have been conducted on SCNT preimplantation embryos because of the limited availability of samples. Besides, these observations are limited to chromatin- and cytoskeletal-related molecules such as intermediate filaments [19], histone variants [20], and microtubules [21]. The present results show that EIF1A exists abundantly in SeCNT embryos. We assume that a large portion of EIF1A is maternally derived, because *Eif1a* mRNA was almost totally absent from the SeCNT embryos (Fig. 1A), and maternally derived proteins are thought to be very stable and persist beyond a few cleavages in mouse embryos [22].

Our results suggest that some abnormal gene expression in the SeCNT embryos does not affect the developmental potency if the protein necessary to support development at least until the end of preimplantation stages, is inherited from the oocytes. Thus, our results imply the importance of protein-level analysis in the search for candidate genes responsible for developmental failure of SCNT embryos, which would lead to a better understanding of molecular mechanisms underlying nuclear reprogramming.

Declaration of Interest

The authors declare no competing financial interests.

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