Journal of Genetics and Genomics 45 (2018) 677–680

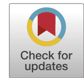
Contents lists available at ScienceDirect

Journal of Genetics and Genomics

Journal homepage: www.journals.elsevier.com/journal-of-genetics-and-genomics/

Letter to the editor

Individual blastomeres of 4- and 8-cell embryos have ability to develop into a full organism in mouse



Following fertilization in mammals, the zygote initiates the developmental program, which has a transient capacity to generate cell types of both embryonic and extraembryonic lineages, which is defined as totipotency (Condic, 2014). In mice, only zygotes and blastomeres of 2-cell stage embryos are considered totipotent, since they have the ability to develop into a full organism without the requirement of carrier cells (Tarkowski, 1959; Ishiuchi and Torres-Padilla, 2013). However, the blastomeres of the 4-cell or later stage embryos quickly lose this ability (Rossant, 1976). Recent studies showed that heterogeneous gene expression, at as early as the 4-cell stage, initiates cell-fate decisions by modulating the balance between pluripotency and differentiation (Fujimori et al., 2003; Burton et al., 2013). At the 8-cell stage, key morphogenetic processes such as compaction and polarization occur, leading to the establishment of the apical domain and subsequent generation of outer and inner cells (Johnson and Ziomek, 1981a; b).

Mouse embryos originating from individual blastomeres at the 2-cell stage successfully develop into term with a survival rate at 65% (Tsunoda and McLaren, 1983). Furthermore, individual blastomeres from 2-cell stage mouse embryos give rise to healthy adults that are similar in size to control mice (Papaioannou et al., 1989). However, mouse embryos originating from individual blastomeres of the 4-cell stage embryo only develop to embryonic day 5.5 (E5.5) (Rossant, 1976). On the one hand, it has been shown that the developmental ability of individual blastomeres varies among different mammalian species. Individual blastomeres from 4-cell stage cattle embryos and 8-cell stage embryos of human, pig, and sheep are totipotent (Willadsen, 1981; Saito and Niemann, 1991; Johnson et al., 1995). Because zygotic genome activation time differs among different species, it is generally thought that activation of the zygotic genes is an important cause of the loss of totipotency in early embryonic blastomeres. On the other hand, highly regulative capacity is a unique characteristic of preimplantation mammalian development. Early embryos can withstand changes such as the removal, addition, and rearrangement of blastomeres (Tarkowski and Wroblewska, 1967; Hillman et al., 1972; Hogan and Tilly, 1978). However, it remains unknown whether blastomeres at the 4-cell or later stages are also totipotent and at which stage the fate of blastomere development is determined.

In this study, we utilized gene knockout and gene knockdown approaches to silence *Cdx2* in mouse zygotes, combined with the tetraploid complementation technology, to establish a shell system for assessing the totipotency of individual blastomeres at the 2-, 4-, 8-, and 16-cell stages. Our results showed that individual blastomeres at the 4- and 8-cell stages still retained totipotency and could generate all embryonic and extraembryonic cell types.

It has been reported that epigenetic differences among blastomeres at the 4-cell stage result in the loss of potential to develop into a complete individual; blastocysts derived from individual 2-, 4-, and 8-cell stage blastomeres exhibit inconsistent development patterns (Tarkowski and Wroblewska, 1967). We biopsied individual blastomeres from 2-, 4-, and 8-cell stage embryos and inserted them into the original zona pellucida. Blastocysts derived from individual 2-cell stage blastomeres showed slightly smaller inner cell mass (ICM) and trophectoderm (TE) compared to the control group (Fig. S1A and B). However, blastocysts derived from individual 4- and 8-cell stage blastomeres showed significantly smaller ICM and TE than the control group (Fig. S1A and B). The blastocyst development efficiencies of the control and 2-, 4-, and 8-cell embryo groups were 91.4%, 76.8%, 63.5%, and 22.1%, respectively (Table S1). These results indicated that the blastocysts derived from individual 4- and 8-cell stage blastomeres could not undergo embryo incubation and implantation. An insufficient number of cells may be an important cause of the failure of 4-cell or later stage blastomeres to hatch from the zona pellucida followed by development to term. Thus, cells without development ability were required to assist in the process of blastocyst implantation of totipotency assessment system.

Blastocyst stage embryos contain ICM and TE. ICM gives rise to the pluripotent epiblast and future fetus, and TE forms the placenta. Embryos used as a system for testing totipotency should contain ICM and TE and have no developmental potential. *Cdx2* is important in mouse embryo development, which is required for TE specification and formation. TE development could be prevented by decreasing the maternal and zygotic expression of *Cdx2* (Jedrusik et al., 2010). Therefore, we simultaneously inhibited the maternal and zygotic expression of *Cdx2*. We adopted gene knockout (i.e., CRISPR-Cas9) to remove zygotically expressed *Cdx2* and gene knockdown (i.e., RNAi) to remove maternally deposited *Cdx2* mRNA (Fig. S2A). Two highly effective (90% and 100%) sgRNAs were designed according to the *Cdx2* CDS1 (Fig. S2B), and the biallelic knockout percentage was 76.7% (Fig. S2C and D). The blastocyst development efficiency of the control group ($n = 179$, 92.4%) was significantly higher than that of the sg group (*Cdx2* knockout; $n = 173$, 61.6%), si group (*Cdx2* knockdown; $n = 180$, 36.8%), and si+sg group (*Cdx2* knockout and knockdown; $n = 236$, 8.2%) (Fig. S2E). The mRNA level of *Cdx2* was significantly decreased in si+sg embryos compared to the control group (Fig. S2F). We further examined the expression of Cdx2 (the TE marker) and Oct4 (the ICM marker). We found that Cdx2 expression was nearly absent in *Cdx2* si+sg embryos (Fig. S2G).

https://doi.org/10.1016/j.jgg.2018.07.012
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Next, we co-injected two siRNAs and two sgRNAs into zygote stage embryos and electrofused the embryos at the 2-cell stage to establish the shell system (Figs. 1A and S2A). Most blastocyst stage shell embryos (*Cdx2* si+sg/4N embryos) showed abnormal blastocoels (Figs. 1B and S2H). To determine whether the system was functional, we monitored the developmental ability of the shell embryos. Tetraploid embryos (4N embryos) lose the development potential of ICM. To detect the lack of functional TE in the shell system, we analyzed the expression of Cdx2 and Oct4 *in vitro* and the development potency. We found that the expression of the TE marker Cdx2 was nearly absent in shell embryos (Fig. 1C), as observed in the *Cdx2* si+sg/2N embryos described above (Fig. S2G). Further, we did not observe implantation at E6.5 among the 253 shell embryos, while 86.6% (97/112) of implantations were observed in the control group at E6.5 (Fig. 1D and Table S1). These results suggested that the shell embryos lack functional TE and ICM.

To confirm the effectiveness of the shell embryo system, we tested the system using the 2-cell stage blastomeres that were proved totipotent. We aggregated individual 2-cell stage blastomeres with 2- or 4-cell stage shell embryos expressing GFP (Fig. S2I). Live pups and placentas were obtained (Fig. S2J and K). We detected a very small number of shell cells with GFP expression in the placenta (Fig. S2K). Thus, our system was effective for assessing cell totipotent.

Next, we investigated whether the blastomeres at the 4-cell stage are totipotent. Because the zona pellucida promotes or protects embryo development (Illmensee et al., 2006), we injected individual 4-cell stage blastomeres into the GFP-expressing shell

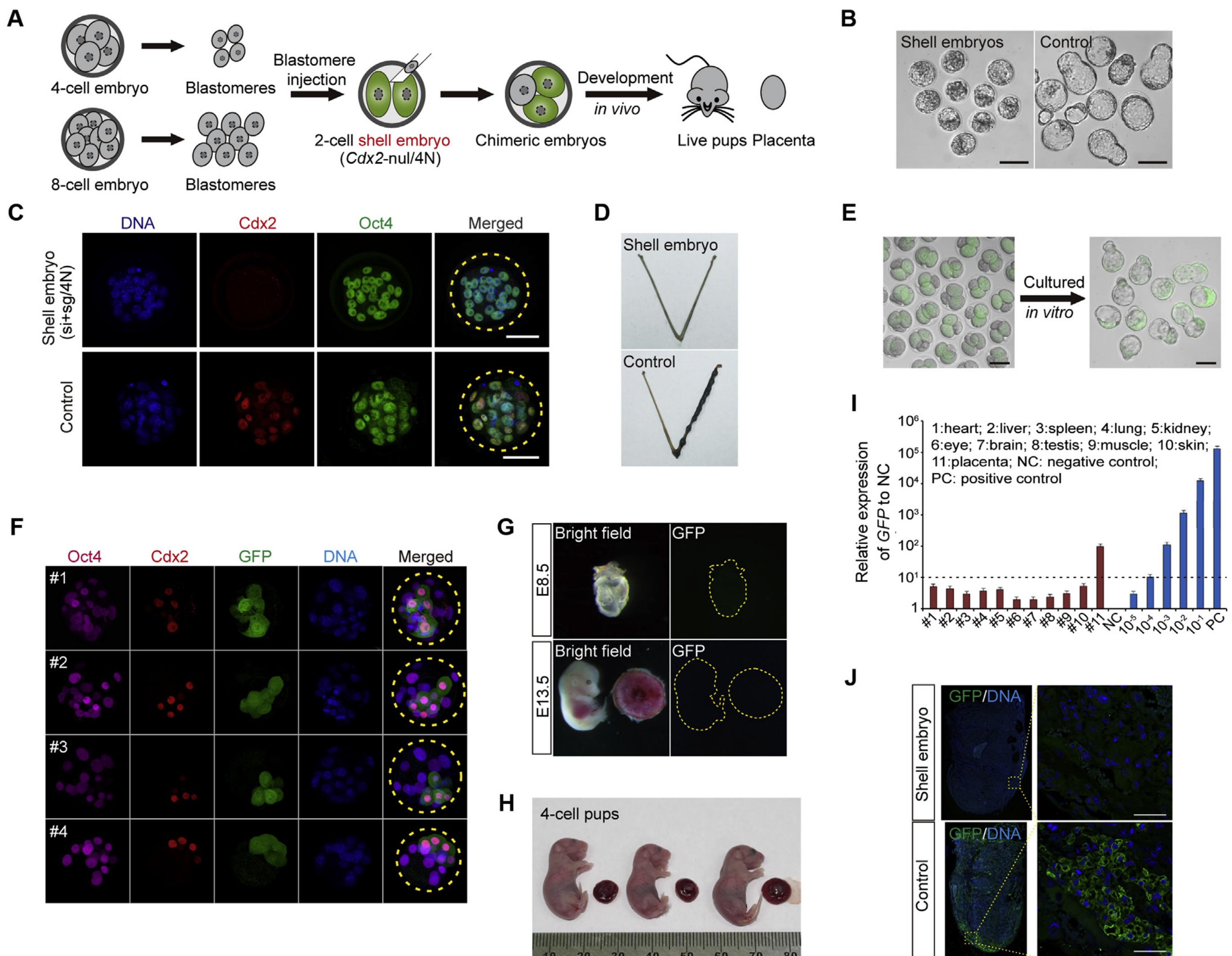

Fig. 1. Blastomeres at the 4- and 8-cell stages are totipotent. **A**: Process to evaluate the totipotency of 4- and 8-cell stage blastomeres. The shell embryo expresses GFP. **B**: Representative phase contrast images showing mouse shell embryos. Wild-type embryos injected control siRNA and sgRNA were used as controls. Scale bars, 100 μm. **C**: Immunofluorescence staining of Oct4 (a specific marker of the ICM lineage) and Cdx2 (a specific marker of the TE lineage) in shell embryos. Wild-type embryos injected control siRNA and sgRNA were used as controls. Shell embryos, $n = 50$; control, $n = 22$. Scale bars, 100 μm. **D**: Morphology of the uterus after shell embryo (upper panel) and control embryo (bottom panel) implantation at E6.5. **E**: Morphology of chimeric embryos at the 2-cell stage (left panel) and blastocyst embryo stage (right panel). Blastomeres from the shell system expressed GFP. Scale bars, 100 μm. **F**: Immunofluorescence staining of Oct4, Cdx2 and GFP (specific to cells derived from individual 4-cell stage blastomeres) in four chimeric embryos. The GFP-expressing blastomeres of the four chimeric embryos were derived from the same 4-cell stage embryo. Scale bars, 100 μm. **G**: Representative phase contrast images of E8.5 and E13.5 chimeric embryos generated by injecting individual 4-cell stage blastomeres into GFP-expressing shell embryos. GFP was nearly undetectable in the fetus and placenta (areas marked by yellow dashed lines). Scale bars, 1 mm (E8.5) and 5 mm (E13.5). **H**: Three living pups were produced from a single 4-cell stage blastomere by shell embryo complementation. **I**: Detection of *GFP* levels in various tissues and placentas of 4-cell stage blastomere-derived pups by real-time PCR. Wild-type ICR mice served as negative controls, and ICR-GFP mice further diluted by 1-, 10-, 100-, 1000-, 10,000-, and 100,000-fold served as positive controls. **J**: Immunofluorescence staining of individual 4-cell stage blastomere-derived full-term development placenta. ICR-GFP mouse placenta was used as a control. Scale bars, 100 μm.

embryos rather than aggregation (Fig. 1A). The blastocoels of the blastocyst stage chimeric embryos were much larger than those of shell embryos and similar to control embryos (Fig. 1B and E). Moreover, most chimeric embryos could hatch from the zona pellucida, indicating their implantation potential (Fig. 1E).

We further examined whether the blastomeres of 4-cell stage embryos have developmental potential to differentiate into both ICM and TE. To highlight the location of cells derived from the blastomeres of 4-cell stage embryos, we injected individual GFP-expressing 4-cell stage blastomeres into shell embryos without GFP expression. We selected four chimeric embryos in which the individual GFP-expressing blastomeres were derived from the same 4-cell stage embryo and performed immunostaining with Oct4 (specific to ICM cells), Cdx2 (specific to TE cells), and GFP (specific to cells derived from individual 4-cell stage blastomeres) antibodies (Fig. 1F). The results showed that some of the cells that arose from 4-cell stage blastomeres could differentiate into ICM and TE at the same time.

Next, chimeric embryos generated by injecting individual 4-cell stage blastomeres into GFP-expressing shell embryos were transferred into pseudo-pregnant mice. Fetuses evaluated at E8.5 and E13.5 both developed normally (Fig. 1G). Finally, we obtained live pups and placentas (Fig. 1H). In 339 chimeras, we obtained 37.5% (127/339) of implantations and 6.8% (23/339) of live pups (Table S1). These newborns and their placentas exhibited similar morphology to that of typical fetuses (Fig. 1H), but had lower weight (Fig. S3A). We detected a small number of shell cells in both bodies and placentas by examining the GFP expression using real-time PCR in ten mice (Fig. 1I). We further performed immunostaining of the sections of placentas, again, a very small number of GFP positive cells were found in them (Fig. 1J).

Four-cell stage blastomere-derived animals developed into adults, and their weights were similar to those of the control group (Fig. S3B). Their offspring were normal and similar to the control group (Fig. S3C). An open field test revealed that their psychology and physical strength were similar to those of the control group (Fig. S3D). These data indicated that 4-cell stage blastomeres are totipotent. Finally, we obtained live pups from individual 8-cell stage blastomeres (Fig. S3E), with 33.7% (100/297) of implantations and 3.7% (11/297) of live pups (Table S1). These results indicated that 8-cell stage blastomeres are totipotent. However, fetuses were not obtained from 16-cell stage blastomeres, and the implantation rate was very low (Table S1).

It is widely accepted that only zygotes and blastomeres of 2-cell stage embryos are totipotent in mice. In this study, by using gene knockout, gene knockdown, and tetraploid complementation technology, we established a shell system that could not develop into functional ICM and TE. Using the shell system, 4- and 8-cell stage blastomeres were shown to be totipotent. Our study greatly advances the understanding of totipotency maintenance and regulation in mammalian early stage embryos.

Stem cells are classified according to their developmental potential: totipotent, pluripotent, multipotent, and unipotent. Mouse embryonic stem cells, a type of pluripotent stem cells, can be tested by tetraploid complementation (Nagy et al., 1990; Eggan et al., 2001), and multipotent and unipotent stem cells can be tested by committed differentiation. However, because of the lack of functional tests, there are rarely appropriate methods for evaluating the development potential of totipotency stem cells. Our shell system provided a valuable method for detecting the capacity of totipotency stem cells to differentiate into the embryonic and extraembryonic lineages.

“Shell system”, we established, could detect totipotency because a sufficient number of cells were provided for the embryos to assist their implantation. Although the shell did not possess developmental potential, it played a supportive role in connecting the embryos to the uterus to improve the implantation rate. Therefore, the shell system established in this study is applicable in the field of assisted reproduction.

Totipotent cells have great potential for applications in regenerative medicine and disease modeling. Only blastomeres from 2-cell stage embryos are commonly used as the standard model to study totipotency. In the present study, we showed that individual blastomeres of 4- or 8-cell stage embryos still possess totipotency. Thus, more cell types can be used to study totipotency and its clinical applications. Further studies will provide insights into the mechanisms of pluripotency regulation at different early embryo developmental stages. Taken together, these findings not only improve the understanding of the acquisition and maintenance mechanisms of cell totipotency, but also provide an effective method for assessing cell totipotency, which may greatly improve the selection and application of totipotent cells.

Acknowledgments

We thank Qing Meng and Shi-Wen Li for their help with FACS and confocal laser-scanning microscopy. This study was supported by the National Natural Science Foundation of China (Nos. 31621004 and 81571356), the Key Research Projects of the Frontier Science of the Chinese Academy of Sciences (QYZDY-SSW-SMC002), the Key Deployment Projects of the Chinese Academy of Sciences (ZDRW-ZS-2017-5), and the Youth Innovation Promotion Association CAS (No. 2017113).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgg.2018.07.012.

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21 March 2018
Available online 19 October 2018

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