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Technical advance

Generation of rat-mouse chimeras by introducing single cells of rat inner cell masses into mouse blastocysts

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In the field of developmental biology and regenerative medicine, mammalian interspecific chimeras have been proved very useful for investigating early embryonic development and the immune system establishment, and extended to a promising potential for human organ generation (Rossant et al., 1982). In the early research, the production of interspecific chimeric animal has been achieved through mixing the blastomeres of early stage embryos from two independent species or subspecies (Rossant and Frels, 1980; Fehilly et al., 1984). Chimeras produced from *Mus musculus* and *Mus caroli*, two mouse subspecies, were described by Rossant and Frels. The famous interspecific live chimera was generated by aggregation of preimplantation embryos of sheep (*Ovis aries*) and goat (*Capra hircus*), reported as the "geep" (Fehilly et al., 1984). However, the attempts of producing interspecific chimeras between mouse and rat failed in various laboratories, since little or no rat cells were detected in resulting live offspring after injecting rat inner cell masses (ICMs) into mouse preimplantation embryos (Rossant, 1976).

Recently, the authentic embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) were successfully derived not only from mouse but also from rat by "2i" culture system (Buehr et al., 2008; Li et al., 2008; Ying et al., 2008; Jiang et al., 2013). By injecting mouse pluripotent stem cells (PSCs) into rat blastocysts or injecting rat PSCs into mouse blastocysts, the viable interspecific chimeras between mouse and rat were generated (Liao et al., 2009; Kobayashi et al., 2010). Then, combining blastocyst complementation, functioning rat pancreas and thymus formation were achieved in xenogeneic chimeras by ESCs/iPSCs interspecific chimeric technique (Kobayashi et al., 2010; Isotani et al., 2011). These works, proof of principle, may provide a solution for the generation of xenogeneic organs for human regenerative medicine.

Although interspecific chimeras have been successful achieved by injecting PSCs into blastocysts, the developmental paths of pluripotent cells under the xenogeneic environment and the mechanisms are not clearly understood. So far, the old issue that which embryonic developmental stages should be chosen for two individual species in generation of interspecific chimeras is still an intriguing question to answer. Here, we report the successful generation of rat-mouse interspecific chimeras via injecting single cells of rat ICMs into mouse blastocysts upon certain technical improvements. The findings will facilitate the understanding in the field of interspecific chimeras and improve the progress of xenogeneic organ generation for human organ regeneration.

In earlier attempts, embryo aggregation between rat ICMs derived from blastocysts and mouse embryos at the morula stage have been used to produce interspecific chimeras which can only develop to embryonic day 5.5 (E5.5) *in vivo*, while no viable offspring could be obtained (Rossant, 1976). In this study, we designed an immunosurgery-trypsinization method to separate rat ICMs into single cells as the donors (Fig. 1A). First, we obtained rat blastocysts carrying GFP fluorescent markers from 4.5 dpc (days post-coitus) DA strain rats which were GFP-labeled in the whole body by the piggyBac system as we reported recently (Li et al., 2016) (Fig. 1B). Moreover, the GFP-positive ICMs were separated into single cells from blastocysts by preforming trypsinization followed by immunosurgery (Fig. 1C). After injecting the single cells of

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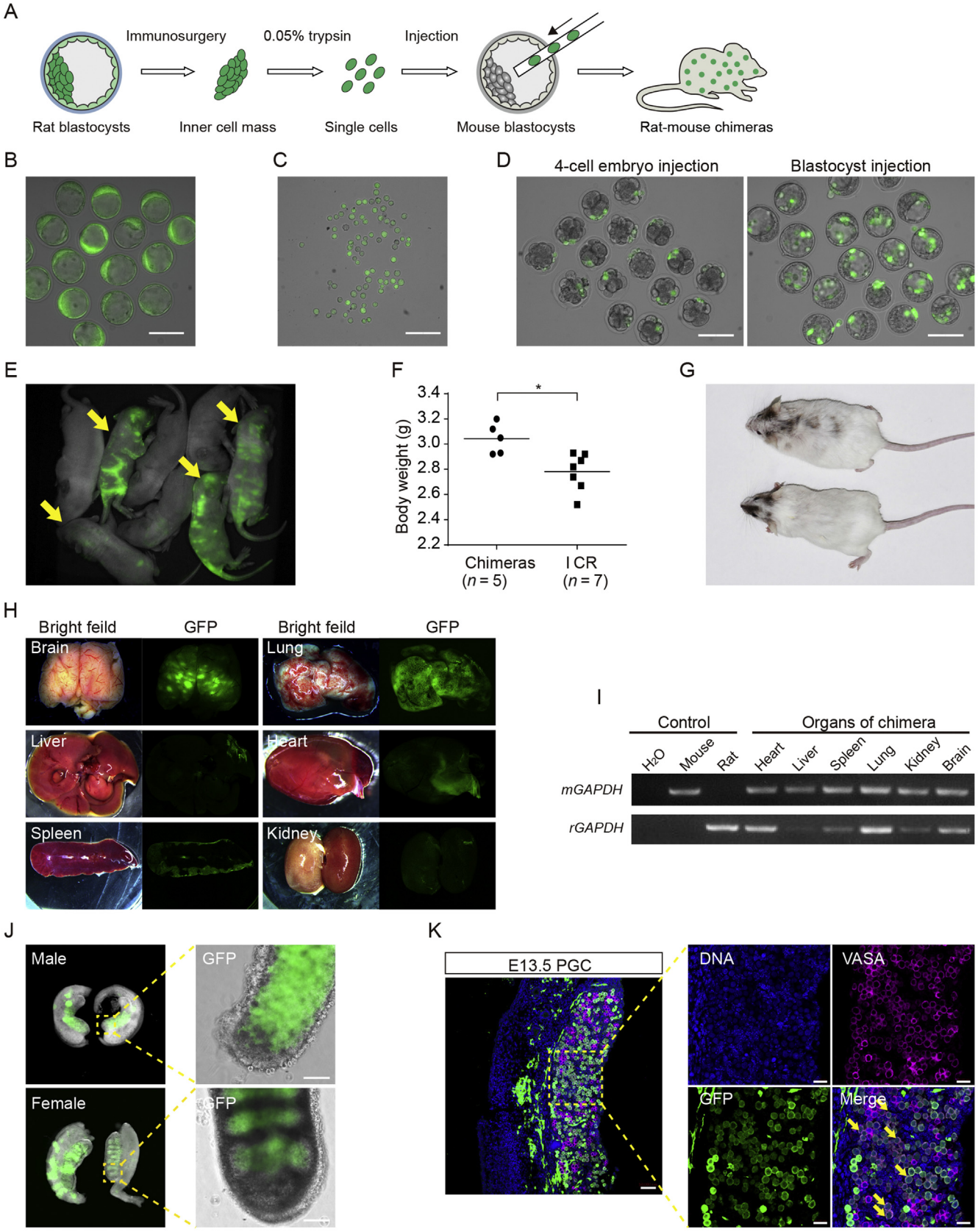
A
Immunosurgery
0.05% trypsin
Injection
Rat blastocysts
Inner cell mass
Single cells
Mouse blastocysts
Rat-mouse chimeras
B
C
D
4-cell embryo injection
Blastocyst injection
E
F
Body weight (g)
3.4
3.2
3.0
2.8
2.6
2.4
2.2
*
Chimeras (n = 5)
ICR (n = 7)
G
H
Bright feild
GFP
Bright feild
GFP
Brain
Lung
Liver
Heart
Spleen
Kidney
I
Control
Organs of chimera
H2O
Mouse
Rat
Heart
Liver
Spleen
Lung
Kidney
Brain
mGAPDH
rGAPDH
J
Male
GFP
Female
GFP
K
E13.5 PGC
DNA
VASA
GFP
Merge

rat ICM into 4-cell stage embryos or blastocysts of CD-1(ICR) mice, alive E13.5 rat-mouse interspecific chimeric fetuses were generated by only injecting into mouse blastocyst (Figs. 1D and S1A). We found that the rat cells with GFP expression aggregated in the body of the E13.5 chimeric fetuses which were detected by fluorescence imaging (Fig. S1A). The PCR analysis also detected the existence of the rat derived cells in several dissected E13.5 chimeric embryos (Fig. S1B). In order to analyze the contribution of rat cells in chimeric fetuses, we next derived the embryonic fibroblasts from the whole body (Fig. S1C). Then we detected GFP-positive cells which came from rat ICMs by fluorescence-activated cell sorting (FACS) analysis. It was found that there were about 5% GFP-positive cells from rat ICMs (Fig. S1D). Also, karyotype analysis of the fibroblast cells confirmed the normal rat diploidy with 42 chromosomes in GFP-positive cells (Fig. S1E).

To investigate the full-term developmental potential of generated chimeras, the single ICM cells derived from two different rat strains, DA strain and BN mated with SD hybrid strain, were used as donner cells. After being transferred into pseoudopregnancy mice, a total of six rat-mouse chimeras were generated from 220 injected embryos with an efficiency at 1.8% in DA and hybrid strain ICM injection (Fig. 1E and Table 1). However, no chimeric animal development to full-term was obtained from the 4-cell stage embryo injection group (Table 1). We also compared the developmental potential between rat ICMs and rat ESCs for interspecific chimera generation. Two rat ESC lines showed a higher chimeric efficiency (8.1% and 15.6%) in chimera generation, which might be explained by the higher cell proliferation (Table 1). We next confirmed that the rat ICMs contributed to the body of these chimeras as detected by GFP fluorescence observation. We also found that the weight of chimeric pups increased significantly than wild-type mice (Fig. 1F). Moreover, these chimeric animals could grow to their adulthood (Fig. 1G). The rat-derived cells also contributed to several organs of the chimeric animals based on the fluorescence detection (Fig. 1H). After dissecting the chimeras, we found that the rat cell contribution differed in organs including the brain, lung, liver, heart, spleen and kidney of the chimeric animals, as detected by gene identification (Fig. 1I).

Another important issue in the field of interspecific chimeras was whether pluripotent cells, including ICMs or ESCs/iPSCs, can make contribution to the germline or generate functional gametes. In this study, E13.5 male/female genital ridges were inspected and we found that there were GFP-positive rat cells in the genital crest of chimeras for both males and females (Fig. 1J). Further, we observed GFP-positive rat cells with VASA expression in the genital ridge of E13.5 chimeric fetuses detected by immunofluorescent staining, indicating that rat ICMs were transmitted to the germline of the interspecific chimeras (Fig. 1K). Next, we investigated whether rat cells could differentiate into gametes in these adult chimeras. More than 10 adult chimeras were dissected, including males and females, and we found GFP-positive rat cells contributed in the testis and ovary, respectively (Fig. S1F and G). However, there were no GFP-positive mature sperms or oocytes found in male or female adult chimeras, analyzed by immunofluorescence staining by VASA and GFP co-localization (data not shown). These results might attribute to the impaired germ cell development or more limited genital ridge environment in the xenogenic embryos.

Chimeras generated from more than one individual species have been widely used for study of cell lineage and cell fate during embryonic development. As the most widely used rodent animal models, generation of the rat-mouse interspecific chimera was first tested in 1970s (Rossant, 1976). As reported, rat ICMs derived by immunosurgery were injected into early mouse embryos and transferred into mouse uteri, and the contribution of rat-derived cells could be detected in sections of the fetuses but not in the full-term pups. We believe that acquiring rat ICMs as donor cells with high cell activity and proliferative ability may act as a critical factor to produce interspecific chimeras. In this study, we developed an immunosurgery-trypsinization approach to derive rat single-cell ICMs with high activity and injected them as donor cells into mouse blastocysts. The mouse embryos at both the 4-cell stage and the blastocyst stage were used as recipients, but only the blastocysts injected with rat ICMs could generate interspecific chimeras. The stage-matching compatibility was reported as a critical factor to generate interspecific chimeras (Masaki et al., 2016). Furthermore, the rat ICM cells are at a more similar pluripotency stage as the recipient mouse blastocysts, which enables them to successfully integrate into mouse blastocysts rather than the 4-cell stage embryos. Fortunately, we successfully generated viable rat-mouse interspecific chimeras after the chimeric blastocysts were transferred into the mouse uterus.

We found that rat ICMs integrated into many tissues and organs of interspecific chimeras including the brain, lung, liver, heart, spleen and kidney as detected by GFP fluorescence protein. Our results also showed that there were about 5% rat derived cells contributed in the rat-mouse chimeras, which was similar with the rat-mouse chimera generation by mouse blastocyst injection with rat ESCs or iPSCs as previously reported (Kobayashi et al., 2010; Isotani et al., 2011). Most importantly, GFP-positive rat cells integrated into primordial germ cells, which implied that rat ICMs have the capacity of germline contribution in rat-mouse interspecific chimeras. Our results suggest that ICMs can be used to generate interspecific chimeras in species, with derived PSCs having a limited developmental potential, such as in humans and non-human primates.

In summary, this approach described here not only provides a choice to generate xenogeneic organs especially in the species with no ESCs or IPSCs derived, but also provides a better understanding of interspecific chimeric ability of pluripotent cells of early mammalian embryos as an initial step towards the ultimate regenerative medicine in the future.

Fig. 1. Generation of rat-mouse chimeric fetuses produced by injecting single cells of rat ICMs into mouse blastocysts **A**: Schematic of isolating single cells from ICMs of rat blastocysts by using immunosurgery-trypsinization. Isolated ICMs from DA rat blastocysts were dissociated in 0.05% trypsin, and segregated single cells could be achieved, which would be injected into mouse blastocysts to generate rat-mouse chimeras. **B**: GPF positived E4.5 blastocysts were harvested from GFP transgenic rats. Scale bar, 100 μm. **C**: Single cells isolated from ICMs of rat blastocysts to be injected into 4-cell or blastocyst stage mouse embryos. Scale bar, 100 μm. **D**: 4-cell or blastocyst-stage mouse embryos injected with single cells isolated from the ICMs of rat blastocysts. Scale bar, 100 μm. **E**: Interspecific chimeric pups (day 3 after postpartum), generated by injecting single cells of rat ICMs into mouse embryos. The arrows point out the interspecific chimeric parts. **F**: Body weight of new born interspecific chimeras (left panel) and ICR mice (right panel). **G**: Two rat-mouse interspecific chimeras survived to the adulthood. The fur of dark color and white color was originated from rat ICMs and mouse embryos (CD-1 strain), respectively. * represents $P < 0.05$. **H**: Phase contrast and fluorescence images of organs derived from chimeras. Rat cells with GFP expression were found in organs of brain, lung, liver, heart, spleen and kidney. **I**: Determination of the cell contribution of rat ICMs by PCR analysis of rat and mouse *Gapdh*. Rat and mouse genome were used as control. **J**: Immunofluorescence detection of gonads in E13.5 male and female chimeric fetuses. GFP positive cells in female genital ridge reflected contribution of rat ICM cells. Scale bar, 100 μm. **K**: Immunofluorescence staining of genital ridges isolated from E13.5 interspecies chimeras. Co-localization of VASA and GFP was observed in the genital ridges of rat-mouse interspecific chimeras (yellow arrows indicated). Scale bars, 50 μm (left panel) and 20 μm (right panel)..

Table 1
Generation of rat-mouse interspecific chimeras by injecting rat ICMs or rat ESCs into mouse embryos.

Donner cell	Rat strain[a]	Cell line	Cell passage	Recipient embryo	Number of embryos transferred	Number of E13.5 development fetuses		Number of full-term development pups	
						Fetuses (%)	Chimeras (%)[b]	Pups (%)	Chimeras (%)[b]
ICM	DA-GFP	/	/	4-cell	67	16 (23.8)	0	–	–
	DA-GFP	/	/	Blastocyst	82	34 (41.5)	5 (6.1)	–	–
	DA-GFP	/	/	4-cell	42	–	–	7 (16.7)	0
	DA-GFP	/	/	Blastocyst	135	–	–	23 (17.0)	4 (2.96)
	BS-GFP	/	/	Blastocyst	85	–	–	12 (14.1)	2 (2.35)
rESC	DA-GFP	DA G-1	P13	Blastocyst	45	–	–	23 (51.1)	7 (15.6)
	DA	DA 5-3	P28	Blastocyst	62	–	–	28 (45.2)	5 (8.1)

[a] DA-GFP and DA represent Dark Agouti strain carried with or without GFP protein; BS-GFP represents the rats of Brown Norway strain (carried with GFP protein) mated with Sprague Dawley strain. [b] The percentages of chimeras were calculated from the number of injected embryos.

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Supplementary data

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