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# Methods to Generate Chimeric Mice from Embryonic Stem Cells

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## 1. Introduction

A chimera is an animal that has two or more populations of genetically distinct cells that originated in different embryos, fetuses, or individuals of the same or different species. During recent decades, embryos, the inner cell mass (ICM), teratocarcinoma stem cells, embryonal carcinoma stem cells, embryonic stem (ES) cells (ESCs), primordial germ cells, spermatogonial stem cells, extraembryonic endoderm (XEN) cells (Kunath et al., 2005), induced pluripotent stem cells (iPSCs) (Boland et al., 2009; Kang et al., 2009; Takahashi & Yamanaka, 2006; Zhao et al., 2009), epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007), and other cells have been used to generate chimeric embryos that can develop into chimeras once transferred to a foster mother.

To date, three methods can be applied to produce ESC-derived chimeric embryos: (1) aggregation, (2) microinjection, and (3) coculture. This review uses mouse ES cells as an example to describe and compare existing methods for generating chimeric embryos.

## 2. Methods for generating chimeric embryos

Long before successful generation of chimeric embryos using mouse ES cells (Evans & Kaufman, 1981; Martin, 1981), large chimeric morulae were first generated using zona pellucida (ZP)-free (denuded) pre-implantation embryos aggregated mechanically in a small drop of medium (Tarkowski, 1961). However, Tarkowski's mechanical method (via a pipette) is technically difficult and tedious for broken and removed the ZP one by one. Later, a study indicated that mouse ZP can be digested and removed easily using pronase (~1 min) and pipettes (Mintz, 1962). A subsequent study demonstrated that using acidic Tyrode's solution (pH 2.5) to dissolve mouse ZP is a relatively simpler and cheaper method (Nicolson et al., 1975). A batch of intact whole embryos (~25) submerged in the acidic Tyrode's solution for approximately 10 seconds is sufficient to partially dissolve the ZP, and the embryos can then be transferred to a buffered medium to wash away the denuded embryos via pipettes. Aggregation has since become one of the major methods for generating chimeric embryos.

Unfortunately, once blastocysts form, generating chimeric blastocysts via the aggregation method is generally impossible. However, one may introduce cells into the cavities of blastocysts to obtain chimeric embryos. It was the first report that chimeric embryos

produced using the five-instrument microsurgical method to introduce ICMs or cells into the blastocyst cavity (Gardner, 1968). Apparently, the five-instrument method is too complex for routine operation. Thereafter, the two-micropipette microinjection method was developed (Moustafa & Brinster, 1972).

Since aggregation and microinjection methods are commonly used to produce chimeric embryos; techniques, equipment, and protocols have been modified and improved. For technical details of current methods, including cells, embryos, instruments, and equipment for making micropipettes and generating chimeric embryos, see previous articles (Bradley, 1987; Nagy et al., 2003; Nagy et al., 2010; Papaioannou & Johnson, 2000; Papaioannou & Dieterlen-Lievre, 1984; Pluck and Klasen, 2009). The website (<http://www.mshri.on.ca/nagy/default.htm#>) of Professor Nagy at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada, also provides technical protocols for generating chimeric mouse and ESC-derived mouse via aggregation between ES cells and diploid (2n) or tetraploid (4n) 2.5-day post-coitum (dpc) embryos.

Although the aggregation and microinjection methods are effective for producing chimeric embryos, due to instrumental and technical limitations, they are unsuited to mass production. Thereafter, the coculture method was developed (Wood et al., 1993a; Wood et al., 1993b).

## 2.1 Comparison of conventional methods for generating chimeric mice

Currently, the most common technique for generating chimeric embryos is direct microinjection of ES cells into the cavity of 3.5-dpc blastocysts. Microinjection is a highly stable and reproducible method that can generate good germline transmitted chimeras. However, this method has various limitations. First, an expensive micromanipulation system is required. Second, intensive training is needed to master micromanipulation skills. Third, the microinjection step is time-consuming, averaging only 20–40 blastocysts/h, limiting production to 50–100 blastocysts daily (Bradley, 1987; Hogan et al., 1994; Nagy et al., 2003). Therefore, generating chimeras usually requires pay-based services. Although using Piezo-driving (Kawase et al., 2001) to introduce ES cells into the cavity of blastocysts may have relatively better efficiency in producing chimeras, it is rarely applied as it requires a high skill level and an extra expensive device.

Well sandwich aggregation is the second most popular method for generating chimeric embryos. This method is also a highly stable, reproducible, and easy method for generating chimeras and has a germline transmission efficiency nearly equivalent to that of blastocyst microinjection (Bradley, 1987; Hogan et al., 1994; Nagy et al., 2003; Papaioannou & Johnson, 2000). Well sandwich aggregation does not require expensive and sophisticated instruments, and is easily learned and implemented. Those familiar with using a mouth pipette can use this method routinely in a laboratory. However, two or more embryos (either XX or XY) are required for aggregation to create a single reconstructed embryo, which is disadvantageous for inbred mice, as only 6–10 embryos can be recovered from each mouse through superovulation. Although single embryo aggregation is a viable option, its efficiency in generating chimeras varies and is inferior to methods using two or more embryos. Therefore, very few studies have used single embryo aggregation to generate chimeric embryos.

Another alternative for generating chimeric embryos is coculturing 2.5-dpc denuded single 4-cell embryos to morulae with ES cells on dish surfaces (Shimada et al., 1999; Wood et al., 1993a) or in droplets (Ueda et al., 1995). However, the efficiency of generating chimeras via this method is far inferior to that of microinjection and well sandwich aggregation. Only a few studies have used this method to generate chimeric embryos.

Table 1 summarizes and compares conventional methods for generating chimeric embryos.

	Microinjection	Well sandwich aggregation <sup>1</sup>	Single embryo aggregation <sup>1</sup>	Coculture <sup>2</sup>
Equipment	Very expensive	Inexpensive	Inexpensive	Very cheap
Skill level	Very high	Low	Low	Very low
Time needed to learn the technique <sup>3</sup>	2–3 months	2–3 weeks	2–3 weeks	1–2 weeks
Time needed to produce chimeric embryos <sup>4</sup>	20–40 blastocysts/h and not more than 50–100 blastocysts/d	~30 pairs/h	~40 embryos/h	>100 embryos/h

<sup>1</sup>Cultured overnight then reconstructed chimeric embryos are recovered for transfer or other treatments.

<sup>2</sup>Cocultured for 3–4 h, the embryo-ESC aggregates are recovered and cultured overnight to produce chimeric embryos.

<sup>3</sup>Persons must be familiar with cell and embryo culturing as well as mouth pipetting.

<sup>4</sup>Enriched ES cells and recovered intact embryos are ready for use.

Table 1. Comparison of conventional methods for generating chimeric embryos

## 2.2 Vial coculture method for generating chimeric mice

Although the microinjection method produced good and reliable results, it is hard to practice by a laboratory. Therefore, outsourcing to a core facility or commercial company is common. Unfortunately, service charges are high at approximately US\$1,000–3,000/case. Conversely, the aggregation method is easily applied and inexpensive. However, this method must be applied in a one-by-one manner and is tedious. The conventional coculture method is also easily applied and is inexpensive. Furthermore, this method facilitates routine mass production of chimeric embryos. Unfortunately, outcomes are not as reliable and good as those of the microinjection and well sandwich aggregation methods. In conventional coculture protocols, denuded embryos on a dish surface have only two-dimensional ES-cell contact surfaces, resulting in only 55–64% of denuded embryos adhering to ES cells (Ueda et al., 1995; Wood et al., 1993a). Obviously, an improved coculturing method is needed that can achieve results as good as or better than those by the microinjection and well sandwich aggregation methods. Moreover, an improved coculturing method should be easily applied, cheap, and suited to mass production.

Recently, my laboratory developed an alternative simple, very cheap, and reproducible method for mass production of chimeric embryos by coculturing 2.5-dpc denuded 8-cell embryos and compacting morulae with ES cells in 1.7-mL Eppendorf vials (micro test tube); this method has fewer technological and instrument-based limitations than conventional methods. Although depressed microwells made by a darning needle had three-dimensional possibility for denuded embryos and ES cells to contact each other, however, in the vial coculturing system, the large number of enriched ES cells surrounding the denuded embryos from every direction may improve the overall adherence. Furthermore, gravity may also contribute to enhanced ES cell adherence via this method. The resulting chimeras show significantly high levels of chimerism and high germline transmission rates (Lee et al., 2007). Table 2 lists an example schedule and protocol for the vial coculture method. Figures 1 and 2 show vial coculturing results.

Date	Time	Target	Treatment
Friday	~16:30	Donor females	PMSG 5–10 units/ip
Sunday	~16:30	superovulated	hCG 5–10 units/ip; donor females are mated with studs
Monday	am	Donor females	Plug checked
		ES cells	Thawed or pass in high density to 0.1% gelatin-coated dishes
Tuesday	~16:30	Recipients (ICR, CD-1, or F1 hybrid)	Estrus females mated with vasectomized males
	am	Recipients	Plug checked
	~08:30	ES cells	Enriched via the double plating method
	~08:40	35-mm cell culture dish	HK (20.85 mM hepes-buffered KSOM, 285 ± 10 mOsm/kg H <sub>2</sub> O) droplets under light-weight mineral oil prepared; room temperature (RT)
	~09:00	Donor females	Recovery of the 2.5-dpc embryos, which are kept in HK at RT until used
	~10:00	ES cells	The first round of enriched cells harvested and re-suspended to a cell medium for the second standing
	~10:25	Enriched ES cells	Cells are harvested and stored at 4°C until the concentration is adjusted for coculturing
Wednesday	~10:40	6-cell embryos to compacting morulae	ZP are removed using the acidic Tyrode's solution
	~11:00	1.7-mL vials	Approximately 0.8 mL enriched ES cells are transferred to vials; after ~5 min, denuded embryos are added for coculturing; 5% CO <sub>2</sub> , 37°C incubator
	~11:15	60-mm bacteriological dish	10-μL droplets of KSOM-AA or KSOM-AA containing 1% FBS are prepared and put in an incubator under 5% CO <sub>2</sub> at 37°C
	~14:00	1.7-mL vials	Vial coculturing ends, embryo-ESC aggregates are recovered
	~14:30	60-mm bacteriological dish	embryo-ESC aggregates are washed to droplets of KSOM-AA or KSOM-AA containing 1% FBS for culturing overnight; 5% CO <sub>2</sub> , 37°C incubator
Thursday	am or pm	Recipients	Chimeric embryos are transferred to uterus horns of 2.5-dpc pseudopregnant recipients
Sunday		Recipients	Pups are born after ETed for 17 days

<sup>1</sup>One person can finish the vial coculturing easily.

Table 2. Schedule and protocol for the vial coculture method for generating chimeric mice<sup>1</sup>

### 2.2.1 Technical considerations for vial coculture method

To ensure that cells adhered to the denuded embryos are the ES cells mainly, any enrichment method for ES cells can be used. When using the double-plating selection method, approximately 96% of harvested cells expressed bright-green fluorescence, and approximately 92% of these cells were  $<12\ \mu\text{m}$  in diameter (Lee et al., 2007). The method is easily implemented, and is selective, effective, and reproducible in removing debris, dead cells, and feeder cells from the prepared ES single-cell suspension.

Different volumes of Eppendorf vials are available for coculturing. I recommend the 1.7-mL vial due to the size is good for handling. Approximately 0.8-mL aliquots of enriched ES cells, either fresh or thawed;  $\sim 5.0 \times 10^5$  cells/mL in KSOM-AA alone or KSOM-AA containing 1% fetal bovine serum (FBS) are added to sterile polypropylene 1.7-mL vials with snap caps. After left to stand for 5 min,  $\leq 200$  denuded 6-cell embryos to morulae are gently and circularly blown from beneath the medium surface into the vial via a mouth pipette, and then coculture in an incubator under 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  for  $3 \pm 1$  h. Denuded 2- to 4-cell embryos are unsuitable for coculturing, as blastomeres sometimes can easily separate during coculturing. Additionally, the relatively smaller diameters of blastomeres make recovery difficult. However, adherent cells are consistently observed in separated blastomeres. Conversely, segregation of blastomeres of electrofused tetraploid (4n) 3- to 4-cell embryos does not occur.

After the coculturing is ended, precipitate in vials is aspirated gently and loose cells on embryonic surfaces are removed by washing using a mouth pipette. Denuded embryos adhered to ES cells are recovered. Following coculturing for 3 h in 1.7-mL Eppendorf vials,  $>90\%$  of denuded 6-cell embryos to morulae adherent ES cells can be recovered. Moreover, approximately 90% of recovered embryos adhered tightly to ES cells and approximately 5–10% (the percentage increases as the number of embryos in the same vial increases) of recovered aggregates had 2 or 3 embryos clustered around and adhered to ES cells. Aggregates have  $>3$  embryos, if necessary, can be separated by gentle pipetting using a mouth pipette. During coculturing, approximately 10% of denuded 8-cell embryos developed into compacting morulae. Experimental data show that cell adherence could reach 100% when ES cell concentrations are increased, or when the coculturing period is extended. The embryo-ESC aggregates are washed directly in either KSOM-AA or KSOM-AA containing 1% FBS droplets (10  $\mu\text{L}$ ) under light-weight paraffin oil on bacteriological dishes. Two to four embryo-ESC aggregates are carefully allocated to different corners of the same droplet to prevent possible adhesion and are cultured overnight in an incubator at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  until transfer to uterine horns of 2.5-dpc pseudopregnant recipients.

Mouse preimplantation embryos and ES cells require different in vitro culture requirements. Essentially, ES cells require at least 5% FBS (Wakayama et al., 1999) to maintain survival and possible pluripotency unless cultured in knockout serum replacement (KSR) (Goldsborough et al., 1998). It has been showed that poor ES cell viability after overnight coculturing in M16 or KSOM media (Huang et al., 2008; Kondoh et al., 1999). Although, M16 supplemented with FBS, could enhance chimera generation (Kondoh et al., 1999). Unfortunately, the viable fetuses derived from the FBS (5–15%) groups were significantly fewer than that derived from the FBS-free control group (Arny et al., 1987; Caro and Trounson, 1984; Khosla et al., 2001). Khosla et al. (2001) indicated that FBS exerts a direct adverse effect on genes responsible for postimplantation development.

To summarize, coculturing 2.5-dpc denuded 6-cell embryos to morulae with ES cells in 1.7-mL Eppendorf vials for approximately 3 h is a simple and effective alternative method for mass production of chimeric embryos. Table 3 compares conventional methods and the vial coculture method for generating chimeric embryos and germline transmitted chimeric mice.

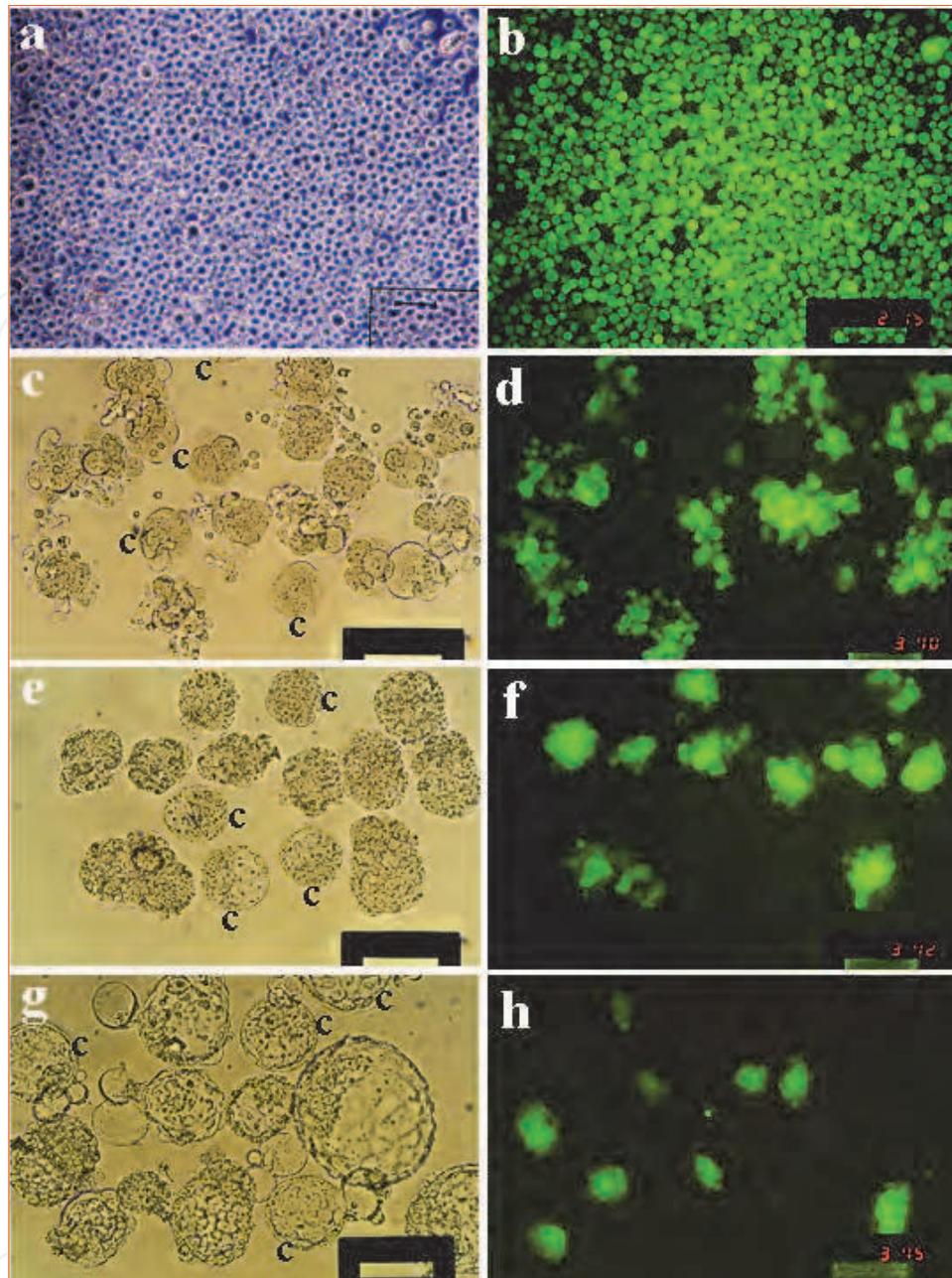


Fig. 1. Enrichment of ES cells and development of denuded embryo-ESC aggregates. a: Attaching and/or attached cells were recovered after the original single-cell suspension of the mouse ES cell, ESC 26GJ9012-8-2, cultured on a 100-mm dish in a 5% CO<sub>2</sub> incubator at 37°C for 80 min. b: Over 94% of cells expressed the green fluorescence protein (GFP). c, d: After denuded 8-cell embryos, morulae, and ESC 26GJ9012-8-2 cells were cocultured in an 1.7-mL Eppendorf vial for 2 h, recovered embryos had adherent green fluorescing ES cells on their surfaces. The left bright and right green fluorescent images show the same view of 14 embryos, including 2 8-cell embryos, 2 compacting morulae with zona pellucida (as the control; c), 5 single-embryo-cell aggregates, 3 2-embryo-cell aggregates, and 2 3-embryo-cell aggregates (some kind sandwich aggregation). e, f: After culturing aggregates from panels c and d overnight in droplets of KSOM-AA containing 1.0% FBS, the aggregates had cells with surface green fluorescence mingling in the developing compacting and compacted

chimeric morulae. g, h: After further overnight culturing, chimeric morulae from panels e and f developed into chimeric blastocysts displaying green fluorescence cells primarily in the ICM. Scale in panel a: bar = 50  $\mu\text{m}$ . Scale in panels b–h: bar = 100  $\mu\text{m}$ . (Reproduced with permission from Lee et al., 2007. *Theriogenology* 67:228–237.)

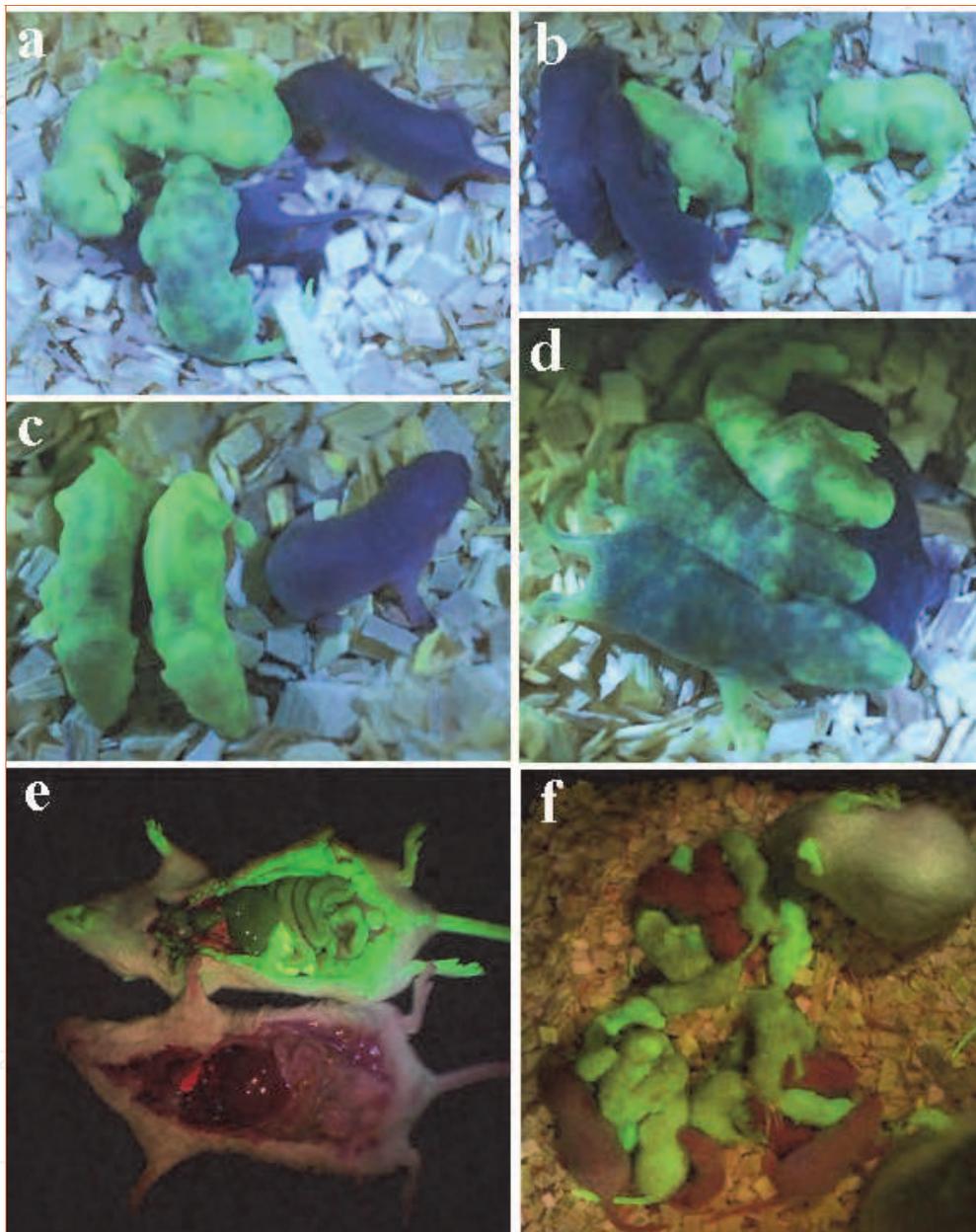


Fig. 2. Chimeras with high percentages of coat color distribution and germline transmission generated using the Eppendorf vial coculture method. In this experiment, chimeric morulae and/or blastocysts developed from aggregates of ICR  $\times$  B6CBAF1 embryos and ESC 26GJ9012-8-2 cells were transferred to pseudopregnant ICR 2.5-dpc uterine horns. The pups born alive had high percentages of coat color and green fluorescence expression. a, b: Pups derived from fresh ESC 26GJ9012-8-2 cells. c, d: Pups derived from thawed ESC 26GJ9012-8-2 cells. e: Green fluorescence was widely expressed on internal organs of mature chimeras. The control mouse had no green fluorescence expression. f: Pups expressing green

fluorescence, following phenotypically normal male chimeras, were naturally mated with ICR to achieve high germline transmission rates. (Reproduced with permission from Lee et al., 2007. *Theriogenology* 67:228–237.)

	Microinjection	Well sandwich aggregation	Single embryo aggregation and coculture	Vial coculture
Pups born alive after chimeric embryos ETed <sup>2</sup> (A)	~60%	~30%	~20%	~25%
Chimeras (B)	~50% (B/A)	~55% (B/A)	~45% (B/A)	~60% (B/A)
Male chimeras (C)	~50% (C/B)	~50% (C/B)	~40% (C/B)	~60% (C/B)
Male chimeras with germline transmission (D)	~30% (D/C)	~40% (D/C)	~30% (D/C)	~50% (D/C)
Total efficiency of germline transmission <sup>3</sup> (A × B × C × D)	~4.5%	~3.3%	~1.1%	~4.5%

<sup>1</sup>Data are compiled from previous studies.

<sup>2</sup>Pups born alive/chimeric embryos transferred.

<sup>3</sup>Germline transmitted male chimeras/chimeric embryos transferred. The total efficiency of germline transmission is highly variable and depends on ES cells used, donor embryos used, persons doing, and mouse facilities managed.

Table 3. Comparative efficiency of different methods for generating germline transmitted chimeric mice<sup>1</sup>

### 2.3 Generation of ESC-derived mice

Authentic ES cells are defined by three cardinal properties: unlimited symmetrical self-renewal in vitro; comprehensive contribution to primary chimeras; and generation of functional gametes for genome transmission (Buehr et al., 2008). However, using 4n complementation for generating ESC-derived mice is regarded as the most solid criterion for ES cell pluripotency. The criterion is also accepted for generation of iPSC-derived mice (Boland et al., 2009; Kang et al., 2009; Zhao et al., 2009).

The mouse pluripotent ES cells can be established from zygotes, blastomeres, and ICMs (Lee et al., 2011). However, the originate of ES cells limit its developmental potential to embryo proper mainly (Beddington & Robertson, 1989). Tetraploid embryos typically stop their development during the early stage of gestation and do not develop beyond the mid-gestation period due to a lack of the embryo proper, but persist in extraembryonal membranes (Eakin & Behringer, 2003; Kaufman & Webb, 1990; Snow, 1975; Tarkowski et al., 1977). Combining 4n embryos and 2n ES cells is logical as 4n embryos may complement the deficient extraembryonal differentiation of ES cells while allowing full expression of their potential for fetal development (Nagy et al., 1990). Thereafter, a viable and fertile ESC-derived mouse was generated (Nagy et al., 1993).

Since ESC-derived mice were generated successfully using 4n 4-cell embryos aggregated with 2n ES cells (Nagy et al., 1990; Nagy et al., 1993), well sandwich aggregation is the main choice to do that (Eggan et al., 2002; Li et al., 2005; Ohta et al., 2008; Schoonjans et al., 2003; Ueda et al., 1995). The second popular method is microinjecting ES cells into 4n blastocysts (Eggan et al., 2001; Kirchain et al., 2008; Li et al., 2005; Lin et al., 2010; Schwenk et al., 2003; Wang et al., 1997; Wang & Jaenisch, 2004).

Most tetraploid-ESC neonates derived from hybrid ES cells developed into fertile adults (Eggan et al., 2001). Conversely, most studies revealed that ESC-derived pups from inbred ES cells died shortly after delivery. However, one study demonstrated that inbred ES cells can generate ESC-derived mice (Schoonjans et al., 2003). Furthermore, the Caesarean section is required to overcome the failure of respiratory problems of ESC-newborns (Nagy et al., 1993). Apparently, this is a tedious work and is not a practical protocol for routine operation. Previous studies suggested that the recipient mothers can be subject to natural delivery instead of Caesarean section (Lee et al., 2003; Li et al., 2005). However, this is not suitable for any ES cell.

Previous studies also revealed that an ESC-derived mouse have host embryo contamination (Eggan & Jaenisch, 2003; Li et al., 2005), poor viability, and other minor abnormalities such as altered growth rate and body weight. However, adults had normal morphological, physiological, and neurological characteristics (Schwenk et al., 2003).

Using 4n complementation method, 4n embryos require produced of 2n 2-cell embryos usually by electrofusion and are less viable than normal 2n embryos. Thus, the generation efficiency of ESC-derived mice is relatively low at approximately 1-5%. One reason for this low efficiency may be the low cell number of 4n blastocysts. Using 3-5 aggregated 4n embryos, the efficiency in generating ESC-derived mice can be increased 2-3 times (Ohta et al., 2008). Additionally, the modified method would be applicable to any ES cell, including general ES cells used for gene targeting (Ohta et al., 2008).

Although ES cells can produce viable and fertile ESC-derived mice, this is an inefficient process; many tetraploid-ESC aggregates die before reaching term, even when early passage ES cells are used (Nagy et al., 1993). Therefore, this approach cannot be considered as a feasible approach for routinely achieving germline transmission from ES cells (Nagy et al., 1993). Reasonably, other alternatives might be developed.

#### **2.4 Generation of ESC-derived F0 mice**

After the blastocyst microinjected with ES cells that can adhere to and mingle in ICMs, then co-develop to an embryo proper including germ cells. The same phenomenon was also observed when 2.5-dpc embryos aggregated with ES cells. The mechanisms underlying this phenomenon have been investigated. Unfortunately, this mechanism remains unclear. However, data from studies of chimeric embryos suggest a combined contribution of multiple factors, including geometrical effects of cell size and polar or apolar positioning (Hillman et al., 1972; Johnson & Ziomek, 1981; Saburi et al., 1997; Tarkowski & Wroblewska, 1967). Notably, this phenomenon may also reflect the possibility that ES cells are naturally more committed to an ICM fate (Wood et al., 1993a).

Previous studies have demonstrated that ES cells adhering to the surfaces of 8-cell embryos or compacting morulae are generally localized in the ICM of blastocysts following culturing (Lee et al., 2007; Shimada et al., 1999; Wood et al., 1993a; Wood et al., 1993b). Repentigny and Kothary (2010) recently microinjected ES cells into the perivitelline space (PVS) of

zygotes. They showed that at the 2- to 4-cell stage embryos, injected ES cells remain in the PVS and are not incorporated into embryos. The ES cells begin partial blastomere incorporation into an embryo at the 8-cell embryo. Finally, at the compacted morula, ES cells are almost completely incorporated into an embryo. At the blastocyst, ES cells form an ICM (Repentigny & Kothary, 2010). Whether ES cells can replace ICMs completely and develop thereafter as an ESC-derived mouse is worthy of investigation.

Compared with 3.5-dpc blastocyst microinjection, an alternative method that microinjects ES cells into the tight space between ZP and blastomeres (the subzonal cavity) of 2.5-dpc 8-cell embryos has been reported (Tokunaga & Tsunoda, 1992). Experimental results showed that the proportion of male chimeric mice capable of germline transmission increased significantly. Furthermore, 100% coat color chimeric mice with germline transmission were produced. Unfortunately, the meaning of 100% coat color chimerism was not investigated (Tokunaga & Tsunoda, 1992). Papaioannou and Johnson (1993, 2000) have been mentioned that the result of microinjecting ES cells into 2.5-dpc 8-cell embryos was comparable to but not better than microinjection of 3.5-dpc blastocysts. Notably, microinjecting 2.5-dpc embryos are more difficult than microinjecting 3.5-dpc blastocysts due to the tight and small subzonal cavity and possible damage to blastomeres, explaining why only a few follow-up studies exist.

Laser-assisted microinjection of 7-9 ES cells into the subzonal cavity of 2n 8-cell embryos may enhance microinjection and efficiently yield F0 generation mice (100% coat color chimerism) that are fully ESC-derived and healthy, exhibiting 100% germline transmission and containing no more than 0.1% host embryo contamination (Poueymirou et al., 2007). They suggested that the F0 mouse is equivalent to the ESC-derived mouse. A subsequent study indicated that 8-10 ES cells Piezo (toxic mercury in a microinjection pipette used) microinjected into the subzonal cavity of 2n 4- or 8-cell embryos also generated F0 ES cell offspring (Huang et al., 2008).

The efficiency of generating ESC F0 mice is much better than using ESC-derived mice via ES cell assemble with 4n embryos (Eakin & Hadjantonakis, 2006; Eggan et al., 2001; Li et al., 2005; Nagy et al., 1993; Ueda et al., 1995; Wang & Jaenisch, 2004). However, these methods need an expensive laser or Piezo-driving equipment and additional training is required to acquire the necessary skills. The disadvantages of both methods limit their applications. Recent studies, which used conventional microinjection to introduce ES cells into 2- to 8-cell embryos, produced 100% coat color chimeras (Kraus et al., 2010; Ramirez et al., 2009). However, the technical problems still exist.

The Eppendorf vial coculture method can generate massive amounts of chimeric embryos. The resulting chimeric mice show approximately 40% of pups born alive with almost 100% ES cells coat color distribution (Lee et al., 2007). The major disadvantage of the coculture method is variable adhesion of ES cells onto the surfaces of denuded embryos. In practice, 4-cell embryos to morulae are recovered from superovulated 2.5-dpc donor mice. The denuded 8-cell embryos and morulae are good for vial coculturing. However, the 4-cell embryo is not suitable for vial coculturing, because blastomeres usually separate during coculturing.

The 2n ES cells microinjected into, aggregated with, or cocultured with 2.5-dpc denuded 2n 8-cell embryos and/or morulae can generate germline transmitted F0 mice. New methods with the higher efficiency may be worth developing to overcome the limitations and disadvantages of existing approaches.

### 2.5 The hypertonic microinjection method for generating chimeric mice

For conventional microinjecting ES cells to 2.5-dpc 3-cell embryos to morulae, the tight subzonal cavity is a major technical hurdle. Theoretically, increasing the space of the subzonal cavity can solve this problem.

Zona pellucida is a rigid glycoprotein that resists both hypertonic and hypotonic solutions. In contrast, the volume of an embryo proper changes in proportion to osmolarity of solutions (Leibo, 1980). In other words, the space of the subzonal cavity increases when embryos are in hypertonic solutions. Notably, a high sucrose concentration is virtually non-toxic to embryos and oocytes (Kasai et al., 1983; Kasai et al., 1992; Kuleshova et al., 1999). Therefore, pre-blastocyst embryos in a microinjection medium can increase the space of the subzonal cavity proportionally to the added sucrose concentration and that may pose no threat to embryos for hours. Accordingly, my laboratory is developing a method in which ES cells are hypertonically microinjected into 2.5-dpc embryos. Table 4 gives an example schedule and protocol for this method. The hypertonic microinjecting ES cells into 2.5-dpc embryos and fertile chimeras are shown at Fig. 3.

Date	Time	Target	Treatment
Friday	~16:30	Donor females superovulated	PMSG 5–10 units/ip
Sunday	~16:30		hCG 5–10 units/ip; Donor females are mated with studs
Monday	am	Donor females	Plug checked
		ES cells	Thawed or pass in a high density to 0.1% gelatin-coated dishes
Tuesday	~16:30	Recipients (ICR, CD-1, or F1 hybrid)	Estrus females mated with vasectomized males
	am	Recipients	Plug checked
	~08:00	ES cells	Enriched via the double plating method
	~08:20	60-mm cell culture dish	HK, EK <sup>2</sup> , and KSOM-AA droplets under light-weight mineral oil prepared; RT
	~08:30	Donor females	Recovered 2.5-dpc embryos and stay in HK at RT until used
Wednesday	~09:30	Making microinjection and holding pipettes	Microinjection pipette with a beveled tip and spike: outer diameter, 18–20 $\mu\text{m}$ ; inner diameter, 13–15 $\mu\text{m}$ . Holding pipette: outer diameter, 50–70 $\mu\text{m}$ ; inner diameter, 22–25 $\mu\text{m}$
	~09:50	ES cells	The first round of enriched cells harvested and re-suspended in a cell medium for the second standing
	~10:20	Enriched ES cells	Harvested cells are kept at 4°C until re-suspended in high density in a hepes-buffered microinjection medium <sup>3</sup> containing 0.2 M sucrose <sup>4</sup>
	~10:40	Microinjection chambers	A ~80- $\mu\text{L}$ droplet of 0.2 M sucrose microinjection medium under light-weight mineral oil is prepared.

Date	Time	Target	Treatment
			Thousands of enriched ES cells, then 3-cell embryos to compacting morulae added to the droplet of microinjection medium in a row.
	~10:55	60-mm cell culture dish	HK, EK, and KSOM-AA droplets under light-weight mineral oil to 5% CO <sub>2</sub> , 37°C incubator
	~11:00	Microinjection chamber and micropipettes	Setup for microinjection
	~11:20	Microinjection chamber	Sucking hundreds of ES cells into a microinjection pipette within 5~10 min <sup>5</sup>
	~11:30	Microinjection begins	Approximately 5–30 ES cells are microinjected at RT into the large subzonal cavity of 3-cell embryos to compacting morulae
	~12:30	Microinjection finishes	After 60–150 2.5-dpc embryos are microinjected within 1 h <sup>6</sup> , injected embryos are washed to and culture in EK droplets for ~1 h; 5% CO <sub>2</sub> , 37°C incubator
	~13:50	microinjected embryos	Wash to fresh EK or KSOM-AA droplets for overnight culturing; 5% CO <sub>2</sub> , 37°C incubator
Thursday	am or pm	Recipients <sup>7</sup>	Chimeric embryos are transferred to uterus horns of 2.5-dpc pseudopregnant recipients
Sunday		Recipients <sup>7</sup>	Pups born after ETed for 17 days

<sup>1</sup>Base on one person finish the hypertonic microinjection. However, two persons are more efficiently and practically.

<sup>2</sup>EK (6.5% KSR without FBS): 37.5% KSR ESC medium (20% KSR) and 62.5% KSOM-AA (285 ± 10 mOsm/kg H<sub>2</sub>O)

<sup>3</sup>Any hepes-buffered microinjection medium can be used. My laboratory usually uses EHK (37.5% KSR ESC medium and 62.5% HK) as the microinjection medium no matter how ES cells are culturing either in FBS- or KSR-ESC media.

<sup>4</sup>Most enriched ES cells in 0.2 M sucrose EHK (500 ± 10 mOsm/kg H<sub>2</sub>O) are approximately 9–11 μm in diameter. Therefore, the diameter of microinjection pipette is smaller than a conventional pipette.

<sup>5</sup>Cleaned and enriched ES cells are very important when sucking cells into a microinjection pipette. Otherwise, a single cell suspension, which has many cells with large diameters or sticky debris, will slow loading, generating a microinjection bottleneck.

<sup>6</sup>The randomly sucked embryos by a holding micropipette do not need to adjust the position for microinjecting ES cells into the subzonal cavity, which can be completed in less than 30 seconds.

<sup>7</sup>Embryos with ZP can be transferred into the oviducts of 0.5-dpc recipients (Ramirez et al., 2009). Pups will be born after being subjected to ET for 19 days.

Table 4. Schedule and protocol for the hypertonic microinjection method for generating chimeric mice<sup>1</sup>

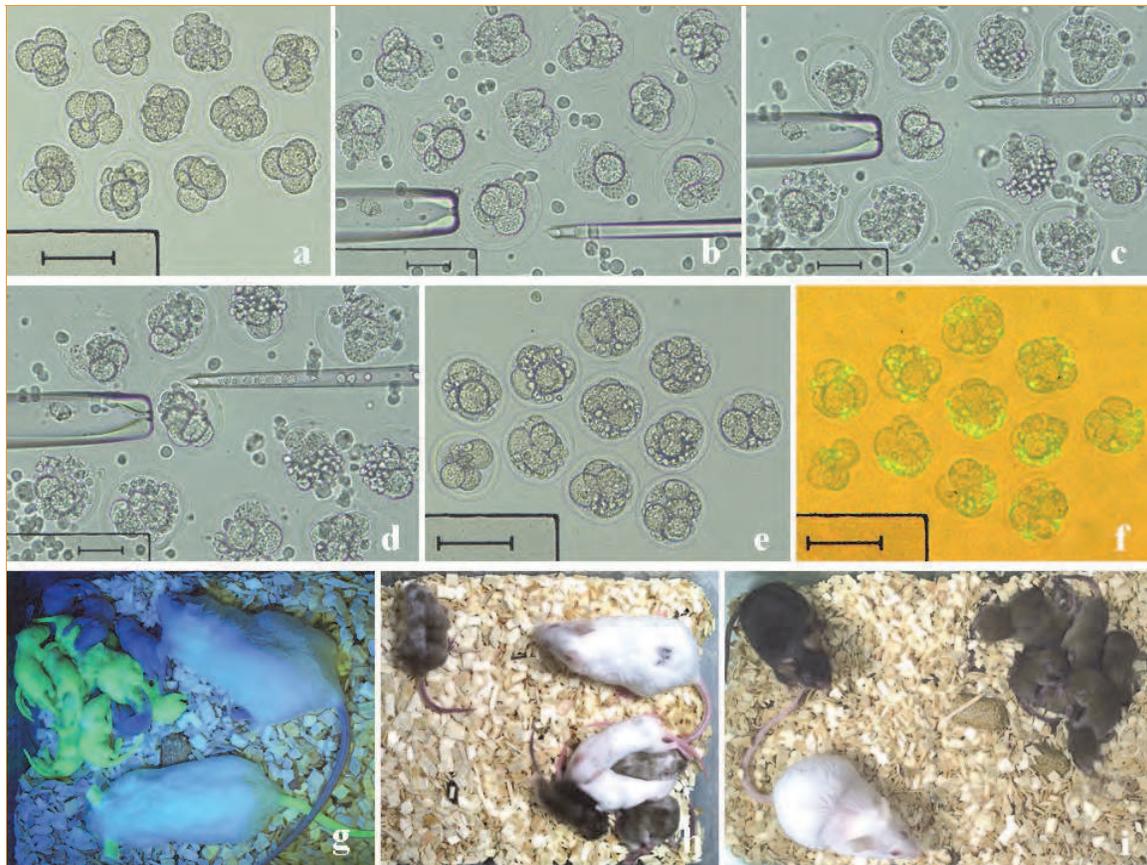


Fig. 3. The generation of germline transmitted chimeric mice via the hypertonic microinjecting ES cells into a subzonal cavity of ICR  $\times$  ICR 2.5-dpc embryos. The chimeric embryos were transferred into the pseudopregnant ICR 2.5-dpc uterine horns. a: The 2.5-dpc 4- to 8-cell stage embryos in isotonic KSOM-AA ( $285 \pm 10$  mOsm/kg  $H_2O$ ) have tight subzonal cavities. b: Embryos and enriched ES cells (ESC 26GJ9012-8-2, P14) in 0.2 M sucrose EHK (37.5% KSR ESC medium and 62.5% hepes-KSOM) hypertonic injection medium ( $500 \pm 10$  mOsm/kg  $H_2O$ ) show large subzonal cavities. c, d: Injecting and injected embryos in hypertonic injection medium. e, f: After injected approximately 25 ES cells into embryos, which were washed to isotonic EK (37.5% KSR ESC medium and 62.5% KSOM-AA). The bright (e) and bright plus green fluorescent (f) images show the same view of 11 injected embryos. g: The green fluorescence expressing germline transmitted chimeric mouse was generated by injecting ESC 26GJ9012-8-2 cells to 4-cell embryos. h: Chimeric pups born alive after approximately 15 ESC98B33 cells (P5) were injected into 4-cell embryos and cultured overnight in EK. The ESC98B33 cell was derived from C57BL/6J 0.5-dpc denuded zygotes cultured on human foreskin fibroblast (Hs68) feeders and KSR ESC medium containing 2i (0.5  $\mu$ M PD0325901 and 3  $\mu$ M CHIR99021) and 10  $\mu$ M ACTH fragments 1-24. i: A chimeric mouse with 100% coat color distribution was generated after approximately 20 ESC98B27 cells (P8) were injected into compacting morulae and cultured overnight in KSOM-AA. The ESC98B27 cell was derived from an isolated single blastomere of a C57BL/6J 1.5-dpc 2-cell embryo cultured on Hs68 feeders and KSR ESC medium containing 2i. Scale in panels a, e, f: bar = 100  $\mu$ m. Scale in panels b-d: bar = 50  $\mu$ m.

Preliminary data demonstrate that ES cells microinjected into the subzonal cavity of 2.5-dpc embryos in a microinjection medium containing 0.2 M sucrose ( $\sim 500$  mOsm/kg  $H_2O$ ) can

generate chimeric embryos with high percentages of chimerism (including 100% coat color and/or GFP expression) and viable, healthy, germline transmitted mice (Fig. 3). These preliminary results also indicate that hypertonic microinjection is at least comparable to conventional, laser, and Piezo microinjection methods for generating germline-transmitted chimeras.

Technically, the differences between conventional microinjection and hypertonic microinjection are that later method uses the 0.2 M sucrose microinjection medium, a microinjection pipette with a smaller diameter, and very fast microinjection. The developing hypertonic microinjection method may be an useful alternative for generating chimeric or F0 mice.

### 2.6 Optimal method of generating chimeras depends on embryo stage

Depending on embryo stage, germline transmitted chimeric mouse or F0 mouse are generated by microinjection, well sandwich aggregation, or coculture methods. Typically, embryos recovered from 0.5-dpc (zygotes) and 1.5-dpc (2-cell embryos) mice for use in generating chimeras are usually no better than 2.5-dpc and 3.5-dpc (Repentigny & Kothary, 2010). In practice, therefore, 3.5-dpc and 2.5-dpc embryos are more commonly used. For blastocysts, conventional microinjection is the only choice. For pre-blastocyst embryos, almost all methods are possible. However, the optimal method may differ. The comparative efficiency of methods for generating germline transmitted chimeric mouse or F0 mouse is summarized at the Table 5, which also might be adopted for generation of ES cell-derived mouse via 4n embryos.

Embryo stage	2.5-dpc			3.5-dpc	
	3-cell to 5-cell embryo	6-cell to 8-cell embryo	Compacting morula <sup>3</sup>	Compacted morula <sup>3</sup>	Blastocyst
Conventional microinjection	+	+	+	+	+++
Hypertonic microinjection	++	+++	+++	+	+–
Laser-assisted microinjection	+	+	+	+	+–
Piezo-driving microinjection	+	+	+	+	+–
Well sandwich aggregation	++	+++	+++	–	–
Single embryo aggregation	+–	+	+	–	–
Conventional coculture	+–	+	+	–	–
Vial coculture	+	+++	+++	–	–

<sup>1</sup>Methods are compared in terms of efficiency in generating germline transmitted chimeras, required equipment, required skills, and operational time.

<sup>2</sup>+++; best; ++; 2nd choice; +; 3rd choice; +–; not suggested –; not good at all.

<sup>3</sup>Compacting and compacted morulae can be cultured in Ca<sup>++</sup>-, Mg<sup>++</sup>-free PBS for 30–60 min to reveal the blastomeres. In some cases, approximately 10 ES cells can be directly microinjected into an embryo proper.

Table 5. Comparative efficiency of methods for generating germline-transmitted chimeric mouse or F0 mouse<sup>1,2</sup>

### 3. Conclusion

Currently, the most common techniques for generating chimeric mice or ESC-derived mice are microinjection or well sandwich aggregation. Single denuded embryo aggregation or coculturing with ES cells are less common alternatives because the efficiency in generating chimeras is inferior to that of microinjection and well sandwich aggregation. Thus, both methods are rarely employed. However, due to systemic limitations and the disadvantages of conventional microinjection, aggregation, and coculturing, new methods are needed.

Recently, my laboratory developed an alternative simple, inexpensive, and reproducible method for mass production of chimeric embryos by coculturing 2.5-dpc denuded 8-cell embryos and/or compacting morulae with ES cells in 1.7-mL Eppendorf vials (micro test tube). This vial coculture method has significantly fewer technological and instrumental problems than existing methods. The resulting chimeras have significant levels of chimerism (including 100% coat color chimerism) and high germline transmission rates.

Previous studies showed that microinjecting ES cells into 2.5-dpc 8-cell embryos could produce 100% coat color chimerism. However, due to the tight space between ZP and blastomeres, one must be very careful to avoid damaging blastomeres while microinjecting. Thus, the method is rarely adopted. Using a laser pulse or Piezo-driving equipment to assist introducing ES cells into the subzonal cavity of 8-cell embryos could have superior efficiency in generating ESC-derived F0 chimeras (100% coat color chimerism), which are equivalent to ESC-derived mice. However, only few studies have adopted either method due to the skill and/or extra expensive instruments needed.

Recently, my laboratory revealed that ES cells microinjected into the subzonal cavity of 2.5-dpc embryos in a microinjection medium supplemented with 0.2 M sucrose could efficiently generate chimeric embryos with high percentages of chimerism and viable, healthy, germline transmitted F0 ES-cell mice.

Both vial coculture and hypertonic microinjection methods are useful alternatives for producing germline chimeric or F0 mice effectively, efficiently, and reliably.

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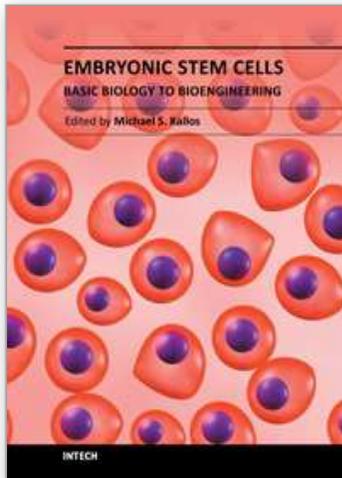
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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

### **How to reference**

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