Generation of embryonic stem cells derived from the inner cell mass of blastocysts of outbred ICR mice

Na Rae Han a, Song Baek a, Hwa-Young Kim a, Kwon Young Lee b, Jung Im Yun c, Jung Hoon Choib, Eunsong Lee b, Choon-Keun Parka,d and Seung Tae Lee a,d,e

aDepartment of Animal Life Science, Kangwon National University, Chuncheon, Korea; bCollege of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon, Korea; cInstitute of Animal Resources, Kangwon National University, Chuncheon, Korea; dDepartment of Applied Animal Science, Kangwon National University, Chuncheon, Korea; eKustoGen Inc., Chuncheon, Korea

ABSTRACT

Embryonic stem cells (ESCs) derived from outbred mice which share several genetic characteristics similar to humans have been requested for developing stem cell-based bioengineering techniques directly applicable to humans. Here, we report the generation of ESCs derived from the inner cell mass of blastocysts retrieved from 9-week-old female outbred ICR mice mated with 9-week-old male outbred ICR mice (ICRESCs). Similar to those from 129/Ola mouse blastocysts (E14ESCs), the established ICRESCs showed inherent characteristics of ESCs except for partial and weak protein expression and activity of alkaline phosphatase. Moreover, ICRESCs were not originated from embryonic germ cells or pluripotent cells that may co-exist in outbred ICR strain-derived mouse embryonic fibroblasts (ICRMEFs) used for deriving colonies from inner cell mass of outbred ICR mouse blastocysts. Furthermore, instead of outbred ICRMEFs, hybrid B6CBAF1MEFs as feeder cells could sufficiently support in vitro maintenance of ICRESC self-renewal. Additionally, ICRESC-specific characteristics (self-renewal, pluripotency, and chromosomal normality) were observed in ICRESCs cultured for 40th subpassages (164 days) on B6CBAF1MEFs without any alterations. These results confirmed the successful establishment of ESCs derived from outbred ICR mice, and indicated that self-renewal and pluripotency of the established ICRESCs could be maintained on B6CBAF1MEFs in culture.

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Introduction

Embryonic stem cells (ESCs) with self-renewal and pluripotency properties have attracted a great deal of interest as a model of organogenesis during embryogenesis in developmental biology (Dvash et al. 2006; Prajumwongs et al. 2016) and as a source of cells for cell therapies in regenerative medicine (Trounson and McDonald 2015; Duncan and Valenzuela 2017). Furthermore, they have been used for the generation of genetically modified animals, screening of drugs without clinical experiments, and for the development of personalized drug treatment regimens (Kawamata and Ochiya 2010; Lou and Liang 2011; Lee et al. 2020). Therefore, they have been actively applied not only in basic research in the fields of regenerative medicine, transgenic animal research, and pharmacetics (Prajumwongs et al. 2016; Ukai et al. 2017), but also in clinical research (Illic et al. 2015; Duncan and Valenzuela 2017).

In the early stages of stem cell research, ESCs derived from blastocysts of mice with a variety of genetic backgrounds were widely used for the development of stem cell-related techniques (Arufe et al. 2006; Ouyang et al. 2007). Commencing with generation of the first mouse ESCs derived from the 129/SvEa strain in 1981 (Evans and Kaufman 1981; Martin 1981), attempts have been made to establish mouse ESCs (mESCs) derived from a variety of strains (Schoonjans et al. 2003; Tanimoto et al. 2008; Nichols and Smith 2011). However, successful establishment of mESC lines have been limited to a few permissive strains, such as 129 and C57BL/6 sub-strains (Schoonjans et al. 2003; Tanimoto et al. 2008; Nichols and Smith 2011). Simultaneously, the generation of mESCs derived from non-permissive strains that are refractory to ESC generation, such as ICR, CBA, NOD, DBA, and BALB/c, showed extremely low efficiency (Kawase et al. 1994).
As the genetic identity between mice and humans is approximately 99%, various laboratory mouse strains, including inbred, hybrid, and outbred mice, have been widely used for research purposes (Fox et al. 2006). Consistent results can be obtained from inbred mice with good genetic and phenotypic stability (Yoshiki and Moriwaki 2006; Choi et al. 2017), whereas the impaired homeostasis regulation-related genes are trouble in recovery because of their genetic homology at chromosomes (Fox et al. 2006). Hybrid mice generated by deliberately crossing mice of two inbred strains maintain genetic and phenotypic uniformity, similar to inbred strains (National Research Council 1999). However, acquisition of data related to genetic background may be difficult (Schauwecker 2011). On the other hand, outbred mice have genetic characteristics similar to humans, including undefined genetics and phenotypic variation, and a high degree of heterogeneity (Chia et al. 2005; Jensen et al. 2016). They have also been shown to be useful as base populations for selection in producing new or improved humanized mouse models (Zuluaga et al. 2006). The results obtained from outbred stocks are generally considered more valuable than those from inbred or hybrid strains for application of the results to humans (Shin et al. 2017). Therefore, toxicology, pharmacology, and fundamental biomedical research continue to be performed using outbred mice (Chia et al. 2005).

To date, there have been a few reports regarding the establishment of ESC lines derived from outbred ICR mouse blastocysts (Meng et al. 2003; Lee et al. 2012). These establishment of ESC lines derived from denuded intact embryos or blastomeres of ICR mouse was mainly conducted under microenvironments specialized by the addition of diverse extrinsic factors such as knockout serum replacement (KSR), differentiation inhibitors and proliferation stimulators as an alternative for enhancing derivation efficiency (Lee et al. 2012). However, any characterization and long-term culture of the established ICR mouse-derived ESCs have not been reported (Lee et al. 2012). In addition, the usage of extrinsic factors in the establishment of ESCs resulted in reduction of ESC viability (Naujok et al. 2014) and alteration of ESC characteristics (Wu et al. 2015). Therefore, with establishment of ESC lines derived from outbred ICR mouse under extrinsic factors-free microenvironments, their characterization and long-term culture system development should be required for enhancing their usability.

Here, we report the establishment of ESC lines derived from outbred stocks of ICR mice. ICR stock mESCs were isolated and cultured in vitro from the inner cell mass of blastocysts derived from outbred ICR mice, and their identity was confirmed based on parameters related to self-renewal and differentiation potential.

Materials and methods
Detailed information of all experimental procedures and statistical analysis performed in this study can be found in the supplementary information.

Results
Establishment of ICR ESCs
Establishment of ICR ESCs was performed according to the procedure presented in Figure 1. Of the 218 blastocysts produced from outbred ICR mice, 115 blastocysts were adherent to ICR-MEF feeder cells and 106 colonies grew out from the inner cell masses of the 115 ICR-MEF-adherent blastocysts. However, in establishing ESCs from 106 outgrown colonies, only one ESC was successfully maintained over the 14th subpassage. Subsequently, the established ICR ESCs were characterized between the 15th and 20th subpassages. Similar to E14 ESC colonies (upper right in Figure 2(A)), colonies of ICR ESCs showed well-defined boundaries and dome-shaped morphology (Figure 2(A)). AP protein expression (Figure 2(B)) and activity (Figure 2(C)) were observed partially and weakly in a portion of ICR ESC colonies, unlike E14 ESCs showing strong AP protein expression (upper right in Figure 2(B)) and activity (upper right in Figure 2(C)) throughout the colonies. Additionally, the established ICR ESCs showed the same expression pattern as E14 ESCs with regard to the transcription and translation of self-renewal-related genes. With the successful transcriptional expression of Oct4, Sox2, Nanog, Tert, and AP (Figure 2(D)), positive expression of Oct4 (Figure 2(E)), Sox2 (Figure 2(F)), and Nanog (Figure 2(G)), and negative expression of Tra-1-60 (Figure 2(H)) and Tra-1-81 (Figure 2(I)) were detected in both the established ICR ESCs (Figure 2(E–I)) and the E14 ESCs (upper right in Figure 2(E–I)). In addition, the EBs formed from ICR ESCs (Figure 2(J)) showed lineage-specific differentiation into endoderm, mesoderm, and ectoderm. The spontaneously differentiated EBs showed positive staining for neurofilaments as an ectodermal marker (Figure 2(K)), α-smooth muscle actin as a mesodermal marker (Figure 2(L)), and cytokeratin 18 as an endodermal marker (Figure 2(M)). The teratomas formed from ICR ESCs transplanted into nude mice included ducts with simple columnar epithelial cells (endodermal lineage; Figure 2(N1)), blood vessels (endodermal lineage; Figure 2(N2)), simple cuboidal cells (endodermal lineage; Figure 2(N3)), chondrocyte
Differentiation of ICRESCs into germ cells induced successful generation of oocyte-like cells with ZP (Figure 2(O), arrowhead). The established ICRESCs had a normal diploid karyotype of 40 (Figure 2(P)) and their sex was confirmed as female by identifying the presence of X-chromosome-specific Xist and the absence of Y-chromosome-specific Zfy1 in the genome (Figure 2(Q)). Subsequently, to determine whether ICRESCs originated from embryonic germ cells or pluripotent cells that may co-exist in ICRMEF feeder cells, ICRMEF feeder cells used in the process of ESC establishment were cultured for 14 days in standard ESC culture medium. Throughout the culture period, no dome-shaped colonies were formed on the cultured ICRMEF feeder cells (Supplementary Figure S1A) and the yield of cells positive for pluripotent stem cell-specific proteins (Oct4, Sox2, and Nanog) and embryonic germ cell-specific protein (VASA) was extremely low (< 1%) in the cultured ICRMEFs (Supplementary Figure S2D). These results indicated that MEF feeder cells derived from ICR and B6CBAF1 mice were useful for maintaining the self-renewal of ESCs derived from ICR mice. Furthermore, as the genetic background of feeder cells used for in vitro culture should be different from the cultured ESCs for eliminating cellular contamination derived from feeder cells, we confirmed that the usage of MEF feeder cells derived from B6CBAF1 mice were useful for maintaining the self-renewal of ESCs derived from ICR mice. To examine the usefulness of the ICRESC-optimized MEF feeder cell-based culture system for long-term maintenance of ICRESCs, the ICRESCs at the 21st subpassage were cultured on hybrid B6CBAF1 MEFs until the 34th subpassage and long-term cultured ICRESCs were characterized.

**Establishment of MEF feeder cell-based culture system customized to ICRESCs**

Subsequently, the strain of MEF feeder cells sufficiently supporting the in vitro maintenance of ICRESC self-renewal was determined by analyzing the doubling time, colony size, and number, and self-renewal-related protein expression among ICRESCs cultured on MEF feeder cells derived from outbred ICR, inbred C57BL/6, and hybrid B6CBAF1 mice. The results indicated that ICRESCs maintained on C57BL/6 MEFs showed significantly longer doubling time (Supplementary Figure S2A), smaller colony size (Supplementary Figure S2B), and fewer colonies (Supplementary Figure S2C) than those on ICRMEFs and B6CBAF1 MEFs, which did not differ significantly from each other in each of the above parameters. Moreover, there were no significant differences in expression of self-renewal-related proteins (Oct4, Sox2, and Nanog) among ICRESCs cultured on ICREMFs, C57BL/6 MEFs, and B6CBAF1 MEFs (Supplementary Figure S2D). These results indicated that MEF feeder cells derived from ICR and B6CBAF1 mice were useful for maintaining the self-renewal of ESCs derived from ICR mice. Further-
between the 35th and 40th subpassages. All colonies derived from E14ESCs (upper right in Figure 3(A)) and ICRESCs (Figure 3(A)) had well-defined boundaries and dome-shaped morphology. Strong AP protein expression (upper right in Figure 3(B)) and activity (upper right in Figure 3(C)) were detected throughout all colonies derived from E14ESCs, whereas a portion of the colonies derived from ICRESCs showed partial and weak AP protein expression (Figure 3(B)) and activity (Figure 3(C)). Moreover, no significant differences were observed in Oct4, Sox2, Nanog, Tert, and AP expression at the transcriptional level in ICRESCs cultured for a long time on B6CBAF1 MEFs compared to ICRESCs at an early subpassage (Figure 3(D)). The long-term cultured ICRESCs (Figure 3(E)) showed an equivalent expression pattern to E14ESCs with regard to Oct4, Sox2, Nanog, Tra-1-60, and Tra-1-81 (upper right in Figure 3(E–I)) as follows: positive for Oct4 (Figure 3(E)), Sox2 (Figure 3(F)), and Nanog (Figure 3(G)), and negative for Tra-1-60 (Figure 3(H)) and Tra-1-81 (Figure 3(I)). With successful formation of EBs from long-term cultured ICRESCs (Figure 3(J)), neurofilaments as an ectodermal marker (Figure 3(K)), α-smooth
muscle actin as a mesodermal marker (Figure 3(L)), and cytokeratin 18 as an endodermal marker (Figure 3(M)) were detected in spontaneously differentiated EBs. Additionally, following transplantation into nude mice, the long-term cultured iCRiESCs-derived teratomas showed gut epithelium (endodermal lineage; Figure 3(N1)), double-layered apocrine duct (endodermal lineage; Figure 3(N2)), ducts consisting of simple cuboidal cells (endodermal lineage; Figure 3(N3)), adipocytes (mesodermal lineage; Figure 3(N4)), striated muscle (mesodermal lineage; Figure 3(N5)), smooth muscle cells (mesodermal lineage; Figure 3(N6)), epithelium with keratinization (ectodermal lineage; Figure 3(N7)), nerve bundles (ectodermal lineage; Figure 3(N8)), and neural epithelium (ectodermal lineage; Figure 3(N9)). Differentiation of long-term cultured iCRiESCs into germ cells induced successful generation of oocyte-like cells with ZP (Figure 3(O), arrowhead). The long-term cultured
ICRESCs showed a normal diploid karyotype of 40 (Figure 3(P)), and the presence of X-chromosome-specific Xist and the absence of Y-chromosome-specific Zfy1 in their genome (Figure 3(Q)), indicating that their sex was female. Moreover, alleles detected in ICRESCs by the microsatellite markers D1Mit16 and D17Mit124 were equally observed in ICRESCs on B6CBAF1MEFs (Figure 3(R)), whereas alleles of ICRESCs were different from those of CS7BL/6MEFs and B6CBAF1MEFs (Figure 3(R)). The hypomethylated status of Oct4 and Nanog promoter regions were observed in the long-term cultured ICRESCs (Figure 3(S)), indicating that these gene promoters in ICRESCs are active and the ICRESCs retain pluripotency. These results clearly indicated that ICRESCs were derived from blastocysts of outbred ICR mice. Despite long-term culture of ICRESCs on B6CBAF1MEFs, self-renewal, pluripotency, and chromosomal normality could be successfully maintained without any alterations, indicating that in vitro culture of ICRESCs on B6CBAF1MEFs is a MEF feeder cell-based culture system customized to established ICRESCs.

Discussion

In this study, ICRESCs were successfully derived from one of 218 blastocysts retrieved from outbred ICR mice. Unusually, weak AP protein expression and activity were observed in the derived ICRESCs. Nevertheless, the derived ICRESCs had a normal female karyotype and showed characteristics of stem cells: colonies with well-defined boundaries and dome-shaped morphology, transcriptional and translational expression of self-renewal-related genes, in vitro and in vivo differentiation into three germ layers, and germ cell differentiation. Moreover, ICRESCs maintained for a long time on B6CBAF1MEFs showed no loss of stem cell-related characteristics and no abnormalities of female karyotype. Therefore, we established ESCs derived from blastocysts of outbred ICR mice and developed a system for culture of ICRESCs using B6CBAF1MEFs. A pathway for deriving more human-similar results in a mouse model could be developed.

In addition to Oct4, Sox2, and Nanog, AP is conventionally used as an ESC-specific marker regardless of species (Tielens et al. 2006). Interestingly, as shown in Figures 2(B, C) and 3(B, C), weak protein expression and activity of AP were detected in ICRESCs in comparison to E14ESCs, which is a widely used ESC line. In previous studies, the regulation of tissue nonspecific AP was shown to be affected by p38 mitogen-activated protein kinase (MAPK) (Suzuki et al. 2002; Rey et al. 2007), and decreased protein level and activity were detected in p38−/− mouse ESCs with no changes in pluripotent marker expression (Štefková et al. 2015). Accordingly, it is possible that expression of p38 kinase in ICRESCs may be decreased more than other ESCs, resulting in weak protein expression and activity of AP. Subsequently, for verifying the hypothesis, we quantified expression of p38 MAPK proteins, and there was no significant difference in the amount of p38 MAPK proteins expressed in between the E14ESCs and the ICRESCs (Supplementary Figure S3). These results demonstrate that weak protein expression and activity of AP in ICRESCs result from not expression of p38 MAPK proteins but another unknown factors. Of course, studies on the another unknown factors should be conducted in the future.

Although human ESCs differentiated into numerous types of cells are considered valuable tools for cell therapy (Gerecht-Nir and Itskovitz-Eldor 2004; Ryu et al. 2019), their maintenance and manipulation are costly and difficult, and require high-end facilities of good manufacturing practice (GMP) level (McKee and Chaudhry 2017; Ye et al. 2017). By contrast, maintenance and manipulation of the established ICRESCs are cheaper and easier than those of human ESCs and advanced facilities are not required. Additionally, the results derived from ICRESCs with similar genetic characteristics or heterogeneity to humans (Choi and He 2015) could be used directly for clinical applications without any preclinical tests. Therefore, ICRESCs have a great deal of potential for significantly reducing the costs and time required for specific clinical applications, such as toxicity evaluation or development of pharmaceuticals and stem cell therapy.

In conclusion, we established ESCs derived from the inner cell mass of blastocysts derived from outbred ICR mice as well as a culture system specific for the established ICRESCs. The established ICRESCs will contribute to studies related to unknown characteristics of ESCs derived from outbred ICR mice and will yield results comparable to human ESCs in preclinical studies alone.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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