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An Efficient Method for the Derivation of Mouse Embryonic Stem Cells

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ABSTRACT

Mouse embryonic stem cells (mESCs) represent a unique tool for many researchers; however, the process of ESC derivation is often very inefficient and requires high specialization, training, and expertise. To circumvent these limitations, we aimed to develop a simple and efficient protocol based on the use of commercially available products. Here, we present an optimized protocol that we successfully applied to derive ESCs from several knockout mouse strains (Wnt-1, Wnt-5a, Lrp6, and parkin) with 50%–75% efficiency. The methodology is based on the use of mouse embryonic fibroblast feeders, knockout serum replacement (SR), and minimal handling of the blastocyst. In this protocol, all centrifugation steps (as well as the use of trypsin inhibitor) were avoided and replaced by an ESC medium containing fetal calf serum (FCS) after the trypsinizations. We define the potential advantages and disadvantages of using SR and FCS in individual steps of the protocol. We also characterize the ESCs for the expression of ESC markers by immunohistochemistry, Western blot, and a stem cell focused microarray. In summary, we provide a simplified and improved protocol to derive mESCs that can be useful for laboratories aiming to isolate transgenic mESCs for the first time. STEM CELLS *2006;24:844–849*

INTRODUCTION

Embryonic stem cells (ESCs) were first isolated in 1981 from mouse blastocysts [1, 2] and subsequently from human blastocysts [3, 4]. These cells were found to exhibit unique properties, including the capacity to self-renew and to differentiate into all cell types in the individual. Shortly after their discovery, mouse ESCs (mESCs) became an indispensable tool in biomedical research. Homologous recombination in mESCs revolutionized developmental biology by enabling genetically modified mice. The growing knowledge of signals and mechanisms that regulate development in mutant mice has subsequently allowed for improved protocols to guide differentiation of mouse and human ESCs. This has resulted in two major effects: first, it provided a technique to recapitulate and study development in vitro, and second, it provided a unique tool for cell replacement therapy.

ESCs derived from homozygous knockout or knockin blastocysts represent important tools for analysis of mutant mice, especially when the phenotype is embryonic-lethal at early stages of development. The derivation of mESCs from blastocysts is a process that is often very inefficient, and even in the most favorable strain (129 mouse strain), a success rate of 30% is regarded as high [5]. Derivation of ESCs is strongly mouse strain–dependent [6], and in practice the efficiency of derivation in strains other than 129 strain does not usually exceed 10% [7]. Moreover, the process of ESC derivation from blastocysts requires expertise and skills in handling early mouse embryos. To overcome these limitations, researchers have introduced many improvements to the original protocol. Such improvements include use of specifically conditioned medium [8], genetically modified blastocysts [7], microdissection of the blastocyst [6], treatment with pharmacological drugs [9], and use of serum replacement (SR) [10]. However, despite these significant improvements in the generation of ESCs from mouse blastocysts, there is currently no protocol available that combines high efficiency, simplicity, commercial availability of all reagents, and no special training. For these reasons, and despite the undoubted need of ESCs as experimental models, ESC derivation from mouse blastocysts is not a commonly used method.

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We hereby present an optimized protocol for the derivation of ESCs from mouse blastocysts. The protocol is optimized for efficiency and simplicity, and in our group, investigators without previous mESC experience successfully used it to derive several knockout mESC lines. The protocol does not require special equipment, genetically modified feeder cells, or special training other than regular tissue culture and animal handling skills.

Materials and Methods

The derivation of mESCs from blastocyst is still not a fully defined process, and this may explain the substantial variation in success rates among different laboratories. The factors influencing the success rates are unknown but may be associated with cell culture reagents/methods. To guarantee the reproducibility of the derivation procedure, we specifically mention all experimental details, including catalogue numbers of commercial products.

To derive ESCs we modified the generally used protocol described by Hogan [11]. When the original experimental protocol was modified, the following points were kept in mind: All reagents should be commercially available, micromanipulation should be avoided, an inexperienced person should be able to perform the derivation, only standard cell culture equipment should be used, and the efficiency of ESC derivation should be improved. Only one protocol, schematized in Figure 1, fulfilled all these criteria.

In this protocol, we used a feeder layer of mouse embryonic fibroblasts (MEFs) that was obtained from embryonic day 12.5 (E12.5) or E13.5 mouse embryos as described before [11]. We have used MEFs from several mouse strains, and they did not differ in their ability to support the process of ESC derivation. We used first- or second-passage MEFs because we observed that later passages of MEFs (passages 3–5) have lower viability during the derivation protocol and hence do not support the ESC derivation to the extent that early passages of MEFs do. MEF feeders were mitotically inactivated by treatment with mitomycin C for 2 hours (10 μg/ml, no. 107409; Roche, Basel, Switzerland, http://www.roche.com), or alternatively 1 μg/ml of mitomycin C can be used overnight. After mitomycin C inactivation, cells were washed with phosphate-buffered saline (PBS), trypsinized and seeded (75,000 cells/cm^2) into 12-well plates (MULTIWELL 12 well, no. 353043; BD Falcon, Franklin Lakes, NJ, http://www.bectondickinson.com), and coated with gelatin (no. G1890; Sigma, St. Louis, http://www.sigmaaldrich.com). To gelatinize the culture dishes, the bottom was covered with an autoclaved solution of 0.1% gelatin in Mili-Q water and incubated for 5 minutes at 37°C. The excess gelatin solution was removed just prior to adding culture medium.

To obtain blastocysts, time-mated females were killed at E3.5 and their uteri were immediately transferred into Knockout Dulbecco's modified Eagle's medium (DMEM) (no. 10829–018; Gibco, Grand Island, NY, http://www.invitrogen.com) buffered with 1× HEPES buffer solution (no. 15630-056; Gibco) (HEPES/DMEM) preheated to 37°C in an incubator. Remnants of fat were removed, the uteri were transferred into 2 ml of fresh preheated HEPES/DMEM, and the blastocysts were flushed out of the uterine horn under a dissection scope using a 2-ml syringe with 0.6-mm needle as described earlier [11]. Blastocysts were collected under an inverted microscope (Axiovert 25; Carl Zeiss, Oberkochem, Germany, http://www.zeiss.com) at low magnification and transferred individually using Diamond D10 precision tips and a 20-μl pipette to a well of a 12-well plate containing a feeder layer of MEFs mitotically inactivated 1 day prior. The cultivation medium (SR–embryonic stem [ES] medium) was added to MEFs 1–3 hours before blastocysts were added. SR-ES medium was composed of Knockout DMEM supplemented with 20% Knockout SR (no. 10828-028; Gibco), penicillin (100 U/ml)/streptomycin (100 μg/ml) (no. 15140-122; Gibco), 2 mM L-glutamine (no. 25030-024; Gibco), 1× minimal essential medium nonessential amino acids (no. 11140-035; Gibco), 100 μM β-mercaptoethanol (to prepare 100× stock solution, 7 μl of β-mercaptoethanol [no. M7522; Sigma] was diluted in 10 ml of PBS), and recombinant mouse leukemia inhibitory factor (1,000 U/ml of ESGRO, no. ESG1107; Chemicon International, Temecula, CA, http://www.chemicon.com). The blastocysts were allowed to attach to supportive MEFs and hatched and expanded without any further experimental interference for 6 days. (This process was microphotographed and discussed later in Fig. 2A.) After that time, all cell clumps originating from the blastocysts were trypsinized in drops of 2.5% trypsin as detailed below.

The procedure of the first trypsinization was as follows: (a) appropriate numbers of 12-well plates with confluent layers of mitotically inactivated MEFs were prepared 1 day in advance; (b) 2 hours before trypsinization, the MEF medium was replaced by 1 ml per well of fetal calf serum (FCS)–ES

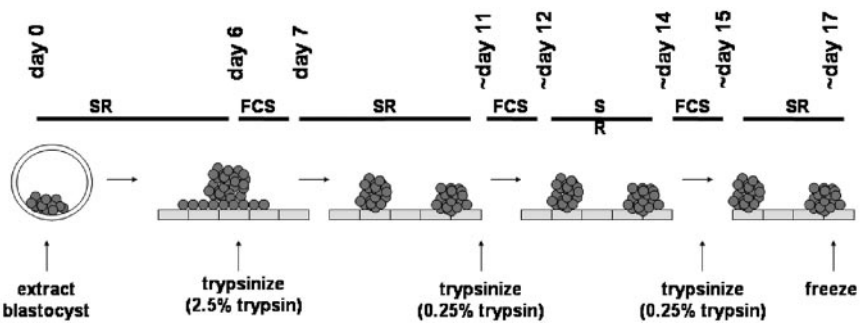

Figure 1. Scheme of differentiation protocol. Embryonic day 3.5 blastocysts are extracted at day 0 and allowed to attach and expand on MEF feeder layer in SR-ES medium (20% SR). At day 6, the expanded blastocyst is trypsinized using 2.5% trypsin and left to attach to fresh MEFs in FCS-ES medium (20% FCS) overnight. The next day, FCS-ES medium is replaced with SR-ES medium and cells are grown without medium changes for an additional 4 days. At approximately day 11, the well is trypsinized using 0.25% trypsin and transferred onto fresh feeder in FCS-ES medium, which is replaced with SR-ES medium the following day. Depending on the cell density and size of the colonies, one or two additional rounds of trypsinization are required before confluent 5 cm–diameter dish of newly established mESC line can be frozen (at approximately day 17). Abbreviations: ES, embryonic stem; FCS, fetal calf serum; MEF, mouse embryonic fibroblast; SR, serum replacement.

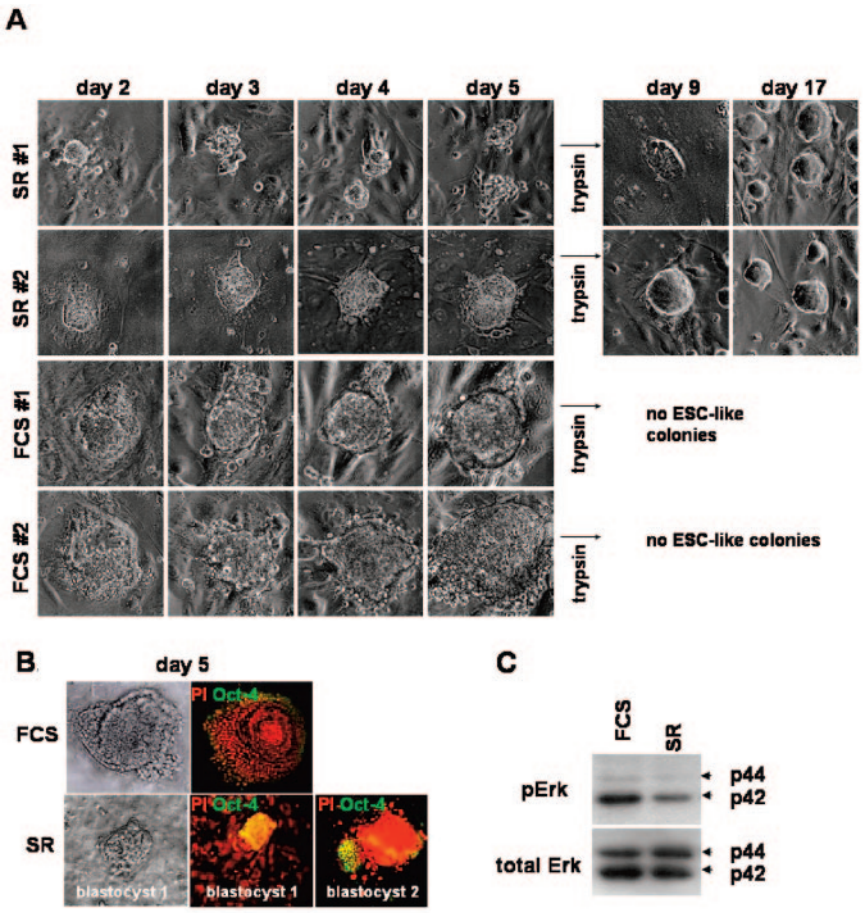

Figure 2. The effects of FCS and SR on the process of ESC derivation. **(A):** Blastocysts grown in SR-ES (blastocyst 1, SR no. 1; blastocyst 2, SR no. 2) and FCS-ES (blastocyst 1, FCS no. 1; blastocyst 2, FCS no. 2) medium were microphotographed at days 2, 3, 4, and 5 after extraction and were trypsinized. ESC-like colonies detected in the blastocysts SR no. 1 and SR no. 2 were expanded and microphotographed at days 9 and 17. No ESC-like colonies were detected in the blastocysts grown in FCS-ES medium. **(B):** Blastocysts grown in SR-ES or FCS-ES medium stained for Oct-4 expression at day 5. PI was used to visualize cell nuclei. **(C):** mESCs grown in the absence of feeders for 24 hours in FCS- and SR-ES medium were lysed and analyzed for the activation (phosphorylation) of p42/44 Erk1/Erk2 kinase by Western blotting using phospho-Erk– and total-Erk–specific antibodies (sc-7383 and sc-93; Santa Cruz Biotechnology). Data are representative of three independent replicates. Note the decreased phosphorylation of p42/44 Erk1/2 kinases in SR-ES medium. Abbreviations: ES, embryonic stem; ESC, embryonic stem cell; FCS, fetal calf serum; mESC, mouse embryonic stem cell; PI, propidium iodide; SR, serum replacement.

medium, a medium identical in composition to SR-ES medium, in which SR was replaced by 20% ESC-pretested FCS (no. A15-080; PAA Laboratories, Linz, Austria, http://www.paa.at); (c) 20 μl drops of 2.5% trypsin (no. 15090-046; Gibco) were made on a 5 cm–diameter culture dish; and (d) the SR-ES medium was sucked off the wells containing expanded blastocysts on MEFs, and then 0.25% trypsin-EDTA (no. 25300-54; Gibco) was added to the well and after 15 seconds was removed, leaving only a wet surface. This procedure reduced stickiness of the cells. Next, the expanded blastocyst and surrounding MEFs were mechanically detached from the dish, using a 20-μl pipette and Diamond D10 precision tip that contained 10 μl of 2.5% trypsin solution aspirated from the drops. Note that MEFs cultured in SR-ES medium for 6 days produce high amounts of extracellular matrix embedding the cells; therefore, detaching blastocysts from the remaining feeder layer was difficult even after trypsinization washing. Using the pipette tip, we made several circles around the blastocyst in order to separate the blastocyst, along with some MEFs, from the rest of the feeder layer. Then, 10 μl of 2.5% trypsin was carefully pipetted on each blastocyst. As the blastocyst floated, it was rapidly aspirated back into the tip and transferred into the remaining 10 μl drop of 2.5% trypsin in a 5 cm–diameter culture dish. Cells in a drop were trypsinized for approximately 5 minutes in the 37°C incubator and then, by pipetting up and down approximately 10 times with a Diamond D10 precision tip, dissociated into individual cells and small cell clumps. This process was monitored under the dissection microscope, and when the blastocyst disaggregated into several smaller cell clumps, the cell suspension was pipetted directly into a well of a 12-well plate containing MEFs and FCS-ES medium. FCS-ES medium was used primarily for inactivation of trypsin, but better cell attachment and survival after trypsinization were also observed when compared with SR-ES medium and soybean trypsin inhibitor. The following day, the FCS-ES medium was replaced by SR-ES medium and the cells were allowed to grow without further medium changes for another 4–5 days.

Approximately 2 days after trypsinization, compact cell colonies resembling ESC colony morphology could be detected (Fig. 2A). The total number usually varied from one to 20 colonies. The colonies were monitored daily, and we found that if they grew rapidly and maintained a typical undifferentiated morphology, they resulted in successful derivation of ESCs in all cases. These cells could be subsequently expanded and trypsinized according to needs. All cells in a well were trypsinized at approximately day 11 with 0.25% trypsin-EDTA, and the resulting single-cell suspension was directly mixed with FCS-ES medium in a dish containing fresh feeder cells. The next day, the medium was replaced by SR-ES medium and ESCs were allowed to grow with medium changes every second day. Usually, an additional trypsinization step is needed before

the cells can be grown on 5 cm–diameter dishes to subconfluency (several hundreds of thousands of cells) before being frozen at approximately day 17 as described [12].

Results and Discussion

High Efficiency of ESC Derivation and Potential Mechanisms

The protocol described above was applied in our laboratory to blastocysts from several mouse strains in order to derive ESC lines from various genetically modified mice. In summary, we isolated 28 ESC lines from 52 blastocysts of four transgenic mouse strains (Wnt-1 [13], Wnt-5a [14], Lrp6 [15], and parkin [16] null mutants) in six independent attempts (Table 1). In all attempts, the efficiency was never lower than 50% and varied between 50% and 75%. Usually, the blastocysts were allowed to hatch and expand for 6 days; however, we noted that earlier trypsinization (at day 4 or 5) did not affect the efficiency of derivation. In our hands, the typical duration of the described process of ESC isolation (from blastocyst flushing to freezing of subconfluent 5 cm–diameter plate) was approximately 17 days (ranging from 15 to 20 days; Table 1). Earlier trypsinization (day 4 or 5 after hatching) did not seem to affect efficiency but prolonged the period needed for ESC derivation up to 28 days. As shown in Table 1, ESCs were derived from embryonic-lethal strains by crossing heterozygous animals. The genotypes of the ESCs isolated followed a mendelian distribution.

The protocol schematized in Figure 1 combines the use of SR-ES and FCS-ES medium. To define the effects of SR and FCS in more detail, we allowed the blastocysts extracted from a single female to attach and expand either in SR- or FCS-containing ES medium. As we demonstrate in Figure 2A, the process of attachment and expansion differs between blastocysts cultured in SR (SR no. 1 and SR no. 2) and FCS (FCS no. 1 and FCS no. 2) in several aspects. First, FCS promoted growth of cells originating from the blastocyst to a higher extent than did SR; as a result, the total cell number expanded from the blastocysts were several times higher after 5 days in vitro in ES medium containing FCS compared with SR-containing ES medium (compare day 5 in Fig. 2A). Second, SR-ES medium did not support growth of trophoblast giant cells, and after 5 days of culture very few trophoblast cells were detected in SR-ES medium (in contrast to blastocysts cultured in FCS-ES medium). The slower growth and increased degeneration of trophoblast giant cells can be an important factor favoring growth of ESC-like cells within the blastocyst. Third, it appears that SR-ES medium selectively favored the growth of ESCs at the expense of other cell types within the blastocyst. Despite the fact that in some cases only small clumps of few cells were visible after 5 days in vitro (Fig. 2A, SR no. 1, day 5), these cells clearly retained the capacity to expand and differentiate in an ESC manner and expressed ESC markers. Blastocysts cultured in FCS-ES medium, on the other hand, did not form expandable ESC-like colonies after the first trypsinization.

To directly test the effect of SR and FCS on the ESC-forming population derived from the blastocyst, we cultured blastocysts in SR- and FCS-ES medium for 5 days and analyzed the proportion of cells that retained ESC properties, such as expression of the ESC marker Oct-4 [17]. As shown in Fig. 2B, colonies grown in FCS showed only few positive cells with weak nuclear staining for Oct-4 at the edge of the expanded blastocyst. In contrast, the majority of cells derived from blastocysts expanded with SR showed positive nuclear staining for Oct-4. In some cases, when SR expanded blastocysts were larger (e.g., Fig. 2A, SR no. 2, day 5), it was possible to distinguish a highly Oct-4–positive cell cluster budding off the cluster of Oct-4–negative cells (Fig. 2B, blastocyst 2). However, in all cases, Oct-4–positive cells were selectively propagated (Fig. 3C).

Interestingly, it was recently reported that decreased Erk kinase activation enhances the derivation of mESCs [9]. To explore the possibility that SR-ES medium has lower Erk-inducing activity, we tested the activation of Erk kinase in ESCs that were derived by the abovementioned protocol and cultured for 24 hours in FCS- or SR-ES medium. As shown in Figure 2C, Erk activation (measured by phospho-Erk–specific antibodies) was lower in ESCs cultured in SR-ES medium, suggesting that this may contribute to the increased efficiency of the SR-ES medium in the derivation of ESCs compared with FCS-ES medium. Indeed, Erk inhibition was reported to allow the derivation of ESCs even from nonpermissive mouse strains [9]. Similarly, the predominant genetic background of all mouse strains that were used as a source of blastocysts was C57Bl/6 [13–16], which is not generally considered to be permissive in terms of ESC derivation. Thus, our results suggest that SR-ES medium enhances the derivation of ESCs from mouse strains usually resistant to ESC isolation.

However, we believe that use of SR is unlikely per se to be the only factor influencing the efficiency of ESC derivation when using protocol described above. A similar protocol using

Table 1. Embryonic stem cell–derivation summary

Strain [reference]	No. of blastocysts	No. of embryonic stem cell lines (+/+, +/−, −/−)	Efficiency	Time [days] (mean; [minimum–maximum])	Notes
Wnt-1 [13]	16	8 (2,4,2)	50%	16.8 (16–19)	
Wnt-5a [14]	5	3 (2,1,0)	60%	17.0 (16–18)	
Wnt-5a [14]	10	5 (0,5,0)	50%	16.5 (15–17)	
Wnt-5a [14]	6	3 (0,2,1)	50%	20.7 (16–24)	Trypsinized at D4
Lrp6 [15]	11	6 (2,2,2)	54.5%	19.6 (19–20)	
parkin [16]	4	3[a]	75%	23.3 (21–28)	Trypsinized at D5
Total	52	28 (6,14,5)	53.8%	17.6 (15–20)[b]	

[a]Parkin mutation is not embryonically lethal, and all blastocysts were obtained from −/− intercrossings.
[b]Only blastocysts trypsinized at D6 are included here.

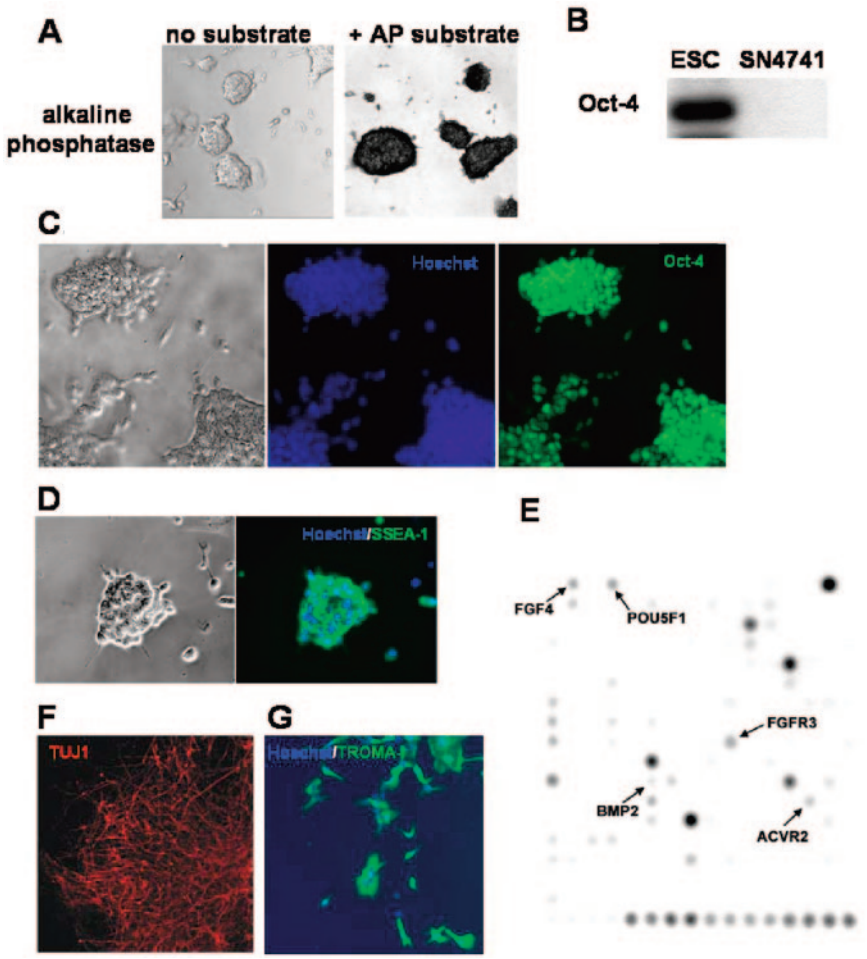

Figure 3. Properties of the mESCs derived by the new protocol are consistent with previously derived ESC lines. Wild-type mESCs were grown in the absence of feeders for several passages. **(A):** ESCs showed strong AP activity as determined by enzyme activity assay. **(B):** The level of Oct-4 is also high in ESCs as determined by Western blotting [22]. The neuronal SN4741 cell line was used as a negative control. **(C):** The homogeneity of ESCs was monitored by immunohistochemistry with anti–Oct-4 staining (green). The nuclei were counterstained with Hoechst (blue). **(D):** ESCs were also positive for the expression of mESC antigen SSEA-1 (green; antibody from R&D Systems), which was determined by immunohistochemistry. The nuclei were counterstained with Hoechst (blue). **(E):** The transcriptome of the newly derived ESCs was analyzed by a stem cell focused microarray. Genes typical for mESCs are indicated by arrowheads. A full list of genes expressed by this array is available on supplemental online Table 1. **(F):** The ESCs differentiated into cell types originating from all three germ layers, including cardiomyocytes (data not shown) and neurons (neuron-specific β-III-tubulin, TUJ1 shown in red). **(G):** The ESCs differentiated into cell types originating from all three germ layers, including cardiomyocytes (data not shown) and TROMA-I–positive (green) endodermal cells. The cultures were counterstained with Hoechst (blue). Abbreviations: AP, alkaline phosphatase; ESC, embryonic stem cell; mESC, mouse embryonic stem cell.

MEFs and SR was applied to derive ESCs from blastocysts of the C57Bl/6J mouse strain, but the yield of ESCs was only 8.3%–26% [10]. Our protocol differs from the mentioned protocol in several ways, including alternating SR and FCS, higher SR/FCS concentration, and a reduced manipulation of the blastocyst, all of which may further improve the efficiency. Shortening the period of time that the cells were outside the incubator and eliminating the centrifugation steps are also likely to contribute to enhanced yields of ESCs. Finally, it is worth noting that although SR significantly promotes proliferation of undifferentiated ESCs, it does not contain the full spectra of growth and survival factors that FCS contains and the cells do not attach as efficiently in SR. In contrast, FCS contains pro-differentiation factors that can affect the propagation of undifferentiated ESCs. Thus, we think that the alternation between these two mediums allows greater trophic support after trypsinization (FCS-ES medium) and selective propagation of ESCs (SR-ES medium).

Properties of ESCs Derived by SR-Based Protocol

All ESC lines described by our optimized protocol showed typical ESC morphology, rapid growth, and the ability to remain undifferentiated in the absence of MEFs for at least 20 passages after thawing. A brief characterization of ESCs is shown in Figure 3. ESCs derived by the protocol described above showed typical mESC characteristics, including high alkaline phosphatase activity [11] assessed by Vector Blue Alkaline Phosphatase Substrate Kit III (no. SK-5300; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). The level of transcription factor Oct-4 [17] (Fig. 3B) was determined by Western blotting using an Oct-4–specific antibody (no. sc-9081; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com) and was found to be high in ESCs (compared with the neuronal cell line SN4741). Immunocytochemical staining confirmed the expression of Oct-4 and showed that almost all cells, even when cultured in the absence of MEF feeders, are positive for Oct-4 (Fig. 3C) and SSEA-1 (Fig. 3D). To obtain a broader view of the properties of the mESCs derived by the protocol described above, we processed cDNA for microarray analysis. The expression profiles of two independently derived mESCs grown in the absence of feeders were analyzed by a stem cell focused microarray (GEArray S Series Mouse Stem Cell Gene Array, no. MM-601.2; SuperArray Bioscience Corporation, Frederick, MD, http://www.superarray.com). The overall expression profile was similar to that observed for the undifferentiated D3 mESC line [18]. Differences in gene expression could be due to differences in culture conditions, labeling conditions, or array methodologies. The microarray showed positivity for genes typically expressed in mESCs, including *Oct-4*, *FGF4*, *FGFR3*, *BMP2*, and *ACVR2* [18–20] (Fig. 3E; a full list of genes is

available on supplemental online Table 1). Thus, our results indicated that mESCs isolated by this new protocol exhibited similar characteristics to those of previously isolated mESCs. Moreover, we confirmed that mESCs derived by this new protocol retained in vitro developmental potential similar to that of established cell lines and gave rise to mesodermal derivatives (as demonstrated by formation of contractive cardiomyocytes from embryoid bodies [data not shown]), ectodermal derivatives (as demonstrated by differentiation into neurons [Fig. 3F] [21]), and endodermal derivatives (as demonstrated by differentiation into TROMA-I–positive endodermal cells [Fig. 3G] [22]).

SUMMARY

We present an optimized, simple, and efficient protocol for the derivation of mESCs from blastocysts that can be used for establishing new mESCs from transgenic mouse strains. We believe that the protocol described above will demystify the process of mESC isolation and will contribute to the expansion and popularization of ESC technology.

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AUTHORS' NOTE

ES cells derived by this method contributed to chimaeras after blastocyst injections and showed pluripotency in vivo.

DISCLOSURES

The authors indicate no potential conflicts of interest.

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