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# Conditions and Techniques for Mouse Embryonic Stem Cell Derivation and Culture

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## 1. Introduction

Stem cells, characterized by their ability for self-renewal and differentiation, have been derived from the embryo and from various postnatal animal sources. They are usually classified according to their developmental potential. Totipotency is defined as the ability of a single cell to replicate and produce all differentiated cells in an entire organism, including extraembryonic tissues that will develop and differentiate into the fetal placenta and fetal membranes [1,2]. In plants, spores are totipotent cells. In some cases, cells can de-differentiate and regain their totipotency. For instance, a plant cutting or callus can be utilized to grow an entire functional plant [3]. In mammals, only the zygote and early blastomeres are totipotent cells [4-7]. In other words, an individual cell is capable to generate a functionally normal animal with fertile ability [8-10]. Mouse embryonic stem (ES) cells, typically derived from inner cell masses (ICMs) or corresponding earlier blastomeres or later epiblasts (develop to embryo proper), are an example of pluripotent cells that can self-renew and generate all types of body cells *in vivo* and *in vitro*, but cannot generate the extraembryonic trophoblast lineage [11-14]. Under some particular conditions, an ES cell-derived mouse with germline transmission can be generated routinely [15-21]. Multipotent cells, such as hematopoietic stem cells, can give rise all cell types within a particular lineage. Spermatogonial stem cells are unipotent stem cells, as they can only form sperm [22].

In recent years, major improvements in deriving mouse ES cell (ESC) lines have dramatically increased success rates. Therefore, this chapter reviews and discusses the conditions and techniques for derivation and cultivation of mouse ES cell (mESC) lines. Thereafter, a proposed novel and user-friendly protocol that is efficient, reproducible, easy to carry out and relatively cheap is presented.

## 2. Conditions for derivation of mouse embryonic stem cells (mESCs)

Since the first mouse ES cell lines were described [23,24], various empirical combinations of conditions and techniques for derivation and cultivation of mESCs from blastocysts and isolated ICMs have been developed [25-27]. Of which, selected batches of fetal bovine serum (FBS), inactivated STO (a SIM mouse embryonic fibroblast line resistant to 6-thioguanine and ouabain) or murine embryonic fibroblast (mEF) feeder cells, conditioned media, mouse strains, embryo status, and different small growth areas of wells to initiate cultivation are the principal concerns when deriving mESCs [27].

To support fetal growth and development, FBS contains mixed combinations of cell replication stimulators and cell differentiation inducers. Notably, FBS is a biological product, such that its biopotency to support mESCs varies from batch to batch. Therefore, to circumvent interference from differentiation factors and other disadvantages associated with FBS, chemically defined KnockOut™ serum replacement (KSR) [28] and N2B27 [29,30] were developed to replace FBS. That is, when culturing established mESCs, KSR and N2B27 are usually as effective as FBS. Unfortunately, embryos in the KSR ESC medium do not result in effective derivation of ESCs [28,31]. However, a chemically defined ESC medium containing differentiation inhibitors has much better efficiency than the FBS ESC medium when deriving mESCs [31].

Zygotes to hatched embryos and blastomeres, ICMs, or epiblasts of early-stage embryos can be used to establish mESCs [31,32]. These cells have extremely high capability for cell division and differentiation. Theoretically, inhibiting endogenous differentiation and maintaining or enhancing proliferation of pre-implantation embryos can be helpful for the establishment of ES cell lines. In 1988, researchers have found that leukemia inhibitory factor (LIF) assists in the derivation and maintenance of mESCs pluripotency [33,34]. However, when protocols and media containing LIF for mESCs derivation are applied to mouse strains other than 129s, efficiency declines from about 20% to less than 5% [35-38]. Furthermore, ESC media supplemented with LIF are not good for deriving ESCs other than mESCs [39,40]. Since then, regulatory mechanisms, pathways, and signal transduction of self-renewal, differentiation, proliferation, and apoptosis have been investigated [41,42]. Additionally, the corresponding synthesized inhibitors and/or stimulators/inducers/enhancers of stem cells [43-47] have been investigated intensively.

In the early 1990s, as the specificity of developed inhibitors was not sufficiently strong, their effectiveness in inhibiting differentiation and then helping to establish mESCs were not satisfactory and culturing results were inconsistent. More recent year, a breakthrough result was reported. Following the progresses, using the novel mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor PD0325901 or SU5402 to eliminate differentiation-inducing signaling from MAPK and using the glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021 to enhance ESC growth capacity and viability helped dramatically in establishing mESC lines [48]. Accordingly, ES cell lines of the second mammalian species (rat) with germline transmission have been reported [49,50]. The combination of MEK inhibitor PD0325901 and GSK3 inhibitor CHIR99021 (2i) also appears to improve the generation efficiency of induced pluripotent stem cells (iPSCs) [51].

To date, the success rate in establishing mESCs via whole embryos is usually more than 50%, regardless of the mouse strains used [31,52-58]. Further progress in the derivation of mESCs from whole early-stage embryos seems limited. Conversely, the success rate in establishing mESCs via isolated single blastomeres is relatively low and highly variable [31,59]. Although the success rates in some reports are approximately 30% [59,60], it cannot be the routine yet due to the variable results.

### **2.1. Fetal bovine serum (FBS) vs. serum replacement (SR)**

To date, FBS, with its excellent nutrient mixture, remains the most important and universal component for propagating cells. Additionally, FBS contains growth factors that support ESCs. However, FBS also contains potential differentiation factors for ESCs [61-64]. Therefore, testing and then selecting batches of FBS to support the growth of undifferentiated ESCs is necessary. Otherwise, qualified ESC-grade FBS, which is more costly than conventional FBS, can be used.

To support mESCs, the biopotency of FBS (a biological product) varies from batch to batch. Additionally, its supply worldwide is sometimes limited and it is expensive. Furthermore, animal-originated materials risk introducing adventitious agents into a cell culture system. Therefore, to circumvent interference from differentiation factors and other disadvantages of FBS, chemically defined KSR was developed for use in place of FBS [28].

Although KSR is a commercial product, its formula remains unknown. With its well-defined chemical formula, N2B27, can replace KSR and achieve almost the same deriving and maintaining ESCs results [48,65]. Originally, N2B27 is an empirically mixed formulation that provides optimum cell viability and efficient neural differentiation [29,30].

Conventional ESC media usually contain 15–20% FBS or 10% FBS plus 10% newborn serum. For a chemically defined ESC medium, FBS can be replaced completely by KSR or N2B27; otherwise, 5–15% FBS can be replaced by SR. When changing FBS to a new batch, the ESCs sometimes have to adapt gradually to the new batch. For example, one can mix 50% old FBS with 50% new FBS and allow the ESCs to acclimatize to the new medium. Generally, ESCs can be changed easily from serum replacement (SR) to FBS ESC media by sequential adaptation with approximately three passages. Reversely, acclimatizing ES cells from the FBS to the SR ESC medium is sometimes difficult and can fail. When one is switching to a serum-free cell culture, sequential adaptation for approximately 4–5 passages is required.

Notably, FBS, a good buffer, is a complex solution that contains many chemicals and proteins with different molecular weights. Therefore, mESCs in FBS-free medium are more sensitive to extremes of pH, osmolarity, enzyme treatment, and mechanical forces. Furthermore, a 5- to 10-fold lower antibiotics concentration is used in an FBS-free medium because serum proteins typically bind a certain amount of the antibiotic; without these serum proteins, the antibiotic concentration may be toxic to mESCs.

After passing mESCs in a SR ESC medium, centrifugation is necessary to remove trypsin from the cell suspension to avoid further digestion of cells due to the lack of trypsin inhibitors in the medium. For convenience, enzymes that can stop digestion due to the decreased concentration will be a good alternative to trypsin. TrypLE™, a recombinant enzyme derived from

microbial fermentation, can stay at room temperature (RT) before expiration. My laboratory routinely uses TrypLE™ Express to pass mESCs during derivation and maintenance with good outcomes [31].

## 2.2. Feeder cells

The ESCs are extremely sensitive to culture conditions, including properties of culture media and dishes, when maintaining pluripotency without differentiation. Unfortunately, commercially available plates and dishes are not adequate for ESCs.

In the 1970s, pluripotent teratocarcinoma (stem) cell lines were established after introduction of a cell feeder layer [66-68]. Feeder (helper) cells have since been used to help study teratocarcinoma stem cells and embryonal carcinoma (EC) cells. The STO feeder cells then demonstrated to help derive the first mESCs [23] and maintain mESCs at the undifferentiated state.

Feeder cells are usually inactivated via mitomycin C or  $\gamma$ -irradiation treatment. While  $\gamma$ -irradiation leads to breaks in DNA strands, mitomycin C has the extraordinary ability to crosslink DNA with high efficiency and is specific for the CpG sequence [69,70]. Although feeder cells are alive, they do not replicate but gene transcription and protein synthesis are not affected.

It has been speculated that feeder cells support embryos and ESCs attachment through the physical matrix [71]. Furthermore, feeder cells may release embryo trophic factors, reduce inhibitory or toxic factors in FBS, or may be beneficial by lowering concentrations of ions and/or glucose in medium, and thereby overcoming the developmental blockage of embryos mediated by the release of growth factors essential for activation of the embryonic genome and for normal embryonic development. Additionally, feeder cells may protect embryos from oxygen toxicity [63].

Various feeder cells, which differ in their ability to support ESCs, have been utilized to establish, propagate, and maintain the pluripotency of ESCs [35,72]. Conventionally, STO and mEF are the most popular feeder cells for deriving and maintaining ESCs. However, human foreskin fibroblast (hFF) feeder cells also support propagation and self-renewal of human [73-76] and mouse ESCs [31,77].

As feeder cells at earlier passages are used, their ability to support ESCs is increased [78]. Reports have demonstrated that mEF, STO, and hFF feeder cells secrete different growth factors to support ESCs [76,77,79-81].

To help establish mESCs, the STO, mEF, and hFF feeder cells might have roughly equal efficiency [31,82]. Recently, a study reported that hFF supported mESC self-renewal superiorly to mEFs due to the convenience. Using the hFF system, multiple lines of mESCs have been successfully derived without addition of exogenous LIF and any inhibitors. These mESCs have capacities to self-renew for a long period of time and to differentiate into various cell types of the three germ layers both *in vitro* and *in vivo* [76]. The STO is a cell line (ATCC No. CRL-1503) for unlimited propagation. The hFF may be a cell line (CCD-1112Sk, ATCC No. CRL-2429; Hs68, ATCC No. CRL-1635; HFF-1, ATCC No. SCRC-1041) or primary cells. The mEF is



primary cells harvested from fetuses approximately 12.5–13.5 days post coitum (dpc). However, mEF can be passed and propagated only for a short period [83-85]. Therefore, they must be prepared continuously. Additionally, the traits of mEFs differ from batch to batch, and quality control of mEFs, especially for mycoplasma contamination, may be the problems [86,87]. Moreover, hFFs are more durable than mEFs in that they remain in healthy condition more than 2 weeks after inactivation by radiation. In contrast, mEF deteriorates within 1 week after the inactivation [76].

To derive and maintain mESCs, feeder cells and ESC medium supplemented with LIF (typical concentration is 1,000 unit/mL) are usually chosen. The reason is due to the LIF produced by mEFs and STO is not enough to maintain ESC properties most of the time. However, recombinant murine LIF is expensive. Therefore, STO that expresses a high level of LIF (SNL76/7, ATCC No. SCRC-1049; SNLP 76/7-4, ATCC No. SCRC-1050) has been developed [88]. This cell line also can be used as a feeder layer supporting the derivation and growth of mESCs and iPSCs [89]. The disadvantage of the SNL76/7 as a feeder cell is the highly variable level of LIF in culturing medium, that might have different effects on mESCs [24,56,57,90-93].

### 2.3. Conditioned media

Both teratocarcinoma stem cells and EC cells were established in the 1970s [66,94-96]. Both pluripotent cells usually undergo extensive differentiation *in vivo* and *in vitro* to generate a wide variety of cell types [97]. Mouse teratocarcinoma stem cells can condition themselves. Therefore, medium conditioned with teratocarcinoma stem cells, which is equivalent to a 5-fold concentration of LIF [92], was used to help establish mESCs [24]. Thereafter, the propagation of mESCs in high densities reduces possible differentiation [26,98].

Feeder cells secrete many different factors, including growth factors, to support ESCs [76,77,79-81]. The recovered conditioned medium is a complex solution containing many unidentified chemicals. However, as conditioned media are exhausted media prepared in a batch-by-batch manner, their biopotency might vary. The question is whether conditioned medium is still needed to derive ES cells when chemically defined ESC media supplemented with differentiation inhibitors and growth factors are available.

A few different conditioned media have been used to establish mESCs. Of which, an FBS ESC medium conditioned by a rabbit fibroblast cell line transduced with genomic rabbit LIF (10 ng/mL) allows efficient derivation and maintenance of mESC lines from all 10 inbred mouse strains tested, including some that were presumed nonpermissive for mESC derivation [56]. This commercialized conditioned medium, RESGRO™ Culture Medium (Millipore), can establish and rescue established mESCs that have started drifting, and either generate low-percentage chimeras or lose their germline transmission capability [53,92].

### 2.4. Mouse strain and embryo status

Teratomas (benign) and teratocarcinomas (malignant) are tumors composed of an ectoderm, endoderm, and mesoderm mixtures of adult tissues [99]. Most are found in gonads and rare in mammals, including experimental animals. In the 1950s, mouse strain 129 had found an

incidence of spontaneous testicular teratoma of about 1% [100]. After progress and refinement of cell culture techniques, most notably the introduction of the cell feeder layer, allowed the reliable cultures of pluripotent teratocarcinoma (stem) cell lines [66-68].

The mESCs were first derived from 129SvE [23] and then (ICR × SWR/J) F1 hybrid embryos [24]. However, due to the demand for animal models of human diseases, strains other than 129s have been used to establish new mESC lines. Unfortunately, the following mESCs were derived mainly from 129 strains due to the permissive nature of the genetic background [27,32,35,38]. The derivation of mESCs from blastocysts is a process that is often very inefficient, and even in the most favorable 129 strains, a success rate of 30% is regarded as high [26]. Derivation of mESCs is strongly mouse strain-dependent [32], and in practice the efficiency of derivation in strains other than 129 strains does not usually exceed 10% [36].

Strains other than 129s, such as FVB, CBA/Ca, and the non-obese diabetic (NOD) mouse [101], have the extremely low derivation rates of mESCs using conventional conditions. They are traditionally regarded as highly refractory (nonpermissive) for derivation of mESCs. Moreover, they also produce chimeras either incapable of germline transmission [102] or restricted in their germline competence [37].

Mouse strain C57BL/6 (B6) is not usually considered as a permissive strain for mESC derivation. Although the B6 mES cell lines have been available since the early 1990s [35,103], the efficiency in establishing B6 mESCs via FBS or KSR ESC medium containing LIF varies and is typically less than 10% [32,38,56,61,82,103-109]. When establishing C57BL/6J mESCs using FBS (15%) ESC medium containing LIF, the highest success rate was 40% (36/89) [56]. Notably, this study used mEF feeder cells coated on 96-well cell plates for initiation of blastocysts and thereafter digested ICM outgrowths cultured at 39°C under 5% CO<sub>2</sub> in an incubator. In establishing B6 mESCs using KSR (20%) ESC medium containing LIF, the derivation rate of (C57BL/6N × C57BL/6J) F1 mES-like colony can be as high as 40% (10 of 25 blastocysts) [109]. Other studies also demonstrated that the efficiency in establishing mESCs is significantly higher for the C57BL/6N strain (53%) than for the C57BL/6J strain (20%) [109]. Recently, my study showed that C57BL/6J blastocysts in KSR ESC medium did not lead to effective derivation of mESCs; however, the success rate in 2i medium was as high as 75%. Furthermore, the efficacy of 2i medium was also demonstrated when using morulae (60%) and 8-cell embryos (50%) for mESCs derivation [31].

Although previous results suggested that true ES cells can be derived from embryos explanted at any stage of preimplantation development in the mouse [110]. My laboratory was the first to report success in deriving mESCs from mouse zygotes. These zygote-derived mESCs are morphologically indistinguishable from mESCs derived from fertilized embryos and blastomeres. Moreover, the generation of germline transmitted chimeras confirmed that the established mES-like cells are pluripotent mESCs [31].

In recent years, the mESC lines of nonpermissive strains have been established routinely from 3.5 dpc blastocysts when SR ESC medium containing differentiation inhibitors and/or proliferation enhancers was used.

Typically, diploid male mESCs capable of a high percentage generation of chimeras with germline transmission are selected for further utilization. However, some unusual mESCs have been reported including the androgenetic [111], germline transmitted female [112], adult somatic cell nuclear transferred [113], XO [114], parthenogenetic [115], haploid [54], and androgenetic haploid [116] mESCs.

## 2.5. Single blastomere

To date, most available ES cell lines were derived from the outgrowth of ICMs of blastocysts. However, due to ethical concerns over the derivation of human ES (hES) cells for regenerative medicine, a single blastomere (usually from 8-cell embryos) has been utilized to derive ESCs. Unfortunately, conventional methods used to establish mESCs directly from an isolated single blastomere, which is extremely sensitive to culture conditions, are unsuccessful. Actually, most of the isolated blastomeres divide to form small sheets of cells with a trophoblastic-like morphology or small blastocysts with or without visible ICM-like cells [6,31,117]. In 1996, mES-like cells were first reported from single blastomeres [118]. Since then, no ESCs were established from blastomeres until 2006 [117].

The mESCs can condition themselves to inhibit or prevent differentiation. Therefore, blastomeres aggregated with established mESCs for initial co-culturing to help in the derivation of mESCs is a logically reasonable alternative. In this manner, the single blastomere-derived mESCs have been established but overall success rates are less than 5% when using the conventional FBS ESC medium [117-119]. In spite of that, a possibility may exist for deriving personalized hES cells without destruction of 8-cell embryos.

On 2004, the study showed that the KSR ESC medium do not support mES single cell culturing. Contrary, single mESCs were propagated without loss of pluripotency when the adrenocorticotrophic hormone (ACTH) was added to KSR ESC medium [62]. The authors of that study hypothesized that ACTH may be integrated via a weak cross interaction with an unknown, non-physiological inhibitory G protein coupled receptor. A signaling system other than the cAMP-PKA pathway or PKA pathway may play an important role in propagation of mESCs [62]. Later, simple and efficient establishment of mES cell lines from a single blastomere of 2- to 8-cell embryos with KSR ESC medium containing ACTH fragments 1-24 (ACTH 1-24) on mEF feeders was reported [59].

Wakayama *et al.* (2007) developed a novel protocol and established mESCs via blastomeres and polar bodies. In their experiment, isolated B6D2F2 blastomeres were cultured on KSR (20%) ESC medium containing ACTH 1-24 in 96-well plates coated with mEFs. The mESC establishment rates were 33%, 8%, and 8% for blastomeres derived from 2-cell (1/2), late 4-cell (1/4), and 8-cell embryos (1/8), respectively. However, they did not aggregate blastomeres with other mESCs, suggesting that success was likely attributable to the KSR ESC medium containing ACTH 1-24 [59]. Other studies then demonstrated that mESCs can be established from 1/2, 1/4, 2/4, 1/8, 2/8, 3/8, and 4/8 blastomeres in medium containing ACTH 1-24 [31,120-122]. For blastomeres, as the developmental stage of embryos decreases and the number of isolated blastomeres used increases, the derivation efficiency of mESCs increases. Moreover, whole embryos always have better derivation efficiency than corresponding blastomeres. Further-



more, KSR ESC medium containing differentiation inhibitors and/or proliferation stimulators is better than FBS ESC medium in establishing mESCs.

For ethical concerns, such as maintaining the developmental potential of embryos and establishing corresponding hES cells, a 1/8 blastomere is one of the best candidates. Unfortunately, derivation efficiency of mESCs via a 1/8 blastomere is usually approximately 5–10%, such that it cannot be a standard protocol for the routine operation. Obviously, reliable and efficient protocols for ESCs derivation should be developed.

Previous studies have indicated poor derivation efficiency of mESCs from a 1/8 blastomere, partly due to the low division rate of single blastomeres when compared to that of their counterparts with a higher number of blastomeres (2/8, 3/8, and 4/8). Communication and adhesion between blastomeres, from which the derivation process begins, are likely important aspects to efficiently deriving mESC lines. Therefore, an approach consisting of chimeric E-cadherin (E-cad-Fc) adhesion to the blastomere surface has been devised to recreate the signaling produced by native E-cadherin between neighboring blastomeres inside an embryo. Via this approach, the 1/8 blastomere incubated with E-cad-Fc for only 24 h can significantly improve the mESC derivation efficiency from 2.2% to 33.6% [60]. To date, this novel method via 1/8 blastomere has the best derivation efficiency for mESCs. However, its reproducibility must be confirmed by other laboratories.

## 2.6. Pluripotent signaling pathways

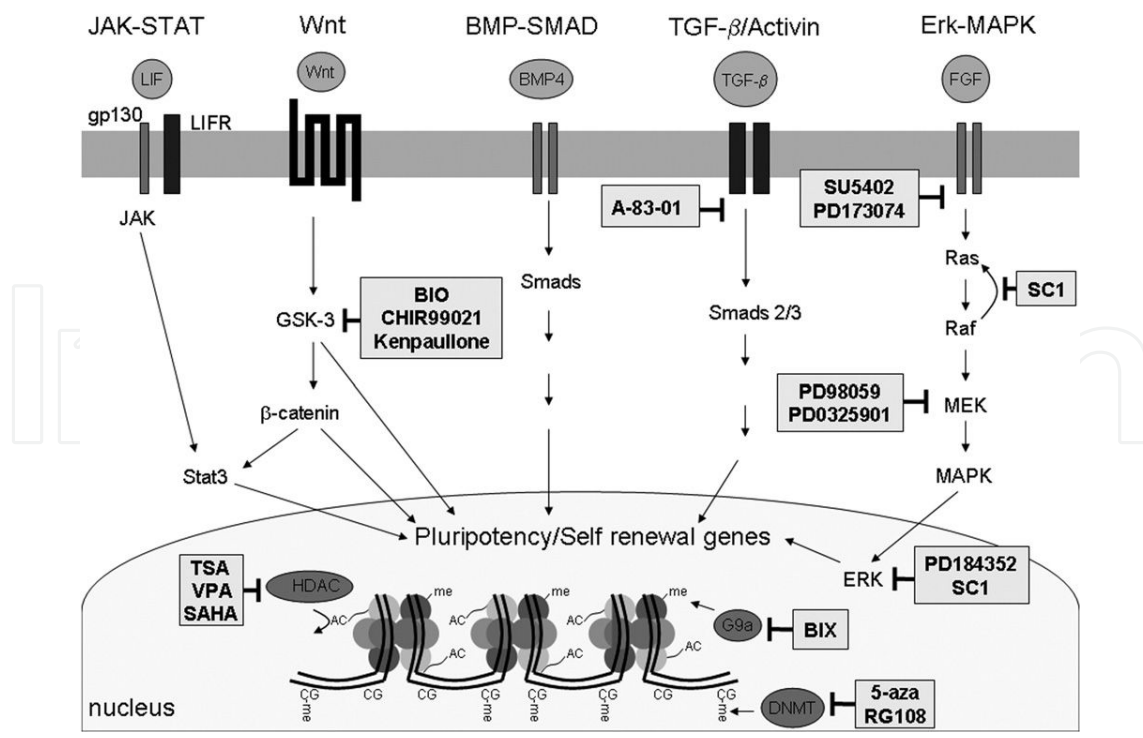
In 1988, two studies demonstrated that LIF could assist in derivation and maintenance of the pluripotency of mESCs [33,34]. Unfortunately, following studies revealed that LIF is not as effective or good for mammals other than mice in establishing ESCs. These experimental results implied that different mammals might have different regulatory mechanisms for ESCs. Actually, distinct signaling pathways have been shown to regulate the pluripotency of mouse and human ESCs [41,42,123].

The regulatory mechanisms and signal transductions of self-renewal, differentiation, proliferation, and apoptosis [41,42,124], as well as the corresponding inhibitors of stem cells [43-47, 125] have been investigated (Figure 1).

To date, self-renewal, pluripotency, and the propagation signaling transduction pathways of ESCs includes the LIF/signal transducer and activation of transcription 3 (STAT3), Wnt/ $\beta$ -catenin, phosphatidylinositol 3-kinase (PI3K), bone morphogenic protein 4 (BMP4)/Smad1/5, and basic fibroblast growth factor (bFGF) [42,45]. Conversely, the Ras/Raf/MEK/ERK pathway is central to the signaling networks that govern proliferation, differentiation, and cell survival [126]. The active Ras/Raf/MEK/ERK pathways induce differentiation of mESCs [42,124,126]. The mESCs have high ERK activity when they undergo differentiation. Suppression of the ERK pathway promotes self-renewal of mESCs. Moreover, BMP4 activation inhibits differentiation of mESCs in medium containing LIF due to inhibitor of differentiation (Id) genes expression and ERK inactivation [127-129].

Of the many pathways, self-renewal of mESCs largely depends on LIF/interleukin 6 (IL-6) family members [130] and BMP4 [65,127]. LIF binds to a cell surface complex composed of the

LIF receptor and the transmembrane signaling molecule gp130, resulting in activation of transcription factor STAT3, which is essential and sufficient to promote self-renewal and inhibit mesoderm and endoderm differentiation of mESCs [125,130-132]. Additionally, Wnt signaling inhibits GSK3 $\beta$  and results in the protein stabilization of cytoplasmic  $\beta$ -catenin ( $\beta$ -Ctnn). The GSK3, a constitutively acting multi-functional serine threonine kinase, derives its name from its substrate glycogen synthase, a key enzyme involved in conversion of glucose to glycogen. Although GSK3 is kept inactive by phosphorylation, activated GSK3 enhances synthesis of glycogen and inhibits cell proliferation. The name GSK does not adequately describe the multitude of diverse substrates and functions attributed to GSK3. For instance, it is involved in various cellular processes, ranging from glycogen metabolism, insulin signaling, cell proliferation, neuronal function, and oncogenesis to embryonic development [133]. Additionally, GSK3 is one of the crucial molecules involved in regulation of the Wnt/ $\beta$ -catenin, Hedgehog, and Notch signaling pathways. The undifferentiated pluripotency of both mouse and human ESCs can be maintained by GSK3-specific inhibitor 6-bromoindirubin-3'-oxime (BIO), which prevents phosphorylation of  $\beta$ -Ctnn by GSK3 $\beta$  and activates Wnt [134,135]. The Wnt signaling is endogenously activated in mESCs and is down regulated upon differentiation [135]. The target genes of the Wnt signaling pathway, such as c-Myc and CyclinD1, promote cell proliferation and self-renewal. The LIF/STAT3 pathway combines with the Wnt/GSK3 $\beta$ / $\beta$ -catenin pathway to enhance self-renewal by activation of pluripotency genes, including transcription factors Nanog, Oct3/4, and Klf4. Nevertheless, LIF also activates the MAPK/ERK pathway, which induces mESC differentiation [45].



**Figure 1.** The use of signalling pathway inhibitors and chromatin modifiers for enhancing pluripotency. (Reproduced with permission from Sumer *et al.*, 2010. *Theriogenology* 74:525–533.)

As a protein, transforming growth factor beta (TGF $\beta$ ) controls proliferation, cellular differentiation, and other functions in most cells. This protein is a secreted protein of cytokines. The TGF $\beta$  family is part of the large TGF $\beta$  superfamily, which has more than 40 members, including TGF $\beta$ , activin, nodal, BMPs, inhibins, anti-müllerian hormone, decapentaplegic, and Vg-1. All of these ligands are associated with ESCs. Although BMP4 is a member of the BMP family, it functions via a different signaling pathway with TGF $\beta$ . Notably, BMP4 induces expression of Id genes and inhibits MAPK signaling as well as neuroectoderm differentiation [65,127]. Although activation of STAT3 is sufficient for self-renewal of mESCs, a study showed that LIF-STAT3 do not maintain mESCs in serum-free ESC medium. The combination of LIF and BMP4 maintained the self-renewal of mESCs in the absence of both feeder cells and FBS [65,127]. Thus, BMP4 and LIF have synergistic effects on the self-renewal of mESCs [127]. In contrast to mESCs, BMP4 does not maintain the self-renewal of hES cells; rather, it induces trophoblast or primitive endoderm differentiation of hES cells [136].

The PI3K pathway is also important for proliferation, survival, and maintenance of pluripotency, as well as inhibiting apoptosis in ESCs. The ESC-expressed Ras (ERas) is specifically expressed in ESCs, stimulating PI3K. This PI3K activation promotes ESC proliferation [137] and self-renewal [138]. Inhibition of PI3K and Akt induces differentiation of mESCs in the presence of LIF and feeder cells [139], suggesting that PI3K/Akt signaling is necessary for maintenance of the pluripotency of ESCs.

The ESC can maintain its pluripotency with feeder cells. Exogenous supplementation of LIF is sufficient to sustain mESCs at undifferentiated state in a feeder cells free condition. However, LIF is insufficient for maintaining the pluripotency of hES cells. Human ESCs have been most commonly cultured in the presence of bFGF either on fibroblast feeder layers [140] or in fibroblast-conditioned medium. The bFGF signaling pathway appears to be important to the self-renewal of hES cells [141,142]. Reports have shown that bFGF (40 ng/mL) combined with noggin (inhibitor of BMP4) supports the undifferentiated proliferation of hES cells in the absence of feeder cells [143-145]. Furthermore, a high bFGF concentration (100 ng/ml) alone is sufficient to maintain human ESCs [145]. The post-implantation epiblast-derived stem cell (EpiSC) lines [14] express transcription factors that regulate pluripotency and robustly differentiate into the major somatic cell types as well as primordial germ cells [12]. Furthermore, the EpiSCs and hES cells share patterns of gene expression and signaling responses in the epiblasts [12]. In fact, the similarities (FGF and activin growth factors for self-renewal; *in vitro* pluripotency; chimera formation; and spontaneous trophoblast differentiation) between hES cells and mouse EpiSCs have led to the suggestion that hES cells are equivalent to early post-implantation epiblasts, rather than their ICM progenitor [146,147].

## 2.7. Differentiation inhibitors help to derive mouse ES cells

In the early 1990s, as the specificity of developed inhibitors was insufficient, the effectiveness of these inhibitors to inhibit differentiation and then help to establish mESCs was not satisfactory and outcomes are varied. Since the late 1990s, using MAPK/ERK kinases (MEKs) inhibitors PD098059 [128] and UO126 [41], or by dephosphorylating ERKs by mitogen-

activated protein kinase phosphatase 3 (MKP-3) [41] enhanced the self-renewal of mESCs and inhibited their differentiation.

It was the first report showed that the combination of PD98059 (MEK 1 inhibitor) with LIF enhances the establishment of mESCs from the refractory CBA strain [148]. A combination of the MEK1/2 inhibitor, U0126, with LIF further promoted the efficiency of mESC derivation from CBA [129]. These small molecules, PD98059 and U0126, play positive roles in the self-renewal of mESCs, but they are incapable of maintaining the pluripotency of mESCs in long-term culture without LIF [48,128,135].

Notably, BMP4 inhibits both ERK and p38 MAPK pathways in mESCs. The inhibitors of the ERK and p38 MAPKs mimic the effect of BMP4 on mESCs. Inhibition of the p38 and MAPKs by SB203580 overcomes the roadblock in deriving mESCs from blastocysts lacking a functional Alk3, the BMP4 type-IA receptor [127].

The self-renewal of mESCs is generally dependent on multifactorial stimulation. To support the growth and development of fetuses, FBS contains cell replication stimulators and cell differentiation inducers. Serum and serum substitutes contain various inductive stimuli that may activate commitment and differentiation programs [49]. However, simple withdrawal of serum or other exogenous stimuli cannot prevent differentiation of ESCs due to endogenous autoinductive differentiation of fibroblast growth factor 4 (FGF4) via the MEK/ERK pathway [48,149]. Therefore, to suppress endogenous autoinductive differentiation and to maintain high viability and growth rates, one must inhibit the MEK/ERK pathway and/or provide LIF, or restrict the activity of GSK3, which acts mainly via the Wnt/ $\beta$ -catenin signaling pathway to suppress cellular biosynthetic capacity, and subsequent cell growth and viability [134,150]. Those studies have demonstrated that inhibition of GSK3 via BIO dramatically augment mESC derivation from isolated ICMs of both C57BL/6 (76%) [135] and BALB/c (31%) [134].

Through dual inhibition of the Ras GTPase-activating protein (RasGAP) and ERK1, a function-oriented and novel small molecular pluripotin, also called SC1, was developed. By using this novel pluripotin, one can propagate mESCs in an undifferentiated and pluripotent state under chemically defined conditions in the absence of feeder cells, FBS, and LIF. Moreover, long-term pluripotin-expanded mESCs can generate germline-transmitted chimeric mice [43]. By combining pluripotin and LIF for the derivation of mESCs, the successfully isolated mESCs from five strains of mice; efficiency was 57% for NOD-scid, 63% for SCID beige, 80% for CD-1, and 100% for two F1 strains from C57BL/6 x CD-1. Pluripotin combined with LIF improved the efficiency of mESC isolation by selectively maintaining Oct4-positive cells in outgrowths. This is the first work to efficiently derive mESCs from immunodeficient mice (NOD-scid on an NOD background and SCID beige on a BALB/c background) on refractory backgrounds [57].

Recently, a novel protocol involves an unusually long initial incubation of 12 days for blastocysts seeded in 12-well plates coated with mEFs and containing LIF and pluripotin-supplemented KSR ESC medium (15% KSR), which results in the formation of large spherical outgrowths. These outgrowths are morphologically distinct from classical ICM outgrowths and can be picked easily and trypsinized. Importantly, pluripotin needs to be omitted after the



first trypsinization because it blocks the attachment of mES-like cells to the mEF feeder layer; its removal facilitates the formation of mESC colonies. In addition, pluripotin is harmful to the mEFs and it is not unusual that half of the mEFs die during the first week. However, this massive death of mEFs does not affect the mES cell derivation efficiency [55]. I also found that STO and Hs68 feeder cells were dying when culturing in KSR ESC medium supplemented with 3  $\mu$ M pluripotin (unpublished observation). The modified protocol gives rise to mESCs (more than five passages) in a robust and reliable manner with an extremely high success rate of 94% (78/83) [55]. Surprisingly, 10 mESCs derived with 4  $\mu$ M pluripotin showed the chromosomal instability. All of these cells generated weak chimeras. Thus, these lines are only suitable for *in vitro* analysis. In contrast, mES-cell lines derived with 2  $\mu$ M pluripotin during the blastocyst outgrowth phase were generated with unusual high efficiency (100%) and these lines had a normal karyotype. In addition, strong chimeras could be derived from these mESCs [55]. Yang and colleagues (2009) derived mESCs with 3–5  $\mu$ M pluripotin. Their mESCs showed some chromosomal abnormalities and were not proven to be germline competent [57].

Although the derivation of novel mESCs have been improved significantly via differentiation inhibitors and/or proliferation stimulators, no germline-transmitted ESCs other than the mESCs have been reported. In recent years, the MEK inhibitor PD0325901 or SU5402 has been used to eliminate differentiation-inducing signaling from MAPK and the GSK3 inhibitor CHIR99021 has been used to enhance mESC growth, enabling derivation and propagation of germline-competent mESCs from CBA and 129 strains in an N2B27 chemically defined ESC medium. These findings reveal that self-renewal is enabled by the elimination of differentiation-inducing signaling from MAPK. Additional inhibition of GSK3 consolidates biosynthetic capacity and suppresses residual differentiation. Complete bypass of cytokine signaling is confirmed by isolating *stat3*<sup>-/-</sup> mESCs [48]. Accordingly, ES cell lines of the second mammalian species (rat) with germline transmission have been established [49,50].

Recently, my report showed that two C57BL/6J mESCs were derived from two morulae in KSR ESC medium supplemented with 2i but without LIF [31]. These experimental results confirmed previous reports indicating that inhibitors that block the MEK/ERK differentiation pathway can support self-renewal of mESCs, even without LIF signaling [43,48].

The efficiency of establishing mESCs with 2i was not reported [48]. Recently, my experimental results showed that the efficiency in establishing C57BL/6J ES cells in KSR ESC medium supplemented with 2i and LIF could be high as 65% for morulae and 70% for blastocysts [31]. Thus, the efficacy of 2i in helping establish mESCs is confirmed.

## 2.8. Self-renewal and proliferation stimulator

Although a previous study showed that extrinsic stimuli are dispensable for derivation, propagation, and pluripotency of mESCs [48], the self-renewal and derivation of mESCs largely depends on LIF [130] and BMP4 [65,127]. When LIF is present at concentrations higher than those used in routine cultivation (500 unit/mL for the mEF feeder and 1,000 unit/mL for the STO feeder), the derivation efficiencies of mESCs from refractory strains are improved significantly [57,91,93]. Indeed, strain difference in response to soluble growth factors is evident from two original reports of mESC derivation. Evans and Kaufman (1981) established



mESC lines from permissive 129SvEv delayed blastocysts, solely by culturing them on STO feeder cells. Notably, Martin (1981) was unable to establish mESC lines from immunosurgically isolated ICMs of (ICR × SWR/J) F1 and (C3H × C57BL/6) F1 fully expanded blastocysts unless the teratocarcinoma stem cell-conditioned medium, equivalent to a 5-fold LIF concentration [92], was added. A previous report revealed that a mESC medium containing 10 ng/mL rabbit LIF or conditioned by a rabbit fibroblast cell line transduced with genomic rabbit LIF facilitates efficient derivation and maintenance of mESC lines ( $\geq 10$  passages) from all 10 inbred mouse strains tested, including some that were presumed nonpermissive for ESC derivation [56]. A more recent study demonstrated that SR ESC medium containing high concentrations of LIF (2,500 and 5,000 unit/mL) could establish mES cell lines from C57BL/6, Balb/K, nonpermissive CBA/Ca, and NOD mice [92].

Additionally, another report shows that KSR ESC medium does not support single mESC culture, likely because this medium lacked some important growth factors or such undefined factors, such as “stem-cell autocrine factors,” are secreted by mESCs themselves [62]. However, when KSR ESC medium is supplemented with ACTH, single mESCs are propagated and their pluripotency is maintained. Accordingly, mESC lines have been established efficiently using single blastomeres from 2- to 8-cell embryos in KSR ESC medium containing ACTH 1–24 [59].

The CHIR99021 pathway and proposed ACTH pathway likely differ. Therefore, synergetic effects of 2i (PD0325901 + CHIR99021) and ACTH 1–24 may exist in deriving mESCs. My experimental results reveal that neither STO nor Hs68 feeder cells coated on 10- $\mu$ L droplets and cultured in KSR ESC medium supports effective derivation of mESCs from embryos or blastomeres of ICR or C57BL/6J. However, by supplementing KSR ESC medium with 2i or ACTH 1–24, efficiency in establishing mESCs increased dramatically. Experimental results also demonstrate that inhibiting cell differentiation and increasing cell growth/viability (in the presence of 2i) simultaneously is better than increasing only cell survival and/or proliferation (with ACTH 1–24) when deriving mESCs [31]. Additionally, experimental results also suggest that GSK3 inhibitor CHIR99021 and ACTH 1–24 likely have different pathways in synergistically enhancing the establishment of mESCs because 2i with ACTH 1–24 is much more effective than 2i or ACTH 1–24 alone [31].

## 2.9. Developing more powerful differentiation inhibitors

A previous study has demonstrated that blastomeres from 2- to 8-cell embryos developed into blastocysts within 3–5 days in KSR ESC medium containing ACTH 1–24 [59]. Another report indicates that 2- to 4-cell embryos and blastomeres, which developed into blastocysts in medium containing ACTH 1–24, PD98059 (MAP2K1 inhibitor) or SB203580 (MAPK14 inhibitor), yields developmental rates comparable to those of the control embryos [151]. My previous study showed that approximately 60% of 1.5–2.5 dpc denuded whole embryos developed into typical blastocysts or small blastocysts and approximately 80% of the corresponding blastomeres developed into small blastocysts within 2–4 days in KSR ESC medium containing 2i or ACTH 1–24. These experimental results imply that 2i and ACTH 1–24 might have little or no adverse effect on cell proliferation or the development of embryos and blastomeres. Furthermore, these observations indicate that 2i, MAP2K1, and MAPK14

inhibitors are not strong enough to inhibit differentiation of embryos and blastomeres entirely [31]. I also note that 0.3–3  $\mu\text{M}$  pluripotin did not inhibit differentiation of ICR 2.5 dpc embryos and blastomeres completely (unpublished observation).

These experimental findings suggest that other more powerful inhibitors, via the Ras-MEK-ERK signaling pathway or other signaling pathways, may be worth developing to further enhance the success rate of deriving ESCs. Theoretically, chemical cocktails that completely inhibit endogenous differentiation, increase cell division, and decrease apoptosis of pluripotent cells should maximize the derivation efficiency of ESCs. Furthermore, is it possible to develop novel differentiation inhibitors that maintain the totipotency of very-early-stage blastomeres?

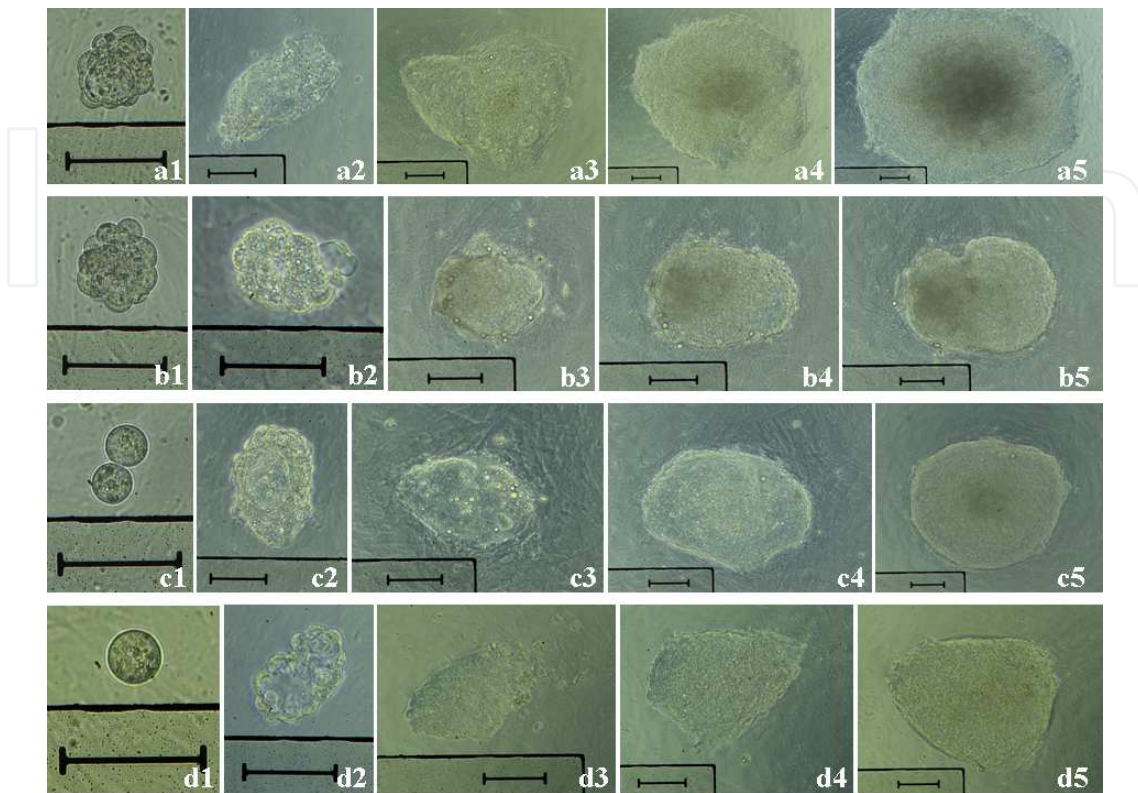
### **3. Novel efficient, reproducible, and user-friendly protocol for deriving mouse ES cells**

An excellent protocol for deriving mESCs must be efficient, reproducible, easy to perform, and relatively cheap.

The following protocol, adopted and minor modified from my previous study [31], has been used in my laboratory for more than 3 years with reproducible high derivation efficiency (always more than 50%, occasionally reaching 100%) for 2.5 dpc whole embryos, regardless of which strains (ICR, B6, ICRB6F1, and B6CBF1) are tested. Moreover, all other pre-implantation embryos can be used. Although the same protocol can be used to derive mESCs via single blastomeres, derivation of mESC varies in efficiency, which depends on the origin of blastomeres (approximately 10–30%).

#### **3.1. Growth area for initial cultivation**

For initial cultivation of embryos, ICMs, and blastomeres to establish mESCs, different growth surface areas in multi-well cell plates are used. Of which, the 24-well and 96-well plates are used mainly. In practice, the amount of medium needed to half fill one well in a 24-well plate is approximately 0.5 mL and 150  $\mu\text{L}$  for a 96-well plate. Both volumes are enormous to the mouse embryo (diameter, 85  $\mu\text{m}$ ; volume, 320 pL = 0.00032  $\mu\text{L}$ ) or 1/8 blastomere (diameter, 20  $\mu\text{m}$ ; volume, 4 pL = 0.000004  $\mu\text{L}$ ). Furthermore, the height of wells is not user-friendly when taking photographs or picking growing three-dimensional outgrowths. Therefore, to reduce the amount of expensive ESC media that is usually exchanged every other day, ease handling, and increase the disaggregation efficiency of growing three-dimensional outgrowths, different volumes of micro-droplets were tested. I hypothesize that homemade micro-droplets on cell culture dishes coated with feeder cells are as effective as cell plate wells for initial cultivation to establish mESCs. Finally, 10- $\mu\text{L}$  and 20- $\mu\text{L}$  droplets are chosen for initial and for disaggregated outgrowths cultivation, respectively. Up to 32 10- $\mu\text{L}$  or 24 20- $\mu\text{L}$  feeder droplets on a 60-mm cell culture dish covered with heavy weight paraffin oil can be prepared and used routinely. My culturing results show that 10- $\mu\text{L}$  droplets support the growth of 2.5 dpc embryos for at least 7 days and 1/8 blastomeres for at least 10 days (Figure 2).

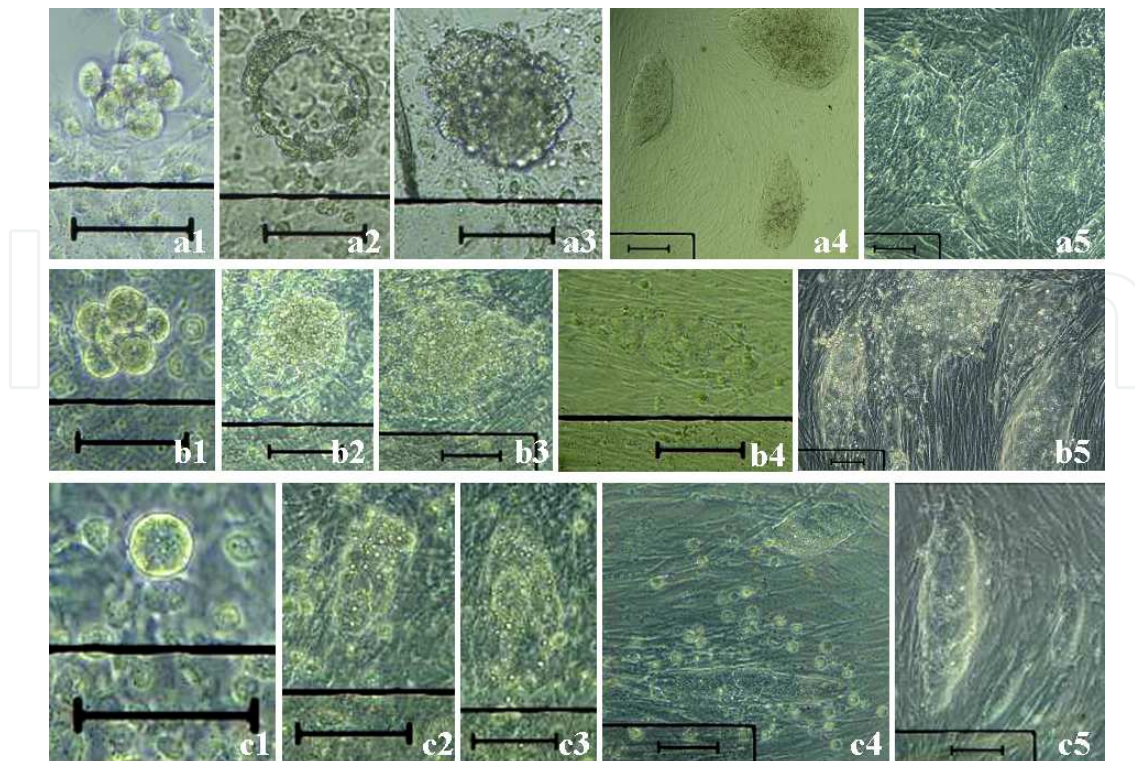


**Figure 2.** The 10- $\mu$ L droplets are excellent for initial cultivation to derive mESCs. Embryos and blastomeres seeded in 10- $\mu$ L droplets coated with Hs68 feeder cells in KnockOut™ serum replacement (KSR) ESC medium supplemented with 0.5  $\mu$ M PD0325901 (MEK inhibitor), and 3  $\mu$ M CHIR99021 (GSK3 inhibitor) (2i), 10  $\mu$ M ACTH-24 (adenylyl cyclase inhibitor), as well as 1,000 unit/mL LIF support the growth of 2.5 dpc embryos for at least 7 days and 1/8 blastomeres for at least 10 days. a1, early blastocyst; b1, morula; c1, 2/16—isolated single blastomere that originated from late 8-cell embryos were divided; d1, 1/8—isolated single blastomere that originated from 8-cell embryos. a2–d2, cultured for 3 days after initiation of cultivation; a3–d3, cultured for 7 days; and a4–d4, cultured for 10 days; a5–d5, cultured for 12 days. The bar represents 100  $\mu$ m.

The previous study reported that the culturing blastocysts allow to hatch and expand for approximately 6 days; however, the earlier trypsinization (at day 4 or 5) of outgrowths do not seem to affect efficiency but prolonged the time needed for mESCs derivation [58]. Very interestingly, blastocyst outgrowths can be cultured in 2  $\mu$ M pluripotin-containing KSR ESC medium for 12 days (or up to 18 days) without losing their ability to form mESCs. The main advantage of a long initial cultivation is to greatly increase the number of cells, which including undifferentiated cells, before the first trypsinization. The derivation efficiency could be as high as 94% (78/83) [55].

Further, my experimental results demonstrated that mESCs are established efficiently from C57BL/6J denuded whole embryos culturing in a 10- $\mu$ L droplet coated with Hs68 feeder cells of KSR ESC medium supplemented with 2i, ACTH-24, and LIF (Figure 3) [31].





**Figure 3.** The C57BL/6J ES cells derived from 2.5-day post-coitum (dpc) denuded 8-cell embryos or single blastomeres cultured in 10- $\mu$ L droplets. a1: An 8-cell embryo cultured in KSR ESC medium containing 0.5  $\mu$ M PD0325901 (MEK inhibitor) and 3  $\mu$ M CHIR99021 (GSK3 inhibitor) (2i) with STO feeders. Images show 2 (a2), 4 (a3), 11 (a4; passage #1, P1) and 14 (a5; ESC98B01, P2) days after initiation of cultivation. b1: An 8-cell embryo cultured in KSR ESC medium with Hs68 feeders. Images show 3 (b2), 4 (b3), 9 (b4; P1), and 18 (b5; ESC98B05, P4) days after initiation of cultivation. c1: A single blastomere isolated from an 8-cell embryo cultured in KSR ESC medium containing 2i with Hs68 feeders. Images show 3 (c2), 6 (c3), 9 (c4; P1), and 14 (c5; ESC98B04, P3) days after initiation of cultivation. The bar represents 100  $\mu$ m. (Reproduced from Lee *et al.*, 2012. *Stem Cells and Development* 21:373–383.)

### 3.2. Feeder cells and medium

The cell lines of STO and Hs68 (Caucasian human newborn foreskin fibroblast; ATCC No. CRL-1635) as well as primary mEF cells can be used as feeder cells. These cells share the same growth medium, which comprises DMEM (glucose, 4.5 g/L) containing 10% FBS, penicillin (50 unit/mL), and streptomycin (50  $\mu$ g/mL).

After mitotic inactivation by 10  $\mu$ g/mL mitomycin C for 2–3 h, single trypsinized cells via 0.25% trypsin-EDTA are used to prepare feeder layers directly or are frozen until thawed for feeder preparation. Up to 32 10- $\mu$ L feeder droplets on a 60-mm cell culture dish, covered with heavy weight paraffin oil, is prepared 1–2 days before denuded embryos or isolated blastomeres are seeded.

No difference existed between STO and Hs68 feeders in the derivation of mESCs [31]. However, as STO feeders occasionally detached and curled up during cultivation in 10  $\mu$ L and 20  $\mu$ L droplets, Hs68 is usually chosen as the feeder cells. However, propagation of Hs68 is slow

compared to that of STO. Therefore, STO feeder cells are adopted for the large growing areas in commercially available cell plates and dishes.

### 3.3. KSR ESC medium supplemented with 2i and ACTH 1–24

The basal KSR ESC medium contained KnockOut™ DMEM (glucose 4.5 g/L) supplemented with 20% KSR, 0.1 mM non-essential amino acids, 1.75 mM GlutaMAX™-I supplement, 0.1 mM  $\beta$ -mercaptoethanol, penicillin (15.62 unit/mL), streptomycin (15.62  $\mu$ g/mL), and LIF (1,000 unit/mL). The KnockOut™ DMEM can be replaced by conventional DMEM or (KnockOut™) DMEM/F12.

Although media prepared from powder are far cheaper than using liquid directly, experiments always use embryo- or cell culture-tested grades liquid media or solutions, as they are more consistent and efficient for derivation of mESCs, especially when KSR ESC media are used in cultivations. The variable quality of homemade ultrapure ddH<sub>2</sub>O might prove problematic. However, once the mESCs established, the media prepared from powder could be used for routine cultivation.

To enhance the successful derivation of mESCs, the basal KSR ESC medium is supplemented with 0.5  $\mu$ M PD0325901 (MEK inhibitor), 3  $\mu$ M CHIR99021 (GSK3 inhibitor) (2i) [48], and 10  $\mu$ M ACTH 1–24 (adenylyl cyclase inhibitor) [59].

### 3.4. Derivation of mouse ES cells

Natural or superovulated 3.5–4.5 dpc morulae and blastocysts are used. Recovered embryos are washed and placed in the KSOM medium supplemented with 20.85 mM HEPES (HK) at RT until the next treatment. The zona pellucida of embryos is removed (denuded) in seconds using acidic Tyrode's solution. Blastomeres of 2- to 8-cell embryos are separated by incubating denuded embryos in 0.25% trypsin-EDTA for approximately 3–4 min in a 37°C incubator with humidified atmosphere of 5% CO<sub>2</sub> in air, followed by gentle pipetting using a mouth pipette. The blastomeres and denuded embryos or hatched (naked) blastocysts are washed and then plated into 10- $\mu$ L feeder droplets (P0) in KSR ESC medium supplemented with 2i and ACTH 1–24, which is exchanged 1–2 h earlier. Embryos and blastomeres are cultured in a 37°C incubator with 5% CO<sub>2</sub> in air. Following attachment of embryos or blastomeres to the feeder cells, the media are exchanged at the second or third day. Thereafter, the media are exchanged every 1 to 2 days.

After culturing for  $6 \pm 2$  d (for whole embryos) or  $9 \pm 2$  d (for blastomeres), individual three-dimensional outgrowth is identified visually. The feeder cells and flat growing cells are removed mechanically by a mouth pipette. The outgrowth in the same droplet is washed with TrypLE™ Express once and then incubated with TrypLE™ Express in a 37°C incubator for approximately  $13 \pm 3$  min. The outgrowth is washed with KSR ESC medium once and then disaggregated into clumps and single cells, which are reseeded onto fresh 20- $\mu$ L droplets (P1). After  $4 \pm 1$  d, only morphologically mES-like colonies are then passed (via TrypLE Express) to fresh 1 or 2 wells of 4-well cell plates (P2), and thereafter to either 4-well plates or 35-mm cell culture dishes (P3) depending on the number of cells. Once mES-like cells are growing in 4-



well plates and larger dishes, KSR ESC medium (2i + ACTH 1–24 is option) is used for subsequent propagation. At passages  $5 \pm 2$ , mES-like cells are frozen in FBS supplemented with 10% (v/v) dimethyl sulfoxide (DMSO). The typical duration of the above-described process of mESC derivation (from embryos to freezing of subconfluent 35 mm-dishes) is ranging from 14 to 20 days.

Practically, the first 2–3 passages are critical for successful derivation of mESCs. This protocol suggests that one use KSR ESC medium containing 2i + ACTH 1–24 only for the first 10- $\mu$ L droplets for embryo outgrowth (P0) and second (for single cells and clumps of digested outgrowths, P1) and/or third 20- $\mu$ L (for mES-like cells, P2) droplets. Once mES-like cells are growing on 4-well plates (P2–3), KSR ESC medium is used thereafter for all following cultivations.

The success rates in establishing B6 mESCs by this simple protocol are always greater than 50% for 2.5–4.5 dpc embryos. This efficiency is comparable to that achieved in two other studies [56,109] and much better than those in many other studies [32,38,61,82,103–108]. This proposed protocol has a simple layout, is easy to operate, is highly efficient, is reproducible, and can be an alternative method for establishing mES cell lines routinely.

## 4. Conclusions

Zygotes to hatched embryos and blastomeres, ICMs, and the epiblasts of early-stage pre-implantation embryos can be used to establish mESCs. Both embryos and blastomeres have an extremely high capability for cell division and differentiation. Theoretically, chemical cocktails that can completely inhibit endogenous differentiation, increase cell division, and decrease apoptosis of pre-implantation embryos can be helpful to maximize the derivation efficiency of ESCs.

Culturing pre-implantation embryos, no matter what strains, on a very small surface area coated with feeder cells in a chemically defined medium supplemented with differentiation inhibitors (*e.g.*, 2i) and/or proliferation enhancers/stimulators (*e.g.*, LIF and ACTH 1–24) can be used as a routine protocol to establish mESCs efficiently and reproducibly (always more than 50%, occasionally reaching 100%). Unfortunately, the same protocol when using 1/8 blastomeres to derive mESCs is merely acceptable (10–25%). Obviously, the possibility to increase the efficiency of deriving mESCs from whole embryos is limited and unexciting unless novel differentiation inhibitors or proliferation enhancers/stimulators reveal the effectiveness of using 1/8 blastomeres and embryos from species other than rodents.

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