EFFECTS OF MURINE STRAIN, BLASTOCYST STAGE AND INNER CELL MASS ISOLATION TECHNIQUE ON THE EFFICACY OF MURINE EMBRYONIC STEM CELLS

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ABSTRACT The aim of this study was to compare the effects of murine strain, blastocyst stage and inner cell mass (ICM) isolation technique on the efficiency of deriving murine embryonic stem cell (mESC) lines. Foetal mouse embryonic fibroblasts (MEFs) were cultured to Passage 2, cryopreserved and thawed at each passage to be used as feeder layer for mESC culture. Five blastocyst stages from *in vivo* and *in vitro* produced blastocysts were cultured on the MEFs by using 3 different ICM isolation techniques. ICM outgrowths were disaggregated by trypsin/EDTA (0.05%) and manual dissociation, cultured on new inactivated MEFs in CO_2 (5%) incubator, 37ºC. The attachment, primary ICM outgrowth and successful consecutive passages rates up to P3 were compared among the murine strains, blastocyst stages and ICM isolation techniques. There were significant differences ($P<0.05$) in successful passage rate at P3 between CBA/ca with ICR and C57BL/6J (19.81% vs. 9.00% and 8.50%), respectively, also mESC at P1 for mid-, expanded- and hatching blastocyst stages versus early- and hatched blastocyst (45.35%, 52.79% and 43.01% vs. 27.88% and 24.53%), respectively. Manual cut ICM isolation technique consistently gave the highest attachment, primary ICM outgrowth and successful mESC P2 and P3 rates compared with whole blastocyst culture and laser dissection techniques (78.03% vs. 66.52% and 71.06%; 78.35% vs. 75.32% and 75.67%; 52.06% vs. 41.62% and 45.06%; 36.52% vs. 25.77% and 30.49%), respectively. In conclusions, the CBA/ca strain, expanded blastocyst stage and manual cut ICM isolation techniques showed the highest results obtained in production of mESC lines.

ABSTRAK Kajian ini adalah untuk membandingkan strain mencit, peringkat blastosis dan teknik-teknik mengasingkan jisim sel dalaman (ICM) terhadap kecekapan dalam memperolehi titisan sel batang embrionik mencit (mESC). Fibroblas embronik mencit (MEF) dikultur sehingga pasaj 2, dikrioawet dan dinyahsejukbeku pada setiap pasaj untuk digunakan sebagai lapisan sel pembantu bagi pengkulturan mESC. Lima peringkat blastosis diperolehi daripada *in vivo* dan *in vitro* telah dikultur atas MEF dengan 3 teknik pengasingan ICM. Pertumbuhan ICM telah dipisahkan dengan cara trypsin/EDTA (0.05%) dan penceraian secara manual, dikultur di atas MEF baru tak teraktif dalam inkubator CO_2 (5%) pada 37ºC Pelekapan, pertumbuhan ICM primer dan kadar pasaj turutan yang berjaya sehingga ke P3 telah dibanding antara strain mencit, peringkat blastosis dan teknik pengasingan ICM. Terdapat perbezaan signifikan ($P<0.05$) dalam kadar pasaj yang berjaya pada P3 antara CBA/ca dengan ICR dan C57BL/6J (19.81% vs. 9.00% dan 8.50%), masing-masing, juga mESC pada P1 bagi peringkat blastosis pertengahan, pengembangan dan penetasan berlawan dengan peringkat blastosis awal- dan menetas (45.35%, 52.79% dan 43.01% vs. 27.88% dan 24.53%), masing-masing. Teknik penceraian ICM secara pemotongan manual adalah dengan konsisten memberi kadar-kadar dalam pelekapan, pertumbuhan ICM primer serta P2 dan P3 bagi mESC yang berjaya yang paling tinggi berbanding dengan kultur seluruh blastosis dan teknik pembedahan laser (78.03% vs. 66.52% dan 71.06%; 78.35% vs. 75.32% dan 75.67%; 52.06% vs. 41.62% dan 45.06%; 36.52% vs. 25.77% dan 30.49%), masing-masing. Secara ringkasnya, strain CBA/ca, peringkat pengembagan blastosis dan teknik pemotongan secara manual bagi teknik pengasingan ICM menunjukkan hasilan optimal diperolehi dalam menghasilkan titisan-titisan mESC.

(**Keywords**: Blastocyst stage, ICM isolation technique, mESC, murine strain)

INTRODUCTION

Murine serves as a preferred human therapeutic model for embryonic stem cell (ESC) research as this species has close similarities in terms of molecular biology, physiology and developmental process. Since murine species has relatively short generation interval and prolific,

easy to handle, bred and managed as well as capable of reproducing in large numbers, it is suitable to establish a large number of both MEF stock and to obtain the blastocysts needed for stem cell culture. ESC could differentiate into any cell type in the body, including gametes [1, 2]. It is defined functionally as cells that have the capacity to self-renew and the ability to generate differentiated cells [3, 4]. Isolation and culture of embryo-derived cell lines have been reported in many mammals such as murine [2, 4], ovine [6, 7], hamster [8, 9], porcine [10, 11, 12, 13], mink [14], rabbit [15, 16], bovine [17, 18], including primates [19] and humans [20].

mESC isolated from both whole embryos [21] and isolated ICM [2, 22], have similar morphological and biochemical properties to cells from the early murine embryos [23]. mESC have generate chimeric murine showing pluripotency in nature [24] and cultured ICM on MEF in the presence of leukaemia inhibitory factor (LIF) [25]. Blastocysts are either plated intact of feeder layer [26, 27] where they hatch and attach to feeder cell layer or the ICM is isolated from blastocysts either by immunosurgery [27, 28], enzymatic digestion using trypsin [26, 27] or mechanical isolation [26].

Feeder cell is one of the important factors affecting ESC culture [29]. Feeder cell of various types have been used for ESC culturing; STO fibroblasts in murine [2], bovine foetal fibroblasts, bovine uterus epithelial cells, MEF, human lung fibroblasts for ESC culturing in bovines [28, 30, 31] and buffalo foetal fibroblasts in buffalo [26]. It has been reported that feeder layer could secrete some kinds of cytokines, such as LIF [32], which may stimulate ESC growth and inhibit their differentiation. Without a suitable culture medium or feeder layer, ESC would spontaneously differentiate. The original protocol for deriving mESC by Evans and Kaufman [2] is highly inefficient. Even though improvements have been made [33], an efficient protocol is still needed to be developed.

The production of blastocysts as s source for mESC production involves either natural mating or superovulation of murine females to produce a large amount of fertilisable eggs, followed by timed-mating. The desirable blastocysts could also be obtained directly via uterine flushing or indirectly by culturing early stages oviduct-flushed embryos up to blastocyst stage. Many regard the 129 strain as the most favourable and efficient strain [33]. Experiments using other strains showed varying success rates [34, 33]. It has been established that strain difference has an effect on the efficiency of establishing mESC lines [35]. The reason behind this is still poorly understood. Although the 129 and C57BL/6J strains are commonly used, relatively few researchers used the ICR or CBA/ca strain to develop ESC lines.

Most studies ignore the blastocyst stage as a factor when deriving ESC [35, 33]. Movassagh-Pour *et al*. [36] found that the efficient of deriving ESC from hatched blastocysts were low. This study indicates that blastocyst stage may be a crucial factor affecting ESCs derivation.

mESC could be obtained by culturing the whole blastocyst, manual cut or laser dissection technique on a suitable culture medium with MEF as feeder cell layer. Besides that, other methods of deriving ESC include microdissection of blastocysts [34], isolation of the ICM through micromanipulation and culturing of single blastomeres [37]. The cells could then be used for subsequent steps in research, or cryopreserved for future usage.

Culture of ICM of murine blastocysts was carried out for production of mESC lines and characterised with marker expressed as alkaline phosphatase, Oct 4, SSEA 1, SSES 3, TRA-1-60 and TRA-1-81.

In the present study, we evaluated the effects of murine strain (ICR, CBA/ca and C57BL/6J), blastocyst stage (early-, mid-, expanded-, hatching- and hatched blastocyst) and ICM isolation technique (whole blastocyst culture, manual cut, laser dissection) on efficiency of mESC culture.

MATERIALS AND METHODS

Preparation of feeder cell

The feeder cells (MEF) were prepared from 13.5-14.0 d.p.c. murine foetuses. The pups were processed by removing the head, tail, limbs and internal organs, then transferred to a fresh PBS(-) solution for washing and minced it by using a sterile blade in small amount of trypsin/EDTA (0.25%) solution. Mincing was done for a few

more minutes to further reduce the size of the pieces. The large pieces were filtered out through a sheet of sterilised nylon and then centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant was removed and the pellet was re-suspended in around 2 to 4 ml of 3x MEF culture media. The cells were then seeded into the culture dishes coated with 0.1% gelatine with culture medium DMEM (Invitrogen) supplemented with 10% foetal bovine serum (FBS; Hyclone), 200mM L-glutamine (Invitrogen) and 1x penicillin/streptomycin (PS) (Invitrogen). The MFF were inactivated with 5 μg/ml mitomycin C (Sigma) for 2-3 hr followed by a thorough wash before plating.

When the cells from the explants reached 80% confluence, they were harvested using trypsin/EDTA (0.25%) and then sub-cultured to P1 or P2. The MEF at P1 or P2 were cryopreserved using DMSO (20%) mixed in the tissue culture medium and stored in liquid nitrogen. The frozen cells were thawed and cultured up to 80% confluence to use ready used as feeder cells for mESC cultured.

Embryo collection

Three pure-strains of murine (ICR, CBA/ca and C57BL/6J) blastocysts were collected by *in vivo* oviduct (2-cell stage embryos) or uterine (blastocysts) flushing. Murine females (6-8 weeks old) were superovulated with an intraperitoneal (i.p.) injection of pregnant mare's serum gonadotrophin (PMSG; 10 IU) followed by an intraperitoneal injection of human chorionic gonadotrophin (hCG; 10 IU) at 46-48 hr later. Each female murine was then placed in a cage with a stud male and copulation plug was checked on the next morning (day 1 pregnancy).

For recovery 2-cell stage murine embryos, oviducts of superovulated murine females were flushed with Hepes Whitten's medium (HWM) using a flushing needle (32 G) connected to a syringe (1 ml). Collected embryos were washed 3 times in equilibrated Whitten's medium (WM) and cultured under mineral oil at 37.5ºC in CO_2 (5%) in humidified air for *in vitro* development until blastocyst stage prior to use for producing mESC. Murine blastocyst stage will be located in the uterus. By using a method described by Hogan *et al*. [38], blastocysts were flushed from the uterus between days 3.5-4.5 d.p.c.

Isolation of inner cell masses from blastocysts

Three different ICM isolation techniques were used in present study, namely whole blastocyst culture, mechanical dissection or manual cut (30 G needle) and laser dissection, and the cells consecutively cultured to obtain primary ICM outgrowths (**Figure 2**). In whole blastocyst culture, the efficiency of early-, mid-, expanded-, hatching- and hatched-blastocysts for deriving mESC were compared. The zona pellucida of each blastocyst stage was removed by using pronase (0.5%). Then, the whole blastocysts were plating on inactivated MEF in a humidified atmosphere of CO_2 (5%) in air at 37˚C for culturing. After 6-8 days later, the growing colonies were individually dissociated into clumps after treating with trypsin/EDTA (0.05%). The resultant small clumps containing approximately 20-50 cells were transferred to a new well with a fresh feeder cell and medium. The new colonies were inspected daily and sub-cultured at an interval of approximately 6-10 days according to their size and growth rate, and medium were changed on every other day.

For manual cut ICM isolation technique, the zona pellucida of blastocyst was removed similar as previously described. A single murine blastocyst was transferred onto a droplet of ESCs culture medium. A cut was performed between the ICM and trophectoderm (TE) on blastocyst by using 2 needles (30 G). The murine ICM was isolated after the cutting and transferred onto an inactivated feeder cell and finally was placed onto the feeder cell with sufficient gap in between them.

In laser dissection techniques, the zona pellucida of blastocyst was not removed. This technique was carried out on the micromanipulator system where the holding pipette (left side) and biopsy needle (right side) were used in manipulated blastocyst. The ICM was located at the 9 o'clock position. The blastocyst was held by holding pipette and the laser was shot along the way between the ICM cells and TE cells. After shooting, the biopsy needle was sucked on the other side of the blastocyst to remove the zona pellucida and TE from the ICM. The isolated murine ICM was cultured onto the inactivated feeder cells and the primary outgrowth of the culture was observed.

Isolation and passages of embryo-derived cell lines

The primary ICM outgrowths were sub-cultured using trypsinisation or mechanical dissection procedures by trypsin/EDTA (0.05%). After around 4-6 days of culture, the primary ICM outgrowths were picked and sub-cultured into new feeder cells. The ICM that were selected had characteristics included dome shape colony surrounded with primitive endoderm, homogeneous and higher nuclear to cytoplasm ratio (bigger nucleus). They had a slightly refracted and were located directly next to the outgrowths (**Fig. 1**). The suitable outgrowths were picked before differentiation occurred.

Alkaline phosphatase (AP) staining

For alkaline phosphatise (AP) staining, the mESC was fixed with paraformaldehyde (4%) for 30 min. After 30 min, they were washed with PBS(-) for 5 times. The AP was added to the substrate just to cover the cell and incubated for 15-30 min in dark environment. After 15-30 min, it was washed again with PBS(-) for 2 times. AP staining was carried out to determine the AP activities that found in mESC lines. The mESC were purplish and differentiated cells were colourless after staining (**Figure 4**).

Characterisation mESC by ESC protein markers

The mESC can be confirmed by immunofluorescent staining. They were fixed with paraformaldehyde (4%) for 30 min and the cell was washed with PBS(-) for 5 times and blocking solution (10% FBS+PBS(-)) was added and leave it for 2 hr at room temperature. The primary antibody (such as Oct 4, SSEA 1, SSEA 3, SSEA 4, TRA-1-80 and TRA-1-60) was prepared at the optimal concentration with the ratio (1:250). After 2 hr of blocking solution, the blocking solution was removed and the diluted primary antibody was added and incubated overnight at 4°C. After that, the primary antibody was removed out and washed 5 times with PBS(-). The washing step took 10-15 min/time. Next, secondary antibody (diluted in ratio 1:1000) was added just to cover the cell and incubated 2 hr in dark environment. After 2 hr, the secondary antibody was discarded out and washed again 5 times with PBS(-). Lastly, Hoechst 33342 (5 μg/ml) was added and incubated 5 min in dark condition. The staining cell was observed under fluorescent microscope.

Statistical analysis

Data were analysed by analysis of variance (ANOVA) and Duncan's multiple range tests (D-MRT), using the SPSS statistical software package version 16. A probability of $P<0.05$ was considered significant for all statistical tests. Values were presented as mean±SEM.

RESULTS AND DISCUSSION

A total of 864 and 212 murine were used *in vivo* oviduct flushing and in *vivo* uterine flushing with representative numbers of 297 and 121 (ICR), 278 and 37 (CBA/ca), 289 and 54 (C57BL/6J), respectively. After *in vitro* culture, a total of 6324 (84.36%) murine blastocysts were obtained from 7544 two-cell stage murine embryos through *in vivo* oviduct flushing. Out of 6324 blastocysts obtained, 2589 (86.39%), 1988 (84.26%) and 1747 (81.84%) blastocysts were obtained from the ICR, CBA/ca and C57BL/6J murine pure-strains, respectively.

The percent successful attachment of blastocysts and consecutive passages of mESC lines up to P3 derived from 3 pure-strains of murine (ICR, CBA/ca and C57BL/6J) are summarised in **Table 1** and **Figure 1**. There were no significant differences ($P>0.05$) among the 3 pure-strains of murine in percent successful consecutive passages mESC lines up to P3. However, CBA/ca gave the highest percent attachment of blastocysts and successful consecutive passages mESC lines with the values of 62.68% (attachment rate), 41.32% (P1), 31.00% (P2) and 19.81% (P3), respectively. ESC derived from different strains of murine generally have different success rates [35, 33]. Our results have shown that there were significant differences between CBA/ca and ICR with C57BL/6J, whereby mESC derived from CBA/ca strain have higher success rate of passaging up to P3 compared to ICR and C57BL/6J. Therefore, it is suggested that CBA/ca strain may be more suitable for deriving mESC.

Table 1: Percent successful attachment of blastocysts and consecutive passages of mESC (%, mean±SEM) up to P3 from 3 pure-strains of murine.

Strain	No. of females	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
				P1 (n)	P2 (n)	P3 (n)
ICR	63	971	56.74±3.18^{d,x} (n=777)	35.20±2.61^{c,x} (n=443)	23.77±2.48^{b,x} (n=207)	9.00±1.65^{a,x} (n=71)
CBA/ca	34	758	62.68±20.90^{b,x} (n=429)	41.32±3.49^{ab,x} (n=237)	31.00±3.56^{ab,x} (n=109)	19.81±3.51^{a,y} (n=38)
C57BL/6J	39	709	44.68±2.53^{c,x} (n=359)	40.71±4.49^{c,x} (n=160)	22.10±3.75^{a,x} (n=64)	8.50±2.59^{a,x} (n=22)
Total	136	2438	55.18±5.55	38.15±1.93	25.18±1.80	11.62±1.39

^{xy}Mean values within a column within a group with different superscripts was not significantly different (P>0.05).

^{abcd}Mean values within a row within a group with different superscripts were significantly different (P<0.05).

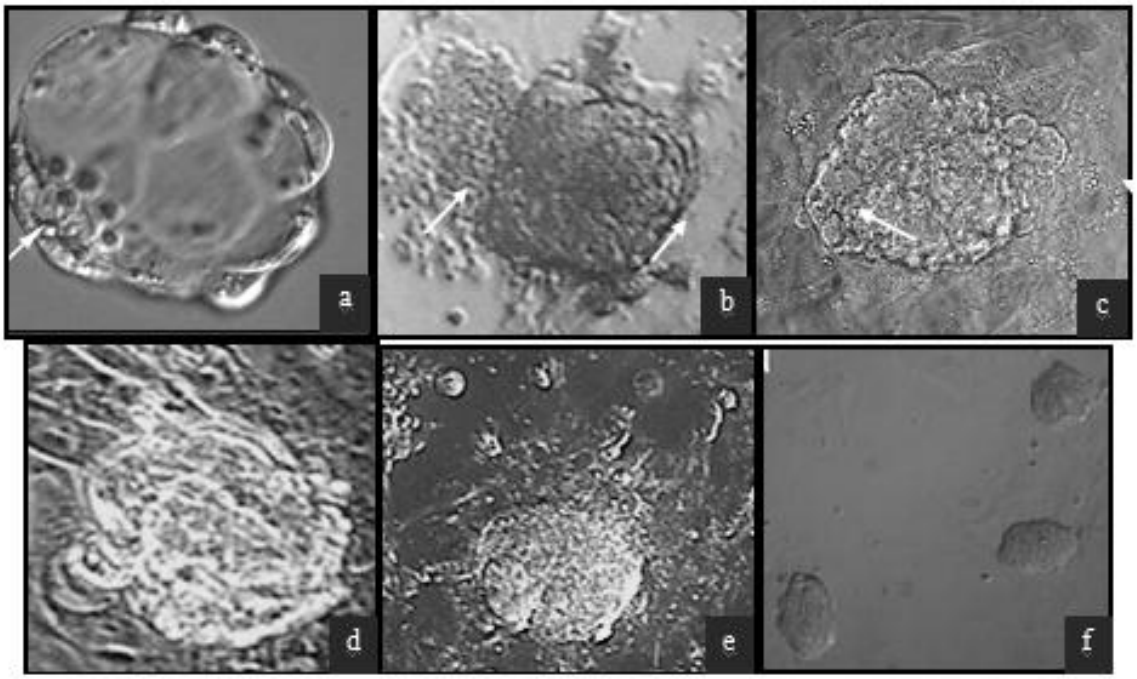

Figure 1: a) Blastocyst without the zona pellucida. b) Attachment and primary ICM outgrowth ICM at day3. c) Primary ICM outgrowth was sub-cultured by 0.05% trypsin/EDTA (P1). d) P2 of mESC was sub-cultured by manual dissociation before differentiation occurred. e) Undifferentiated mESC at P3 with the sharp and clear edge as well as dome-shape. f) ESC colonies obtained. Arrow: ICM.

Previously, it was found that blastocysts from C57BL/6J and CBA/ca have an efficiency of deriving ESC of 58% and 66% [39], which was considerably higher than the findings of this study. One possible factor is the use of ESC medium conditioned with rabbit fibroblast cell line which may have increased the amount of LIF and other support factors in the culture medium, thus improving efficiency [39] in their studies. However, it is noteworthy to mention that only 12 blastocysts were used for each strain in their reports; whereas in the present study 758 and 709 blastocysts from CBA/ca and C57BL/6J were used. No data were found on the performance of blastocysts derived from ICR strain. The overall low efficiency in deriving

mESC (11.62%) compared to other researchers may be attributed to the use of 35 mm culture dishes instead of multi-well plates which provide greater contact between the blastocysts and feeder cells [40, 33].

Percent successful attachment of blastocysts and consecutive passages of mESC lines up to P3 from 5 blastocyst stages (early-, mid-, expanded-, hatching- and hatched blastocyst) are summarised in **Table 2**. There were no significant differences (P>0.05) in percent attachment for early-, mid-, expanded-, hatching- and hatched blastocyst with the values of 65.17%, 48.08%, 68.78%, 57.01% and 37.45%, respectively. Although each blastocyst stage could passage up to P3 in producing mESC lines, expanded blastocyst stage showed the highest percent successful attachment of blastocysts and successful consecutive passages compared to other blastocyst stages with the values of 68.78% (attachment rate), 52.79% (P1), 37.55% (P2) and 17.24% (P3), respectively. The low success rate of deriving mESC from hatched blastocysts is in agreement with the findings of Movaassagh-Pour *et al*. [36]. Up to now, no reports were found comparing the 5 different blastocyst stages in murine species on the mESC culture. The reason for this phenomenon is not known as this time. A possible explanation could be the biochemical and morphological events taking place during the development of blastocysts from early to hatched stage. In early blastocysts, the ICM and TE have not fully separated. Thus, when cultured on the feeder cell, the consecutive outgrowth would have a high mixture of both ICM and TE derived cells. The interaction among the cells possibly makes the mESC more likely to differentiate. Meanwhile, hatched blastocysts have ICM that were already beginning to differentiate into the epiblast. This is supported by the fact that Oct4 expression in blastocysts decreases sharply at 4.5 d.p.c. [41]. Oct 4 expression is crucial in maintaining the pluripotency and undifferentiated state in ESC [42]. Thus, ESC derived from them may have a higher tendency to differentiate. Therefore, hatched blastocysts were not suitable for deriving mESC.

Table 2: Percent successful attachment of blastocysts and consecutive passages of mESC lines up to P3 (mean±SEM) from 5 blastocyst stages.

Blastocyst stage	No. of females	No. of blastocysts	Percent attachment (n)	Percent successful consecutive passages (n)		
				P1	P2	P3
Early	82	256	$65.17\pm24.88^{b,x}$ (n=198)	$27.88\pm3.79^{ab,x}$ (n=105)	$20.28\pm3.70^{a,xy}$ (n=46)	$9.18\pm2.98^{a,x}$ (n=14)
Mid	115	488	$48.08\pm4.00^{c,x}$ (n=278)	$45.35\pm5.60^{c,y}$ (n=164)	$28.11\pm4.29^{b,yz}$ (n=81)	$15.16\pm3.47^{a,x}$ (n=30)
Expanded	129	666	$68.78\pm3.38^{d,x}$ (n=465)	$52.79\pm3.92^{c,y}$ (n=262)	$37.55\pm4.36^{b,z}$ (n=138)	$17.24\pm3.67^{a,x}$ (n=55)
Hatching	122	682	$57.01\pm3.65^{d,x}$ (n=418)	$43.01\pm3.67^{c,y}$ (n=224)	$28.02\pm3.81^{b,yz}$ (n=89)	$11.60\pm2.95^{a,x}$ (n=25)
Hatched	97	346	$37.45\pm4.58^{c,x}$ (n=206)	$24.53\pm3.53^{b,x}$ (n=85)	$11.92\pm3.26^{a,x}$ (n=26)	$8.08\pm2.94^{a,x}$ (n=7)
Total	545	2438	5.44±5.48	38.71±1.94	25.18±1.80	2.27±1.44

^{xyz}Mean values within a column within a group with different superscripts were significantly different (P<0.05).
^{abcd}Mean values within a row within a group with different superscripts were significantly different (P<0.05).

A significant finding in this experiment is that expanded blastocysts are better sources of mESC lines. The use of expanded blastocysts could be applied in future experiment involving ESC to improve efficiency, rather than ignoring the blastocyst stages when deriving ESC lines [33, 34, 35, 43].

A total of 6831 murine blastocysts were used in this experiment consisting of 2438, 2062 and 2331 for whole blastocyst culture, manual cut and laser dissection techniques, respectively. There were significant differences (P<0.05) in percent attachment of blastocysts for 3 different ICM isolation techniques with the values of 66.52%, 78.03% and 71.06% for whole blastocyst culture, manual cut and laser dissection techniques, respectively (**Table 3**; **Figure 2**). Manual cut ICM isolation technique gave the highest percent primary ICM outgrowth

with the values of 78.35%, followed by laser dissection (75.67%) and whole blastocyst culture (75.32%). There was a significant decrease in percent successful consecutive passages from P1, P1, P2 and P3 for 3 different ICM isolation techniques.

Table 3: Percent attachment of blastocysts, primary outgrowth inner cell mass and successful consecutive passages of mESC lines (%, mean±SEM) on 3 different inner cell mass isolation techniques

Isolation techniques	No. of females	No. of blastocysts	Percent attachment (n)	Percent primary outgrowth (n)	Percent successful consecutive passages (n)		
					P1 (n)	P2 (n)	P3 (n)
Whole blastocyst culture	241	2438	$66.52\pm2.18^{c,x}$ (n=1565)	$75.32\pm1.73^{d,xy}$ (n=1167)	$72.51\pm2.25^{cd,y}$ (n=841)	$41.62\pm3.23^{b,x}$ (n=380)	$25.77\pm3.34^{a,x}$ (n=131)
Manual cut	494	2062	$78.03\pm0.94^{d,z}$ (n=1624)	$78.35\pm1.24^{d,y}$ (n=1295)	$67.84\pm1.60^{c,y}$ (n=906)	$52.06\pm1.94^{b,y}$ (n=545)	$36.52\pm2.45^{a,y}$ (n=255)
Laser dissection	357	2331	$71.06\pm1.11^{d,y}$ (n=1690)	$75.67\pm0.85^{d,x}$ (n=1275)	$58.75\pm2.25^{c,x}$ (n=843)	$45.06\pm2.35^{b,x}$ (n=457)	$30.49\pm2.52^{a,xy}$ (n=194)
Total	1092	6831	72.97±0.79	75.67±0.85	65.70±1.19	47.23±1.39	31.96±1.57

[xyz] Mean values within a column within a group with different superscripts were significantly different (P<0.05).
[abcd] Mean values within a row within a group with different superscripts were significantly different (P<0.05)

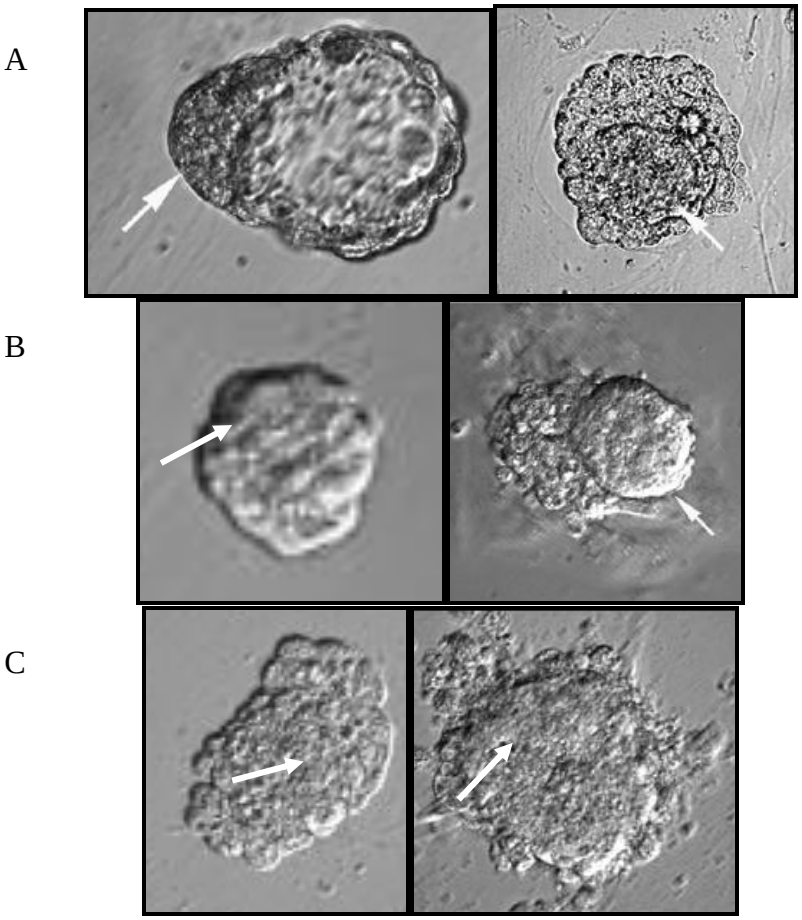

Figure 2: A) Whole blastocyst culture, B) manual cut ICM and C) laser dissection ICM isolation techniques with theirs ICM outgrowth shown by arrow.

ICM isolation technique is an important step to ensure the success of the ESC establishment. In present study, the whole blastocyst culture gave the lowest attachment, primary ICM outgrowth and successful passages up to P3 rates compared to manual cut and laser dissection ICM isolation techniques. This may be due to intact embryos, where trophoblastic cells induce ICM differentiation to be 3 embryonic germ layers by suppressing Oct 4 and Nanog expression level [44]. Therefore, completely removed trophoblastic cells provide more benefit to ICM cells turn to be ESC. The ICM could be separated and isolated from the TE by 5 ways, namely immunosurgery [45], with mechanical processes [46], with whole embryo culture of the blastocysts and partial embryo culture methods [47] or single blastomeres [48] and laser dissection [49].

Mechanical isolation of the ICM has previously been successfully used in the derivation of 2 cell lines, as reported by Genbacev *et al*. [50], Mummery [51] and van de Stolpe *et al*. [52]. Manual cut ICM isolation technique has been proven to improve results (Chanchao Lorthongpanich, personal communication). However, the difficulty of the manual cut procedure limits the rate at which blastocysts can be dissected. Furthermore, some ICM may become damaged due to the use of needles to pull the inner cell mass away from the overlying trophectoderm. The embryo tended to rotate away from the 30 G needle when it was cut towards one pole. The choice of well expanded blastocysts enabled trophoblast to be obtained with little risk of inclusion of ICM. Ideally, more training and practice should be done to master manual cut ICM isolation technique to allow comparisons in terms of efficiency.

Laser dissection method are used in the present study was to evaluate a new method of isolation ICM and derivation of ESC lines in a murine. Laser technology is commonly used for assisted hatching with some applications. One of them consists of making the embryonic membrane weaker to make the exit of the future blastocyst easier and so favour the derivation [53]. Until now, this new laser dissection method has been suggested to derive stem cell lines by Wang *et al*. [54], although without presenting any conclusive results. A recent paper, Tanaka *et al*. [55] reported preliminary results for a murine model using this method; subsequent culture of ESC in a serum/cell-free culture system was achieved. We have compared the ICM isolation method using laser dissection in a murine blastocyst with one of the most commonly used methods, the whole blastocyst culture [47]. However, with good quality blastocysts with a large and distinct ICM, we used the laser drill (Hamilton, USA). In our experiment, the murine blastocyst to be treated with laser shot was positioned at the centre of the field of view under 40x magnification. Blastocysts can be secured by 2 holding pipettes with the ICM positioned at '9 o'clock' if desired [55]. After focusing on the TE cells, the object had to be moved so that the part of the TE to be treated was located at the cross-hair position displayed on the monitor as the impact location of the laser focus. Thus, this new mechanical method destroyed the TE cells by shooting the laser over them carefully without damaging the ICM. Although some reported that the whole blastocyst culture method of the blastocysts is more effective than the laser method (70% versus 52.4%) [56], we have used the laser drill for the good quality blastocysts and that the concealment of the ICM by the TE cells is the only disadvantage of the whole blastocyst culture method [46]. Therefore, we must continue to improve the laser dissection technique so that the TE cells are destroyed and do not interfere with ICM [55].

ESC protein markers were used in determining the ESCs formation. Specific ESC markers were found in mESC such as Oct 4 (green) and SSEA 1(green) in this experiment (**Figure 3**). There were no colours shown for TRA-1-60 and TRA-1-81 ESC markers in murine species. Therefore, it indicates the presence of ESC in murine species. Also, purplish colour was shown in mESC after underwent AP staining. It could be confirmed that the presence of true mESC and pluripotent of mESC have been found. Five widely adopted antibody markers (anti-Oct 4, anti-Sox 2, anti-SSEA 1, 3 and 4, anti-TRA-1-60 and anti-TRA-1-81) are the protein markers that usually found on ESC. Transcription factors (such as Oct 3/4 [57, 58], Sox 2 [59] and Nanog [60, 61]) function to identify pluripotency in both early embryos and ESC. Oct 4 and Sox 2 are transcription factors that are highly expressed in undifferentiated ESC and EGC [62, 63, 64]. In our study, mESC express high levels of membrane alkaline phosphatase (AP) and Oct 4, a transcriptional factor critical to ICM and germline formation. Expression stage-specific embryonic antigen (SSEA 1) only appeared on mESC

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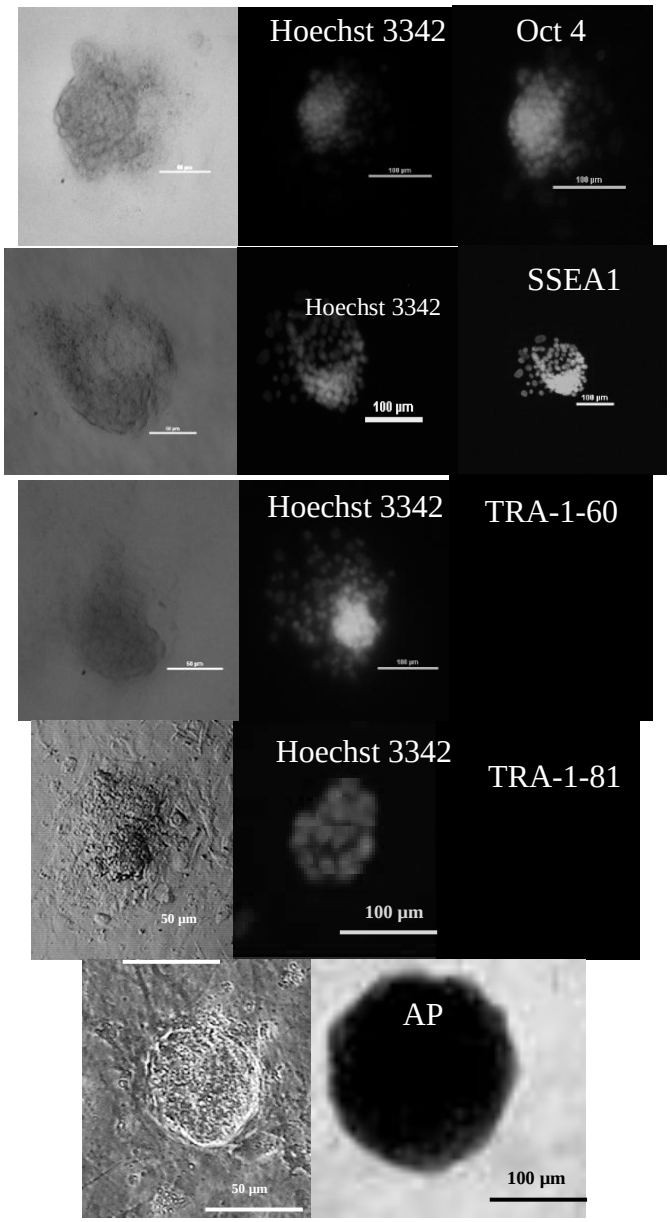

Figure 3: mESC were confirmed by the expression of murine specific ESC markers (Oct4, SSEA1) and human ESC specific markers (TRA-1-60 and TRA-1-81) as negative control. Transmission light images and Hoechst DNA staining were showed in the first and second column. The AP activities were positive and showed in the bottom line of the picture.

For future studies, ESC could be directed towards differentiation pathways by changing growth conditions, leading to development of specialised cells such as heart muscle cells, neurons or insulin-secreting cells, opening the way to the development of new therapies in human regeneration medicine.

CONCLUSION

In conclusion, protocols for the derivation of MEF cell culture and establishment of mESC lines were successfully developed that may serve as the platform for future scientific research in ESC. Three murine strains could produce mESC lines. However, the CBA/ca strain shows

satisfactory rate of consecutive passages mESC lines than ICR and C57BL/6J strains. With the establishment of ESC lines in murine by different stages of blastocyst and ICM isolation techniques, expanded blastocysts gave better growth rate of mESC lines then other stages. Manual cut gave highly significant successful rate in producing mESC lines compared the other 2 techniques. mESC lines were confirmed where it was found that Oct 4 and SSEA 1 protein markers as well as gave positive results in AP staining.

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