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From Microsurgery to Single Blastomere Biopsy for ES cell Establishment

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Review Article

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Abstract

Inner cell mass (ICM) is an important source of embryonic stem (ES) cells. There are several methods that have been developed to increase the efficiency of ICM cell isolation as well as improve the ES cells derivation rate. In conventional ICM isolation methods, the methods currently in use destroy trophectoderm cells of blastocyst stage embryos in order to release the ICM cell clumps. These conventional methods are considered embryo destruction methods as the embryos will be unable to survive after ICM is removed. Recently, an alternative method was reported using single biopsied blastomeres of earlier stage embryos as a source of ES cells. This novel method provides a new, ethically positive option that avoids destroying the embryos. This brief review of the techniques involved in ICM isolation and ES cells establishment, along with methodological comparisons, outlines the development of each technique, which may be used as a resource for choosing a suitable procedure for future experiments.

Keywords: blastocyst, blastomere, embryonic stem cell, immunosurgery, inner cell mass, microsurgery

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บทคัดย่อ

จากเทคนิคจุลศัลยกรรมสู่เทคนิคการใช้เซลล์ตัวอ่อนเพียงหนึ่งเซลล์สำหรับการผลิตเซลล์ต้นกำเนิดตัวอ่อน

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อินเนอร์เซลล์แมส (ICM) เป็นแหล่งของเซลล์ที่สำคัญที่สุดในการผลิตเซลล์ต้นกำเนิดตัวอ่อน การสกัดเซลล์ ICM ออกจากตัวอ่อนสามารถทำได้หลายวิธี จวบจนปัจจุบันหลายวิธีได้ถูกพัฒนาเพื่อให้ได้เซลล์ ICM ที่มีประสิทธิภาพดีและสามารถเพิ่มอัตราความสำเร็จในการชักนำให้เป็นเซลล์ต้นกำเนิดตัวอ่อนได้ ทั้งนี้วิธีสกัดเซลล์ ICM แบบดั้งเดิมเป็นเทคนิคที่ต้องทำลายตัวอ่อน เนื่องจากตัวอ่อนจะไม่สามารถพัฒนาต่อไปได้เมื่อ ICM ถูกสกัดออกไป แต่ล่าสุดมีการพัฒนาเทคนิคใหม่ขึ้นมาเป็นอีกหนึ่งทางเลือกที่จะทำให้ผลิตเซลล์ต้นกำเนิดตัวอ่อนได้โดยไม่ต้องทำลายตัวอ่อน เนื่องจากจะใช้เซลล์ตัวอ่อนที่ระยะต้นๆ ของการพัฒนาเพียงหนึ่งเซลล์มาใช้เป็นแหล่งของเซลล์ต้นกำเนิดตัวอ่อนแทนการใช้ ICM จากตัวอ่อน ระยะบลาสโตซีส เทคนิคการผลิตเซลล์ต้นกำเนิดตัวอ่อนจากเซลล์ตัวอ่อนระยะต้นเพียงหนึ่งเซลล์นี้สามารถลดข้อจำกัดทางจริยธรรมลงได้ เนื่องจากตัวอ่อนที่ถูกสกัดเซลล์ออกมาเพียงหนึ่งเซลล์จะยังสามารถพัฒนาต่อไปได้เช่นเดียวกับตัวอ่อนปกติ ในเอกสารฉบับนี้ได้รวบรวมเทคนิคต่างๆ ที่เกี่ยวข้องกับการสกัดเซลล์ ICM และผลิตเซลล์ต้นกำเนิดตัวอ่อน นอกจากนี้ยังมีข้อมูลสำหรับเปรียบเทียบแต่ละเทคนิค รวมถึงการพัฒนาเทคนิคแต่ละวิธีจากอดีตจนถึงปัจจุบันเพื่อจะได้เป็นประโยชน์ ในการวางแผนการทดลองของผู้ที่สนใจต่อไปในอนาคต

คำสำคัญ: บลาสโตซีส บลาสโตเมียร์ เซลล์ต้นกำเนิดตัวอ่อน immunosurgery อินเนอร์เซลล์แมส microsurgery

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Introduction

The blastocyst contains trophectodermal (TE) and inner cell mass (ICM) cells. TE cells develop into the placenta after the blastocyst implants to the endometrium layer of a mother's uterus. ICM cells develop into three embryonic germ layers and a yolk sac. The three embryonic germ layers eventually incorporate into a fetus. In other words, the ICM is differentiated into the three embryonic germ layers of the fetus. Therefore, the ICM, which can only be observed when the embryo develops to the blastocyst stage, is the only source for establishing an ES cell. High quality blastocysts with distinct ICM cells are the most appropriate for ES cell establishment. In general, one blastocyst contains only one ICM. If the ICM is removed to establish an ES cell line then the blastocyst has no further chance to implant and develop into a fetus. Since 1981, there have been several methods of ICM cells isolation that have been developed, but among those techniques immunosurgery seem to be the most favored technique among researchers because it is the most effective and the simplest method to isolate the clear ICM cells (Solter and Knowles, 1975; Martin,1981; Piedrahita et al., 1990; Chen et al., 1999; Anderson et al., 1994; Thomson et al., 1995; Moore and Piedrahita, 1997; Thomson et al., 1998; Reubinoff et al., 2000; Suemori et al., 2001; Vrana et al., 2003; Cowan et al., 2004; Heins et al., 2004; Brevini et al., 2005; Ock et al., 2005; Mateizel et al., 2006; Mitalipov et al., 2006; Shiue et al., 2006). This could explain why almost all ES cell lines have been established from the ICM cells of blastocyst embryos since then. Unfortunately, immunosurgery required animal products in the process, such as anti-sera and guinea pig complement, which adds a risk of pathogen contamination, especially when using this technique to establish human ES cell lines. Recently, a novel method for ES cell establishment has been developed by using single blastomeres of early stage embryos as a source of ES cells (Chung et al., 2006). It has been introduced as another method of choice to address ethical issues, especially those of embryo destruction. Additionally, it could be used as a method for establishing the patient specific ES cells. This review will summarize the methods involved in ICM cells isolation including

the techniques that destroy embryos (conventional techniques) and those that do not (novel methods). However, each procedure has specific characteristics that need to be considered before their use with your embryos.

1. Conventional methods

Since 1972, ICMs have been isolated by mechanical techniques (Gardner, 1972). Several methods have since been established, such as immunosurgery, calcium ionophore (A23187), whole or partial blastocyst culture, laser dissection and digestion methods. Each method has been used in ICM isolation for ES cell establishment. However, these methods are considered embryo destruction techniques, which clearly raise ethical issues. Yet some of these techniques such as immunosurgery and whole or partial embryo culture have widely still been in use.

1.1. Microsurgery

This technique was first reported by Gardner (1972) for TE and ICM cells separation in his investigation of the function of TE and ICM cells of mouse blastocysts (Gardner, 1972). The separation was performed by micromanipulator. The blastocysts were placed in a drop of medium hanging from the coverslip of a chamber filled with heavy liquid paraffin. Then, the blastocysts were held by suction pipette against the underside of the coverslip of the hanging drop. A piece of micro-blade was attached and arranged vertically on one manipulator unit, so that the micro-blade surface was parallel with the coverslip of the chamber. Then the micro-blade was slowly moved parallel to the ICM cells surface to cut the TE cells (Gardner, 1972; Rossant, 1975). This method was later modified for isolating ICM cells from blastocysts and used as a source of ES cells. The manipulation technique was modified by using only two fine needles for ICM dissection under stereomicroscope which was much more practical and easier to perform. It worked very well in several *in vivo* produced embryos such as cat (Yu et al., 2008) and a human embryo (Kim et al., 2005) because the *in vivo* produced embryos had more distinct ICM cells than the embryos produced *in vitro*. Later in 2007, Strom and colleagues reported a new mechanical technique for ICM isolation by using a specially made flexible tungsten needle, with a diameter of 0.125 mm. The tip was thin and sharpened using electrolysis. Another blunter needle was used to hold the blastocyst during the cutting out of the ICM. Both needles were fixed to hand-pieces of pencil-thickness for manual operation under stereomicroscope. The blastocyst was moved to the operation drop then drawn out with the needle so that the blastocyst became attached to the surface of the well. With the blastocyst attached to the plastic, it was possible to make a hole in the zone pellucida with the needle to open up the blastocyst and by making two to three cuts, to remove the ICM from the trophectoderm. The procedure took about two to three minutes per embryo (Strom et al., 2007).

1.2. Immunosurgery

Immunosurgery can be used to obtained large quantities of pure ICM masses in relatively short periods of time (Solter and Knowles, 1975). It relies on the compatibility of antibody to the cell surface antigen of trophoblastic cells of the blastocyst stage embryo. This compatibility causes a selective killing of trophoblastic cells after the complement is added into the system. Briefly, the blastocysts are cultured with a proper concentration of antigen for about 30 minutes (Solter and Knowles, 1975). The antigen is usually derived from serum of rabbit anti spleenocytes of the target embryo's species. Then the blastocysts are subsequently washed several times to discard the unbound antibodies before incubation with a suitable concentration of complement for 15-30 minutes. During the incubation with complement, trophoblastic cells swell, and finally loose their semipermeability. Pilz and colleagues (1970) suggested that the mouse blastocyst was impermeable for molecules larger than 40A°. This makes the passage of an immunoglobulin molecule (antibody) with a larger diameter impossible. Therefore, to intensively wash out the unbound antibodies will secure ICM cells, because there will be no antibodies left over to bind with the antigen on the ICM cells surface after the TE cells are destroyed in the complement. To do so, only TE cells will be destroyed, thus leaving the ICM intact. One of the advantages of this method is that it allows the recovery of many ICMs without the risk of mechanical damage that might occur in a microsurgical method (Solter and Knowles, 1975). To assess the purity of ICM cells after immunosurgery, Handyside and Barton (2007) studied the failure to detect fluorescent-conjugate antibodies in IgGs or trophoblastic-type outgrowths *in vitro* and found that the dissected ICMs showed a negative result. Moreover, they found that the protein synthetic profile of these ICMs was similar to microsurgically dissected ICMs, and in particular, trophoblast specific spots were absent. Additionally, when the ICM cells were transferred to the uterus of the pseudopregnant mice for evaluation of the implantation capacity, they only found the ICM derived tissues. This finding demonstrates the lack of TE cell contamination and functional viability of these ICMs.

In 1981, Martin, G.R. established the first ES cell lines by using immunosurgery as a method for ICM cells dissection before culturing ICM cells in teratocarcinomas conditioned medium. Several days after culture, the ICMs showed remarkable resemblance to pluripotent morphologies as embryonal carcinoma (EC) stem cell lines. Martin named the pluripotent cell lines derived from ICM cells as embryonic stem (ES) cells to denote their origin directly from embryos and to separate them from EC cells which were derived from teratocarcinomas (Martin, 1981). After the success of the first mouse ES cell lines established by immunosurgery, several attempts in other animal species were reported such as pig (Piedrahita et al., 1990; Anderson et al., 1994; Moore and Piedrahita, 1997; Chen et al., 1999; Brevini et al., 2005; Ock et al.,

2005; Shiue et al., 2006), monkey (Thomson et al., 1995; Suemori et al., 2001; Vrana et al., 2003; Mitalipov et al., 2006), and human (Thomson et al., 1998; Reubinoff et al., 2000; Cowan et al., 2004; Heins et al., 2004; Mateizel et al., 2006). Currently, immunosurgery is a commonly used method for establishing ES cells from ICM of blastocyst stage embryo.

1.3. Calcium Ionophore A23187

Calcium ionophore A23187 is a monocarboxylic acid antibiotic, specifically a divalent cation. It increases intracellular ionized calcium (Reed and Lardy, 1972) and also includes mitogenic effects on lymphocytes (Freedman, Raff & Gomperts, 1975; Hesketh et al. 1977), inhibited morphological changes in cells induced by dibutyryl cyclic AMP (cAMP; Henneberry et al., 1975), releases histamine from most cells (Foreman et al., 1973), causes prevention of retinal orientation in developing eyes of Xenopus laevis (Rose and Loewenstein, 1975; Jacobson, 1976), and chemically activated unfertilized eggs such as mouse (Hagemann et al., 1994; Uranga et al., 1996), cat (Grabiec et al., 2007), pig (Wang et al., 1998) and bovine (Liu et al., 1998; Chung et al., 2001; Xu and Yang, 2001; Sedmikova et al., 2003). It also can be used as one of the activation reagents in cloned embryo development in several species such as bovine (Milazzotto et al., 2008), buffalo (Saikhun et al. 2003) and human (Heindryckx et al., 2007). For ICM isolation, calcium ionophore A23187 was accidentally found during the course of experiments to detect the mitogenic action of A23187 on blastocysts. Surani and colleagues (1978) found that $2x10^{-5}$ M calcium ionophore (A23187) caused selective lysis of trophectoderm cells and occurred after approximately 30 min following their swelling and vesiculation, but the ICM apparently remained intact (Surani et al., 1978). Using calcium ionophore A23187 to destroy TE cells is morphologically identical to the lysis of the cell observed after treatment of blastocysts with antibodies and complement similar to that used in the immunosurgery method. The ICM recovery rate after the late expanded blastocysts were treated with ionophore was 100%, but the recovery rate was lower when early stage blastocysts were used (75-82%; Surani et al., 1978). Calcium ionophore A23187 was used in some later reports for ICM isolation of rodent (Harlow and Quinn, 1979; 1980; Piedrahita et al., 1990) and ovine embryos (Piedrahita et al., 1990). The mechanism of TE cells lysis after being treated with calcium ionophore A23187 is still not clear. It can be due to an osmotic phenomenon which can be explained by either the suggestion that sodium (Na+) flows into the cells more quickly than the outward flow of potassium (K^+), that the influx of water then causes swelling and lysis of cells (Green et al., 1959) or the ionophore induces the uncontrolled flux of carbonic anhydrase II (Reed and Larde, 1972) followed by swelling and vesiculation after the entry of water into the cells. This method, however, is not generally used for ICM cell isolation today. This might be because of the uncontrollable osmotic action and mechanism of destroying the TE cells are not clear and, as mentioned above, the calcium ionophore A23187 can be used in several ways which might generate side effects or affect the success rate of ES cells establishment.

1.4. Whole Blatocyst or Partial Blastocyst Culture

The whole blastocyst and partial blastocyst culture methods seem to be the most advantageous to produce pathogen-free ES cell lines especially for human ES cells, because of the absence of animal products such as antibody and complement, which are used in immunosurgery methods. Whole blastocyst culture has been used for ES cell establishment since 1981 when Evan and Kaufman (1981) cultured the delayed blastocysts in culture drops to let the embryos attach to the feeder cells. After attachment, TE cells grew out and differentiated to giant cells, where as ICM cells formed into egg cylinder shapes. The ICM cells were picked off and dispersed by trypsin treatment and reseeded onto feeder cells. By using this technique, they established 15 pluripotent cell lines from an independent embryo (Evans and Kaufman, 1981). This method has been used in particularly with animals in therapeutic studies such as pig (Evans et al., 1990; Piedrahita et al., 1990; Hochereau-de-Reviers and Perreau, 1993; Gerfen and Wheeler, 1995; Miyoshi et al., 2000; Li et al., 2004[a]; Kim et al., 2007) and human embryos as well (Heins et al., 2004, Kim et al., 2005). The whole blastocyst culture method is much simpler than other methods described above because it does not need any special chemical reagent or high instrument platform. For this method, the zona pellucida can be removed from embryos by using any suitable method. The zona-free embryos are then plated onto feeder cells. A few days after plating, the attachment of the whole embryo to feeder layer can be seen. The TE cells collapsed and begin to expand, whereas ICM-like cells form a dome shape surrounded by differentiated TE cells. When the ICM clump looks big enough, a finely pulled pipette is used to picked off the ICM clump and transferred it to fresh feeder cells. The differentiated TE cells are left in the plate or discarded after ICM clumps are picked off. This simple procedure, however, runs a much greater risk of TE cell overgrowth than the other methods, because the entire TE cells are cultured along with the ICM clump for several days. The ICM cells are often covered with differentiated TE cells resulting in unclear observation of ES cell morphologies. The ICM might not grow properly, might degenerate or eventually differentiate (Li et al., 2003). To avoid these problem, partial blastocyst culture is used in some cases instead of whole blastocyst culture. The partial blastocyst culture protocol is similar to whole blastocyst culture, except part of the TE cells will be cut off by a mechanical technique such as fine needles or glass pipette. To do so, parts of the TE cells will be removed resulting in significant reduction of the risk of TE cells overgrowth, which tends to inhibit the growth of ICM cells (Lee et al., 2003; Kim et al., 2005). The zona intact blastocyst culture is another method of choice to establish ES cells. Kim and colleagues (2007) cultured zona-intact pig blastocyst stage embryos and compared them with zona-free blastocysts and ICMs (derived from immunosurgery). They found that the number of attached blastocysts of zona intact embryo

(27.8%; 15/54) was lower than zona-free embryo (42.4%; 36/85) and significantly lower than those from immunosurgery (68.5%; 87/127). The significant differences were also observed in the rates of primary colony formation of ICMs derived by immunosurgery (36.8%; 32/87), which significantly increased in the group of zona-free (19.4%; 7/36) while no primary colony formed in the zona-intact group (0%; 0/15). Even though, zona-intact embryo culture is one of the pathogen free techniques, it is not often used because this technique has resulted in lower success rates of ES cell establishment (Kim et al., 2007). This might be because of the difficulty of the hatching process, which is a problem often found in *in vitro* cultured embryos.

1.5 Laser Dissection

Laser applications have been used in assisted reproductive technology (ART) for several years including assisted hatching (Obruca et al., 1994), embryo or polar body biopsy (Veiga et al., 1997; Montag et al., 2000), sperm immobilization (Montag et al., 2000), and ICSI (Rienzi et al., 2001), all of which resulted in significantly high fertilization and pregnancy rates of *in vitro* produced embryos (Obruca et al., 1994; Tanaka et al., 2006). Laser assisted ICM isolation was first used for mouse ICM cells isolation in 2006 (Cortes et al., 2006; Tanaka et al., 2006). Their results demonstrated that the laser dissection method had no influence on ICM attachment and ES cells derivation when compared with the zona-free whole blastocyst culture and ICMs derived immunosurgery, but was significantly higher than zona-intact blastocyst culture. In 2008, Turetsky and colleagues (2008) used the erbium-yttrium-aluminium-garnet (Er: YAG) laser to isolate ICM cells from human embryos (Turetsky et al., 2008). Eight ICMs were isolated from nine hatched blastocysts, which gave rise to three hES cell lines (37.5%; 3/8). This efficiency is similar to the reported after isolation of the ICM by immunosurgery (Thomson et al., 1998; Cowan et al., 2004; Ludwig et al., 2006; Mateizel et al., 2006). In general, laser assisted ICM isolation is performed as follows: The zona-intact blastocysts are fixed by two holding pipettes with the ICM positioned at either nine o'clock (Tanaka et al., 2006) or three o'clock (Turetsky et al., 2008). In mouse embryos, approximately 10 infrared laser pulses at 300 mW x 1 ms (ZILOS-tk™, Hamilton Thorne Research, Beverly, MA USA) are fired to split the blastocyst into two unequal portions the smaller consisting of ICM, the larger consisting exclusively of trophoblast; whereas in human embryos, 20-30 infrared laser pulses at 200 mW x 0.5 ms are used. However, special attention must be paid to direct the laser beam far enough from the ICM to prevent heating and damage of the ICM. Additionally, during the laser dissection experiment, there is a 40% possibility that some blastocysts will not undergo successful ICM isolation, because the TE cells collapse during the laser drill (Turetsky et al., 2008).

1.6 Digestive Method

The digestive method is also possible for ICM cells isolation (Li et al., 2003; 2004[b]). Currently, two reagents have been used for TE cells digestion, enzymatic (Trypsin/EDTA) and Acidic Tyrode's solution. The enzymatic method has been used with pig embryos to compare the efficiency of isolation among whole blastocyst culture, immunosurgery and the enzymatic method. For the digestive method, blastocyst stage embryos must be treated with pronase or acidic Tyrode's solution to remove the zona pellucida. Then, the zona-free embryos are submerged into a microdrop of 0.25% trypsin-0.04% EDTA solution for several minutes. During the treatment, embryos are observed under stereomicroscope for the dispersion of TE cells. When the TE begins to disperse, the blastocysts are transferred to another drop with culture medium, and ICM cells are isolated from the dispersed TE cells by the aid of two needles and a pulled mouth pipette. However, it should be noted that the whole embryo culture method has a lower attachment than ICM isolated by enzymatic method (Li et al., 2003). Eighty-five percent of the ICM cells are successfully recovered from blastocyst treated by the enzymatic method as opposed to 40% from immunosurgery (Li et al., 2004[b]). The ICMs obtained by the enzymatic method prove to be pluripotent and can be differentiated into other types of cells.

Acidic Tyrode's Solution is a chemical defined solution, which is widely used to remove all or some zona pellucida from embryos (Cowan et al., 2004; Ellerstrom et al., 2006). It is an effective medium and very quickly removes zona pellucida within a few seconds. Because of the chemically defined solution, acidic Tyrode's solution is much more suitable for a pathogen-free system than pronase which is a product of bacteria. Ellerstrom and colleagues (2006) used acidic Tyrode's solution for ICM isolation from human embryos. The blastocysts were incubated in acidic Tyrode's solution and carefully observed until zona pellucida and TE cells were eliminated. They suggested that 30-40 seconds were the optimum time to remove both zona pellucida and TE cells. However, this treatment could not be used to completely destroy TE cells without damaging the ICM cells. Therefore, the initial outgrowth from the treated blastocysts composed of a heterogenous cells population. However, areas of morphologically distinct ES cells appeared which could be picked off and transferred to fresh plates. Homogenously expressed Oct4 and morphologically resembled undifferentiated hESCs, with a small cytoplasm-to-nucleus ratio, could be found at around the fifth passage (Ellerstrom et al., 2006).

2. ES Cell Lines Derivation from Single Blastomeres

Since 1981, ES cells have been successfully established from several species including mice (Evan and Kaufman, 1981; Wakayama et al., 2007), monkeys (Thomson et al., 1995; Suemori et al., 2001), and humans (Baharvand et al., 2006; Heins et al., 2006). Although most of the currently available ES cell lines were derived from the ICM cells of a blastocyst stage embryo, it was noted that only a small number of blastomeres from eight-cell (Delhaise et al. 1996) and

16-cell (Eistetter, 1989) mouse embryos were viable for deriving ES cells. However, those resulted ES cells can still be considered established by an embryo destruction technique. Therefore, they are still faced with ethical issues, and equally important, immune-compatibility can not be expected from ES cell lines derived from ICM of blastocyst stage embryos. Chung and colleagues (2006) reported an alternative method of establishing ES cell lines using a technique of single blastomere biopsy, which is similar to the techniques used in pre-implantation genetic diagnosis (PGD) in IVF clinics. A single biopsied blastomere will not interfere with the developmental potential of the biopsied embryos (Chung et al., 2006). In PGD, the most common technique involves removing a single blastomere from the eight-cell embryo. The blastomere is analyzed to detect genetic abnormalities; the resultant seven-cell embryos that have no abnormalities are then transferred to the mother's uterus for implantation and pregnancy. After the novel report by Chung and colleagues (2006), there were several further attempts in mice, monkey and humans that have been reported (Klimanskaya et al., 2006; Wakayama et al., 2007; Teramura et al., 2007; Chung et al., 2008; Lorthongpanich et al., 2008[a,b]; Narita et al., 2008). However, there are also some new techniques discovered by those people besides culturing the single blastomere as described by Chung and colleagues (2006).

The following summarizes techniques relating to ES cells establishment from a single blastomere. The techniques will be grouped into three categories including (1) single blastomere co-cultured with ES supporting cells, (2) whole blastocyst derived from single blastomere outgrowth and (3) immunosurgery of blastocyst derived from single blastomere. The first technique, co-culturing a single blastomere with ES supporting cells, was reported in mouse (Chung et al., 2006) and human embryos (Klimanskaya et al., 2006; Chung et al., 2008). Single blastomeres were biopsied from eight-cell stage embryos and each separated blastomere was aggregated with a small clump (around 100 cells) of GFP-positive ES cells (ES supporting cells) in a 300μm depression created by pressing a needle into the bottom of a plastic tissue culture plate. After 24-48 hours of incubation in ES cell culture medium supplemented with 2,000 U/ml mouse leukemia inhibitory factor (LIF) and 50mM MEK1 inhibitor, a GFP negative bud was observed on the side of ES supporting cells. Then, the aggregates were transferred to feeder cells and ES culture medium until the GFP negative cells were large enough to subculture. The GFP-negative cells were separated from ES supporting cells by microcapillary under fluorescent microscope. The GFP-negative cells were then expanded and tested for ES cell markers. Not only mouse ES cell lines, but also extraembryonic (TE) stem cell lines could be established by single blastomere culture with ES supporting cells (Chung et al., 2006). The second method, whole blastocysts derived from single blastomere outgrowth, was reported a year later by Wakayama and colleagues (2007). The biopsied blastomeres were individually cultured in 96 well plates precoated with feeder cells in ES culture medium with 20% Knockout Serum Replacement (KSR) and 0.1 mg/ml adrenocortico-tropic hormone (ACTH; fragments 1–24) instead of fetal calf serum (FCS). During the time that the blastomeres were in culture, they could divide and develop into blastocysts. After 10 days or more, proliferation outgrowths were dissociated and replated to expand until stable ES cell lines grew out. Using this technique, they could produce several ES cell lines from single blastomeres derived from two-cell (50-69%), early four-cell (28-40%), late four-cell (22%), and eight-cell (14-16%) stage embryos (Wakayama et al., 2007). The last method is immunosurgery, where the blastocyst is derived from a single blastomere. Since the single blastomere derived blastocyst contains a small amount of ICM cells (Lorthongpanich et al., 2008[a]), there are not many attempts to use immunosurgery with a single blastomere derived embryo. However, there was a paper describing the successful use of immunosurgery with blastocysts derived from single blastomeres of two-cell stage embryos that contained prominent ICM cells (Teramura et al., 2007).

3. Current Situations of ES cell Established from Single Blastomere

Deriving embryonic stem cells from single blastomeres is an effective technique to overcome the ethical concerns for establishing ES cells, especially hES cells, because this is a non-destructive embryo technique. However, as it has only been in use for a few years, there still have many profound problems that need to be studied. Some of the more interesting problems are why, in the more advanced embryonic stage, the success rate of ES cell establishment is less; and how can the success rate of ES cell establishment be improved. In addressing those problems, we may be able to look at two major factors, which are blastomere fate determination and the culture system. We know that during the pre-implantation embryo development, two distinct segregated cell lineages are established late in the morula stage. The outer cells of the embryo will become TE cells and the inside cells will become ICM cells. Lineage segregation has also been studied in early embryos for almost 50 years. Currently, there are two active models that attempt to explain the segregation fate in early embryos. One posits that lineage is not predetermined until late morula stage, whereas another suggests that fate is already determined very early in the embryo development. Up to now, the early embryonic sister blastomeres differentiation competence is still one of the fundamental questions that has not been fully explained (Tarkowski et al., 1959; Takowski and Wroblewska, 1967; Rossant, 1976; Tsunoda and McLaren, 1983; Papaioannou et al., 1989; Chan et al., 2000; Piotrowska et al., 2001; Piotrowska-Nitsche et al., 2005). There have been several attempts at trying to produce a full set of offspring after transfering isolated blastomeres derived embryos and splitting embryos to surrogates. Unfortunately, not all blastomeres were able to develop to term (Rossant,

1976; Tarkowski et al., 1959; Tsunoda and McLaren, 1983; Papaioannou et al., 1989; Heyman et al., 1998; Chan et al., 2000; Mitalipov et al., 2002; Schramm and Paprocki, 2004). Even though the production of monozygotic twins by separating two-cell embryos has been achieved in some species (Willadsen, 1979; Willadsen et al., 1981; Willadsen et al., 1981; Ozil et al., 1982; Allen and Rashen, 1984; Tsunoda et al., 1984; Matsumoto et al., 1989; Papaioannou et al, 1989) only one report showed the pluripotency of four-cell blastomeres which produced quadruplets (four identical calves) (Johnson et al., 1995). It has also been found that although each two-cell stage blastomere gives rise to both the ICM and the TE lineages, one cell tends to contribute more to the embryonic part of the blastocyst, and the other cell contributes to the abembryonic part (Gardner, 2001; Piotrowska-Nitsche et al., 2001; Fujimori et al., 2003; Piotrowska-Nitsche and Zernicka-Goetz, 2005). Additionally, at the four-cell stage, Piotrowska-Nitsche and colleagues (2005) demonstrated that each blastomere had predictable fates. The allocation bias of a two- and four-cell blastomere progeny suggests the differential developmental competence of the sister blastomeres and their lineage fate. As reported by Chung and colleagues (2006), the blastomeres were biopsied from eight-cell stage embryos and were co-cultured with ES supporting cells. They could produce both ES cell lines and extraembryonic (trophoblastic) stem cells lines. These also suggest the bias of the blastomeres of eight-cell stage embryos. However, several reports have demonstrated the non-developmental bias in early stage blastomeres. Therefore, whether or not the lineage fate is committed at an early embryonic stage remains unclear. Another factor that might affect the success rate of ES cell establishment from a single blastomere is the culture system. This technique was originally developed by the culture of a single blastomere in conventional mouse ES medium supplemented with some protein inhibitor reagents and/or co-cultured with ES supporting cells. MAPK family is the common pathway involved in stem cell research because of its roles in regulating proliferation, differentiation and apoptosis (Binetruy et al., 2007). Some of the MAPK inhibitors have been used for the derivation of ES cells from single blastomeres such as MAP2K (I) and MAPK14 (I) (Chung et al., 2006; Lorthongpanich et al., 2008[b]). Although single blastomeres derived ES cell lines have been successfully established from MAP2K (I) supplementation and co-culturing with ES supporting cells, the role of MAP2K (I) and ES supporting cells have not yet been determined (Chung et al., 2006). MAPK14 (I) has been reported to inhibit TE cells differentiation of mouse morula stage embryo. This suggests the potential of enhancing ICM development by suppressing TE cells (Maekawa et al., 2005). MAPK14 (I) has been supplemented into culture medium for single blastomere culture and ES cell derivation. The results demonstrated that MAPK14 (I) could inhibit the development of blastomeres derived embryos and also inhibit TE cell differentiation which is similar to its action on an intact embryo. However, MAPK14 (I) could not enhance ICM development in single blastomeres derived embryos, but instead inhibited ICM formation (Lorthongpanich et al., 2008[b]). Adrenocorticotropic (ACTH) is a peptide hormone produced from the pituitary gland responsible for an important role in early development. ACTH has been found to enhance ES cell proliferation from ICM derived ES cell lines (Ogawa et al., 2004). Recent studies have further confirmed the role of ACTH in supporting the derivation of ES cells from single blastomeres (Wakayama et al., 2007; Lorthongpanich et al., 2008[b]; Narita et al., 2008). However, supplementation with ACTH is not enough to ensure that every blastomere derived from the same embryo become ES cells. The result of ACTH studies also suggested that blastomeres derived from higher stage embryos had lower success rates than those blastomeres derived from lower stage embryos (Wakayama et al., 2007).

Table 1 Comparison of each method for ES cells establishment from ICMs of blastocyst stage embryos

Method	Mechanism	Prominent point	Weak point
Microsurgery	Use micro blade or fine needles to cut TE cells	Simple method	Crude method, Takes time, Difficult to make ICM with clear TE cells, ICM might have losses during processing, Depending on the operator technical's skills
Immunosurgery	Specification of antibody against antigen on TE cells surface	Complete destruction of TE cells	Pathogen-contamination risk, ICM might have losses during processing
Calcium Ionophore (A23187)	Osmotic pressure change in TE cells causes cell lyses	Simple method	Uncontrollable of various effects of A23187
Whole or partial blastocyst culture	Remove ZP or partially cut TE cells and ready to plate onto feeder cells	Most simple method, Pathogen-free	TE cells overgrowth
Laser dissection	Laser drill to destroy TE cells under	Pathogen-free	Need Laser set and operator's micromanipulator skill, Takes time, Heat from laser beam
Digestive method	Use Trypsin/EDTA or Acidic Tyrode's solution to digest TE cells	Simple method	Pathogen-contamination risk (Pronase), ICMs might be hurt from digestion process

Conclusion

ICM isolation is an early step that determines whether ES cells are considered pathogen free. When generating ES cell lines, there are some criteria that need to be considered before choosing one of the methods for ICM cells isolation. First, we need to consider whether the ES cells need to be xeno-free, then, what quality of embryo is needed, and finally, if your laboratory has a manipulator and a skillful operator. As shown in Figure 1, when using the conventional method, one must first decide whether the ES cells need to be xeno-free. If they do, then the embryos need to be separated into (at least) two groups, good and poor quality. The poor quality embryos with unclear and small ICM cells should use zona-free whole blastocyst culture otherwise the tiny ICM cells may be lost during processing in subsequent complicated procedures. For good blastocysts, there are many options depending on your manipulator's skills. For skillful people, laser dissection or mechanical isolation with micro blade or with ultra fine pulled glass needles could be a method of choice. On the other hand, partially cutting off the TE cells with fine needles, calcium ionophore (A23187) or Trypsin digestion methods can be used by a person with lesser skills. However, if the ES cells do not need to be xeno-free, you can also use immunosurgery for ICM cells isolation from a good blastocyst. Even though there are several methods that can be used at present, it is unclear which approach is more efficient. Therefore, the most suitable method needs to be considered if it fits the skills of the operator (Table 1 and Table 2). A novel method, single blastomere derived ES cells, opens a new era for ES cell establishment with less ethical problems. However, since this technique is new, there are many questions such as blastomeres fate, efficiency of each blastomeres from different embryonic stages to derive ES cells and a suitable culture system that need to be clarified. At present, there are several laboratories attempting to overcome these obstacles and developing techniques to derive ES cells that can reach maturity from single blastomeres (Green et al., 2009; Roberts et al., 2010).

Figure 1 Diagram of ICM isolation method consideration

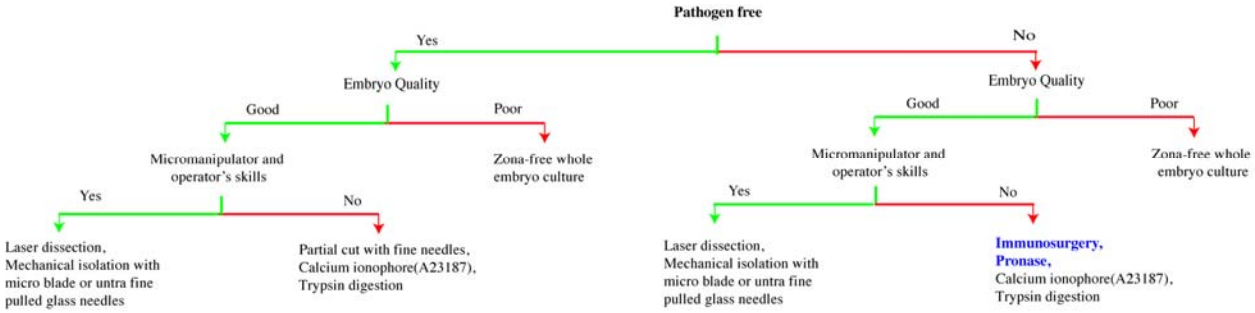

Table 2 Comparison of each method for ES cells establishment from single blastomeres co-culture and non co-culture with ES supporting cells

Method	Mechanism	Prominent point	Weak point
Co-culture	Single blastomere co-culturing with ES supporting cells for several days	Increase chance success	Unclear mechanism of ES supporting cells, Contamination risk from supporting cells, Unequal competence of each blastomere
Non-co-culture	Single blastomeres are cultured individually to blastocysts and outgrowths	Xeno-contamination free	Unequal competence of each blastomere

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