Development of Human Embryonic Stem Cell Derivation

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Objective: *To establish human embryonic stem (hES) cells from human embryos.*
Design: *Experimental study.*
Setting: *Reproductive Medicine Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University.*
Material and Method: *Abnormal and normal fertilization embryos were cultured in vitro until reaching blastocyst stage. Four different methods for isolation of ICMs were used. Immunosurgery, mechanical isolation, laser assists, and whole blastocyst culture were performed. The feeder layers used in the present study were fibroblasts, isolated from either mouse or human. Mechanical splitting of ICM outgrowths or hES-like cells was performed for propagation of cells. Characterization of hES-like cells was conducted by morphology, detection of immunostaining of Oct-4, and enzymatic activity of alkaline phosphatase (AP). HES-like cells were spontaneously differentiated through suspension culture of embryoid body (EB). Subsequent differentiation was done on gelatin-coated dishes.*
Main outcome measure: *Establishment of hES cells*
Results: *By using abnormal fertilization embryos, 80.0% (8/10) of blastocysts were able to attach on the feeder layers, 50% (4/8) formed ICM outgrowths, but no hES-like cells were established. By using normal fertilization embryos, 84.6% (22/26) of blastocysts were able to attach on feeder layers, 18.2% (4/22) formed ICM outgrowths. One hES-like cell line was successfully established by using mechanical isolation of ICMs and human adult skin fibroblasts as feeder layers. This hES-like cells exhibited typical morphology of hES cells, positive staining for Oct-4 and AP. hES-like cells were able to form EB and differentiated into neural-like cells.*
Conclusion: *This is the first report in Thailand that hES-like cells can be isolated from normal development human embryos at blastocysts-stage using mechanical isolation of ICM and culture with human adult skin fibroblast as feeder layers.*

Keywords: *Stem cell, Cultured, Embryo, Fibroblasts, Human, IVF*

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Since the first success of derivation of human embryonic stem (hES) cell lines were reported in 1998[1], several hES cell lines have been derived across the world. hES cells represent a good model for studying development biology, drug discovery as well as cell replacement. These cells exhibit the unique characteristics including indefinitely proliferation, normal karyotypes, high level of telomerase activity, and differentiation ability *in vivo* and *in vitro*[1,2].

hES cells can be isolated from surplus fresh embryos, frozen-thawed embryos, or embryos that carried genetic disorder after being identified by pre-implantation genetic diagnosis (PGD)[1,2]. Derivation of hES cells involve with three major steps, including isolation and culture of inner cell mass (ICM), culture and propagation of hES cells, and characterization

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of hES cells *in vitro* and *in vivo*. The methods for isolation of ICM include, immunosurgery, mechanical isolation, laser assists, or whole blastocysts culture. They were successfully performed for obtaining hES cell lines[3-5]. Culturing of ICM or hES cells can be used as feeder cells derived from mouse, human, or without feeder cells[5]. hES cells derived under the conventional method run the risk of contamination with animal pathogens and make them unsuitable for cell transplantation purposes. Thus, several efforts, for instance, using human feeder layers or feeder free system have been conducted to isolate hES cells under xeno-free conditions.

This preliminary study was aimed to (i) optimize the authors' culture condition for derivation of hES cells using abnormal fertilization embryos in the first phase and (ii) isolate hES cells from normal fertilization embryos using different method of ICM isolation and feeder layers.

Material and Method

Human embryos and ethical approval

Human embryos used for the present study were donated from the couple who underwent the in vitro fertilization (IVF) program for infertility treatment at Reproductive Medicine Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University. Isolation of hES cells was performed after the approval of the Institutional Review Board (IRB number 096/50), Faculty of Medicine, Chulalongkorn University.

Culture of human embryos

Fresh abnormal, frozen-thawed abnormal and frozen-thawed normal fertilization at pronuclear (PN)-stage embryos were transferred to droplets of Global medium (LifeGlobal) supplemented with 10% serum substitute supplement (Irvine Scientific), covered with light oil (LifeGlobal), and cultured at 37°C, 5% O_2, 6% CO_2, 89% NO_2. After being cultured for an additional 3-4 days, embryos developed to blastocyst-stage were then collected for isolation of hES cells.

Preparing of feeder layers

Commercial fibroblast cells including, mouse embryonic fibroblasts (MEFs; SCRC-1008), Sandos inbred mice (SIM) resistant to 6-thioguanine and ouabain (STO; CRL-1503) and human foreskin (HFFs; CRL-1635) from Advance Cell Technology (ATCC; USA) were used.

In-house adult human skin fibroblasts (HSFs) were prepared from the surgical scar tissue of female. Briefly, surgical tissue from caesarean section were immediately washed in phosphate-buffered saline (PBS) containing 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen), cut into 1-mm^2 pieces under dissection microscope, and plated on the serum coated 60-mm culture dishes (Falcon). The tissues were left for 5-10 min under the laminar flow. Then add 2-3 ml of culture medium for fibroblasts into the culture dish and incubate at 37°C, 5% CO_2. After 7-10 days, the growing fibroblasts monolayer was trypsinized by 0.05% Trypsin-EDTA (Invitrogen) and replated on the new culture dish. HSFs were passaged when they reached 70-80% of confluency and frozen for further use. HSFs from passage number 5 to 10 were used for hES cell isolation.

Fibroblasts were cultured in DMEM high glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen). To use fibroblasts as feeder cells, confluent fibroblasts were mitotically inactivated with 10 μg/ml mitomycin C (Sigma) for 2.5-3 h and washed three times with PBS. Inactivated fibroblasts were then dissociated with 0.05% trypsin-EDTA, counted and plated at 5.0 x 10^5 cells per cm^2 for MEFs, 6.0 x 10^5 cells per cm^2 for STO and 7.0 x 10^5 cells per cm^2 for HFFs and HSFs, onto the gelatin-coated dishes to form a confluent monolayer and used as substrate cells on the following days.

Isolation of ICMs

In the present study, four different methods of isolation of ICMs were applied according to their size and morphology.

Immunosurgery

The present method was applied with grade 3 or 4 of blastocysts, according to grading system reported by Gardner[6]. Zona pellucida of expanded blastocysts was firstly removed with 0.1% acidified Tyrode's solution (Specialty media). Zona pellucida-freed blastocysts were incubated in anti-human whole serum (Sigma) and followed by guinea pig complement (Sigma). The remnants of trophectoderm cells were removed from ICM by gentle pipetting[4].

Mechanical isolation of ICMs

Expanded blastocysts were treated with 0.1% acidified Tyrode's solution to dissolve their zona pellucida, and the region in which the ICM was located,

was surgically isolated, with an eye toward excluding trophectoderm as much as possible, by using a finely drawn glass pipette[4].

Laser assisted ICM removal

ICM removal was assisted by micromanipulation techniques through a Zilos-TK Infrared Laser (Hamilton Torne Biosciences). ICMs were dissected from the trophectoderm by a laser beam and removed through the orifice in the zona pellucida as described previously[7].

Whole blastocyst culture

The present method was used for blastocysts which the ICM were unable to visualize because the immunosurgical method, mechanical isolation method or laser micromanipulation was not suitable for the isolation of the ICM. After zona pellucida of blastocysts was removed by 0.1% acidified Tyrode's solution, blastocysts were washed three times in hES culture medium and then directly plated on the feeder layers.

Culture of ICMs or blastocysts

To culture isolated ICM or blastocysts, the culture medium for feeder layers was changed to hES culture medium, consisting of Knock out DMEM (Invitrogen) supplemented with 20% Knock out serum replacement, 1% glutamax®, 0.55 mM β-mercaptoethanol, 1% nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), and 8 ng/ml bFGF (Invitrogen). ICMs or blastocysts were cultured for 7-14 days until the ICM outgrowths were formed. Mechanical splitting by using finely-drawn pipette was performed to propagate primary hES-like cells.

Experimental design for hES cell derivation

In the present study, two separated phases were designed to develop hES cell derivation under different conditions.

The first phase was designed to test the feasibility of hES cell derivation using blastocyst-stage embryos, developed from fresh or frozen-thawed abnormal fertilization embryos. Fibroblasts isolated from mouse or human were used as feeder layers.

The second phase was designed to isolate hES cells using blastocyst-stage embryos, developed from frozen-thawed normal fertilization embryos. Conventional and nearly xeno-free condition for hES cell derivation were applied.

In vitro characterization of hES-like cells

At passage 4, hES-like cells was characterized by immunostaining for measuring the expression of specific pluripotency marker, Oct-4. Briefly, colonies were fixed with 4% paraformaldehyde (PFA) and washed three times with washing buffer. Colonies were treated with 0.2% Triton X-100 (Sigma) and blocked of non-specific site was carried out with 5% bovine serum albumin (BSA; Sigma). Colonies were incubated overnight at 4°C with primary monoclonal antibodies against Oct-4 (Abcam) diluted 1:200 in washing buffer. After washing three times, colonies were incubated with FITC conjugated secondary antibody (Abcam) at room temperature for 1 hour. Colonies were then washed, viewed and photographed under inverted fluorescent microscope.

Histochemical staining for alkaline phosphatase (AP) was carried out using a commercially available kit following the manufacturer's instructions (Sigma).

Embryoid body (EB) formation and spontaneous differentiation

To test differentiation potential of hES-like cells, undifferentiated colonies were manually cut into small pieces, picked up and cultured in ultra low adhesion culture dishes (Corning). Differentiation medium comprised of Knock out DMEM, 10% FBS (Hyclone), 1% Glutamax, 0.55 mM β-mercaptoethanol, 1% nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin without bFGF. After 7-14 days, some EBs were transferred to gelatin-coated dishes and cultured further for spontaneous differentiation.

Results

Derivation of hES-like cells

During the first phase of the present study, four blastocysts developed from fresh abnormal fertilization embryos and six blastocysts developed from frozen abnormal fertilization embryos were used. Only 1 of 10 blastocysts showed prominent ICM and were found suitable for immunosurgery. While ICM was successfully isolated by immunosurgery, this ICM was unable to attach onto the feeder layers. However, whole blastocysts culture was applied to the other nine blastocysts and all of them attached on the feeder layers, ICM outgrowths were formed on both MEFs and HFFs. The results of hES cell isolation using abnormal fertilization embryos are shown in Table 1.

In the second phase, frozen normal fertilization embryos were thawed and subjected to isolation of hES cells. Four different methods for ICM isolation

and four different types of fibroblasts were used as feeder layers. One putative hES-like cells was successfully derived by using mechanical isolation of ICM and cultured on HSFs. The results of hES cell isolation using frozen normal fertilization embryos are shown in Table 2.

The resulting colonies showed typical morphology of hES cells (Fig. 1A-B). These hES-like cells were passaged and frozen for further characterization.

In vitro characterization of hES-like cells

HES-like cells showed positive results for enzymatic activity of AP (Fig. 1C), and immunostaining of transcriptional factor, Oct-4 (Fig. 1D). Furthermore, hES-like cells formed spherical EBs after cultured for 7 days in suspension (Fig. 2A). Preliminary results of spontaneous differentiation showed that hES-like cells were able to differentiate into neuronal like cells (Fig. 2B).

Discussion

Although the methods for derivation of hES cells are well established and repeatable, the efficiency of hES cell derivation still depends on the experience of the researchers. Before the authors started derivation of hES cells, the authors gained experience by establishing of ES cell lines from mouse blastocysts[8]. The skills of establishing and culturing of mouse ES cells can be applied to hES cell derivation even though there are some differences between mouse and human ES cells[9].

The method for isolation of ICM is generally dependent on the size and morphology of ICM in blastocyst. Immunosurgery is normally applied for blastocysts with large and prominent ICM[1,4,10,11]. The trophectodermal (TE) cells will be destroyed by the reaction of antibody and complement, the remnants of dead TE cells were removed by gentle pipetting. In the present study, four blastocysts were subjected to

Table 1. Effect of ICM isolation methods and feeder cells on the derivation of hES cells from abnormal ffertilization embryos

Type of embryos	Method of ICM isolation	Type of feeder cells	No. blastocyst	No. attached	No. ICM outgrowth	No. ES-like cells
Fresh abnormal	Whole	HFFs	2	2	0	0
	Whole	MEFs	2	2	1	0
Frozen-thawed abnormal	Immunosurgery	HFFs	1	0	0	0
	Whole	HFFs	4	3	2	0
	Whole	MEFs	1	1	1	0
Total			10	8	4	0

HFFs = human foreskin fibroblasts; MEFs = mouse embryonic fibroblasts

Table 2. Effect of ICM isolation methods and feeder cells on the derivation of hES cells from frozen-thawed normal fertilization embryos

Type of embryos	Method of ICM isolation	Type of feeder cells	No. blastocyst	No. attached	No. ICM outgrowth	No. ES-like cells
Frozen-thawed normal	Immunosurgery	MEFs	1	1	0	0
	Immunosurgery	HFFs	2	2	1	0
	Mechanical	HFFs	1	1	0	0
	Mechanical	HSFs	4	1	1	1
	Laser	HFFs	2	2	0	0
	Whole	HFFs	11	11	2	0
	Whole	STO	5	4	0	0
Total			26	22	4	0

HFFs = human foreskin fibroblasts; HSFs = human skin fibroblasts; MEFs = mouse embryonic fibroblasts

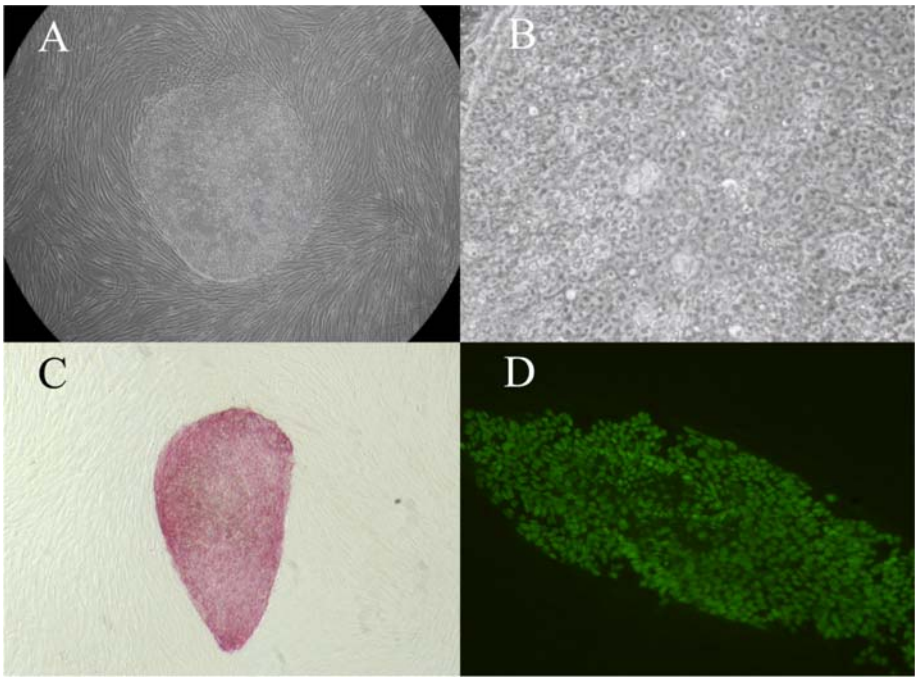

Fig. 1 HES-like cells derived from frozen-thawed normal fertilization embryo
After passaging of the ICM outgrowth, typical hES-like colony appeared within 7 days (A; magnification 40x), these hES-like cells showed typical morphology of hES cells with high ratio of nucleus: cytoplasm (B; magnification 200x). These hES-like cells show positive results for detection of alkaline phosphatase (C; magnification 40x) and transcriptional factor, Oct-4 (D; magnification 100x)

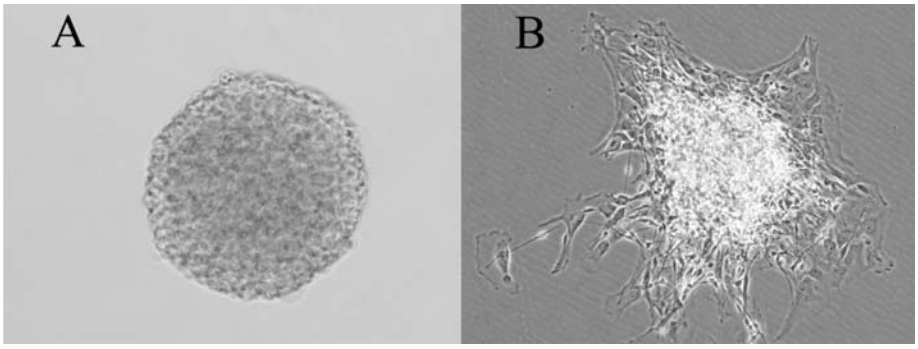

Fig. 2 *In vitro* differentiation of hES-like cells
HES-like cells were able to form three dimension structure, so-called "embryoid body" (A; magnification 100x). After plating of embryoid bodies on gelatin coated dishes and cultured for 10-14 days, hES-like cells differentiated into neuronal-like cells (B; magnification 100x)

immunosurgery and three ICMs were successfully isolated. However, these ICMs disappeared within a few days from feeder layer after initial plating. In case of blastocysts containing small ICM, the mechanical isolation of ICM can be performed in order to avoid the loss of ICM[4,12]. The major advantage of this method is that, ICM will not be in contact with antibody and complement, which are derived from animals. The hES cell lines derived from mechanical isolation of ICMs can be used in clinical trial in the future. At the

beginning of the present study, isolation of ICMs by cutting TE with drawn-glass pipette was time consuming and the success was unsatisfactory. However, after several attempts the authors were able to isolate ICMs with minimal attachment of TE within one minute or less. In the present study, one putative hES cell line was gene rated by the present method.

Moreover, laser micromanipulation was successfully applied for isolation of established ICMs and hES cell lines[3]. In the present study, the authors were able to isolate successfully two ICMs from two blastocysts by using laser system, both isolated ICMs could be attached onto the feeder layers but only differentiated cells grew out from the attached ICMs. The isolation of hES cells by culturing whole blastocysts was used in case of poor quality of blastocysts where ICMs were not easily identified. The major disadvantage of culturing whole blastocysts is that TE might inhibit the growth or induce the differentiation of ES cells. However, several hES cell lines were generated by the present method[4,13,14]. In the present study, there was no hES-like colony that grew out from whole blastocysts culture but only non-hES outgrowth or probably trophectoderm.

HES cell lines were mostly derived and cultured with feeder cells. These feeder cells secrete some factor(s) that enhance the growth and maintenance of the pluripotency of hES cells. However, it has been found that hES cells grow on feeder cells derived from mice bear the subsequent risk of contamination with non-human proteins, which are immunogenic to humans[15]. To use hES cell for cell therapy in the future, it is necessary to eliminate the risk of contamination of animal pathogens in cell culture. Using feeder cells that are isolated from humans are preferable and it was proved that several existing hES cell lines could be maintained in different sources of human feeder cells[16]. To date, hES cell lines can be derived and cultured using several types of human feeder cells[17].

In the present study, the authors used four different types of feeder cells including mouse embryonic fibroblast (MEFs), STO, human foreskin fibroblasts (HFFs) and human skin fibroblasts (HSFs). The results in the present study show that all of the feeder cells supported the attachment of ICMs or blastocysts but differentiated cells appeared after the first passage of ICM outgrowths in most cases. Several factors, for instance, pH, osmolality of culture medium or feeder cells might induce the differentiation of primary ES like cells by decreasing the expression level of Oct-4 and those cells were not able to maintain their pluripotency[18]. However, in the present study hES-like cells can be isolated from the ICM isolated by mechanical isolation and cultured on HSF. These hES-like cells were positive for Oct-4 and AP after characterization by immunostaining and enzymatic activity respectively. Furthermore, by spontaneous differentiation in vitro, they can form three-dimension structure, so called "EB" and differentiated into neuron-like cells. This hES-like cell line is now ongoing for characterization and differentiation processes.

To the authors' knowledge, this is the first report in Thailand that hES-like cells can be established from human embryos at blastocysts-stage using mechanical isolation of ICM and culture with human adult skin fibroblast as feeder cells. These preliminary results will be applied for establishment of xeno-free hES cells in the further study.

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การพัฒนาการสร้างเซลล์ต้นกำเนิดตัวอ่อนมนุษย์

กำธร พฤกษานานนท์, รัฐจักร รังสิวิวัฒน์, ปราณี นำชัยศรีค้า, วิชชุดา อานนท์กิจพานิช, ประมวล วีรุตมเสน

วัตถุประสงค์: *เพื่อสร้างเซลล์ต้นกำเนิดจากตัวอ่อนของมนุษย์*

ชนิดของการศึกษา: *การศึกษาแบบทดลอง*

สถานที่ทำการศึกษา: *หน่วยชีววิทยาการเจริญพันธุ์ ภาควิชาสูติศาสตร์ นรีเวชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

วัสดุและวิธีการ: *เลี้ยงตัวอ่อนที่ได้จากการปฏิสนธิผิดปกติ และปกติ จนถึงระยะบลาสโตซีส แยกอินเนอร์เซลล์แมส และโทรเฟคโตเดิร์ม โดยใช้วิธีทางอิมมูโนวิทยา, ตัดด้วยไปเปต, เลเซอร์ และเลี้ยงทั้งตัวอ่อน เซลล์พี่เลี้ยงที่ใช้คือ เซลล์ไฟโบรบลาสที่ได้จากหนู หรือคน ขยายจำนวนของเซลล์ต้นกำเนิดระยะแรก หรือเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนโดยการตัดแบ่ง พิสูจน์คุณลักษณะของเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อน ด้วยรูปร่าง, การย้อมสีอิมมูโนเพื่อตรวจการแสดงออกของจีน Oct-4, ตรวจการทำงานของเอนไซม์ alkaline phosphatase (AP) กระตุ้นให้เซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนเปลี่ยนแปลงเป็นเซลล์ที่ทำหน้าที่เฉพาะด้วยการเลี้ยงแบบแขวนลอยของ embryoid body (EB) และเลี้ยงบนจานเพาะเลี้ยงที่เคลือบผิวด้วยเจลาติน*

การประเมินผล: *การสร้างเซลล์ต้นกำเนิดจากตัวอ่อนมนุษย์*

ผลการศึกษา: *เมื่อใช้ตัวอ่อนที่ปฏิสนธิผิดปกติ, 80% (8/10) ของตัวอ่อนสามารถเกาะบนเซลล์พี่เลี้ยง 50% (4/8) เจริญเป็นเซลล์ต้นกำเนิดระยะแรก แต่ไม่สามารถสร้างเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดได้ เมื่อใช้ตัวอ่อนที่ปฏิสนธิปกติพบว่า 84.6% (22/26) ของตัวอ่อนสามารถเกาะบนเซลล์พี่เลี้ยง 18.2% (4/22) เจริญเป็นเซลล์ต้นกำเนิดระยะแรก สามารถสร้างเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนได้หนึ่งสายพันธุ์ ด้วยวิธีการแยกอินเนอร์เซลล์แมสโดยการตัดด้วยไปเปต และเลี้ยงร่วมกับเซลล์พี่เลี้ยงที่แยกจากผิวหนังของคนผู้ใหญ่ เซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนที่สร้างได้นี้ แสดงลักษณะที่บ่งชี้ถึงการเป็นเซลล์ต้นกำเนิดตัวอ่อน ให้ผลบวกต่อการย้อมสีทางอิมมูโนของ Oct-4 และ AP เซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนนี้สามารถเปลี่ยนเป็นเซลล์ที่ทำหน้าที่จำเพาะโดยการสร้าง EB และเปลี่ยนเป็นเซลล์ที่มีลักษณะคล้ายเซลล์ประสาท*

สรุป: *รายงานนี้เป็นครั้งแรกในประเทศไทยที่ประสบความสำเร็จในการสร้างเซลล์ที่มีลักษณะคล้ายเซลล์ต้นกำเนิดตัวอ่อนมนุษย์จากตัวอ่อนระยะบลาสโตซิส โดยการตัดด้วยไปเปต แยกอินเนอร์เซลล์แมส และเลี้ยงร่วมกับเซลล์พี่เลี้ยงที่แยกจากผิวหนังของคนผู้ใหญ่*