The establishment of human embryonic stem cell lines demonstrated that chimeric embryos can self-correct

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Article

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Abstract

Background Low-level mosaicism is a common trait of early human development. Although mosaic embryos may lead to healthy live births, the direct effects of mosaicism are unknown. While embryo self-correction was demonstrated in mouse models, humans can only study the effects of chromosomal aberrations and blastocyst mosaicism on the early peri-implantation period by extended *in vitro* embryo culture up to 12 days post-fertilization.

Methods The established culture protocols were followed to generate embryonic stem cells. The 24 high-quality mosaicism / aneuploid mosaicism blastocysts were detected by PGT-A. The isolated ICMs were seeded onto feeder-free dishes, and after 10-12 days of culture, there were six blastocyst ICM-generated stem cell clones. Cells shed during stem cell growth were harvested, and next-generation sequencing was performed on stem cells and the shed. To test pluripotency, a small number of stem cells were isolated and subcultured.

Results The PGD status was confirmed from the results ofnext-generation sequencing of stem cell exfoliated cells and stem cells, the chimeras in five blastocysts were fully repaired while the chimera in one blastocyst was partially repaired. Simultaneously the cells were verified to have pluripotency and the ability to differentiate into three germ layers by immunofluorescence, flow cytometry, and *in vitro* differentiation analyses.

Conclusions Human mosaicism/aneuploid mosaicism blastocysts can self-correction by eliminating chimeric cells.

Introduction

A mosaic embryo is one in which two or more cell lines with different karyotypes coexist in the preimplantation embryo. The International Preimplantation Genetic Diagnosis Society (PGDIS) stipulates that embryos with 20% to 80% of abnormal cells are chimeric, >80% are aneuploid, and <20% are euploid[1]. The first stage of human embryogenesis is the rapid proliferation of cells, during which cell segregation errors often occur, resulting in chromosomal changes. As a result, most human preimplantation embryos exhibit aneuploidy, mainly diploid-aneuploid mosaicism[2]. A large proportion of preimplantation embryos have mosaicism in preimplantation genetic diagnosis (PGD). The detection rate of chimeric embryos depends on the detection method and platform and varies greatly in the literature, ranging from 30% to 90%[3]. Currently, trophoblast ectoderm (TE) biopsy and next-generation sequencing (NGS) are the techniques of choice for preimplantation genetic testing for aneuploidy (PGT-A)[4].

Embryo mosaicism can affect the embryonic developmental potential, leading to implantation failure, miscarriage, and the birth of abnormal fetuses[5]. In the field of *in vitro* fertilization-embryo transfer (IVF-ET) treatment, the selection of chimeric embryos is also controversial, but multiple cases of live births following chimeric embryo transfer have been documented[6]. The reason for this may be that the embryo has a self-correcting mechanism by which it recognizes and excludes abnormal cells from subsequent development[7][8][9].

Based on this hypothesis, we isolated the inner cell masses (ICMs) of human blastocysts to establish an embryonic stem (ES) cell line that can stably self-renew for a long time and has multi-differentiation potential[10]. ES cell lines were cultured, and the chromosomes of dead cells were traced during the growth of the stem cells. The stem cell clone chromosomes were compared to provide evidence that human embryos can self-correct and eliminate cells with chromosomal abnormalities.

Materials And Methods

Institutional approval and informed consent

Embryos classified as chromosomally abnormal by PGT-A were used in this study. The application of human blastocysts in this study was approved by the institutional ethics committee of First Affiliated Hospital, Nanjing Medical University. All methods of this study were performed in accordance with the relevant guidelines and regulations.Each participant was over 18 years old and signed a written informed consent.

Embryo origin and culture

We used high-quality blastocysts donated after PGT-A in the Reproductive Medicine Center of the First Affiliated Hospital of Nanjing Medical University, and a total of 24 embryos from 13 patients (mother's age 29±7) were included in the study. All embryos were subjected to a standard *in vitro* fertilization intracytoplasmic sperm injection cycle and cultured and incubated at 37°C, 5% CO_2, and saturated humidity.

Establishment of embryonic stem cell lines

For embryos detected as A by PGT-A, the embryonic trophectoderm and inner cell mass were carefully separated with a glass needle. The cells were cultured in an incubator with 5% CO_2 and saturated humidity for 12 days and shed cells were harvested with a pipette every day and stored in an EP tube at −20°C. The medium was replaced with a fresh culture medium, and the cell growth state was observed. After 10-12 days, 6 of the 24 ICMs seeded on the dish formed stem cell clones. Some cells were picked with glass needles for a subculture to verify the pluripotency of the ES cells, and the remaining outgrowths were subjected to next-generation sequencing, and the results were compared with the original diagnosis (Fig.1).

Immunofluorescence detection of stem cell pluripotency

A small number of digested clones from human embryonic stem cells (hESCs) were seeded onto coverslips in pre-prepared 24-well plates. The cells were cultured for 4 days, fixed with 4% paraformaldehyde at room temperature for 20 minutes, and washed three times with Dulbecco's phosphate-buffered saline (DPBS). After fixation, the cells were emersed in 100 μl 0.4% TritonX-100/DPBS, broken up on ice for 10 minutes, and washed

with DPBS. We added 5% BSA (200 μl/well) and placed the plates in a 37°C incubator for 30 minutes of blocking. The primary antibody was added and the cells were labeled overnight at 4°C. The primary antibodies used were rabbit anti-OCT4 (1:200 Abcam), rabbit anti-Nanog (1:200 Cell Signaling Technology), mouse anti-SSEA4 (1:200 Abcam), and mouse anti-TRA-1-60(1:200 Cell Signaling Technology). The primary antibody was washed away three times using DPBS, and the cells were labeled with the secondary antibody by incubating at room temperature for 1 hour in the dark. The secondary antibody used was donkey anti-rabbit IgG (H+L) (1:1000 Thermo Fisher Scientific), and donkey anti-mouse IgG (H+L)(1:1000 Thermo Fisher Scientific). After dropping 20 μl of mounting/blocking solution onto the glass slide, the small disc was lifted with a curved needle and flipped so it lay upside down on the glass slide. A fluorescent inverted microscope was used to observe and record micrographs of the cells.

Detection of pluripotency of stem cells by flow cytometry

The cells were collected, and their density was adjusted to 1.0×10^5/10 μl, followed by the addition of 0.1 ml of freshly prepared Fixation Buffer (#420801, Biolegend). After mixing gently, the cells were fixed for 30-60 minutes at room temperature in the dark. Freshly prepared Permeabilization Wash Buffer (#421002, Biolegend) was added to each tube and left at room temperature for 3 minutes to rupture the cell membranes, and then the treatment was repeated. For primary antibody (same as used for immunofluorescence) labeling, 0.1 ml/tube Permeabilization Wash Buffer was used to resuspend the cells, then 3 μl primary antibody was added to each tube, mixed gently, and incubated overnight at 4°C in the dark. Permeabilization Wash Buffer 1.4 ml was added to wash away the primary antibody. For the secondary antibody (same as used for immunofluorescence) labeling, 0.1 ml/tube Permeabilization Wash Buffer was added to each tube to resuspend the cells, followed by 1 μl of secondary antibody, and incubated at room temperature for at least 1 hour in the dark. The cells were washed to remove the secondary antibody, and a 200 μl staining solution was added before the cells were placed into the flow cytometry machine for detection.

In vitro differentiation assay

Collagenase IV and neutral protease were used to digest the hESCs, and the cell clones were collected and placed in an ultra-low adhesion culture dish for suspension culture. A small globular mass of cells called an embryoid body (EB) formed. Gradually, the EB increased in size and the surface became smoother with the passing of culture days EBs were collected on days 0, 3, 7, and 14 of differentiation, and a 1:1 mix of 0.25% trypsin and collagenase II was added and incubated in a water bath at 37°C for 5 minutes, with mixing every 1 minute during the incubation. Cell adhesion molecules were digested to give single cells, which were seeded onto Matrigel-coated discs, cultured in an incubator for 24 hours, fixed with 4% PFA, and detected by immunofluorescence.

Whole-genome amplification of stem cell gDNA and next-generation sequencing

The multiple-annealing SurePlex DNA Amplification Kit (Illumina: catalog # F1006000900) was used to amplify gDNA from the stem cells according to the manufacturer's instructions. Following the protocol for the Veriseq DNA Library Prep Kit (Illumina; F1006001002, F1006001002), sequencing libraries for NGS were constructed for sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Identifying karyotype and copy number variations by NGS genetic screening of stem cells

The read numbers were counted along the 24 chromosomes with a bin size of 4 MB and normalized to the mean of the corresponding bin in all samples. Our earlier pilot data showed that an even distribution of reads along the chromosomes indicates the targeting of chromosomal contents and the cell karyotypes. The pipeline for chromosomal copy number analysis was conducted as described previously[11].

Results

A total of 13 patient mosaicism/aneuploid mosaic embryos were used, all of which were diagnosed as PGT-A. A total of 24 blastocysts were cultured, and all from the trophectoderm were biopsied before the ICM was isolated. The isolated ICM was seeded onto a plate coated with Matrigel hESC-Qualified Matrix (Corning, USA), and after 10-12 days, there were six ES cell clones. The blastocyst morphological grading, appearance, blastocyst NGS results, and their corresponding cell debris and stem-cell clone morphologies are shown in Table 1.

Of the six embryos that formed stem-cell clones, there were two chimeric blastocysts, and all chromosomes of the shed cell fragments were chimeras, while the formed stem-cell clones were euploids(embryos A, B). In addition, of the four aneuploid mosaicism blastocysts, three underwent chimeric repair(embryos C, E, F) and one underwent chimeric part repair(embryo D), but only one chimeric cell was detected in the exfoliated cell fragments(embryo D). The presence of chromosomal mosaicism was also tested for: one aneuploid mosaicism blastocyst had been completely repaired, including its aneuploidy(embryo F).

When we observed under the microscope, the hESCs were seen to have grown clonally, forming compact multicellular colonies with clear borders. The cells were tightly packed and had a high nucleus-to-cytoplasm ratio and prominent nucleoli. The healthy colonies had fused seamlessly into tight clusters of cells with multiple layers of cells in the center. Immunofluorescence and flow cytometry analyses showed that the hESCs expressed high levels of stem cell markers OCT4, NANOG, SSEA4, and TRA-1-60. The *in vitro* three-blast differentiation experiments showed that the three-blast markers SOX17 (endoderm), BRACHYURY (mesoderm), and NESTIN (ectoderm) were highly expressed during differentiation(Fig.2)

Discussion

In this report, we describe the formation of ICMs of mosaicism/aneuploid mosaicism *in vitro* cultured stem cells and their elimination of chimerism by apoptosis or reducing the division of abnormal cells, enabling the

self-repair of embryonic chromosomes. We observed that 24 mosaicism/aneuploid mosaicism blastocysts were separated from the ICM, and six ES cells were obtained. Finally, the chimerism of five blastocysts was repaired, and one blastocyst was partially repaired. During this process, we collected exfoliated cells for next-generation sequencing and found that the exfoliated cells of three stem-cell ICMs contained chimeric cells.

This study explored whether chimeric embryos can self-repair their chromosomes during development. Unlike in previous studies, the embryos were only *in vitro* cultured up to 12-days post-fertilization in this investigation[12]. We established ES cell lines from chromosomally abnormal embryos to explore the genetic characteristics of human embryonic development after the blastocyst stage. To the best of our knowledge, this is the first study to use chromosomal abnormalities and chimeric human embryos using established ES cell lines.

The development of new diagnostic techniques for PGT-A, i.e., rapid low-pass whole-genome next-generation sequencing methods that allow the detection of embryonic aneuploidy in single cells, has led to an increase in reports of embryonic mosaicism[13]. However, how chimeric embryos should be handled is still being debated. Hundreds of chromosomally healthy births have been reported following the transfer of embryos given false-positive chromosomally abnormal diagnoses by PGT-A (“mosaic” and “aneuploid”), confirming that a considerable number of embryos with normal pregnancy potential are unnecessarily discarded, which was recently also pointed out by Paulson[14]. Although the information on neonatal outcomes is limited, more than 100 live births without abnormal phenotypes following chimeric embryo transfer have been documented[15]. Numerous studies have shown that transferring mosaic embryos produces healthy babies because the chimera repairs itself[16]. The mechanism by which this occurs is not well understood, but studies suggest three hypotheses: the embryonic mortality model[17], the clonal depletion model[18], and the preferential assignment of aneuploid cells to the TE model[19].

The incidence of chimerism is known to decrease during development and occurs less frequently in blastocysts than in cleavage stage embryos[20]. This can be explained by the fact that the proliferation of euploid cells competes with that of aneuploid cells, resulting in the death of the aneuploid cells.

Preimplantation embryos from chimeric mouse models were found to have a progressive decrease in aneuploid cells. Chimeras of euploid cells and aneuploid cells showed selective apoptosis of aneuploid cells in the ICM and proliferative defects in aneuploid cells of the TE, leading to a progressive depletion of aneuploid cells from the blastocyst stage onward[21]. This may correspond to the proposed self-correcting mechanism, as aneuploid cells may proliferate slower or undergo apoptosis, whereas euploid cells may compensate by increasing their proliferation rate. Research by Bolton et al. provided direct evidence that apoptosis within the ICM can occur as a mechanism to eliminate chromosomally abnormal cells[22]. The ability of aneuploid embryos to self-correct downstream of the blastocyst stage was first reported in mice and more recently demonstrated in human embryonic cell lineages and the human blastocyst stage. In the current study of extended embryo culture, we validated the self-correcting mechanisms of human embryos and their ability to eliminate/clear abnormal cellular debris[23].

In 2019, Popovich et al. applied next-generation sequencing to study the progression of blastocysts diagnosed as aneuploid, euploid, or mosaic using an expanded *in vitro* culture model. Although the study did not provide

direct evidence of self-correction, a large proportion of mosaic embryos showed euploid growth by day 12 of development, providing evidence for the depletion of abnormal cells throughout the early post-implantation period[11]. In our study, five blastocyst-derived stem cells underwent chromosomal chimeric repair, one blastocyst-derived stem cell underwent partial chromosomal chimeric repair, three of which shed chimeric cells. However, some blastocysts had chromosomal abnormalities that were not found in the debris and stem cells.

The chromosomal abnormality detection methods are significant improvements over previous ones and that NGS technology in PGT-A has significantly improved the identification of diploid/aneuploid mosaicism in multicellular biopsy samples. The accuracy of phenomenon detection is challenged by several biological biases, including sampling and the possibility of mutual errors[24]. Although up to 86.2% of the studied embryos showed consistent PGS conclusions between TE and ICM[25]. In a small study involving array CGH screening of 10 embryos, the concordance between TE and ICM was reported to be as high as 100%[26]. Using the same approach, Johnson et al. showed the agreement between TE and ICM was 96%[27].

However, chromosomal abnormality detection methods continue to result in a clinically significant prevalence of blastocyst mosaicism, given that PGT-A considers the chromosomal composition of an average of 5-6 cells on biopsy to represent the entire embryo, and this brings controversy. Gleicher et al. used a mathematical model to propose that chimeric cells are evenly distributed in the trophectoderm, and at least 27 trophectoderm cells are required in a biopsy to represent the karyotype of the entire blastocyst[28]. Studies have shown there was a 13.8% incidence of inconsistency in PGS conclusions between TE and ICM due to sampling error in embryos with restricted mosaicism, and a 3.4% probability of reciprocal error[29]. In a recent experimental study, Victor et al. reported whole-chromosomal aneuploidy in TE biopsies of 100 human blastocysts with a 96.8% concordance between TE and ICM. However, in the presence of chimeras, the concordance of TE with ICM decreases significantly[30]. In addition, although NGS technology has a high sensitivity and detection rate for chimeric embryos, the length of NGS read data is shorter than that of traditional sequencing data, and the error rate in the sequence assembly process is 0.1% to 15%. The analysis of large amounts of data poses several challenges[31]. This could explain why the final results for three of the six stem cell lines we established did not match the biopsy results perfectly. Again, the sample size of our study was small, and larger studies are needed to confirm or refute our findings.

Conclusion

The findings of this study give insight into the ability of human chimeric blastocysts to self-correct. The method of ICM isolation used to create stem cells provides direct evidence for embryo self-correction via aneuploid cell apoptosis. These findings serve as a reference for chimeric embryo selection in assisted reproduction

Declarations

Ethics approval and consent to participate

The study was approved by ethical committee of Nanjing Medical University

(2012-SR-128)

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' Contribution

CL designed the study, performed experiments, analyzed data, and manuscript writing. JX and CG participated in Whole-genome amplification and NGS. JD assisted in the establishment of stem cell lines. MX and YL contributed to embryo culture and PGT-A. YC,LQ and WW contributed to critical revisions. All authors contributed to the interpretation of the results and reviewed the article.

Acknowledgments

Not Applicable

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Tables

Table 1. Appearance and NGS results of blastocysts PGT-A, their corresponding cell debris and hESC

Embryo number	Embryo score		NGS Results	mosaic correction
A	4AB	Embryo	+4(mos,34%),+6(mos,38%),+14(mos,44%),+Y(mos,29%)	YES
		Debris	+Y(p11.31-q12)(mos,36%,26.17Mb)	
		ES	46,XY	
B	4BB	Embryo	+7(mos,46%),+13(mos,46%)	YES
		Debris	-7(p22.3-p11.2)(mos,37%,57.60Mb) ,-13(q12.11-q34)(mos,30%,95.49Mb)	
		ES	46,XX	
C	4BB	Embryo	+8(p12-qter)(mos,67%,114.96Mb),+19(mos,41%),+20(mos,36%);-5(q14.3-qter)(mos,35%,93.10Mb),-22(q11.21-q13.33)(21.36Mb)	YES
		Debris	+8(p12-q24.3)(114.96Mb),-22(q11.21-q13.31)(28.96Mb)	
		ES	+8(p12-q24.3)(115.56Mb),-22(q11.21-q13.31)(28.96Mb)	
D	4BB	Embryo	+1(q32.3-qter)(34.63Mb) ,+13(q12.11-q12.3)(mos,50%,12Mb);-11(q13.3-qter)(mos,38%,65.14Mb),-13(q12.3-qter)(mos,26%,83.02Mb)	Part correction
		Debris	+1(q32.3-q44)(34.63Mb),+11(p15.5-q13.4)(mos,39%,73.91Mb),-11(q13.4-q25)(mos,40%,60.97Mb), -13(q13.1-q34)(mos,63%,82.29Mb)	
		ES	+1(q32.3-q44)(34.63Mb),-11(q13.4-q25)(mos,48%,60.97Mb),-13(q21.33-q34)(mos,50%,45.09Mb)	
E	4BB	Embryo	+2(p11.2-qter)(mos,29%,154.78Mb),+21;-2(pter-p11.2)(mos,36%,86.53Mb)	YES
		Debris	+21(q11.2-q22.3)(33.78Mb)	
		ES	+21(q11.2-q22.3)(33.78Mb)	
F	4AA	Embryo	+13;-9(q13-q34.11)(mos,34%,62.34Mb)	YES
		Debris	+13(q12.11-q34)(87.62Mb)	
		ES	46 XY	

Figures

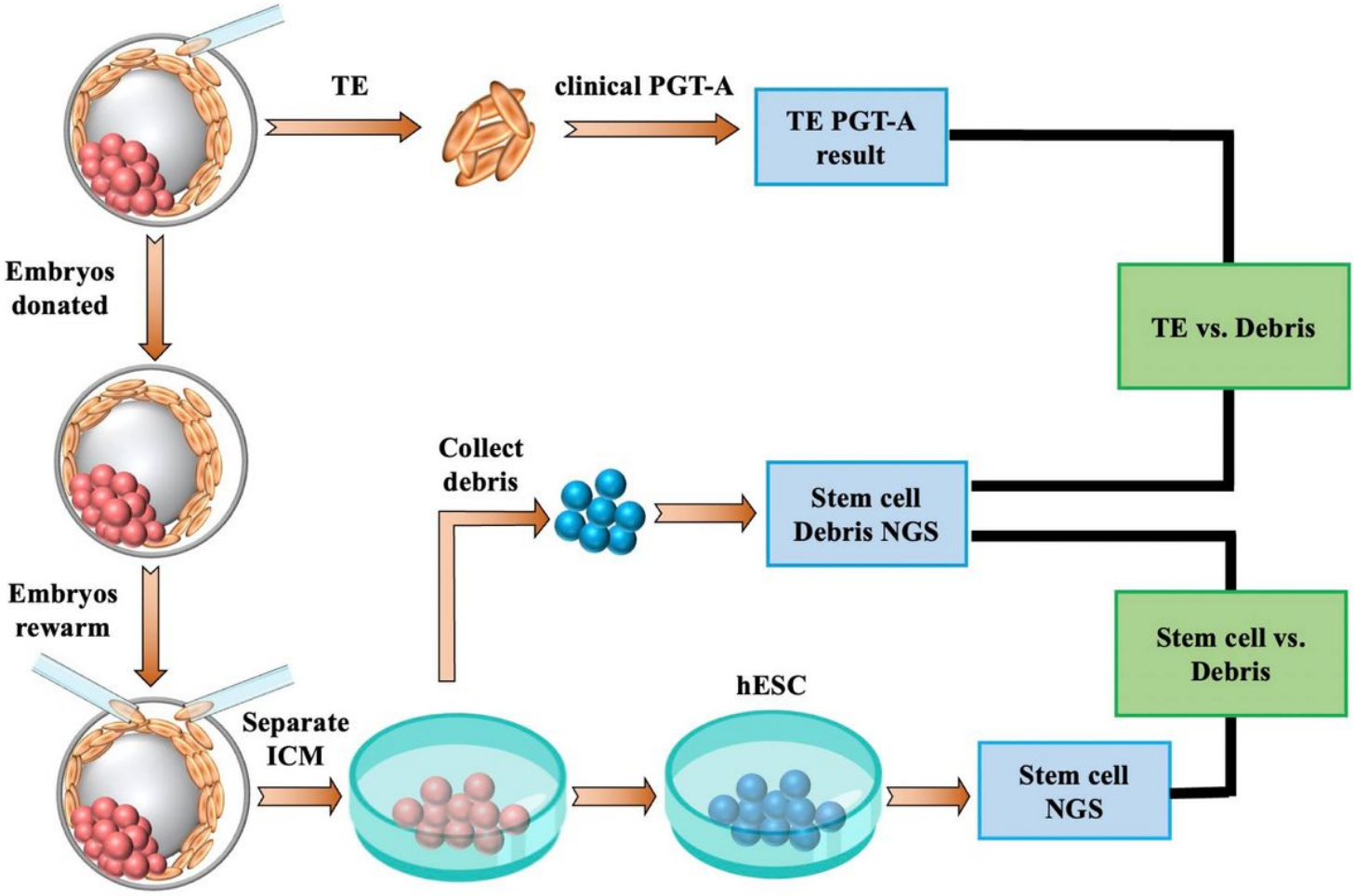

Figure 1

Study design

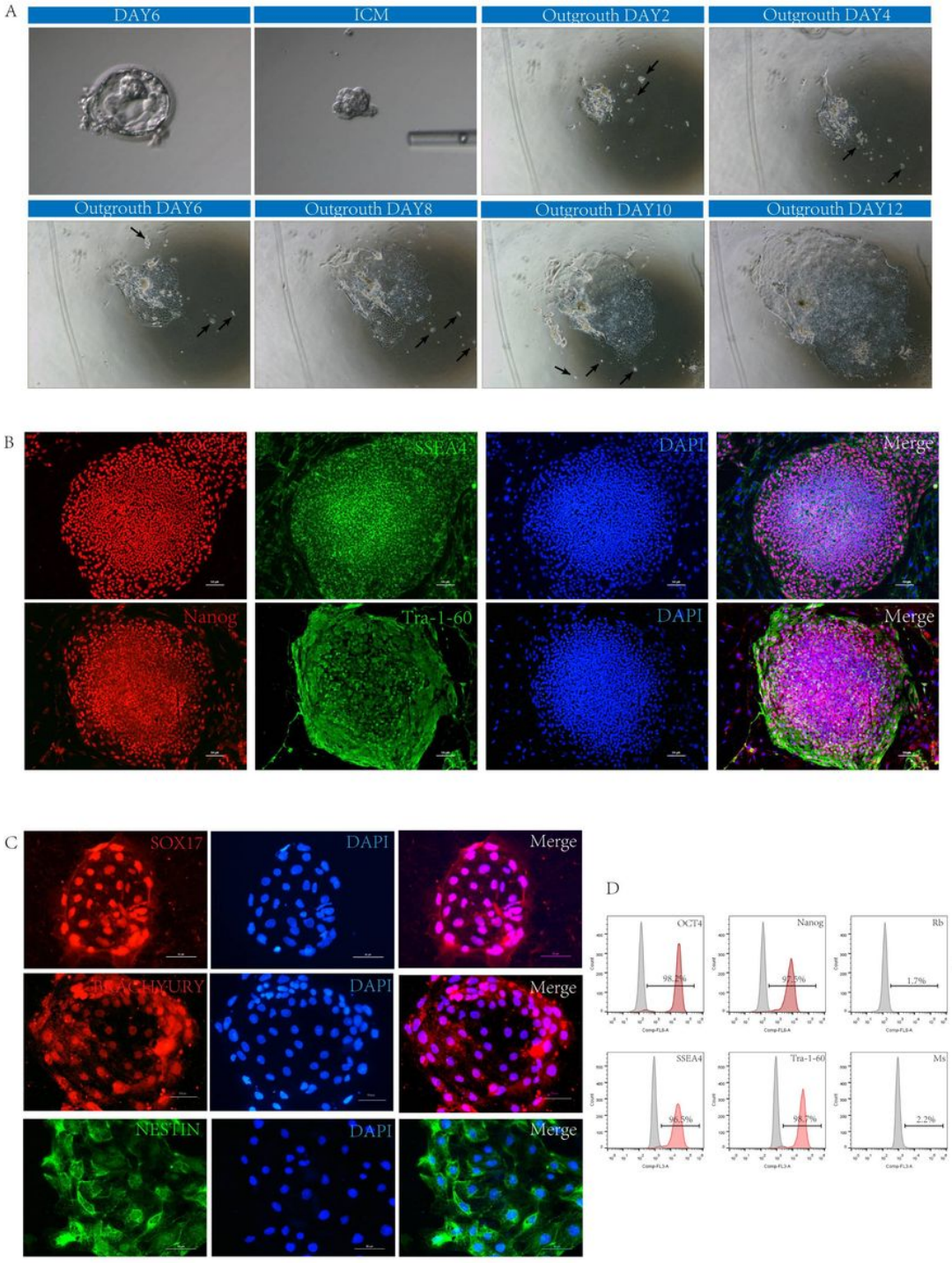

Figure 2

Establishment of human embryonic stem cells. A. Brightfield images of embryo morphology during culture for 2-12 days hES growth process.Arrows are cell debris. B. HES highly expresses pluripotency markers OCT4, SSEA4, Nanog, and Tra-1-60 by immunofluorescence staining. C. hES highly expresses three germ layers markers, SOX17, BRACHYURY, and NESTIN. D. The expression of the pluripotent markers OCT4, NANOG, SSEA4 TRA-1-60 were confirmed by flow cytometry analysis.