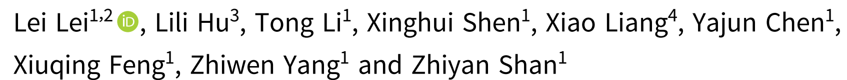
Zygote

cambridge.org/zyg

Establishment of mouse androgenetic embryonic stem cells by double sperm injection and differentiation into beating embryoid body



Lei Lei[1,2], Lili Hu[3], Tong Li[1], Xinghui Shen[1], Xiao Liang[4], Yajun Chen[1], Xiuqing Feng[1], Zhiwen Yang[1] and Zhiyan Shan[1]

[1]Department of Histology and Embryology, Harbin Medical University, Harbin, 150081, Heilongjiang, China; [2]Key Laboratory of Preservation of Human Genetic Resources and Disease Control in China (Harbin Medical University), Ministry of Education, China; [3]Department of Reproductive Medicine, the First People's Hospital of Jining City, Jining, 272100, Shandong, China and [4]Department of Cardiology, the First Affiliated Hospital of Harbin Medical University, Harbin, 150081, Heilongjiang, China

Research Article

Cite this article: Lei L *et al*. (2019) Establishment of mouse androgenetic embryonic stem cells by double sperm injection and differentiation into beating embryoid body. *Zygote* **27**: 405–412. doi: 10.1017/S0967199419000510

Received: 10 June 2019
Revised: 26 July 2019
Accepted: 14 August 2019
First published online: 23 September 2019

Keywords:
Androgenetic embryonic stem cells; Beating embryoid bodies (EB); Double sperms injection; Imprinted genes; Mouse.

Address for correspondence:
Lei Lei. Department of Histology and Embryology, Harbin Medical University, 194 Xuefu Road, Nangang District, Harbin 150081, Heilongjiang, China, Tel: +86 451 86674518. Fax: +86 451 87503325.
E-mail: lei086@ems.hrbmu.edu.cn

Summary

Androgenetic embryonic stem (AgES) cells offer a possible tool for patient-specific pluripotent stem cells that will benefit genomic imprinting studies and clinic applications. However, the difficulty in producing androgenetic embryos and the unbalanced expression of imprinted genes make the therapeutic applicability of AgES cells uncertain. In this study, we produced androgenetic embryos by injecting two sperm into an enucleated metaphase II (MII) oocyte. By this method, 88.48% of oocytes survived after injection, and 20.24% of these developed to the blastocyst stage. We successfully generated AgES cell lines from the androgenetic embryos and assayed the expression of imprinted genes in the cell lines. We found that the morphological characteristics of AgES cells were similar to that of fertilized embryonic stem cells (fES), such as expression of key pluripotent markers, and generation of cell derivatives representing all three germ layers following *in vivo* and *in vitro* differentiation. Furthermore, activation of paternal imprinted genes was detected, *H19*, *ASC12* and *Tss3* in AgES cell activation levels were lower while other examined genes showed no significant difference to that of fES cells. Interestingly, among examined maternal imprinted genes, only *Mest* and *Igf2* were significantly increased, while levels of other detected genes were no different to that of fES cells. These results demonstrated that activation of some paternal imprinted genes, as well as recovery of maternal imprinted genes, was present in AgES cells. We differentiated AgES cells into a beating embryoid body *in vitro*, and discovered that the AgES cells did not show significant higher efficiency in myocardial differentiation potential.

Introduction

Pluripotential stem cells, such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), have prospective application in the clinic due to their unlimited proliferation and multiple differentiation abilities. Some clinical trails have indicated that the transplantation of pluripotential stem cells prognoses well in retina macular degeneration (Chen *et al*., 2014) and neurodegenerative diseases (Chen *et al*., 2019). Myocardial infarction (MI) otherwise known as a heart attack, is the highest risk of death worldwide. It occurs when the coronary artery is occluded, causing the cardiac muscle to die from lack of oxygen. Myocardial cells derived from pluripotent stem cells could replace the damaged cardiac muscle and partially recover the function of the heart (Barad *et al*., 2014). However, stem cell therapies need many preclinical and controlled clinical trials, considering differentiation efficiency, safety and ethical issues.

In mammals, uniparental zygotes with two paternal or two maternal genomes are not able to develop into viable offspring naturally but can form blastocysts from which ES cells can be derived. Uniparental ES cells may represent alternative sources for patient-specific pluripotent stem cells and bypass most ethical concerns regarding derivation from fertilized blastocysts. Alternatively, uniparental ES cells, having uniparental genomes, are very useful models for the study of parental-specific gene expression or for exploring the biological significance of genomic imprinting in mammals (Szabo and Mann, 1994). Parthenogenetic embryonic stem (PgES) cells could be generated by artificially activated oocyte or female pronucleus transplantation (Allen *et al*., 1994; Eckardt *et al*., 2007). AgES cells could be established by *in vitro* fertilization (IVF) of enucleated oocytes, or male pronucleus transplantation (Dinger *et al*., 2008; Eckardt *et al*., 2007). Parthenogenetic and androgenetic haploid ESCs are very useful tools to study functions of the imprinted genes in uniparental mammalian development (Li *et al*., 2018).

© Cambridge University Press 2019.

CAMBRIDGE UNIVERSITY PRESS

Furthermore, haploid AgES cells even could serve as transgenic vehicles to produce multiple genetic manipulations semi-cloned offspring (Zhong *et al*., 2015).

Mammalian androgenetic embryos could be produced by pronuclear transplantation (Mann and Stewart, 1991), however the time to distinguish female and the male pronuclear cells is quite short, and this process must overcome some ethical objections concerning destroying normal fertilized embryos. Obata *et al*. (2000) produced mouse androgenetic embryos by *in vitro* fertilization (IVF) of enucleated oocytes, which allowed the penetration of two spermatozoa (dispermic fertilization). Unfortunately, this process was very inefficient for generating two male pronuclei because polyspermy often occurred. More recently, diploid androgenetic embryos were produced by injection of two round spermatid nuclei into oocytes, followed by female chromosome removal, but the percentage of oocytes surviving the injection was extremely low (<30%) (Miki *et al*., 2009). Here we produced androgenetic embryos by injecting two sperm into an enucleated MII oocyte, while using cytochalasin B to release cytoplasm, which facilitated oocyte survival after dispermic injection (Hu *et al*., 2012). Compared with other protocols, injection of dispermic nuclei after maternal enucleation is an effective alternative method for producing androgenetic embryos (Zhao *et al*., 2010).

Uniparental embryos had aberrant genomic imprinting and unbalanced allele-specific expression of imprinted genes that disrupted fetal development, and was associated with genetic diseases, cancers and neurological disorders (Beygo *et al*., 2018; Jelinic and Shaw, 2007; Khosla *et al*., 2001). In the present research, we established AgES cells from mouse androgenetic embryos produced by injection of two sperm into an enucleated oocyte. In addition, we checked the expression of pluripotent, mesoderm differentiation-related, and imprinted genes in AgES cells. Finally, we compared myocardial differentiation by inducing AgES and normal fertilized ES cells into a beating embryoid body.

Materials and Methods

Animals and reagents

Metaphase II (MII) oocytes were collected from 6- to 8-week-old B6D2F1 (C57BL/6J × DBA/2J) female mice, and sperm were obtained from EGFP–C57BL6 male mice. Here, 8- to 10-week-old CD-1 female mice were used to provide recipients and blastocysts for producing chimeras. Teratomata were induced into 8- to 12-week-old CD-1 nude mice. All mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. Mice were kept under a 14 h light/10 h dark regime; food and water were available *ad libitum*. All reagents were obtained from the Sigma Chemical Company unless stated otherwise.

Generation of androgenetic embryos

Matured oocytes were collected from B6D2F1 female mice that were superovulated using pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotrophin (hCG) injection given 48 h apart in doses of 7.5 IU each, and incubated at 37°C under 5% CO_2 in air before use. Spermatozoa were recovered from the cauda epididymis of EGFP–C57BL6 males in CZB-HEPES medium and were prepared for injection. The spindle of the oocytes was removed using an enucleation pipette (blunt, 10–12 μm in inner diameter) attached to a piezo-drill micromanipulator under a differential interference microscope. Androgenetic embryos were generated by injecting each enucleated MII oocyte with two sperm in CZB-HEPES medium containing 5 μg/ml cytochalasin B. The reconstructed oocytes were activated for 6 h in Ca^{2+}-free CZB medium supplemented with 10 mM $SrCl_2$ and 5 μg/ml of cytochalasin B. Then the embryos were cultured in potassium simplex optimized medium (KSOM) at 37°C in an atmosphere of 5% CO_2 in air.

Derivation of AgES cell lines

The zona pellucida (ZP) of the androgenetic blastocysts was removed in acid CZB-HEPEs medium, and ZP-free blastocysts were then transferred onto feeder layers of mitomycin C-treated mouse embryonic fibroblasts (MEFs). The inner cell mass (ICM)-derived outgrowths were extended for 4–6 days, mechanically harvested and plated onto new feeder cells. Colonies morphologically resembling AgES cells were then picked and disaggregated with 0.25% trypsin–EDTA, and plated onto new feeder cells in ES cell medium consisting of knockout Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 20% knockout serum replacement (KOSR; Invitrogen), 2000 IU/ml leukaemia inhibitory factor (LIF), 0.1 mM NEAA, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 50 IU/ml penicillin, and 50 IU/ml streptomycin following standard procedures. Then, AgES cells were expanded and passaged in high-glucose DMEM supplemented with 15% FBS.

Immunocytochemistry, alkaline phosphatase (AKP) staining and embryoid body formation

To investigate the pluripotent characteristics of AgES cells and the expression of OCT4 and ES-specific surface markers (SSEA1), AgES cells were stained using immunocytochemistry. AgES cells grown on gelatin-coated coverslides were fixed in 4% paraformaldehyde. After permeabilization and blocking treatment, primary antibody anti-OCT4 monoclonal antibody or anti-SSEA1 monoclonal antibody (1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA) incubation was carried out overnight at 4°C, followed by incubation with anti-mouse IgG secondary antibody for 1 h at 37°C. Nuclei of the cells were stained with Hoechst 33342. Images were captured using a fluorescence microscope.

For embryoid body (EB) formation, AgES cells were dispersed and cultured in a bacterial dish under FBS+/LIF conditions. After 2 days, these cells spontaneously formed simple EBs. Immunofluorescence staining was performed to analyze the three-dimensional markers for EBs with antibodies including anti-AFP monoclonal antibody (1:100; Santa Cruz), anti-α-SMA monoclonal antibody (1:100; Santa Cruz), anti-PECAM monoclonal antibody (1:100; Santa Cruz), anti-NF-L monoclonal antibody (1:100; Santa Cruz), and anti-Nestin monoclonal antibody (1:100; Santa Cruz); secondary antibodies were anti-mouse IgG and anti-goat IgG.. An alkaline phosphatase kit was used to detect AKP activity according to the instructions provided (Invitrogen).

For beating EB formation, after 7 days of spontaneously differentiation, EBs were plated onto Petri dishes coated with gelatin and cultured for up to 20 days; beating EBs were counted during the culture.

Karyotype analysis and sry gene detection

Standard G-banding chromosome analysis was carried out, and PCR was used to detect the *sry* gene of genomic DNA according to the

https://doi.org/10.1017/S0967199419000510 Published online by Cambridge University Press

Table 1. Primer sequence and annealing temperature

Genes	Primer sequences	Amplified fragment length/bp	Annealing temperature/°C
Oct4	sense5′-CACGAGTGGAAAGCAACTCA-3′; anti5′-AGATGGTGGTCTGGCTGAAC-3′	246	58
Nanog	sense5′-AAGTACCTCAGCCTCCAGCA-3′; anti5′-GTGCTGAGCCCTTCTGAATC-3′	163	62
Sox2	sense5′-CACAACTCGGAGATCAGCAA-3′; anti5′-CTCCGGGAAGCGTGTACTTA-3′	192	55
Klf4	sense5′-CTGAACAGCAGGGACTGTCA-3′; anti5′-GTGTGGGTGGCTGTTCTTTT-3′	218	60
E-cad	sense5′-CAAGGACAGCCTTCTTTTCG-3′; anti5′-TGGACTTCAGCGTCACTTTG-3′	165	60
Mest	sense5′-CAGAACCGCAGAATCAACCT-3′; anti5′-CGTCTTTGAGGAGCTTTTGG-3′	181	60
Ndn	sense 5′- GAAGAAGCACTCCACCTTCG-3′; anti5′-CCATGATCTGCATCTTGGTG-3′	164	61
Igf2	sense5′-GTCGATGTTGGTGCTTCTCA-3′ ; anti5′-AAGCAGCACTCTTCCACGAT-3′	195	60
Gtl2	sense5′- TTGAAGCTTGGAAAGCCAGT-3′; anti5′-CAGCCCATGGTATCACACAG-3′	239	60
Tss3	sense5′- AGACCTCCGACGAGATCCTT-3′; anti5′-CCTTGAGGATGGAGTGGAAA-3′	165	61
Ins2	sense5′-TTTGTCAAGCAGCACCTTTG-3′; anti5′-TCTACAATGCCACGCTTCTG-3′	206	60
Igf2r	sense5′-GCACCAAGATGAAGCAGTCA-3′; anti5′-ACATCCGGTAGCTGTTGGTC-3′	221	60
Ascl2	sense5′-TGAATGCAAGCTTGATGGAC-3′; anti5′-TGGAAGCCCAAGTTTACCAG-3′	226	62
H19	sense5′-CTCCTCCCCCTACCTTGAAC-3′; anti5′-CCTTGGAGCAGATTCCTGAG-3′	163	66
Nnat	sense5′-CCGGCAGAACTGCTCATCAT-3′; anti5′-CAGCTTCTGCAGGGAGTACC-3′	158	65
GAPDH	sense5′-AGGTCGGTGTGAACGGATTTG-3′; anti5′-TGTAGACCATGTAGTTGAGGTCA-3′	123	60

instructions provided. Primer sequences were as follows, mF: TGTGGTCCCGTGGTGAGA; mR: CAACAGGCTGCCAATAAA.

In vivo differentiation of AgES cells and production of chimeric mice

AgES cells were suspended at 1×10^7 cells/ml in Dulbecco's phosphate-buffered saline (DPBS) and injected subcutaneously into the neck and back of CD-1 nude mice. Four weeks after injection, teratomata were surgically dissected from the mice. Particular cell types were distinguished from the vicinal tissue cells according to their unique morphologies. Blastocyst injection was performed to produce chimeric mice. Fifteen to 20 AgES cells were injected per blastocyst, then the blastocysts were transferred into the oviducts of pseudopregnant CD-1 female mice. Chimeric offspring were delivered naturally, and examined for germline transmission.

Real-time PCR for pluripotential, mesodermal related, and imprinted genes

Total RNA from AgES and fES cells was extracted using TRIzol Reagent and treated with RNase-free DNase, following the manufacturer's instructions (Invitrogen). The absence of genomic DNA contamination was demonstrated by the lack of *Gapdh* amplification by PCR. Total RNA was reverse transcribed into cDNA. The cDNA pool was used to perform relative quantitation of gene expression using SYBR premix Ex *Taq*™ on a real-time PCR system (Roche 480) and according to the manufacturer's instructions. The expression of each gene was evaluated based on *Gapdh* expression in individual samples. All results were obtained from at least three independent experiments, and each assay was performed in triplicate. Specific primers and PCR conditions are listed in Table 1.

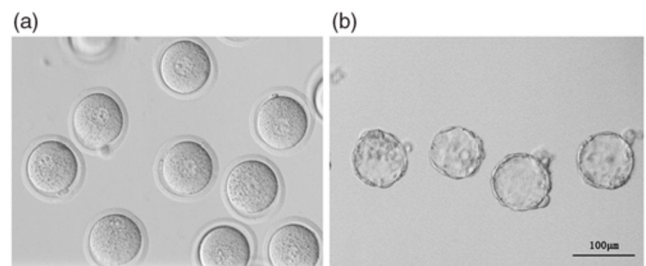


Figure 1 *In vitro* development of androgenetic embryos. (*a*) Pronuclear stage of androgenetic embryos. (*b*) Androgenetic blastocysts without zona pellucida. Bar = 100 μm.

Statistical analysis

Chi-squared test was used to evaluate the difference and a P-value < 0.05 was considered significant.

Results

Reconstruction of diploid androgenetic embryos

Diploid androgenetic embryos were produced by injecting enucleated MII oocytes with two heads of sperm. The survival rate after injection was 84.48%. A big male pronucleus was found in each activated embryo instead of dual pronuclei (Fig. 1a). Although the survival rate, pronuclear formation, and 2-cell development of androgenetic embryos were similar to the control intracytoplasmic sperm injection (ICSI) embryos, the blastocyst rate of androgenic embryos was obviously lower compared with ICSI embryos (20.24% vs 87.62%, $P < 0.05$) (Table 2), and androgenetic blastocysts had small ICMs (Fig. 1b).

https://doi.org/10.1017/S0967199419000510 Published online by Cambridge University Press

Table 2. Survival rate and *in vitro* development rate of androgenetic embryos and ICSI embryos

	Sum total	Survival rate (%)	Pronuclear (%)	2-cell (%)	Blastocyst (%)
AG	116	98 (84.48)	93 (94.90)	84 (90.32)	17(20.24)[a]
ICSI	121	109 (90.08)	106 (97.25)	97 (91.51)	85(87.62)[b]

[a,b]Values in the same columns with different alphabetic superscripts are significantly different ($P < 0.05$). AG: androgenetic embryo; ICSI, intracytoplasmic sperm injection.

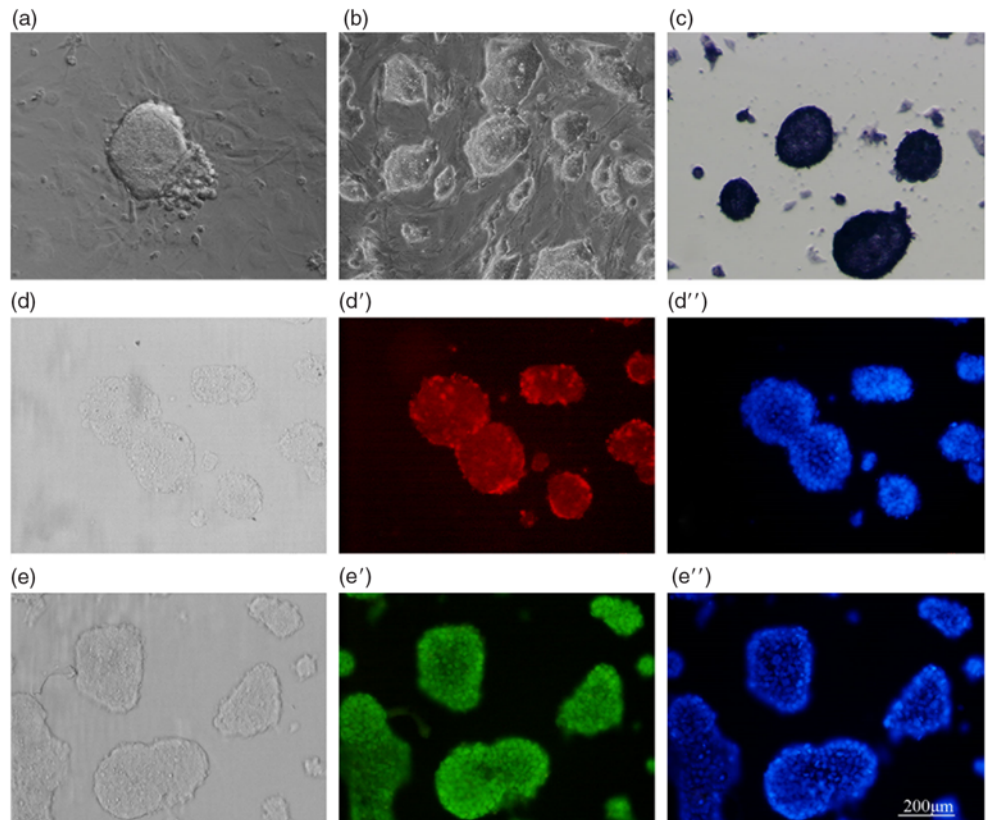


Figure 2. Morphological characteristics of AgES cells established from androgenetic embryos. (*a*) ICM-derived outgrowth. (*b*) AgES cells plated on feeder cells. (*c*) Alkaline phosphatase (AKP)-positive staining. (*d*, *e*) Morphology of AgES cells under a light microscope. (*d*, *e′*) Positive staining of SSEA-1 and OCT4. (*d″*, *e″*) Staining of DNA by Hoechst 33342. Bar = 200 μm.

Establishment and characterization of AgES cells

Outgrowths of androgenetic blastocysts were formed and adhered onto the feeder cells 4 days after plating. They had clear boundaries with some trophoblasts (Fig. 2a). AgES cells possessed the common morphology of fES cells (Fig. 2b). All AgES cells colonies showed high AKP activity (Fig. 2c) and were positive for pluripotency transcription factors OCT4 and ES-specific surface markers SSEA1 (Fig. 2d′, 2d′′, 2e′, 2e′′). AgES cells spontaneously formed simple EBs in the absence of anti-differentiation factors such as LIF that contained endoderm, mesoderm and ectoderm derivation structures (Fig. 3). Histological examination showed that the teratoma possessed tissues that had the three germ layers, including epidermis and nerve cells (ectoderm); respiratory and intestinal epithelium (endoderm); and cartilage and muscular tissue (mesoderm) (Fig. 4c–i).

The karyotype of AgES cells and generation of chimera

Chromosome G-banding results indicated that the AgES cell lines exhibited a normal karyotype of 40/XY (Fig. 5a) and detection of the *sry* gene was coincident with karyotype analysis (Fig. 5b). Chimeras were born and developed to adult, but no germline transmission offspring was obtained (Fig. 5c).

The expression of pluripotent and imprinted genes in AgES cells

Five pluripotency-related genes of stem cells were identified by real-time PCR, including *Oct4*, *Sox2*, *Nanog*, *Klf4* and *E-cad*. We confirmed that mouse AgES cells expressed many examined pluripotent genes at lower levels compared with those in a normal fES cell line except for *Sox2* (Fig. 5d). *Nanog* expression was especially low ($P < 0.01$). Interestingly, paternal imprinted genes were activated in derived AgES cells. *Gtl2* and *Igf2r* were expressed at levels equivalent to those in a normal fES cell line, while expression of *H19* remained deficient in AgES cells (Fig. 6a). The maternal imprinted genes *Igf2* and *Mest* exhibited increased expression, and others, *Nnat*, *Ins2* and *Ndn*, did not have significantly different expression compared with that in fES cells.

Beating EB formation

After 7 days spontaneously differentiation, EBs were plated onto Petri dishes coated with gelatin and cultured for up to 20 days.

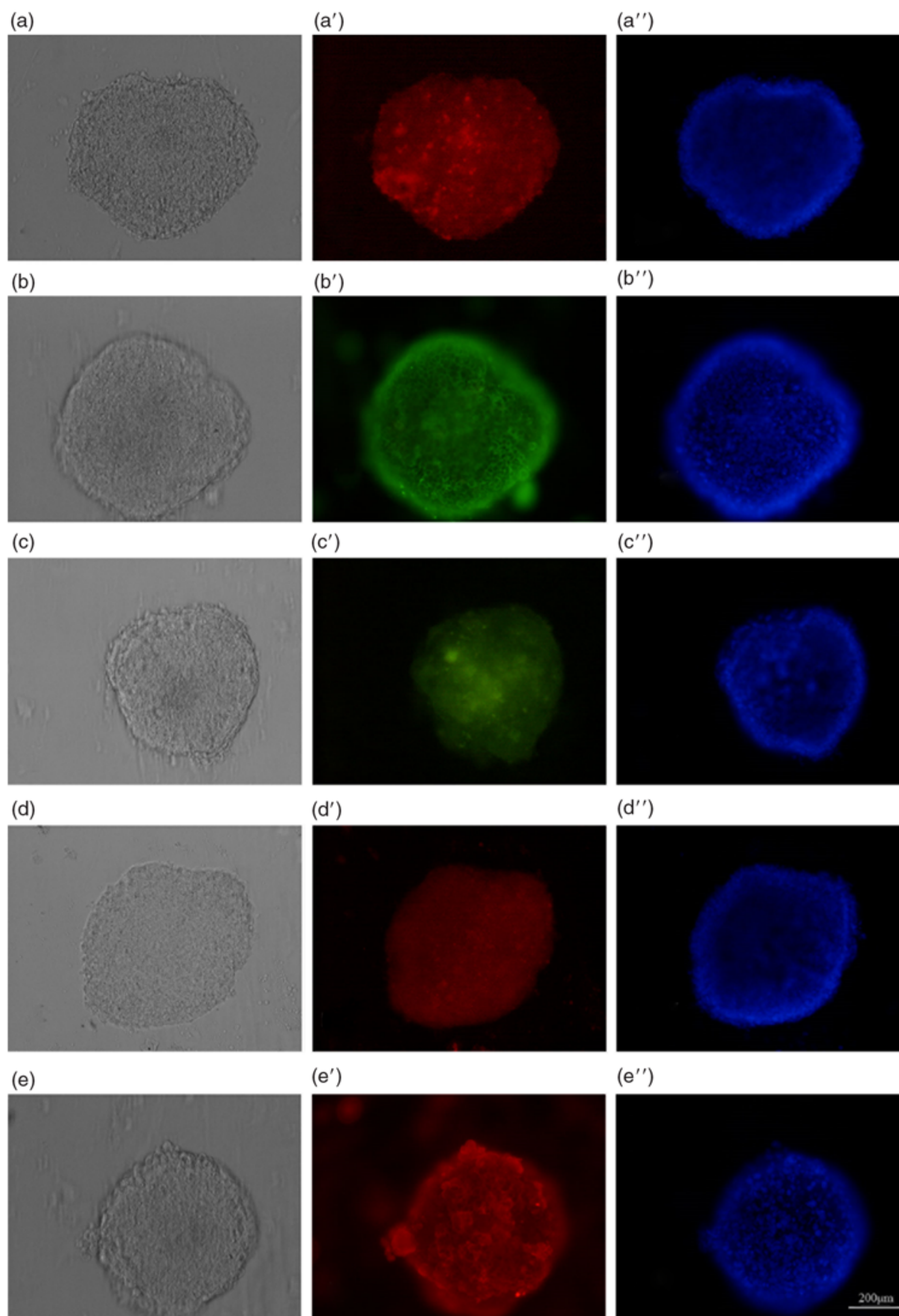


Figure 3. Embryoid body (EB) assays to identify differentiation of AgES cells *in vitro* by immunofluorescence stain. (*a*–*e*) Morphology of EBs by light microscope. (*a*′–*e*′) Positive for AFP, Nestin, NF-L, PECAM and α-SMA, respectively. (*a*″–*e*″) Staining of DNA by Hoechst 33342. Bar = 200 μm.

The rate of beating EB formation between AgES and fES cells did not show any significant difference (7.41% vs. 6.86%) (Table 3).

Discussion

We established a more effective approach to produce mouse androgenetic embryos by injecting enucleated MII oocytes with two sperms that resulted in higher rates of oocyte survival (88.48%) than other methods. Microfilament inhibiting agent CB has been widely used in nuclear transfer experiments to relax the cytoskeleton and enhance flexibility of the oocyte. In our preliminary study, short-time (<30 min) treatment with CB during ICSI manipulation significantly improved survival rates of mouse ICSI embryos, and did not impair the development of ICSI embryos (Hu *et al.*, 2012). Therefore, we performed two sperm injection in CZB-HEPES medium containing 5 μg/ml CB, which also improved the survival rate of androgenetic embryos. In this study, we confirmed that two sperm injection was effective and suitable for the production of a large number of such embryos at one time. Two sperms co-injected using very thin pipette resulted in the influx of a small amount of the medium into the oocytes, and this helped to minimize the damage to recipient

https://doi.org/10.1017/S0967199419000510 Published online by Cambridge University Press

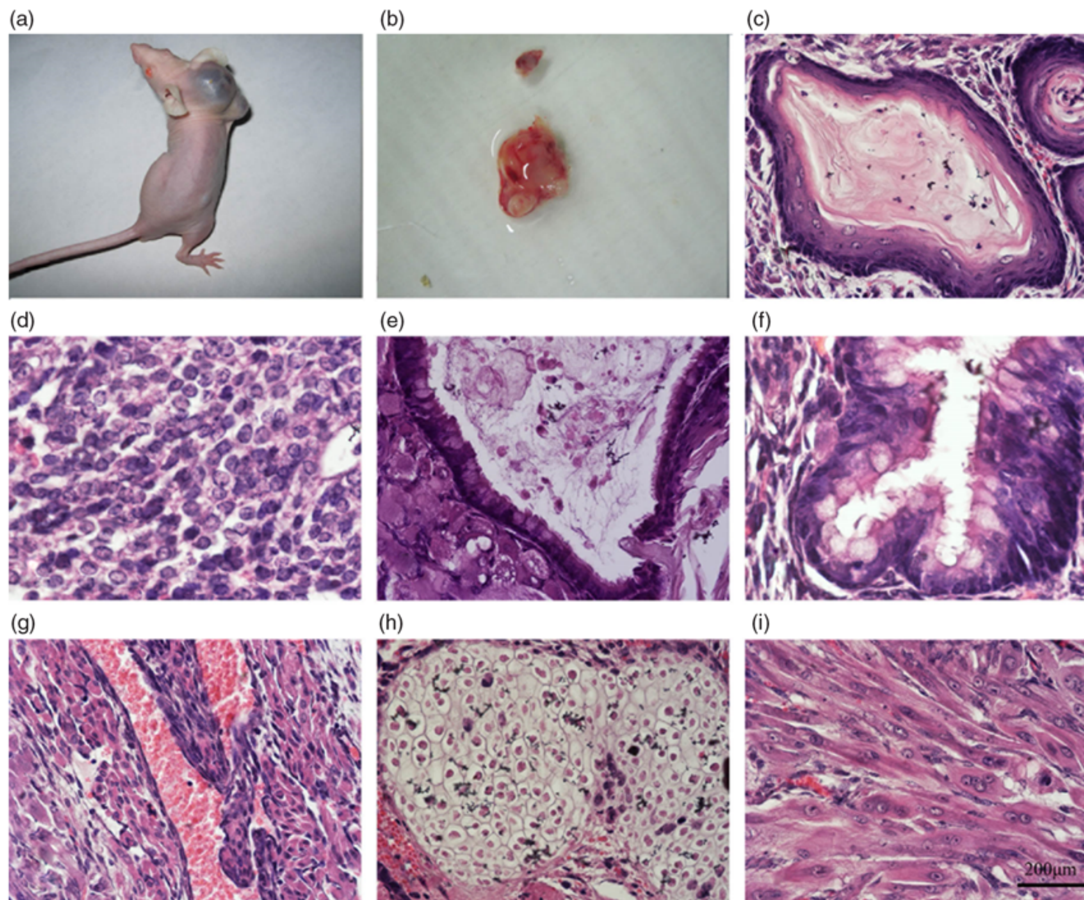


Figure 4. Characterization of AgES cells *in vivo*. Teratoma assays to determine differentiation properties *in vivo*. (*a*) Nude mouse at 4–6 weeks after subcutaneous injection of AgES cells. (*b*) Teratoma. (*c*) Epidermis (ectoderm). (*d*) Nerve cells (ectoderm). (*e*) Respiratory epithelium (endoderm). (*f*) Intestinal epithelium (endoderm). (*g*) Blood (from the host mouse). (*h*) Cartilage (mesoderm). (i) Muscular tissue (mesoderm). Bar in (i) represents 200 μm.

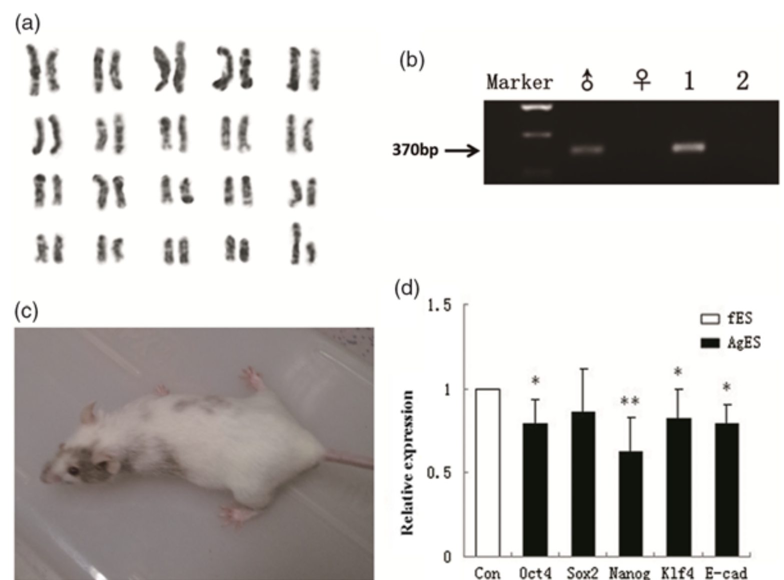


Figure 5. Karyotype analysis, chimera and pluripotent genes expression of AgES cells. (*a*) Karyotype analysis. (*b*) Detection of *sry* gene. Lanes are: marker, ♂: positive control, ♀: negative control, 1, 2: two AgES cell lines. (*c*) Chimera: AgES cells contributed to coat. (*d*) Pluripotent gene expression (*$P < 0.05$; **$P < 0.01$).

https://doi.org/10.1017/S0967199419000510 Published online by Cambridge University Press

Table 3. Comparison of the beating EB rates between AgES and fES cell differentiation *in vitro*

Cell line	Total EB	Beating EB (%)
AgES	108	8 (7.41%)
fES	204	14 (6.86%)

P > 0.05, there is no significant difference. EB, embryoid body.

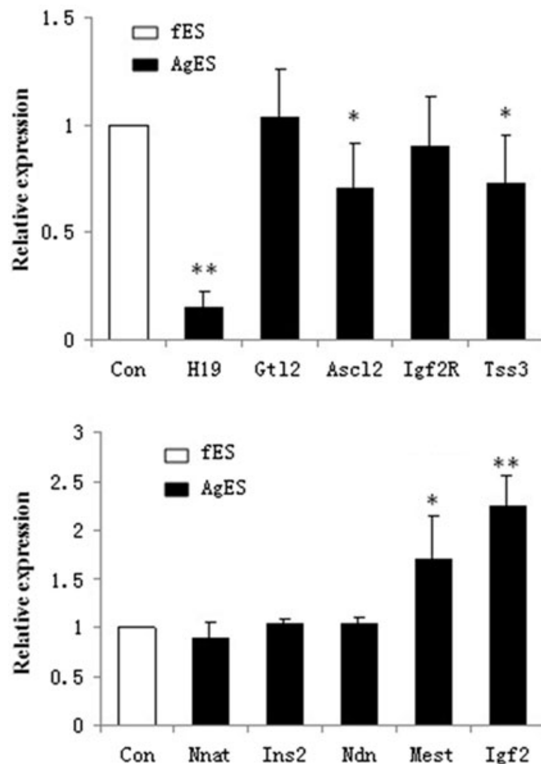


Figure 6. Expression of imprinted genes in AgES cells compared with fES cell counterparts by real-time PCR analysis. (*a*) Maternal expression of imprinted genes. (*b*) Paternal expression of imprinted genes. *Gapdh* served as the internal control. (**P* < 0.05; ***P* < 0.01).

oocytes. Furthermore, a big male pronucleus presented during the process of pronuclear formation of androgenetic embryos due to the small distance between the two sperm.

Parental origins genomic contribution is necessary for completion of embryonic normal development in the mammal. Androgenetic embryos, which are deficient in the expression of key maternal alleles, may have limited developmental potential. In the present study, compared with ICSI embryos, the blastocyst rate of androgenic embryos was obviously low. This result coordinated with previous studies that mouse androgenetic embryos had lower developmental ability even at the early preimplantation stages (Thomson and Solter, 1988). Furthermore, the two sperm were co-injected into oocytes randomly. Theoretically, the sex chromosome constitution of these eggs would be expected to be XX, XY and YY. However, androgenetic embryos containing YY chromosomes would be destined to arrest after a few cleavage divisions, lacking the ability to develop into blastocysts (Obata *et al.*, 2000). Consequently, only XX and XY androgenetic embryos were allowed to develop to the blastocyst stage (Miki *et al.*, 2009). Similar to the previous study, we established AgES cell lines that exhibited normal karyotypes of 40, XY and no cell line with the YY type was found.

Although androgenetic embryos lack full-term developmental potential, they can form blastocysts in which the derivation of AgES cells can be established and passaged *in vitro*. In this study, we successfully established AgES cells and confirmed that characterization of the AgES cells was similar to that of fES cells. However, some pluripotency genes had lower expression levels in AgES, indicating their potential limitation, and this might be responsible for the lower production rate and lack of germline transmission in the chimera. This limitation may be relevant to the aberrant expression of imprinting genes.

Imprints are established during gametogenesis and play important roles in fetal growth and development. As androgenotes have two sets of paternal genomes, the transcription levels of the paternally and maternally expressed genes in AgES cells should theoretically be double or be a negligible fraction of those in normal embryos. However, our real-time PCR analysis indicated that paternal imprinted genes, except for *H19*, had been activated in derived AgES cells. This result was different from findings by Zhao *et al.* (2010), who reported that *H19* expression was not lower in AgES cells. One possible reason was that there might be differences among cell lines and among mouse strains. In this study, we used B6D2F1 mice while Zhao and colleagues used 129S2 (SvPasCrl × C3H/HeNCrl) mice for AgES cell derivation. Furthermore, expression of maternal imprinted genes *Igf2* and *Mest* exhibited increased expression compared with fES cells, but the expression levels of *Nnat*, *Ins2* and *Ndn* were similar to those of fES cells. This result indicated that some imprinted genes were maintained even in the absence of the maternal genome. We presumed that the *in vitro* isolation and culture conditions might influence the expression of paternal and maternal imprinted genes in AgES cells.

The imprinted *Igf2*/*H19* locus plays a causative role in several embryonic growth disorders and various cancers (Nativio *et al.*, 2011). Maternal *Igf2* and paternal *H19* genes on the mouse distal chromosome 7 are co-ordinately expressed during embryonic development, due to shared tissue-specific enhancers (Hu *et al.*, 1997). Coincidently, we found a clear correlation between the expression of *H19* and *Igf2* in AgES cells. Excess concentrations of *Igf2* have been suggested to play a major role in defects in mouse chimeras made with AgES cells, for example these chimeras frequently died at the perinatal stage and exhibited a range of defects, the most noticeable being a pronounced overgrowth of rib cartilage (Mann and Stewart, 1991). Chimeras made with AgES cells or homozygous for an *Igf2* null mutation, do not develop rib cartilage hyperplasia, demonstrating the dependence of this defect on *Igf2* activity produced by androgenetic cells (McLaughlin *et al.*, 1997). *Igf2* transgenic mice appear to die *in utero*, suggesting that overexpression of *Igf2* is deleterious (Efstratiadis, 1994). Here, we did not find any defects such as previously observed in the AgES cell chimera. However, histological analysis of AgES cell teratomata showed that these cells possessed a large quantity of cartilage tissues.

Under normal culture conditions, AgES cells frequently formed spontaneously beating colonies (data not shown), however the rate of beating EB formation was not significant different between AgES and fES cells when performing *in vitro* differentiation. One paternal imprinted gene Dopa decarboxylase (*Ddc*) plays a critical role in heart development (Menheniott *et al.*, 2008), but did not show significant expression in AgES cells (Fig. S1). We

https://doi.org/10.1017/S0967199419000510 Published online by Cambridge University Press

assumed that parental imprinted genes might not maintain high expression during uniparental ES differentiation.

In conclusion, we established a more effective approach for producing mouse androgenetic embryos by injecting enucleated MII oocytes with two sperm in CB-containing medium. This protocol is suitable for the production of a large number of such embryos at one time with a high survival rate. Our findings demonstrated the activation of paternal imprinted genes and the maintenance of maternal imprinted genes in AgES cells. The mechanisms underlying this phenomenon are not clear. Allele-specific DNA methylation is thought to be a major factor in regulating genomic imprinting. Further experiments such as DNA methylation analysis may provide a clearer insight into this complex phenomenon.

Acknowledgements. We thank all the colleagues in our laboratory for useful help during experiments and preparation of the manuscript.

Ethical standards. This study was conducted in accordance with the Code of Practice Harbin Medical University Ethics Committees.

Financial support. This project was supported by the Overseas Scholar Research Funding of Education Department of Heilongjiang Province (grant no. 1151hz031), and the General Grant of Education Department of Heilongjiang Province (grant no. 11521107).

Conflicts of interest. The authors declare that no conflicting financial interests exist.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199419000510

References

Allen ND, Barton SC, Hilton K, Norris ML and Surani MA (1994) A functional analysis of imprinting in parthenogenetic embryonic stem cells. *Development* **120**, 1473–82.

Barad L, Schick R, Zeevi-Levin N, Itskovitz-Eldor J and Binah O (2014) Human embryonic stem cells vs human induced pluripotent stem cells for cardiac repair. *Can J Cardiol* **30**, 1279–87.

Beygo J, Mertel C, Kaya S, Gillessen-Kaesbach G, Eggermann T, Horsthemke B and Buiting K (2018) The origin of imprinting defects in Temple syndrome and comparison with other imprinting disorders. *Epigenetics* **13**, 822–8.

Chen FK, McLenachan S, Edel M, Da Cruz L, Coffey PJ and Mackey DA (2014) iPS cells for modelling and treatment of retinal diseases. *J Clin Med* **3**, 1511–41.

Chen W, Huang Q, Ma S and Li M (2019) Progress in dopaminergic cell replacement and regenerative strategies for Parkinson's disease. *ACS Chem Neurosci* **10**, 839–51.

Dinger TC, Eckardt S, Choi SW, Camarero G, Kurosaka S, Hornich V, McLaughlin KJ and Muller AM (2008) Androgenetic embryonic stem cells form neural progenitor cells *in vivo* and *in vitro*. *Stem Cells* **26**, 1474–83.

Eckardt S, Leu NA, Bradley HL, Kato H, Bunting KD and McLaughlin KJ (2007) Hematopoietic reconstitution with androgenetic and gynogenetic stem cells. *Genes Dev* **21**, 409–19.

Efstratiadis A (1994) Parental imprinting of autosomal mammalian genes. *Curr Opin Genet Dev* **4**, 265–80.

Hu JF, Vu TH and Hoffman AR (1997) Genomic deletion of an imprint maintenance element abolishes imprinting of both insulin-like growth factor II and H19. *J Biol Chem* **272**, 20715–20.

Hu LL, Shen XH, Zheng Z, Wang ZD, Liu ZH, Jin LH and Lei L (2012) Cytochalasin B treatment of mouse oocytes during intracytoplasmic sperm injection (ICSI) increases embryo survival without impairment of development. *Zygote* **20**, 361–9.

Jelinic P and Shaw P (2007) Loss of imprinting and cancer. *J Pathol* **211**, 261–8.

Khosla S, Dean W, Brown D, Reik W and Feil R (2001) Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod* **64**, 918–26.

Li ZK, Wang LY, Wang LB, Feng GH, Yuan XW, Liu C, Xu K, Li YH, Wan HF, Zhang Y, Li YF, Li X, Li W, Zhou Q and Hu BY (2018) Generation of bimaternal and bipaternal mice from hypomethylated haploid ESCs with imprinting region deletions. *Cell Stem Cell* **23**, 665–76, e664.

Mann JR and Stewart CL (1991) Development to term of mouse androgenetic aggregation chimeras. *Development* **113**, 1325–33.

McLaughlin KJ, Kochanowski H, Solter D, Schwarzkopf G, Szabo PE and Mann JR (1997) Roles of the imprinted gene Igf2 and paternal duplication of distal chromosome 7 in the perinatal abnormalities of androgenetic mouse chimeras. *Development* **124**, 4897–904.

Menheniott TR, Woodfine K, Schulz R, Wood AJ, Monk D, Giraud AS, Baldwin HS, Moore GE and Oakey RJ (2008) Genomic imprinting of Dopa decarboxylase in heart and reciprocal allelic expression with neighboring Grb10. *Mol Cell Biol* **28**, 386–96.

Miki H, Hirose M, Ogonuki N, Inoue K, Kezuka F, Honda A, Mekada K, Hanaki K, Iwafune H, Yoshiki A, Ishino F and Ogura A (2009) Efficient production of androgenetic embryos by round spermatid injection. *Genesis* **47**, 155–60.

Nativio R, Sparago A, Ito Y, Weksberg R, Riccio A and Murrell A (2011) Disruption of genomic neighbourhood at the imprinted IGF2-H19 locus in Beckwith–Wiedemann syndrome and Silver–Russell syndrome. *Hum Mol Genet* **20**, 1363–74.

Obata Y, Ono Y, Akuzawa H, Kwon OY, Yoshizawa M and Kono T (2000) Post-implantation development of mouse androgenetic embryos produced by in-vitro fertilization of enucleated oocytes. *Hum Reprod* **15**, 874–80.

Szabo P and Mann JR (1994) Expression and methylation of imprinted genes during in vitro differentiation of mouse parthenogenetic and androgenetic embryonic stem cell lines. *Development* **120**, 1651–60.

Thomson JA and Solter D (1988) The developmental fate of androgenetic, parthenogenetic, and gynogenetic cells in chimeric gastrulating mouse embryos. *Genes Dev* **2**, 1344–51.

Zhao Q, Wang J, Zhang Y, Kou Z, Liu S and Gao S (2010) Generation of histocompatible androgenetic embryonic stem cells using spermatogenic cells. *Stem Cells* **28**, 229–39.

Zhong C, Yin Q, Xie Z, Bai M, Dong R, Tang W, Xing YH, Zhang H, Yang S, Chen LL, Bartolomei MS, Ferguson-Smith A, Li D, Yang L, Wu Y and Li J (2015) CRISPR-Cas9-mediated genetic screening in mice with haploid embryonic stem cells carrying a guide rna library. *Cell Stem Cell* **17**, 221–32.

https://doi.org/10.1017/S0967199419000510 Published online by Cambridge University Press