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Derivation and characterization of putative embryonic stem cells from cloned rabbit embryos

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ABSTRACT

The present study aimed to establish embryonic stem (ES) cell lines, i.e., ntES cells, using rabbit blastocyst stage embryos cloned by somatic cell nuclear transfer. First, we investigated the development of cloned rabbit embryos reconstructed with normal fibroblasts and fibroblasts transfected with enhanced green fluorescence protein (eGFP). Blastocyst rates were 27.4% and 23.9%, respectively, for the embryos reconstructed with normal fibroblasts and fibroblasts transfected with eGFP compared with that from the parthenogenetic group (43.1%). One ntES cell line was established from embryos reconstructed with eGFP-transfected fibroblasts (1 of 17, 5.9%), and three ntES cell lines were derived from those with normal fibroblasts (3 of 17, 17.6%). All the ntES cell lines retained alkaline phosphatase activity and expressed ES cell–specific markers SSEA-4, Oct-4, TRA-1-60, and TRA-1-81. The pluripotency was further confirmed by reverse transcription–polymerase chain reaction analyses of *Oct-4*, *Nanog*, and *Sox-2* expressions in ntES cell lines. The differentiation capacity of ntES cells was also examined *in vitro* and *in vivo*, by which these ntES cell lines were able to differentiate into all three germ layers through embryoid bodies and teratomas. In conclusion, it is apparent that the efficiency of ntES cells derived using eGFP-transfected donor cells is lower than that with nontransfected, normal fibroblasts donor cells. Similar to those from parthenogenetic embryos, all ntES cell lines derived from cloned rabbit embryos are able to express pluripotency markers and retain their capability to differentiate into various cell lineages both *in vitro* and *in vivo*.

1. Introduction

Rabbits (*Oryctolagus cuniculus*) are classical laboratory animals with many advantages over rodents and other species and have been used for studying human diseases including hypertension [1,2], myocardial infarction [3–5],

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bone and cartilage disorders [6,7], arteriosclerosis [8–10], diabetes [11–13], and so forth. With the progress in molecular technology, they have been genetically modified using most recently developed tools including TALEN [14] and CRISPR-Cas9 [15,16] to model several neuronal disorders [17].

Embryonic stem (ES) cells are derived from the inner cells mass of blastocysts, which possess the ability to differentiate into all the cell lineages of animal body. These cells can be potentially used to study and treat various degenerative diseases [18,19]. Disadvantage of embryo-derived ES cells including those from fertilized embryos (f-ES cells) and parthenotes (p-ES cells) is that they are genetically divergent from the donor or the recipient. After transplantation, various degrees of immune response from the recipient are prominent in rejecting the transplanted ES cells [20–23]. One solution to the immune rejection would be to generate isogenic ES cells from the patient [20]. The genetically reprogrammed somatic cells, also known as induced pluripotent stem (iPS) cells, exhibit functional similarities to ES cells, and they have been successfully produced by many laboratories in several species including humans [24–26]. However, more in-depth study on iPS cell biology is indispensable before they can meet the demands for future clinical applications. Moreover, iPS cells differ from ES cells in many ways, such as DNA methylation [27], epigenetic status [28], gene expression profile [29], and response to induced neural differentiation [30]. An ideal alternative to circumvent the immune rejection problem would be to generate patient-specific ES cell lines by somatic cell nuclear transfer (SCNT) [20].

In addition, study on rabbit SCNT has been reported and early-stage embryonic cells from preimplantation embryos were used as donor cells that can support the full-term development of the cloned pubs [31]. Live births of rabbit clones were reported using different types of somatic donor cells including cumulus cells [32–34], fibroblasts [35], and mesenchymal stem cells [17]. However, how to increase rabbit ES (rES) cell cloning efficiencies have not been systemically studied. In our previous study, we found that young oocytes retrieved from preovulatory follicles (follicular oocytes) were better in quality with a better efficiency to support the development of cloned rabbits [36,37]. To continue the study, we further attempt to establish rabbit ES cells from SCNT embryos using follicular oocytes and protocols that we used to derive f-ES and p-ES cells [38,39].

In the present study, we have first successfully generated high-quality cloned blastocysts using donor cells from rabbit ear fibroblasts to establish ntES cell lines, whose morphology, pluripotency, and differentiation potentials are comparable with those of f-ES and p-ES cells.

2. Materials and methods

2.1. Reagents and animals

The care and use of animals for embryo recovery complied with the guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University, Taiwan, ROC (IACUC Permit NO. 96–72). Chemicals and reagents used were mainly purchased from Sigma–Aldrich Co., Ltd., unless otherwise mentioned. The severe combined immunodeficiency (SCID) mice were purchased from BioLasco Taiwan Co., Ltd., and raised in accordance with the IACUC guidelines of National Chung Hsing University. When the sacrifice of animals was essential, all efforts were made to minimize suffering animals.

2.2. Generation and culture of rES cell lines form SCNT embryos

Approximately 12 hours after hCG injection, recipient oocytes were recovered from both the preovulatory follicular oocytes and the ovulated oocytes using flushing medium supplemented with 3.36 g/L $NaHCO_3$ (S5761), 10% fetal bovine serum (FBS; Gibco 26140–079), 20 mM HEPES (H3375), and 1% antimycotics (Gibco 15240). Cumulus cells from *in vivo*–matured rabbit oocytes were removed by repeated pipetting in 0.1% (vol/vol) hyaluronidase in Dulbecco's phosphate-buffered solution (DPBS).

For enucleation, oocytes were transferred to a droplet of HEPES-TCM 199 containing 5 μg/mL cytochalasin B and 10% FBS under inverted microscope (Nikon) equipped with micromanipulation system (Narishige). Oocytes were first hold with holding pipette and the zona pellucida was cut nearby the position of the first polar body (PB) by a fine glass needle. The cytoplasm adjacent to the first PB was then squeezed out, along with the first PB, from the cut with the same glass needle. Success of enucleation was checked by Bisbenzimide (Hoechst 33342) staining for 5 minutes and observed using an inverted microscope equipped with epifluorescence. For nuclear transplantation, donor cells are trypsinized (0.05% trypsin) and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS for 1 hour before transfer. Single donor cells were inserted into the perivitelline space of the enucleated oocytes by micromanipulators. Electro cell fusion (3.2 kv/cm, 20 μs, and three pulses) was applied to fuse the donor cell with the cytoplasm of the reconstructed embryos. Successfully fused embryos were then incubated in the activation medium containing 6-dimethylaminopurine (2 mM) and cycloheximide (5 μg/mL) for 1 hour followed by culturing in the Menezo's B2 medium (Laboratoire CCD, Paris, France) supplemented with 2.5% FBS in an incubator (39 °C, 5% CO_2 with humidified air) for 4 days on the basis of our previous protocols [40].

2.3. Derivation and culture of rES cell lines

After 4 days of culture, cloned rabbit blastocysts were plated on the mitomycin C–inactivated mouse embryonic fibroblast monolayers in the ES cell medium. The medium consisted of 81.5% DMEM (D7777), 15% fetal calf serum (26140–079; Gibco), 4 mM L-glutamine (G8540), 0.5% nonessential amino acids, 0.1 mM α-mercaptoethanol (M7522), and 1000 U/mL murine leukemia inhibitory factor (ESG 1107; Chemicon, Temecula, CA, USA). After embryos attaching to the feeders, culture medium was changed every other day [40]. Seven days after culture, the inner cell mass outgrowths were picked and passaged to fresh feeder cells in the ES cell medium.

For propagation, rES cells were incubated with 0.05% trypsin–EDTA (030515B; Biological Industries, Kibbutz Beit Haemek, Israel) for 1 minute and then washed and resuspended by repeated pipetting into small clumps. These rES cell clumps were then transferred onto fresh feeder cells in the rES cell medium, which was changed every day.

2.4. Labeling of alkaline phosphatase activity and pluripotency markers

The ES cell lines for marker detection were grown on the six-well dish and rinsed with DPBS before fixation in 4% paraformaldehyde (P-6148) solution.

For alkaline phosphatase (AP) staining, fixed ES cells were rinsed with DPBS three times and then stained with AP solution for 15 to 30 minutes for color development. To the AP-staining buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), 0.25 M Trizma maleate (T3128), 0.008 M $MgCl_2$ (M-8266), 0.17 g/L Fast-Red TR salt (F8764), and 0.4 g/L α-naphthyl phosphate (N7255) were added. The stained cells were observed under an inverted microscope after washing with DPBS.

For specific protein marker expressions, after fixation in 4% paraformaldehyde for 2 to 4 days, ES cell colonies were rinsed, then washed with DPBS for 10 minutes, and stained for SSEA-4 (Cat. No. 4304; Chemicon, Darmstadt, Germany), Oct4 (Cat. No. SC8628; Santa Cruz Biotechnology, Inc., TX), and keratan sulfate antigens (TRA-1-60, Cat. No. ab16288; Abcam, Cambridge, MA, USA and TRA-1-81 Cat. No. ab16289; Abcam) as previously described [41]. Nuclei were labeled by staining with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/mL) in Tris-buffered saline with Tween-20 (DPBST), followed by twice DPBST washes before examined by epifluorescence microscopy [41].

2.5. Gene expression analyses for rES cells and embryoid bodies

A small aliquot of the pluripotent cells and embryoid body (EB) suspension were collected and subjected to reverse transcription–polymerase chain reaction (RT-PCR), screening for gene expressions of the pluripotency and the three germ layers (Table 1). Total RNAs were extracted using a total RNA extraction kit (RT050; Geneaid) as previously described [41]. For PCR reaction, a total volume of 25-μL reaction solution containing 2 μL of the RT reaction mix were added along with 18.7 μL of ddH_2O, 2.5 μL of 10× PCR buffer (2.5 mM $MgCl_2$), 0.5 μL of dNTPs (10 mM each; R0182; Fermentas), 0.3 μL of *Taq* DNA polymerase (5 U/μL; Geneaid), 0.5 μL forward primer (10 μM), and 0.5 μL reverse primer (10 μM). After reaction, 4 μL of amplicons was separated on a 1.5% agarose gel and visualized under UV light with ethidium bromide staining. Primer sequences including sense and antisense, annealing temperatures, and the expected product sizes are listed in Table 1.

2.6. In vitro and in vivo differentiation of ntES cells

2.6.1. EB formation

Induction of EBs and RNA extraction were performed as previously described [41,42]. Briefly, rES cell colonies were lifted from feeders with dispase (1 mg/mL; 17105041; Gibco) at 37 °C and split into small clumps by gentle pipetting and then cultured in suspension for 4 to 8 days in an EB medium consisting of 90% DMEM, 10% FBS, and 2 mM L-glutamine. The medium was changed every 3 days until EBs were recovered for histologic examination. For adherent differentiation, ES cells were digested with 1 mg/mL collagenase and resuspended in rES cell culture medium without leukemia inhibitory factor for 4 days until EB formation, and EBs were plated onto gelatin-coated six-well dishes and cultured for 10 days. Expressions of the three germ layer marker genes (Table 1) were assessed by RT-PCR.

For adherent and spontaneous *in vitro* differentiation, Day 7 EBs were mechanically dissociated and plated directly to gelatin-coated four-well dishes in ES cell medium without inhibitors. After 1 week of culture, cells were subjected to immunocytochemical analysis for lineage differentiation and were screened for the expression of differentiation markers troponin I (for mesoderm; GTX113028; GeneTex), neurofilament light (for ectoderm; AB9568; Millipore), and pancreatic and duodenal homeobox 1 (PDX1, for endoderm; 06–1379; EMD Millipore).

Table 1
Primer sequences and conditions for PCR analyses.

Target genes	Primers	Sequences (5′ → 3′)	Annealing temperature (°C)	Product size (bp)
OCT4	Forward	CTCGGCGCAGCGCACGCCCTGGAG	66	575
	Reverse	CAGCTGGTCGCGCAGCGGGCCCAG		
Nanog	Forward	CCCAGCTGTGTGTGCTCAA	52	382
	Reverse	CCAGGCTTGGGAGTACCAGG		
Sox2	Forward	CTGCACATGAAGGAGCACCC	56	228
	Reverse	CTGCATCATGCTGTAGCTGC		
Pax6	Forward	CATGCAGAACAGTCACAGCGG	60	414
	Reverse	CCCATCTGTTGCTTTTCGCTA		
Desmin	Forward	AGCAGGAGATGATGGAATAC	55	276
	Reverse	TCCAGCTTCCGGTAGG		
GATA4	Forward	CTCAGAAGGCAGAGAGTGTG	59	281
	Reverse	CCGCATTGCAAGAGGCCTGG		
GAPDH	Forward	GGAGCCAAACGGGTCATCATCTC	62	233
	Reverse	GAGGGGCCATCCACAGTCTTCT		

Abbreviation: PCR, polymerase chain reaction.

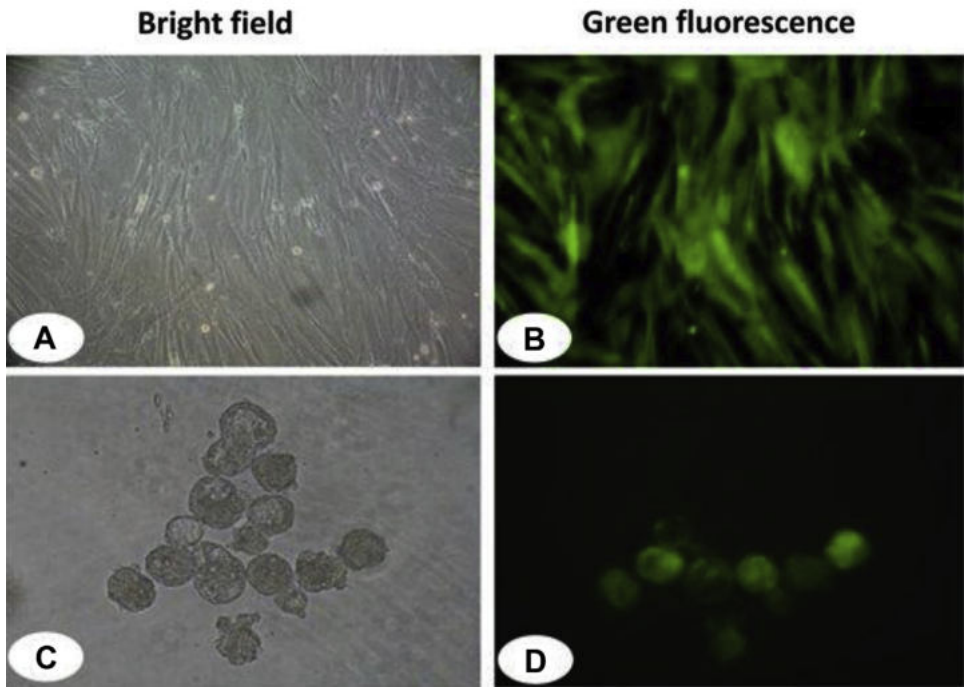

Fig. 1. Morphologies of cloned embryos derived from fibroblasts transfected with *eGFP* and the outgrowths 4 days after cultured on mouse embryonic fibroblast feeders. (A and B) Morphology of fibroblasts under bright-field (A) and green epifluorescence (B) microscopes; cloned embryos are rabbit SCNT-eGFP (C and D). eGFP, enhanced green fluorescence protein.

2.6.2. Induction of teratoma formation

In vivo differentiation potential was determined by induction of teratoma formation in the SCID mouse. Approximately 5×10^6 morphologically undifferentiated nt-rES cells were subcutaneously injected into the intramuscular layer of 7-week-old SCID mice. Six weeks after injection, teratomas were harvested and fixed in 4% paraformaldehyde for histologic examination [41,42].

2.7. Statistical analysis

Percentile data were normalized by arcsine transformation before statistical analysis by ANOVA procedure in SAS, version 9 (SAS Institute, Cary, NC, USA). The results are presented as means ± SEM. Statistically significant level was considered at $P < 0.05$.

Table 2
Development of parthenogenetic, somatic cell nuclear transferred, and SCNT–eGFP rabbit embryos.

Treatments	No. embryos, n	No. cleavage, n (%)	No. blastocyst, n (%)
PA	102	68 (66.7)	44 (43.1)
SCNT (normal)[a]	62	42 (67.7)	17 (27.4)
SCNT (*eGFP*)[b]	71	54 (76.1)	17 (23.9)

Abbreviations: eGFP, enhanced green fluorescence protein; PA, parthenogenetic; SCNT, somatic cell nuclear transferred.
[a] Cloned embryos reconstructed with donor cells from rabbit ear normal fibroblasts.
[b] Cloned embryos reconstructed with donor cells from rabbit *eGFP* transgenic fibroblasts.

3. Results

3.1. In vitro development of cloned embryos and establishment of rabbit ntES cell lines

The *in vitro* development of cloned rabbit embryos reconstructed with fibroblasts transfected with enhanced green fluorescence protein gene (*eGFP*; Fig. 1A, B) and normal fibroblast cells were compared in this study. After reconstruction, cloned embryos were cultured and cleavage (Day 2) and blastocyst (Day 5) rates were observed. As listed in Table 2, the cleavage rates from fibroblasts transfected with *eGFP*, normal fibroblasts, and parthenogenetic embryos were 76.1%, 67.7%, and 66.7%, respectively. Blastocyst rates for fibroblasts transfected with *eGFP*, normal

Table 3
Comparison of the derivation efficiency of rabbit ES cell lines by different embryos (SCNT [normal] vs. SCNT [*eGFP*]) with removal of the trophoblast cells from the primary inner cell mass (ICM) colony.

Treatments	No. of blastocysts plated	No. of ICM colonies formed (%)	Established rES cell lines (%)
SCNT (normal)[a]	17	11 (64.7)^A	3 (17.6)^A
SCNT (eGFP)[b]	17	6 (35.2)^B	1 (5.9)^B

Numbers with different superscript letters (A and B) in the same column differ ($P < 0.05$).
Abbreviations: eGFP, enhanced green fluorescence protein; ES, embryonic stem; SCNT, somatic cell nuclear transfer.
[a] Cloned embryos reconstructed with donor cells from rabbit ear normal fibroblasts.
[b] Cloned embryos reconstructed with donor cells from rabbit ear fibroblasts transfected with *eGFP*.

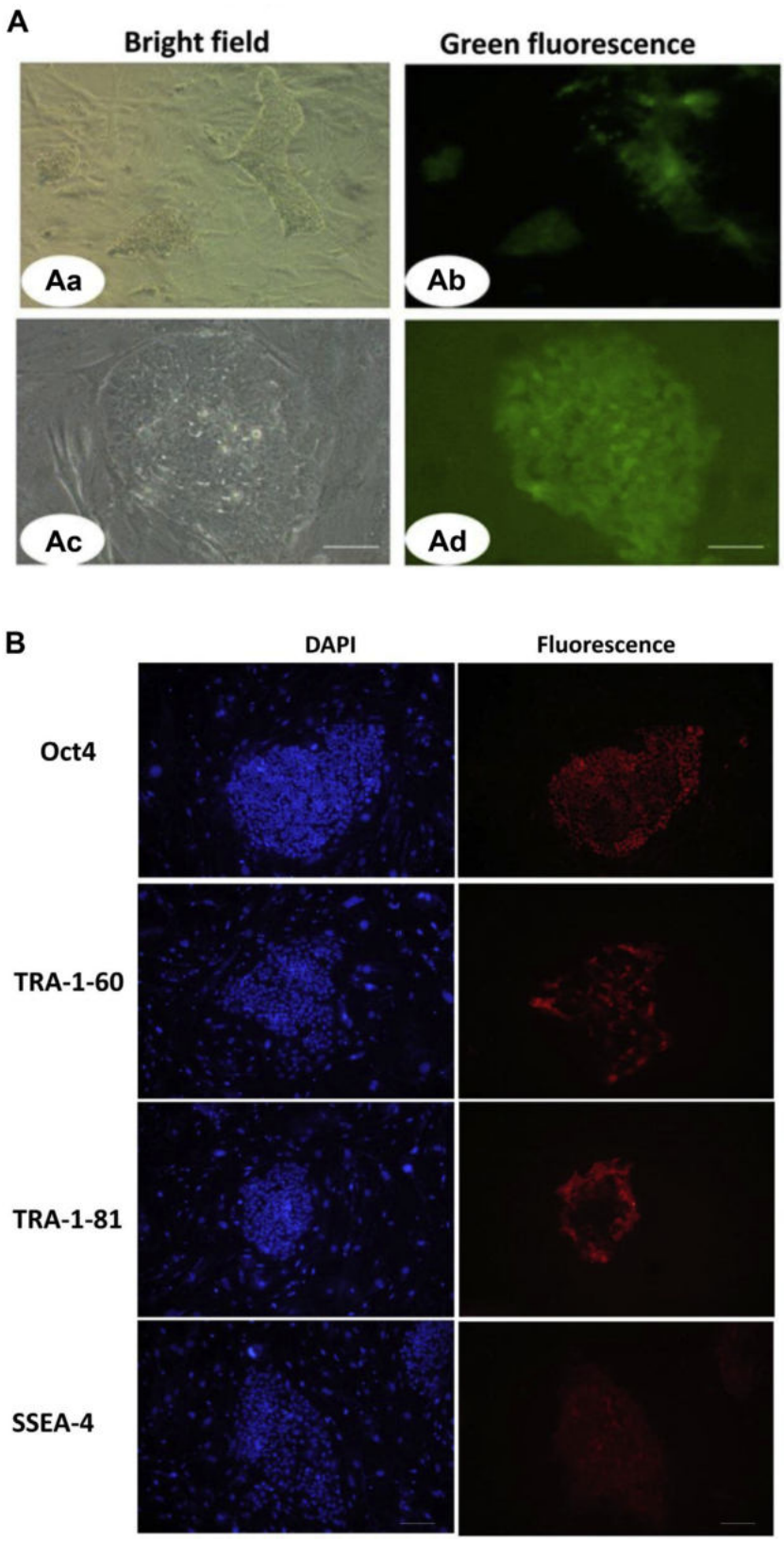

Fig. 2. Morphology of ES cell line, nt-1 (A) and the appearance under epifluorescence microscopy (Aa and Ab). The ES cell colonies are shown at passage 12 after being seeded on mouse embryonic fibroblast feeders (Ac and Ad). The nt-1 also expressed *SSEA-4*, *Oct4*, the keratan sulfate antigens *TRA-1-60* and *TRA-1-81* detected by immunocytochemical staining (B). ES, embryonic stem.

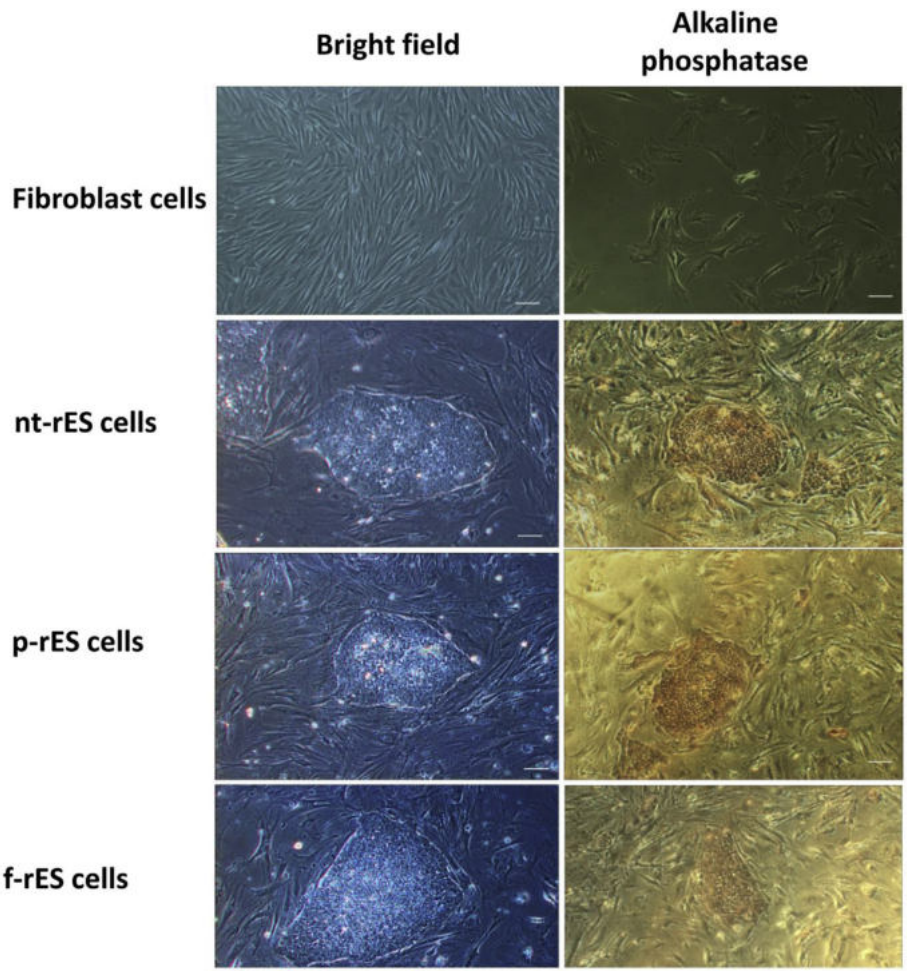

Fig. 3. The morphology of fibroblasts, ntES cells, f-ES cells, and p-ES cells. Rabbit ES cell colonies are shown at passage 15 after seeding on mouse embryonic fibroblast feeders in the culture medium for 3 days. The alkaline phosphatase activity is positive in undifferentiated ntES cells, f-ES cells, and p-ES cells but not in fibroblast cells. Scale bar = 100 μm. ES, embryonic stem; f-ES, ES cells from fertilized embryos; p-ES, ES cells from parthenotes.

fibroblasts, and parthenogenetic embryos were 23.9%, 27.4%, and 43.1%, respectively (Table 2, Fig. 1C, D).

Blastocysts cloned from fibroblasts transfected with *eGFP* (n = 17) and normal fibroblasts (n = 17) were plated on mouse embryonic fibroblast feeders in rES cell medium at 37 °C in 5% CO_2 with humidified atmosphere. One rabbit ntES cell line (nt-1) was obtained from the cloned embryos derived with *eGFP*-transfected fibroblasts that colonized 2 days after plating (Table 3; Fig. 2A, B). These ES cells maintained normal morphology, survived repeated passaging and frozen-thawed procedures, and able to continued proliferation without apparent changes in morphology up to 20 passages. With the same procedure, three rabbit ntES cell lines were also derived from cloned embryos reconstructed with normal fibroblasts.

3.2. Expression of pluripotency markers and karyotyping

Basically, the newly derived ntES cells were of morphology similar to f-ES cells and p-ES cells, with compact colonies and distinct boundaries. When the AP activities of ntES cells, f-ES cells, and p-ES cells were compared, their expression patterns were similar among cell lines as shown in Figure 3. Immunofluorescence staining also demonstrated that the ntES cells expressed ES cell–specific markers SSEA-4, OCT4, Nanog, TRA-1-60, and TRA-1-81 (Fig. 4A). Moreover, RT-PCR results confirmed that the ntES lines expressed the pluripotency genes *Oct-4*, *Nanog*, and *Sox2* similar to those in f-ES cells and p-ES cells (Fig. 4B). Cytogenetic analyses of the ntES cells (ntES cells, passage 18) indicated that 80% of the cells examined were of normal karyotype ($2n = 44$) (Fig. 4C).

3.3. In vitro and in vivo differentiation of rES cell lines

All the ntES, f-ES, and p-ES cells formed EBs readily in suspension culture in the presence of serum (Fig. 5A). The RT-PCR results showed that EBs could be derived from all the three types of ES cells (ntES, f-ES, and p-ES cells) and expressed the three representative germ layer marker genes, *PAX6* (for ectoderm), *Desmin* (mesoderm), and *GATA4* (endoderm) (Fig. 5B) using Day 9.5 rabbit embryos

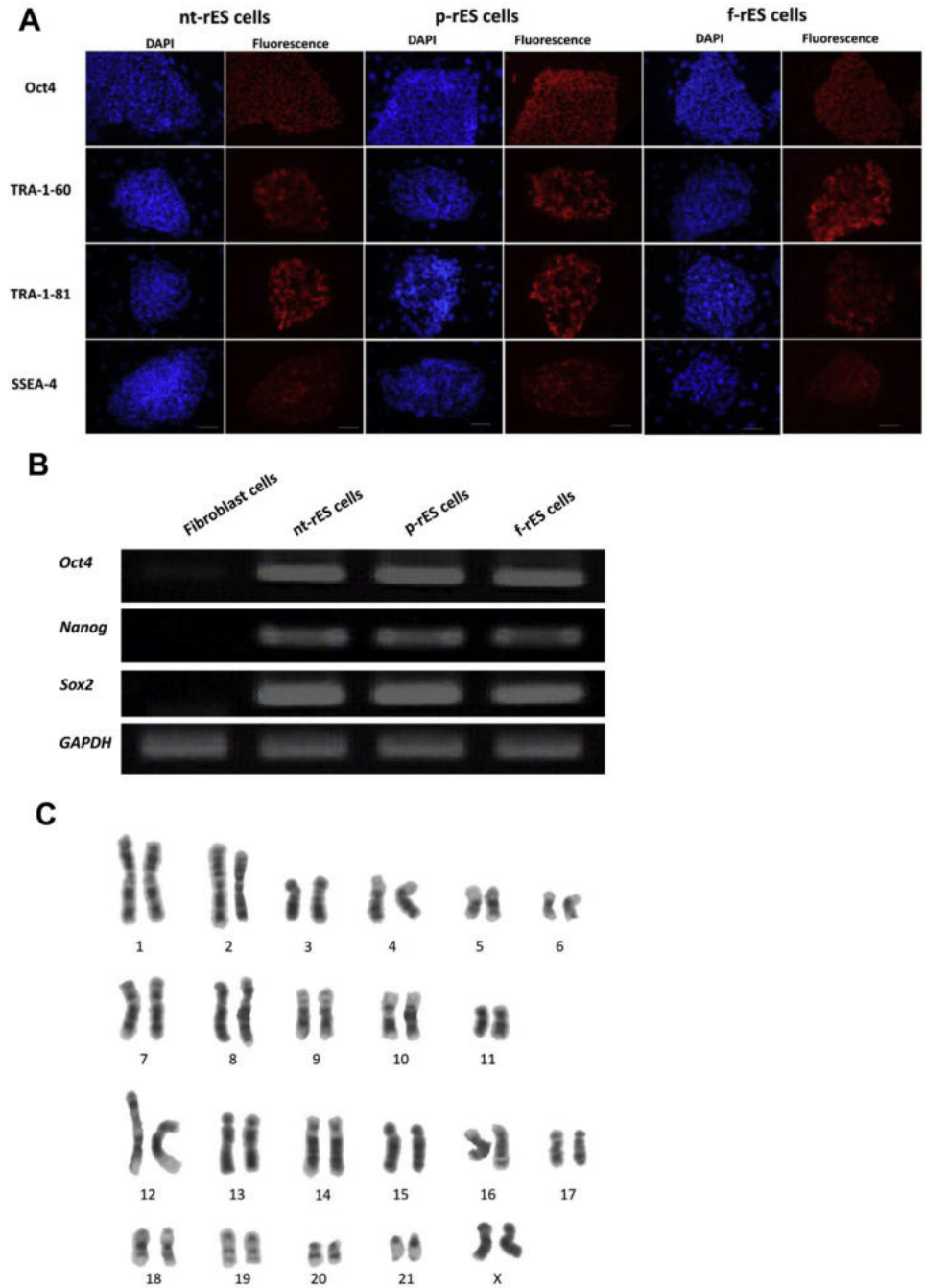

Fig. 4. Immunocytochemical and RT-PCR analyses of marker expression by fibroblast, ntES, f-ES, and p-ES cells. These three types of ES cell lines express markers recognized by antibodies against *Oct-4*, *SSEA-4*, *TRA-1-60*, and *TRA-1-81*; DAPI: for nuclear staining; scale bar = 100 μm (A). *Oct-4*, *Nanog*, and *Sox2* are all expressed in ntES cells, f-ES cells, and p-ES cells markers confirmed by RT-PCR (B). The G-banding karyotype of ntES cells examined are of normal ploidies ($2n = 44$) (C). DAPI, 4',6-diamidino-2-phenylindole; ES, embryonic stem; f-ES, ES cells from fertilized embryos; p-ES, ES cells from parthenotes; RT-PCR, reverse transcription–polymerase chain reaction.

as the positive control. *In vitro* differentiation capacity of ntES cells was tested by plating EBs in culture; when Day 7 EBs (Fig. 6B) were disaggregated and grown in gelatin-coated surface, they attached to culture dishes 1 day after culture (Fig. 6C). The neuron-like cells containing obvious Nissl bodies (Fig. 6D–E, arrow) with epithelium-like morphology (Fig. 6F) were observed 3 days after culture. Differentiated ntES cells expressed three germ layer marker proteins (Fig. 6G–I), as seen in the neuron-like cells expressing cell lineage markers NFL (ectoderm); PDX1 and troponin-1 expressions are for endodermal and mesodermal cell lineages, respectively.

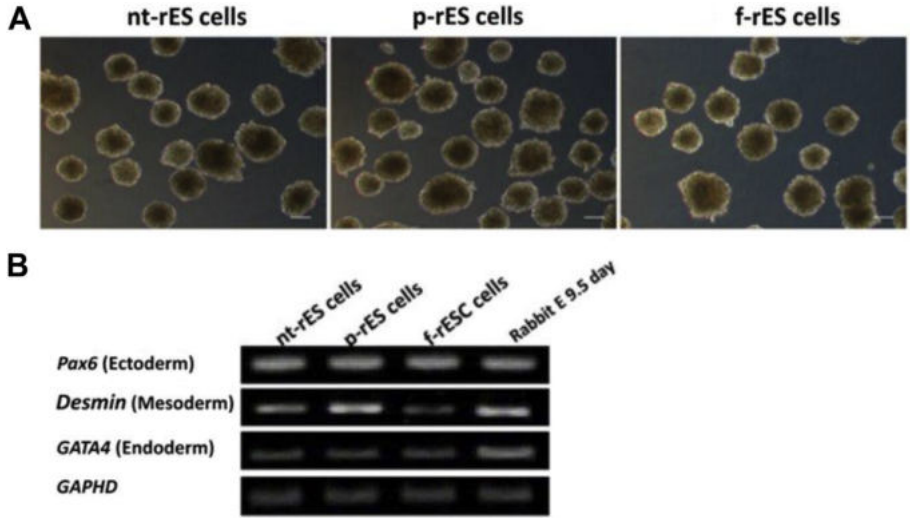

Fig. 5. The rabbit ES cell lines (ntES, f-ES, and p-ES cells) are able to form EBs. (A) EBs derived from the three representative ES lines (ntES, f-ES, and p-ES cells) expressing three marker genes specifically for each germ layers: (B) *PAX6* (ectoderm), *Desmin* (mesoderm), and *GATA4* (endoderm). EB, embryoid body.

To test the *in vivo* differentiation potential of ntES cells, four ntES cell lines were injected under the dorsal skin of SCID mice. A SCID mouse administered with the ntES cells line (nt-4) formed a teratoma 6 weeks after injection. The teratoma was removed from the SCID mouse and measured 3 × 1.5 × 1.0 cm in size (Fig. 7A, B). Histologic examination showed that the derived teratoma revealed a variety of tissue types including apocrine glands (secreting proteinous materials, Fig. 7C), hair follicles (ectoderm, Fig. 7D), cartilage and skeletal muscles (mesoderm, Fig. 7E, F, inset), mucous glands, and ciliated, pseudostratified columnar epithelia (endoderm) (Fig. 7G, H), which originated from the three respective germ layers.

4. Discussion

Cloning technology has been well established and applied in more than 20 animal species, such as cattle, mice, goats, pigs, cats, rabbits, horses, rats, dogs, and so forth [43–51]. Nevertheless, the efficiency and viability of cloned embryos are generally low, and the biology underlying this process remains obscure [52]. It has been known that multiple factors, such as donor cell types and passage numbers, contribute to this inefficiency.

In a previous study, we reported that the efficacy to clone rabbit embryos could be improved using young oocytes [36,37] because young oocytes contain higher maturation-promoting factor and mitogen-activated protein kinase activities than do the aged oocytes [53]. These kinases are associated with the induction of nuclear envelope breakdown and chromosomal modifications fundamental to molecular reprogramming of an activated oocyte [54]. In the present study, we used 12 hours post-hCG–injected oocytes as recipient ooplasm, so called the young oocytes, by which similar blastocyst rates (23%–27%) to the previous study [37] were obtained and, subsequently, gave rise to ntES cell lines. Young oocytes contain factors that enhance nuclear reprogramming and support reprogrammable epigenetic elements in their nuclei up to the ES cell stage [34,36,37].

In porcine SCNT, fetal fibroblasts were the most effective donor cell type to be reprogrammed compared with adult fibroblast, cumulus, and oviductal cells [55]. In rabbits, however, using fresh cumulus cells as donor nuclei provides a better *in vitro* developmental potentiality to cloned embryos than that of using fetal fibroblasts [56], although one report also showed a similar blastocyst development between these two donor cell types (34% vs. 33%) [57]. Another study on *in vivo* development of cloned embryos using fibroblasts and cumulus cells as nuclear donors showed that the pregnancy rate from cumulus cells (60%) is superior to that from fibroblasts (33.3%) [37].

To circumvent the immune rejection and ethical problems of ES cells derived from fertilized embryos, the establishments of ES cell lines from cloned embryos have been reported in primates [20], mice [58], pigs [59,60], and humans [61,62] and one recent study from rabbits [63]. In the present study, ntES cell lines were successfully established on the basis of our previous protocol [41,42]. In the past, typical expression of rabbit ES cell markers, such as Oct4, Nanog, Sox2, SSAE-4, TRA-1-60, and TRA-1-81, had been reported [41,42,64,65]. To date, there is only one report on rabbit ntES cells available; this particular study reported that rabbit ntES cells behaved very similarly to f-ES cells and p-ES cells, and these cells expressed pluripotency markers Oct-4, EBAF2, FGF4, and TDGF1 but not SSEA-1, SSEA-3, SSEA-4, TRA-1-10, and TRA-1-81 [63]. In this study, we established four rabbit ntES cell lines, but three lines could not be maintained infinitively and lost the colonial morphology of ES cells after cultured for more passages. The rabbit ntES cell lines established here were propagated without differentiation for a prolonged period retaining AP activity and expressing pluripotency markers including Oct4, SSAE-4,

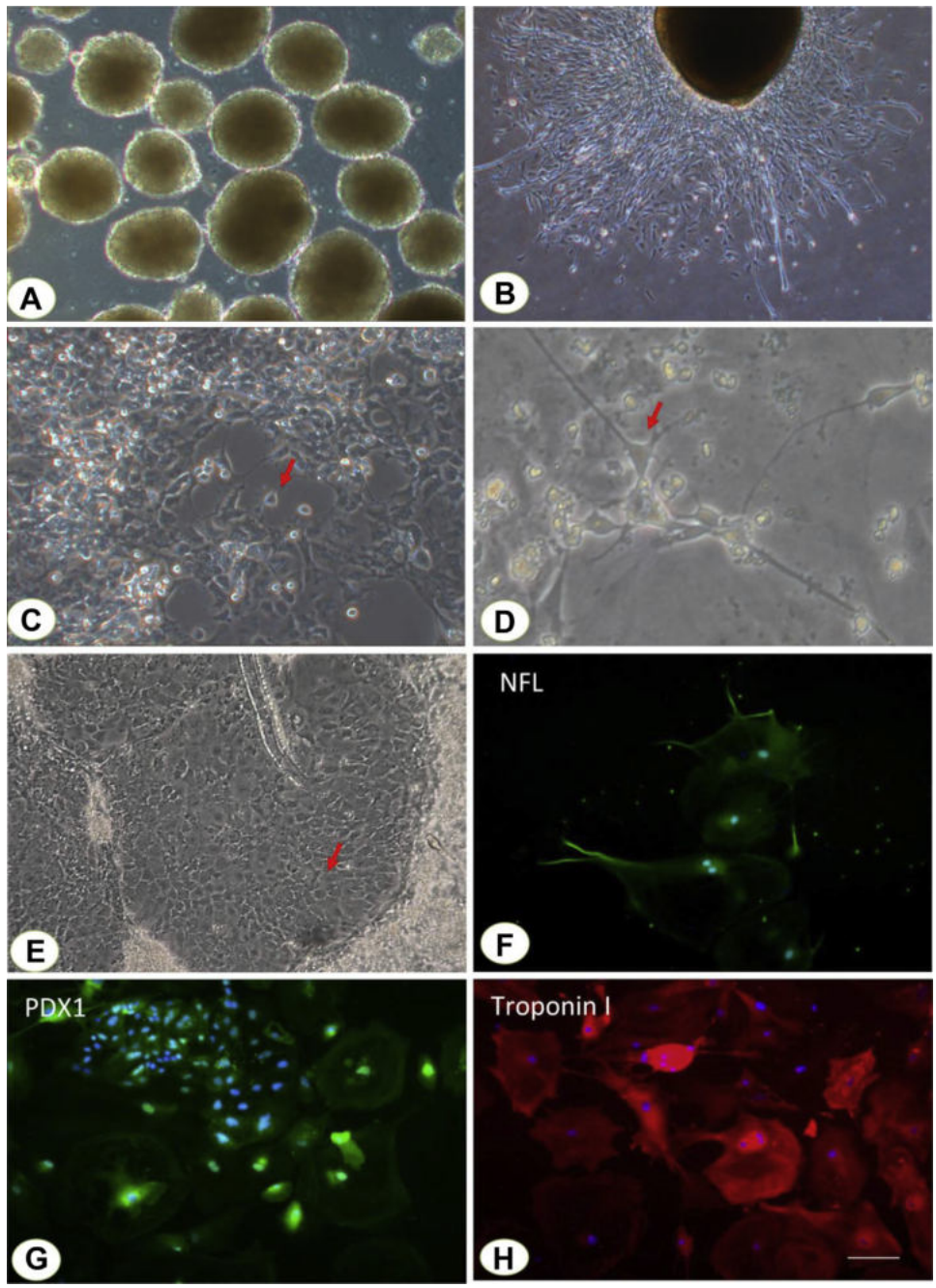

Fig. 6. *In vitro* differentiation capacity of rabbit ntES cells after induction of EB formation. (A) Light microscopic images of undifferentiated ES cell colonies and EBs cultured for 7 days. (B) EB attachment to gelatin-coated surface of culture dishes. (C and D) The neuron-like cells with obvious Nissl bodies (red arrows). (E) The morphology of epithelial-like cells (red arrow). (F–H) Confirmation of the expression of differentiation marker NFL (ectoderm), PDX1 (endoderm), and troponin-1 (mesoderm) from differentiated cells by the immunocytochemical analysis. Scale bar = 100 μm. EB, embryoid body; ES, embryonic stem.

TRA-1-60, and TRA-1-81. They are also very similar to our previously established p-ES and f-ES cells [41,42], which had other additional gene expressions including *Oct4*, *Nanog*, and *Sox2* [65]. Different cell lines might present distinct cellular characteristics, such as their antigens and pluripotency markers. This explains the differential marker expression profiles in different rabbit ES cell lines among laboratories.

Differentiation potential is one important parameter to evaluate the ability of the established pluripotent stem cells. Our previous studies showed that rabbit f-ES cells and p-ES cells had the potential to give rise to all cells and tissue types of the three germ layers both *in vitro* (EB formation) and *in vivo* (teratoma formation) [41,42], similar to the reports from some other groups [64–66]. Consistently, the

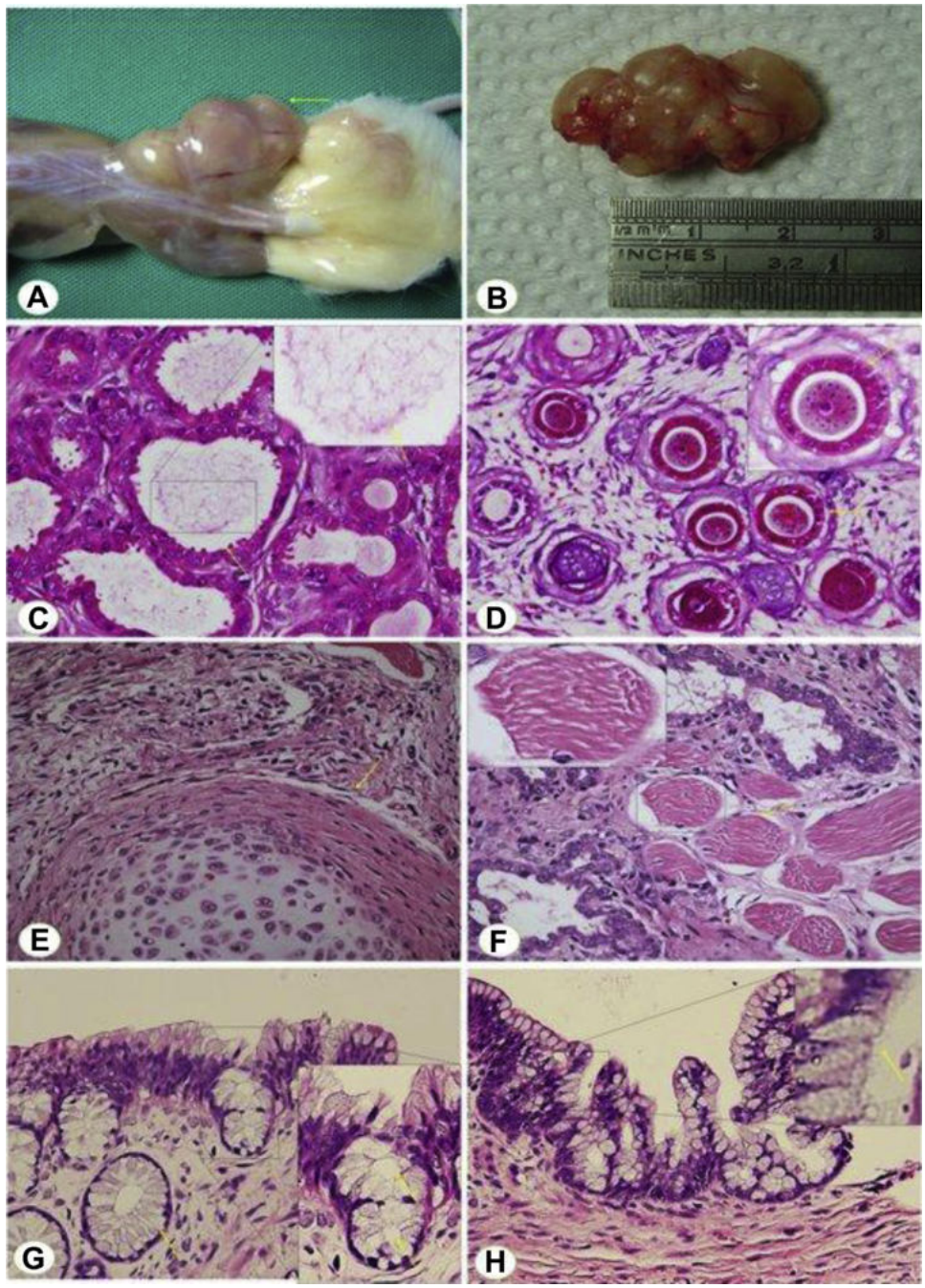

Fig. 7. Teratoma formation of rabbit embryonic stem cells derived from cloned embryos 7 weeks after intramuscular injection into non-obese diabetic (NOD)–SCID mice. (A and B) A representative NOD–SCID mouse was treated with rabbit ntES cells (nt-4, passage 15) and the teratoma (3 × 1.5 × 1.0 cm) was induced. Panels C–H show the cross-section (hematoxylin and eosin staining) of the whole teratoma. All cell type representatives of the three germ layers can be identified and shown by arrows. (C) Apocrine gland that secretes proteinous materials and (D) hair follicles (ectoderm), (E) cartilage, (F) the skeletal muscle shown in the inset (mesoderm), (G) mucous glands, and (H) ciliated, pseudostratified columnar epithelium (endoderm). SCID, severe combined immunodeficiency.

established ntES cells in the present study also exhibited a similar morphology, expression profiles of pluripotency markers, EB formation capability (Fig. 6), and teratogenicity (Fig. 7).

In conclusion, we have successfully established ntES cell lines from cloned blastocyst embryos by SCNT in rabbits. These cells have a similar morphology and characteristics to those rabbit ES cells derived from fertilized and parthenogenetic embryos. For future perspectives, rabbit ntES cell lines are useful as a model system for the study of human diseases and applications in cell transplantation therapy.

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