RESEARCH AND DEVELOPMENT OF PORCINE EMBRYONIC STEM CELLS IN TAIWAN

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ABSTRACT

Embryonic stem (ES), embryonic germ (EG), and induced pluripotent stem (iPS) cells are capable of self-renew, and limitlessly proliferating in vitro with undifferentiated characteristics. They are able to differentiate in vitro, spontaneously or responding to suitable signals, into cells of all three primary germ layers. Consequently, these pluripotent stem cells will be valuable sources for cell replacement therapy in numerous pathologies. However, the promise of human ES and EG cells are cramped by the ethical argument about destroying embryos and fetuses for cell line creation. Moreover, there are still carcinogenic risks existing toward the goal of clinical application for human ES, EG and iPS cells. Therefore, a suitable animal model for stem cell study will benefit the further development of human stem cell technology. The development of embryonic stem cell lines in livestock species, especially in pigs has been achieved in Taiwan. Porcine ES cell lines established have been demonstrated to be pluripotent and can be directed to differentiate into cholinergic and dopaminergic neurons. The studies involving the transplantation of porcine ES cells in animal models of Parkinson's disease, spinal cord injure and periodontal furcation defects have been attempted.

Key words: Pigs, Embryonic Stem Cells, Animal Model.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells firstly isolated from preimplantation mouse embryos (Evans and Kaufman, 1981; Martin, 1981). Thereafter, isolation of putative pluripotent ES cell lines other than murine ES (mES) cells has been attempted in the hamster (Doetschman *et al.*,1988), mink (Sukoyan *et al.*, 1992), rabbit (Giles *et al.*, 1993; Intawicha at al., 2009), rat (Ouhibi *et al.*, 1995; Ueda *et al.*, 2008), primate (Thomson *et al.*, 1995; Thomson *et al.*, 1996), pig (Notarianni *et al.*, 1990; Chen *et al.*, 1999), cattle (Evans *et al.*, 1990; Saito *et al.*, 2005), sheep (Handyside *et al.*, 1987; Saito *et al.*, 2005) and Human (Thomson *et al.*, 1998).

ES cells are able to self-renew and proliferate continuously *in vitro* with the undifferentiated characteristics. In responding to suitable conditions, they can be induced to differentiate into cells of all three primary germ layers. There were many remarkable results in directing the human ES (hES) cells differentiation into neuronal cells

(Thomson *et al.*, 1998; Wichterle *et al.*, 2002), pancreatic β cells (Assady *et al.*, 2001; Moritoh *et al.*, 2003), and cardiomyocytes (Kehat *et al.*, 2001). These results reveal a potential for clinical application of hES cells in the treatment of diseases such as Parkinson's disease, spinal cord injure, diabetes, and heart diseases.

Domestic swine are demonstrated to be very similar to the human in anatomic, immunologic, and physiologic characteristics; and the sizes of their organs are fairly comparable to those of human (Phillips and Tumbleson, 1986; Prelle *et al.*, 1999). Moreover, swine have been demonstrated as excellent animal models in therapeutics development for various human diseases, including congenital heart disease (Swindle *et al.*, 1992), hypertension (Zambraski *et al.*, 1992), organ transplantation (Hall *et al.*, 1986; Flye, 1992), pharmacology, and toxicology (Kurihara-Bergstrom *et al.*, 1986; Feletou and Teisseire, 1992). Although the establishment of pluripotent ES cell lines from domestic species is much more difficult than that in murine species, porcine ES (pES) cell lines have been successfully derived from inner cell mass of the blastocysts (Chen *et al.*, 1999; Li *et al.*, 2003, 2004; Vassiliev *et al.*, 2010). The pES cells were very similar to hES cells in many characteristics, including colony morphology, feeder-dependent and refractory to leukemia inhibitory factor (LIF) in culture, and expression of stem cell markers (Chen *et al.*, 1999; Brevini *et al.*, 2007; Yang *et al.*, 2009; Vassiliev *et al.*, 2010). Therefore, the study of pES cells might serve as an excellent model in development of regeneration medicine in human.

Derivation of pES cell lines from *in vivo* embryos

Isolation and establishment of pES cell lines were firstly accomplished by adapting the ES cell culture system for murine species, with minimum modification (Chen *et al.*, 1999). Briefly, zona-free intact porcine blastocysts and inner cell mass (ICM) collected from sows on day 6 to 8 after the first day of estrus and breeding (day 0) were cultured on mitosis-inactive STO feeder in ES-cell culture medium (ESM, Chen *et al.*, 1991) consisted of Dulbecco's modified eagle medium supplemented with L-glutamine, β-2-mercaptoethanol, MEM non-essential amino acids, adenosine, guanosine, cytidine, uridine, thymidine, antibiotics and fetal bovine serum. The outgrowth was individually detached from the attached blastocyst or ICM, enzymatically or mechanically dissociated into 10- to 15-cells clumps and replaced to fresh feeders in ESM. A total of 14 putative pES cell lines with stable undifferentiated morphology were successfully isolated and established from intact 56 EHB, which resulted in 48 attached outgrowths and 37 primary colonies (Table1). Also, 2 primary pES cultures were derived from isolated ICM from 30 IHB and 40 LHB. Several important characteristics of the pluripotential embryonic stem cells were demonstrated in these cells, including morphology (Fig. 1), normal karyotype, expression of AP activity, in vitro pluripotency based on the behavior of spontaneous and induced differentiation, formation of embryonal body after suspension culture, the ability to direct the development of enucleated metaphase II oocytes to the blastocyst stage after nuclear transplantation, and the capability to participate in the formation of a chimeric offspring.

Table 1. Putative pES cell lines established from porcine *in vivo* blastocysts

Embryonic stage	No. of embryos cultured	No. of embryos attached	No. of primary colonies formed	No. of cell lines obtained
Early blastocyst	16	9	2	0
Expanding blastocyst	32	13	10	0
Early hatched blastocyst	56	48	37	12
Intermediate hatched blastocys	47	41	35	0
Late hatched blastocyst	45	18	10	0

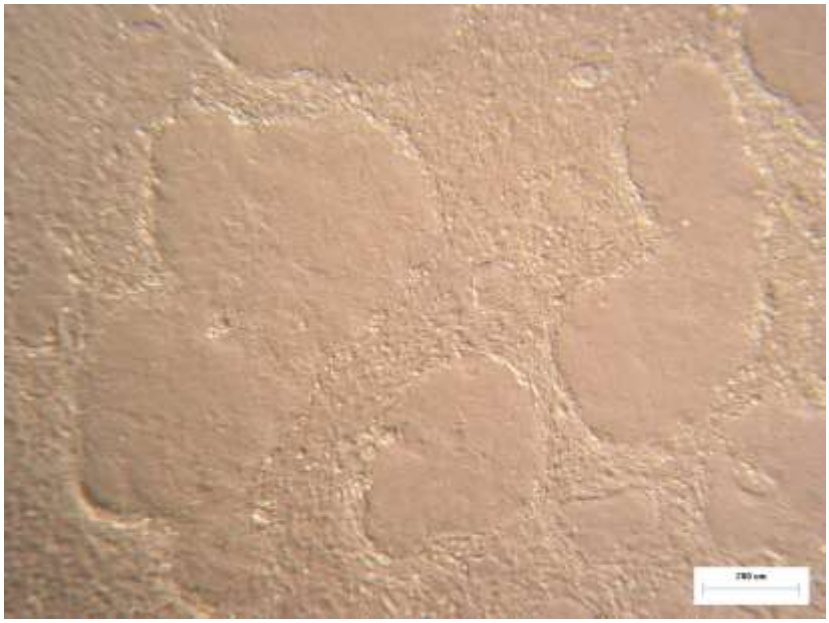

Fig. 1. Morphology of porcine embryonic stem cells cultured on STO feeders.

Porcine ES cells are much similar to hES than mES cells

The development of non-human ES cell lines is crucial for research and development of stem cell therapies and regeneration medicine. Among the species in which establishment of ES cell lines have been declared, the capacity of germline transmission has been demonstrated only in ES cells isolated in mice (Evans and Kaufman, 1981; Martin, 1981) and rats (Ueda *et al.*, 2008). Murine and human ES cell lines have been the two major animal models in the investigation of regeneration medicine. However, considerable differences have been demonstrated between hES and mES cells, especially the gene expression profiles of pluripotency maintenance and differentiation (Przyborski *et al*., 2003; Li *et al*., 2004; Ginins *et al.*, 2004; Darr *et al.*, 2006). In addition, the expressions of pluripotent cell markers were quite different between these species. SSEA1 can be detected in the mES cells but not in hES cells, while SSEA3, SSEA4, TRA-1-60, TRA-1-81 and GCTM-2 are found only in the hES cells (Pera *et al.*, 1988; Henderson *et al.*, 2006). These differences would impair the translation of information gained from mES cells directly to hES cells. Swine has been demonstrated as an excellent animal model in therapeutics development for various human diseases. Furthermore, porcine products including insulin, heart valves and skin have been widely applied to human (Schuurman and Pierson, 2008). Therefore, it will be of great advantage in the production of ESC lines from swine which are more physiologically similar and more relevant for clinical translation to the human compared to these of mice (Vassiliev *et al.*, 2010).

In the previous study, we found that the pES cells had a colony morphology very similar to human ES, and they were both feeder-dependent and refractory to leukemia inhibit factor (LIF) in culture (Chen *et al.*, 1999). They both expressed Oct-4, alkaline phosphatase (AP), SSEA-3/4, TRA 1-60, and TRA 1-81, but not the SSEA1 that was characterized to mouse ES cells (Yang *et al*., 2009). Therefore, the pES cells could provide as a suitable preclinical animal model in the study of regenerative medicine in therapeutic approaches.

Derivation of traceable pES cell Lines for application in regeneration studies

For the application of pES cells as a model in the study and development of regeneration medicine in human, a traceable pES cell line is far beyond necessary, especially in therapeutic approaches involving cell transplantation. Three stable GFP-expressing pES (pES/GFP$^+$) cell lines were established by electroporation with pAAV-hrGFP Control Plasmid (Stratagene, CA, USA.) using 2 DC pulses of 150 V/cm for 10 ms (Yang *et al.*, 2009). Despite repeated freezing and thaw and subcultures for more than 90 passages over 20 months, they retained the defined characteristics of typical ES cells, including continuous proliferation with undifferentiated

status (Fig. 2), maintenance of a normal karyotype, expression of pluripotent cell markers, formation of EBs upon suspension culture (Fig. 3), and the capacity to differentiate into neural and cardiomyocytic lineages (Fig. 4).

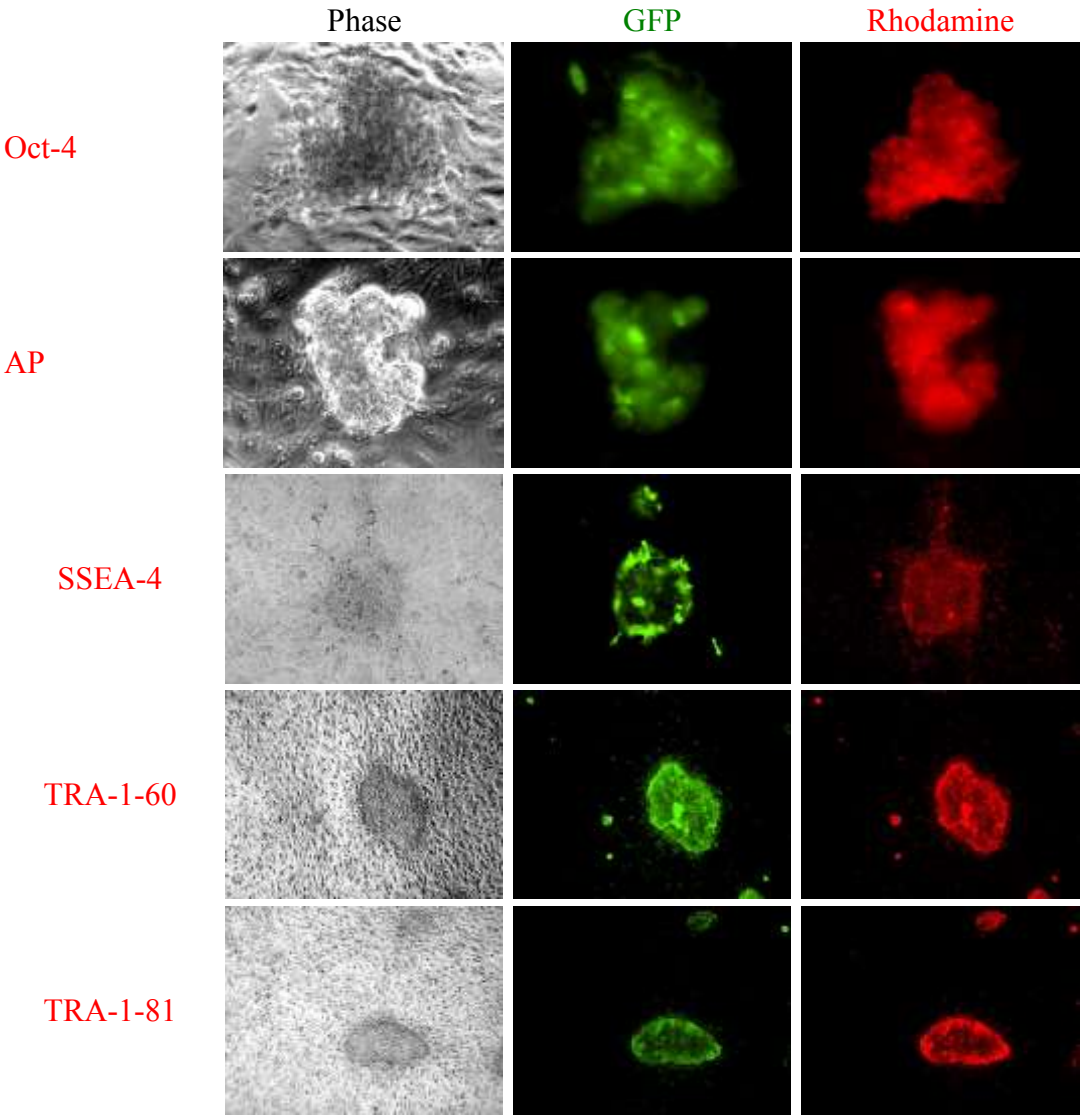

Fig. 2. Expression of Oct-4, AP, SSEA-4, TRA-1-60 and TRA-1-81 of the pES/GFP^{+} colonies determined by immunocytochemistry staining.

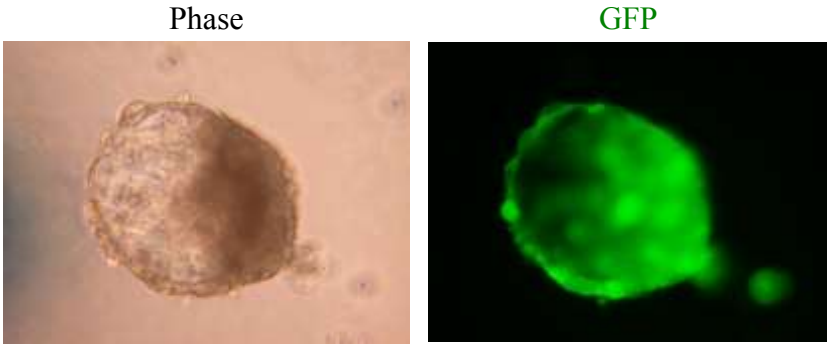

Fig. 3. The embryoid body derived from pES/GFP$^+$ colonies after 12 days of suspension culture.

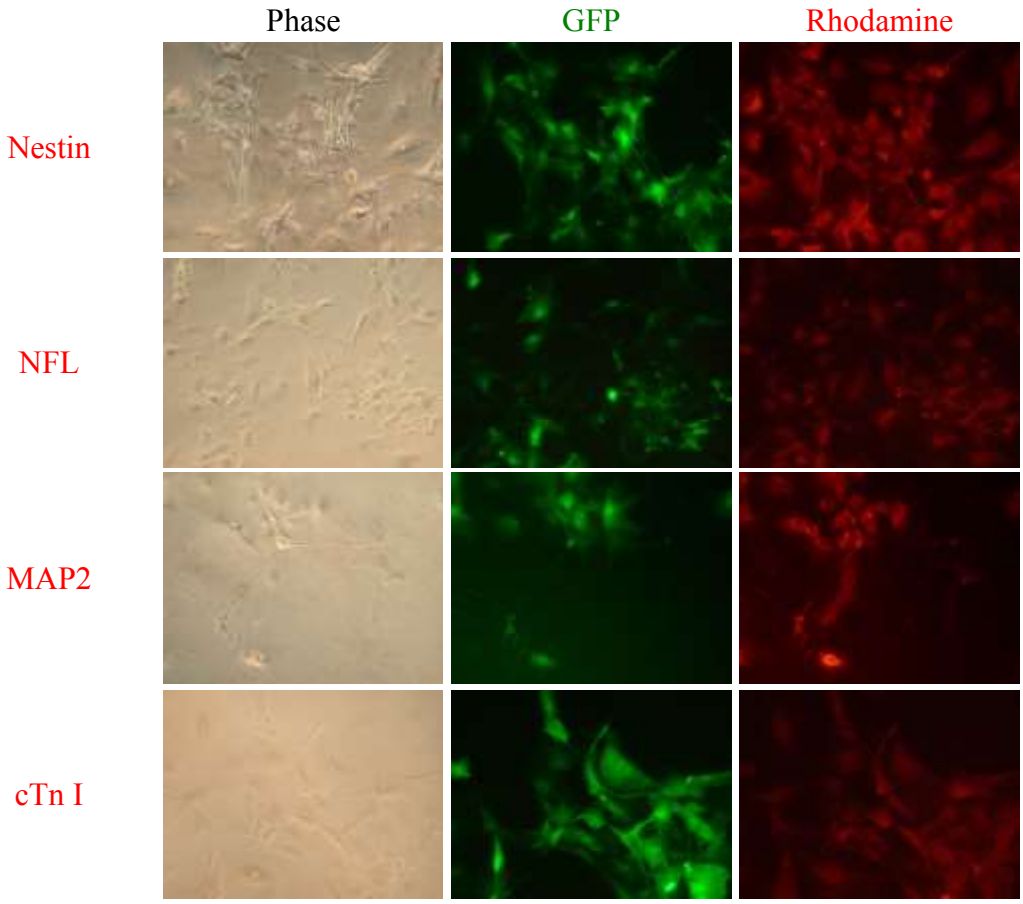

Fig. 4. The expressions of lineage-specific markers in the differentiated cells derived from pES/GFP$^+$ cells. Nestin-positive cells were detected at d 2 of replating culture following suspension culture of the pES/GFP$^+$ cells in RA-containing medium **(A)**. NFL-positive cells were detected at d 6 of replating culture following suspension culture of the pES/GFP$^+$ cells in RA-containing medium **(B)**. MAP2-positive cells were detected at d 6 of replating culture following suspension culture of the pES/GFP$^+$ cells in RA-containing medium **(C)**. cTn I-positive cells were detected at d 8 of replating culture following suspension culture of the pES/GFP$^+$ cells in DMSO-containing medium **(D)**.

Application of pES/GFP$^+$ cell lines in rat Parkinson's disease model

The pES/GFP$^+$ cells were subjected to directed differentiation into neural lineages to investigate therapeutic potential in a rat model of Parkinson's disease (PD) (Yang *et al.*, 2010). Directed differentiation was induced by suspension culture in medium containing RA, SHH, and FGF combinations without going through embryoid body formation. A high yield of nestin-expressing neural precursors was found in all treatments on day 2 after the 12-day induction. On day 6 after re-plating, more than 86.2% and 83.4% of the differentiated cells stained positively for NFL and MAP2, respectively. The expression of TH, ChAT, and GABA specific markers was also observed in these NFL-positive neural cells. The undifferentiated pES/GFP$^+$ cells and their neuronal differentiation derivatives were transplanted into the Sprague-Dawley (SD) rat's brain. The GFP-fluorescent signals from the injection site of SD rats' brain could be detected through the experimental period of 3 months. The PD rats exhibited stably decreased asymmetric rotations after transplantation with pES/GFP$^+$-derived D18 neuronal progenitors (Fig. 5). The dopaminergic differentiation of grafted cells in the brain was further confirmed by immunohistochemical staining with anti-TH, anti-DA, and anti-DAT antibodies. These results suggested that the differentiation approach we developed would direct pES cells to differentiate into DA-neuron lineages and benefit the development of novel therapeutics for Parkinson's disease involving stem cell transplantation.

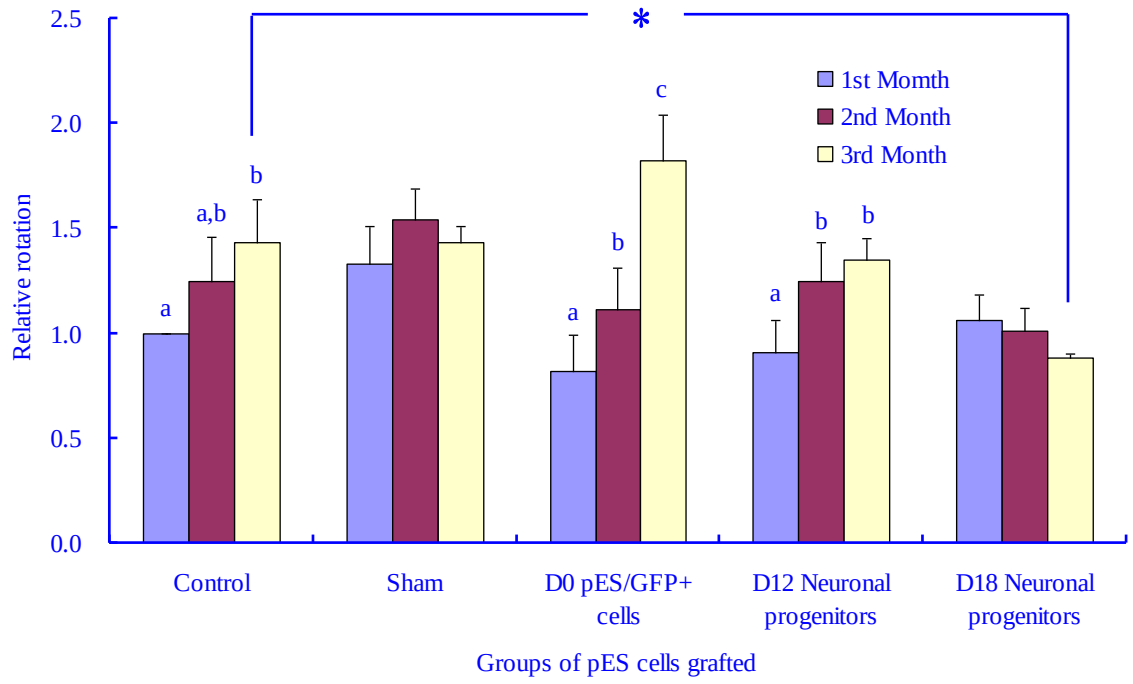

Fig. 5. Functional recovery of the PD rats in the control (n = 4), sham (n = 4), and treatment groups which were grafted with D0 pES/GFP$^+$ cells (n = 5), D12 neuronal progenitors (n = 7), and D18 neuronal progenitors (n = 6) was analyzed by amphetamine-induced rotation test. Relative rotation rates were presented as mean ± S.E.M. for each animal as compared to its pre-transplantation evaluation. The levels of relative rotation rates with different letters in the same group and * symbol between groups were significantly different ($P < 0.05$).

Application of pES/GFP$^+$ cell lines in spinal cord injury rat model

The pES/GFP$^+$ colonies were subjected to a two-step *in vitro* induction protocol to differentiation into neural lineage (Kumagai *et al.*, 2009; Yang *et al.*, 2010) to investigate therapeutic potential in the rat model of spinal cord injury. After suspension culture in neurogenic stimulators consisted of RA, Shh and EGF, the pES/GFP$^+$ cells were replated and cultured in medium containing EGF and FGF to derive neuron progenitors. The Long Evans (LE) rats with contused spinal cord were transplanted with pES/GFP$^+$ cells and their neuronal differentiation derivatives and the functional recovery of rats was assessed with the Basso, Beattie, and Bresnahan Locomotor Rating Scale (BBB scale). As shown in Fig. 6 the rats grafted with D12 and D18 neuronal progenitors resulted in significant recovery of hind limb function compared to control groups ($P < 0.05$). Xenotransplantation of porcine ES cells-derived neuronal progenitors into the spinal cord injured rat model reveal the therapeutic potential of ES cell transplantation in spinal injury cure.

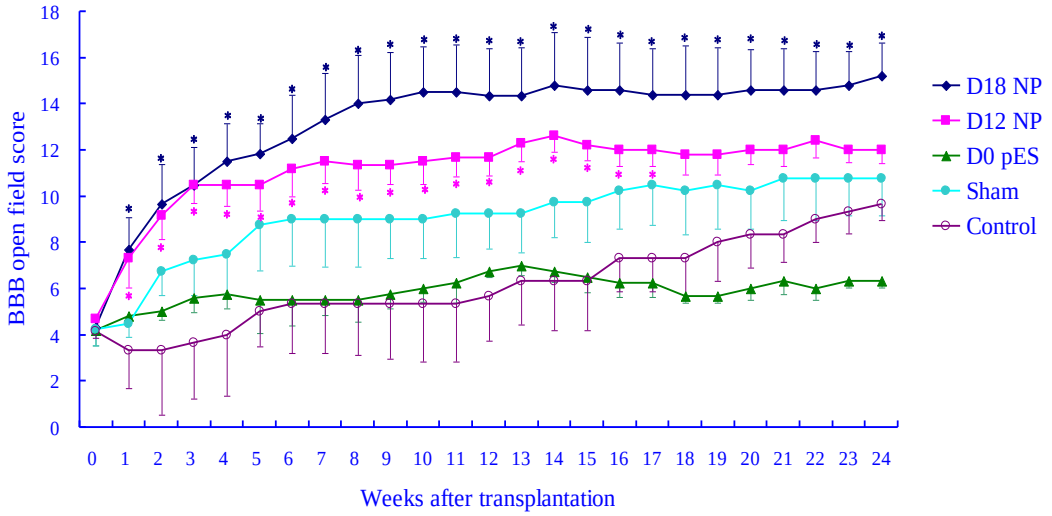

Fig. 6. The BBB locomotor rating scale (mean ± S.E.M.) of control (n = 4), sham (n = 4), D0 pES/GFP$^+$ cells (D0 pES, n = 5), D12 neuronal progenitors (D12 NP, n = 6), and D18 neuronal progenitors (D18 NP, n = 6) transplantation in SCI rats. The * shows a significant difference compared to control groups ($P < 0.05$).

Autotransplantation of pES/GFP$^+$ cells in a periodontal furcation defects model

The pES/GFP$^+$ cells were transplanted to the experimental periodontitis induced in the buccal furcations of bilateral mandibular 2nd premolars of four 5-month-old female Lan-yu pigs. After 3 months of healing, the IHC staining demonstrated that transplanted pES/GFP$^+$ cells differentiated to new periodontal ligament and cementum in the test sites. No obvious teratoma or rejection was seen in any examined animals. The test group had significantly better clinical parameters regarding clinical probing depth and attachment level (Fig. 7), and demonstrated the feasibility of using pES cells to improve the regeneration of periodontal furcation defects (Yang *et al.*, 2013).

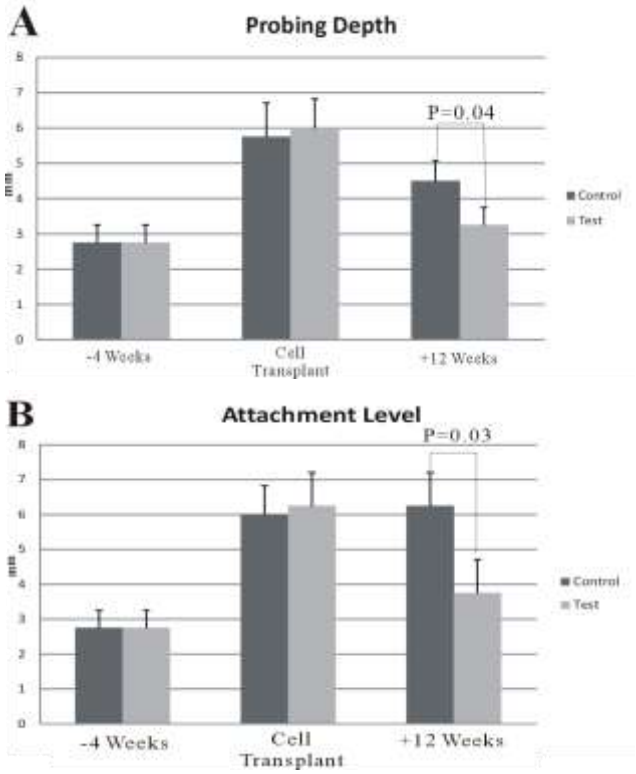

Fig. 7**.** Clinical parameters of the furcations at three different time points. (A) -4 weeks indicated the probing depth at baseline. The middle columns indicated the data measured just before the ES cells were transplanted. +12 weeks indicated the data measured before the sacrifice of animals. (B) The attachment levels were measured at different time points as described in (A).

CONCLUSION

We have successfully isolated and established putative pES cell lines and traceable pES cell lines from in vivo-produced porcine blastocysts. These cells survived repeated subcultures and cryopreservation and maintained typical morphology of ES cells and colonies, normal karyotype, continued expressing pluripotent cell markers of Oct-4, AP, SSEA-3/4, TRA 1-60, and TRA 1-81. They could form embryonal body after suspension culture, and differentiate into several cell lineages representative to three germ layers due to spontaneous and artificial induction. Piglets of somatic but not germline chimera had been obtained from blastocysts injection with pES cells. The gold standard for characterization of embryonic stem cells has almost been fulfilled for the putative pES and pES/GFP^{+} cells described above. In addition, the therapeutic potentials of these cells have also been demonstrated in the regeneration medical studies including xenotransplantation to rat models of Parkinson's disease and spinal cord injury and autotransplanatation to porcine model of periodontitis. The pigs share many similarities to humans in anatomic, immunologic, and physiologic characteristics, and even in the characteristics and behaviors of ES cells. Therefore, the further study of pES cells might directly or indirectly benefit the research and development of regeneration medicine in humans.

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