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Foreword

The horse industry makes a significant contribution to the Australian economy. In 1999 the industry contributed over $6.3 billion to Australia's Gross Domestic Product. The greatest economic contribution comes from the racing sector. Cartilage injuries to race horses are a significant cost to the industry. Improved stem cell technology may provide the opportunity to address cartilage injury problems.

Embryonic stem cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease in humans and valuable animals. However, embryonic stem cell lines have only been well established in mice and humans. It will be a prerequisite to isolate equine embryonic stem cells for such cell therapy.

This report describes the protocols of horse embryonic stem cell-like cell isolation from in vivo fertilised embryos and interspecies somatic cell nuclear transferred embryos. The results showed:

- putative horse embryonic stem cells are partially positive for embryonic stem cell markers
- sheep and horse amniotic epithelial cells are partially positive for human embryonic stem cell markers
- all cells are pluripotent and able to differentiate into some somatic cells spontaneously in vitro.

Further research and clinical trials can flow from the proof of principle results found in this study. If successful, the research reported here could open up a whole new field of veterinary therapeutic application allowing high performance horses to recover from injuries.

This project was funded from industry revenue which is matched by funds provided by the Australian Government

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

Most of RIRDC's publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation

Acknowledgments

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Abbreviations

AEC:	amniotic epithelial cell
AECs:	amniotic epithelial cells
hAECs:	human amniotic epithelial cells
oAECs:	ovine amnion epithelium cells
eAECs:	equine amnion epithelium cells
ESC:	embryonic stem cell
ESCs:	embryonic stem cells
eESCs:	equine embryonic stem cells
SCNT:	somatic cell nuclear transfer
iSCNT:	interspecies somatic cell nuclear transfer
ntESCs:	somatic cell nuclear transfer-derived embryonic stem cells
ICM:	inner cell mass
MHC:	major histocompatibility
FBS:	fetal bovine serum
hLIF:	human leukemia inhibitory factor
hbFGF:	human basic fibroblastic growth factor
MEF:	mouse embryo fibroblast
MEFs:	mouse embryo fibroblasts
ITS:	Insulin-Transferrin-Selenium
D-PBS:	dulbecco’s phosphate buffered saline
DMEM/F12:	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
α-MEM:	Minimum Essential Medium Alpha
DAPI:	4'-6-Diamidino-2-phenylindole
FITC:	fluorescein isothiocyanate
TGFβ-R:	transforming growth factor-β receptor
EB:	embryoid body
AP:	Alkaline phosphatise
iPS:	induced pluripotent stem
MEF:	mouse embryonic fibroblast
KO:	Knockout Dulbecco’s modified Eagle’s medium
NEAA:	Non-essential Amino Acids
hLIF:	human leukemia inhibitory factor
hbFGF:	human basic fibroblastic growth factor
TCM-H:	medium 199-Hepes
TE:	trophectoderm
ED:	endoderm cell-like cells
PA:	parthenogenetic activation
AP:	Alkaline phosphatise
rhTGF-beta 3:	recombinant humanTransforming growth factor-beta 3
IgG:	immunoglobulins G
IgM:	immunoglobulins M
SCID:	severe combined immunodeficiency
SOF:	synthetic oviductal fluid

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Executive Summary

What the report is about

Cartilage injury is the main problem for horse racing. It has a great impact on the equine industry. This report focuses on the new stem cell technology for producing equine stem cells to treat horse cartilage injury and many other diseases. The protocols for isolation of equine embryonic stem cells, ovine and equine amnion epithelium stem cells are described in the report.

Who is the report targeted at?

This report is primarily targeted at scientists who work on veterinary and biomedical sciences. Our findings will attract the interest of veterinarians specialising in both companion and domestic animals and the thoroughbred racing community, which will include thoroughbred horse trainers, breeders and owners.

Background

Joint injuries are a major cause of economic loss in the horse industry. Even a small cartilage injury in a critical spot can end a successful career of a race horse. Some joint resurfacing techniques, such as carticell and mosaicplasty, have been investigated in the past 10 years, however, only limited success was reported. Embryonic stem cells and amniotic epithelial cells both are important potential therapeutic agents due to their pluripotency and plasticity. Although only human and mouse embryonic stem cell lines are well established so far, their capacity to differentiate into all cell types of the body have been proved. Clinical trials have been conducted in humans. Recently, human amniotic epithelial cells have been isolated from the amnion donated by caesarean operation patients and these cells have been differentiated into many cell types including osteogenic and chondrogenic lineage cells. More and more evidence has shown that amniotic epithelial cells will play an important role in stem cell therapy in the future. Therefore, it is essential to develop equine stem cell techniques to produce equine embryonic or amnion epithelium stem cells to meet the clinical requirements for stem cell therapy in horses.

Aims/objectives

This project aimed to develop veterinary stem cell based therapeutic techniques, mainly focused on pluripotent stem cell isolation. Equine embryonic-derived stem cells will be generated from in vivo fertilised and equine-bovine somatic cell nuclear transferred embryos. Such embryonic stem cells were characterised with markers of undifferentiated human embryonic stem cells. Ovine and equine amniotic-derived stem cells will also be isolated and characterised. The differentiation potential of embryonic-derived and amniotic-derived stem cells will be preliminarily assessed.

Methods used

Equine embryonic stem cells were created from inner cell mass cells of blastocysts derived from in vivo fertilised embryos and interspecies somatic cell nuclear transfer embryos. Interspecies somatic cell nuclear transfer was carried out by transplanting equine fibroblast cells into enucleated bovine metaphase II oocytes. The resulting putative equine embryonic stem cells were maintained in the medium containing human leukemia inhibitory factor on mouse feeder cells. The characterisation of the cells was identified by immunochemistry using embryonic stem cell markers. Ovine and equine amnions were collected from sheep undergoing caesarian section near term and horse natural term delivery, respectively. The amniotic epithelial cells were isolated from the amniotic membranes by chemical digestion or subjected to untreated amniotic membrane culture. The characterisation of the amniotic epithelial cells was also identified by immunochemistry using embryonic stem cell markers. Osteogenic and chondrogenic differentiation of the amniotic epithelial cells was induced by culturing

with osteogenic and chondrogenic differentiation medium used for human mesenchymal stem cell differentiation.

Results/key findings

The putative equine embryonic stem cells were obtained from both in vivo fertilised and interspecies somatic cell nuclear transfer embryos. The preliminary results showed that there was expression of embryonic stem cell markers in only some of the putative equine embryonic stem cells. Ovine and equine amniotic epithelial cells are able to be isolated from digested and untreated amniotic membranes. A proportion of the amniotic epithelial cells showed positive for human embryonic stem cell markers.

Implications and Recommendations

This report opens the new area to veterinary medicine although it is just the beginning of research in this field. This report will draw attention to veterinary biotechnology companies to develop new techniques for cell replacement therapy in animals. In terms of results from studies in humans, it would be a new milestone if stem cells could be applied to veterinary clinics. This will also help people to understand the potential of stem cells for human cell therapy. The technology would not have a direct impact on ecological sustainability and biodiversity, however it will improve sustainability and protect biodiversity by allowing high performance horses to recover from injuries and their further utilisation in breeding.

Because this is the first and only study that has focused on stem cells for veterinary clinical medicine, there is a lot of basic research that needs to be investigated further. We recommend government statutory authority and biotechnology companies consider investing research funding to support developing such stem cell therapeutic research in animals. The research should focus on stem cell differentiation in vitro and in vivo and clinical trials in animals.

Introduction

The Australian horse industry represents a wide variety of activities including primary production, as well as sports and recreational interests. The thoroughbred racing industry is one of Australia's top income providers. Many diseases, particularly cartilage injury, can impair athletic function. This has a huge impact on the equine industry. Although joint resurfacing techniques have been studied for over 10 years, both the cell based and cartilage transfer techniques have only had limited success. New treatments for horse cartilage injury need to be developed. Recently, stem cell research brings the hope of potential cures to all these diseases.

Stem cells are originally derived from adult tissue, and are called adult stem cells; and from the inner cell mass (ICM) of early embryos, called embryonic stem cells (ESCs). Although adult stem cells have some similar properties to embryonic stem cells, their differentiation potential is limited, and large quantities of the cells are not available. Unlike embryonic stem cells, adult stem cells are unable to proliferate unlimitedly. Due to the capacity of multipluripotency and self-renewal, ESCs have been proposed for clinical applications of regenerative medicine and tissue replacement after injury or disease in humans. However, as ESC lines have only been well established in mice (Evans and Kaufman 1981; Ginis et al., 2004) and humans (Thomson et al., 1998; Ginis et al., 2004), it is essential to develop ESC techniques to isolate valuable animal ESCs for animal stem cell therapy. Because ESCs are usually generated from fertilised embryos, it is important to create patient specific ESCs to avoid immunorejection after transplantation for cell therapy. Several attempts including, somatic cell nuclear transfer (SCNT), cell fusion and transduction of pluripotency factors (Jaenisch and Young 2008), have been made to isolate patient specific ESCs, but SCNT-derived ESCs (ntESCs) have only been reported in mice (Wakayama et al., 2001) and nonhuman primates (Byrne et al., 2007).

Furthermore, induced pluripotent stem (iPS) cells, believed to be similar to ESCs in pluripotency, were successfully generated from unmodified fibroblasts (Park et al., 2008; Yu etal., 2007) and even from adult dermal fibroblasts without drug selection (Nakagawa et al., 2008). However, iPS cells were generated by retroviral transduction of pluripotency transcription factors. New strategies will need to be developed for clinical purposes to avoid potentially harmful genetic modification. So far, a few reports have been published on ESC isolation in ungulate animals, the sheep (Notarianni et al. 1991; Dattena et al. 2000; Dattena et al. 2006), pig (Notarianni et al. 1990; Notarianni et al. 1991; Wheeler 1994), cattle (Stice et al. 1996) and horse (Saito et al. 2002; Li et al. 2006). These putative ESCs appear to be ESC-like cells. Thus, new protocols need to be improved to culture and maintain ungulate animal ESCs.

Alternatively, amniotic-derived epithelial cells are now recognised as important sources of pluripotent cells. Amnion is the inner of two membranes encasing the amniotic fluid in which the fetus is suspended during gestation. The membranes, together with the placenta are spontaneously delivered soon after birth. The studies have shown that human amniotic epithelial cells (hAECs), derived from term delivered gestational tissue, display key features of pluripotent stem cells. They express the transcription factors Oct-4, Sox-2 and Nanog that maintain the undifferentiated state of pluripotent stem cells as for embryonic stem cells (Ilancheran S et al., 2007). Human amnion derived epithelial cells are capable of self renewal and retain considerable plasticity; differentiating into lineages derived from each of the three primary germ layers *in vitro*, but they do not form teratomas after transplantation into the testes of immunodeficient mice, and they have restricted expression of major histocompatibility (MHC) antigens before, and after, differentiation into several cell lineages *in vitro*. hAECs also appear to suppress lymphocyte proliferation (Li, et al. 2005).

Collectively, these findings indicate that hAECs may elicit minimal immune recognition following transplantation to an allogeneic recipient in their native form, or even after differentiation down a specific lineage. However, there have been no reports on the studiy of amniotic-derived epithelial cells in ungulate animals. Due to such huge available resources of equine, bovine and ovine amnions

in Australia, if animal derived AECs have the similar properties to human AECs, it may be possible to exploit the plasticity, anti-tumor, trophic, anti-inflammatory and immune-privileged features of horse AECs, to develop entirely novel effective veterinary stem cell based therapies in the future.

In this report, we also present research data for the possibility of isolation of equine ESC-like cells derived from in vivo fertilised and equine-bovine somatic cell nuclear transferred embryos and the preliminary results of characterisation of such ESCs. Furthermore, we display the protocols of equine and ovine AEC isolation, and the preliminary results of characterisation of ovine AECs and equine AECs. Differentiation potential of equine and ovine AECs into osteogenic and chondrogenic lineage cells is evaluated.

Objectives

This project aimed to develop veterinary stem cell based therapeutic techniques, mainly focused on pluripotent stem cell isolation. Equine embryonic-derived stem cells will be generated from in vivo fertilised and equine-bovine somatic cell nuclear transferred embryos. Such embryonic stem cells were characterised with markers of undifferentiated human embryonic stem cells. Ovine and equine amniotic-derived stem cells will also be isolated and characterised. The differentiation potential of embryonic-derived and amniotic-derived stem cells were preliminarily assessed.

Methodology

Isolation of equine embryonic stem cells

Generation of equine embryonic-derived stem cells from in vivo fertilised eggs

Horse embryos were collected in Dulbecco's phosphate buffered saline (D-PBS) from the mare by non-surgical flushing on day 6 or 7 post-ovulation in the Goulbourn Valley Equine Hospital (Victoria, Australia) based on the methods described by Squires (1993). The ICMs of blastocysts were isolated mechanically by cutting or pipetting through the fine tip of a pasture pipette. Briefly, the blastocyst cutting was carried out in cell culture, and blastocyst pipetting was performed for the blastocyst treated with TrypLE for 2-3 minutes before pipetting. The ICM cells were then transferred onto a center-well Organ Culture Dish containing γ-irradiated mouse embryonic fibroblast (MEF) feeder cells. MEF cells were prepared from mouse embryos at day 13-14 of pregnancy, and these cells were inactivated by γ-irradiation and frozen down in liquid nitrogen at passage 3-6. A culture dish with MEF feeder cells was prepared freshly on the day before ICM culture. Horse embryonic stem cell culture medium was modified from human ESC culture medium, which is Knockout Dulbecco's modified Eagle's medium (KO) supplemented with 15 % fetal bovine serum, 0.1 mM Non-essential Amino Acids (NEAA), 2 mM L-glutamine, 1% Insulin-Transferrin-Selenium (ITS), 100 μg/ml of streptomycin , 100 units/ml of penicillin, 0.1mM 2β-mercaptoethanol and 40ng/ml hLIF. The culture medium was changed every day after first passage and the passage was performed by mechanical scraping with a needle every 6-8 days until passage 5. After passage 5, horse putative ESC culture conditions were evaluated by culturing under different supplements. Comparisons were made of culture medium without 40ng/ml hLIF, with 4ng/ml hbFGF and 40ng/ml hLIF and with 4ng/ml hbFGF only; as well as with or without feeder cells.

Generation of equine embryonic-derived stem cells from iSCNT embryos

Equine and bovine fibroblast cells were isolated from skin samples taken from adult stallions and bulls, respectively. The cells were cultured with α-MEM supplemented with 10% FBS. Recipient oocytes were prepared from in vitro matured abattoir-derived bovine oocytes. Nuclear transfer was performed according to methods previously described (Tecirlioglu et al. 2005). Briefly, cumulus cells were removed by vortexing in 1 ml of tissue culture medium-Hepes (TCM-H) supplemented with 300IU/ml hyaluronidase at 18-20 h of post-maturation. All oocytes were transferred into pronase solution to remove the zona. Zona-free oocytes were bisected manually to enucleate. Demi–oocytes were assessed for the presence of chromatin with UV-fluorescence microscopy after staining with Hoechst 20μg/ml 33342 and cytoplasts were briefly incubated 200μg/ml phytohaemagglutinin. An actively dividing equine or bovine fibroblast was attached to the demi-oocyte. SCNT embryos were reconstructed by fusion of the triplets (cytoplast-fibroblast-cytoplast) using a single DC, 3.36 kV/cm for 4 μs. Reconstructed equine-bovine, bovine-bovine and parthenogenetic embryos were activated at 3 h of post-fusion by incubating with 5μM calcium ionophore A-23187 for 4min, followed by 2mM 6-dimethylaminopurine for further 3 h of culture. Surviving embryos were then cultured individually or aggregated in pairs in modified SOF medium (Cook Australia) in a "well-of-the-well" system for seven days. Putative ntESCs were isolated from the blastocyst derived from interspecies somatic cell nuclear transfer (iSCNT) as described for in vivo fertilised embryos.

Isolation of ovine and equine amniotic epithelial cells

Ovine amnions were collected from sheep undergoing caesarian section near term at the Department of Physiology, Monash University, and equine amnions were collected from horse spontaneous term

delivery at horse farms and veterinary hospitals around Victoria, Australia. The amnion layer was mechanically peeled off from the chorion. Amniotic epithelial cells were isolated from the amniotic membranes by enzymatic digestion or untreated amniotic membrane was cultured directly. The method of enzymatic digestion was adopted from human amniotic epithelial cell isolation (Ilancheran et al 2007). Briefly, the amnion was removed into a Petri dish and washed with D-PBS to remove blood. The amnion was cut into small pieces and digested by TrypLE at 37◦C for 30 minutes with approximately 100rpm stirring. The supernatant was discarded after centrifugation. Amniotic epithelial cells were then collected from the second and third digestion at 37◦C with approximately 250rpm, stirring for 30min each. Dissociated cells were cultured with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) containing 15% fetal bovine serum (FBS), 0.1 mM NEAA, 2 mM L-glutamine, 1% ITS, 100 μg/ml of streptomycin , 100 units/ml of penicillin, and 0.1mM 2β-mercaptoethanol in T75 flask at 37◦C in a humidified atmosphere of 5% CO2 in air. Ovine amniotic epithelial cells (oAECs) and equine amniotic epithelial cells (eAECs) were passaged between threee to four days and seven days during the first five passages.

Immunohistochemistry of ESCs and AECs

Immunohistochemistry of eESCs

The putative eESCs derived from in vivo fertilised and iSCNT embryos were transferred onto sterile 12-well glass slides on γ-irradiated MEF feeder cells in ESC culture medium at passage 3-6. After 5-7 days of culture, the colonies were fixed with 100 % cold (-20°C) ethanol for 10 minutes and then removed from the ethanol to let the slides air dry at room temperature for 30 minutes. The slides were washed with PBS and then incubated with human-specific antibodies for Oct-4 (1:20; Santa Cruz Biotechnology Inc., CA), the keratan sulphate associated antigen Tra-1-60 (1:20; Chemicon, CA), Tra-1–81 (1:20; Chemicon) and GCTM-2 (provided by ASCC, Australia), stage-specific embryonic antigen-1 (SSEA-1) (1:20; Chemicon, CA), and SSEA-4 (1:20; Chemicon) at room temperature for 30 minutes. After washing, the slides were incubated for 30 minutes at room temperature with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) for Oct-4, SSEA4 and GCTM-2, incubated with goat anti-mouse IgM conjugated with FITC (1:40; Invitrogen) for Tra-1-60, Tra-1–81 and SSEA-1, respectively. The cells were then incubated with 20μg/ml Hoechst 33342 for 5 minutes to stain the nuclei. Mouse IgG was employed as negative control to replace primary antibody. The slides were covered by coverslips with Vectasheild inside, and the edges of the coverslips were sealed with nail polish. Fluorescent images were acquired under an Olympus IX81 microscope.

Immunohistochemistry of AECs

oAECS and eAECs were reseeded onto sterile 12-well glass slides and cultured with AEC culture medium at passage 1. After three to five days of culture, the cells were fixed in 4% paraformaldehyde in PBS for 15-20 minutes at room temperature. The cells were characterised with an ES Cell Marker Sample Kit (Chemicon, SCR002) following the instructions of the manufacturer. Briefly, the slides were washed with PBS and incubated with the antibodies diluted 1:40 for Oct-4, Tra-1-60, Tra-1–81, SSEA-1 and SSEA-4 at room temperature for 30 minutes. After washing, the slides were incubated for 30 minutes at room temperature with goat anti-mouse IgG/IgM conjugated with FITC (1:50; Chemicon). Mouse IgG was employed as a negative control. After immunostaining, the cells were then incubated with 20μg/ml Hoechst 33342 (Sigma) for 5 minutes to stain the cell nuclei. The slides were washed with PBS to remove Hoechst 33342 and covered by coverslips with Vectasheild inside, and the edges of the coverslips were sealed with nail polish. All fluorescent images were acquired by an Olympus microscope IX 81.

Differentiation of ESCs and AECs

General differentiation of eESCs

The putative eESCs was transferred into a petri dish without treatment and cultured with ESC culture medium without ITS, hLIF and in the absence of feeder cells at passage 15. Culture medium was changed every three to five days.

General differentiation of oAECs

Ovine AECs was transferred into a 12-well cell culture plate and cultured with DMEM/F12 medium containing 15% FBS without ITS at passage 1. The culture medium was changed every three to five days.

Chondrogenic differentiation of oAECs

Chondrogenic differentiation of ovine amniotic epithelial cells was carried out at passages 1 and 5 with StemPro Chondrogenic Differentiation Kit (Gibco). The amniotic epithelial cells were dissociated with TrypLE and reseeded into a 12-well plate at approximately $5x10^3$cells/cm^2. The plate was incubated at 37◦C in a humidified atmosphere of 5% CO2 in air, and the differentiation medium was replaced every 3-5 days. After four weeks under differentiation conditions, the culture medium was removed and cells were then fixed. Fixed cells were rinsed with distilled water, and the cells were stained with 1% Alcian Blue solution prepared in 0.1 N HCl for 30 minutes. The cells were observed under the light microscope after rinsing with 0.1 N HCl to neutralise the acidity. The wells were then rinsed with distilled water and observed under the light microscope.

Osteogenic differentiation of oAECs

Osteogenic differentiation of ovine amniotic epithelial cells was carried out at passages 1 and 5 with StemPro Osteogenesis Differentiation Kit (Gibco). The amniotic epithelial cells were dissociated with TrypLE and reseeded onto a 12-well cell culture plate at approximately $5x10^3$cells/cm^2. The plate was incubated at 37◦C in a humidified atmosphere of 5% CO2 in air, and the differentiation medium was replaced every three to five days. After four weeks under differentiation conditions, the culture medium was removed and cells were then fixed using 4% formaldehyde solution for 30 minutes. The wells were then stained with 2% Alzarian Red S solution for 30 minutes. The wells were then rinsed with distilled water and observed under the light microscope.

Results

Equine embryonic-derived stem cells from in vivo fertilised embryos

A total of 25 horse embryos were collected over two years during breeding seasons. ICM is difficult to locate in the expanded blastocyst of the horse (Fig. 1). The horse blastocyst is surrounded by capsule and zona pellucid (Fig. 2). Electron Microscopy of a day 7.5 blastocyst showed that the horse blastocyst typically has three types of cells, which are endoderm, trophectoderm and inner cell mass cells (Fig. 3). ICM can be isolated from the blastocyst by cutting (Fig. 4), and the primary culture of ICM was obtained from blastocyst cutting (Fig. 5) and pipetting (Fig. 6), respectively. Three types of cells were able to be isolated from equine blastocysts (Fig. 7), and the putative ESCs were obtained and passaged without morphology changes at day 6 of passage 3 (Fig. 8).

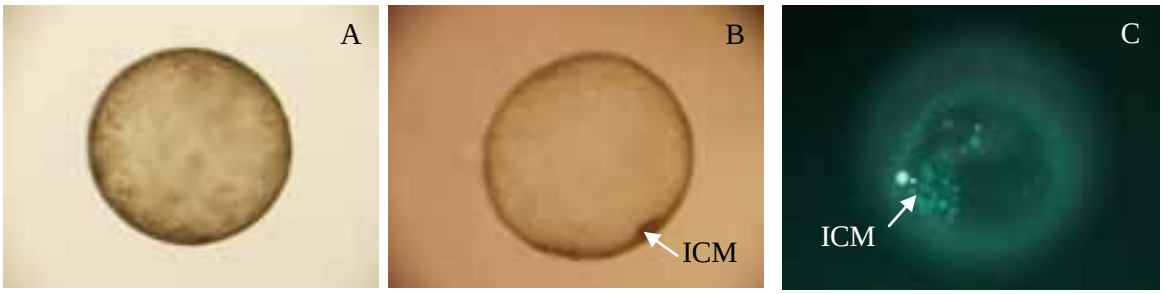

Fig. 1. In vivo-derived horse blastocyst. The expanded blastocyst without obvious ICM (A); the blastocyst with obvious ICM (arrow, B) and ICM was stained with Hoechst 33342 (C).

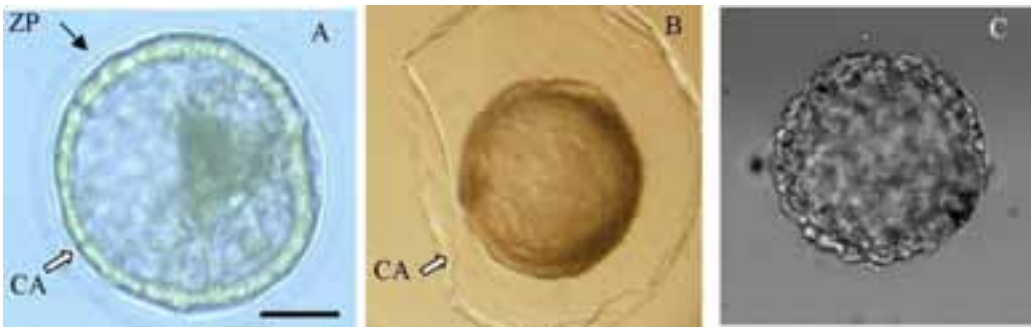

Fig. 2. Horse in vivo fertilised embryos. The expanded blastocyst was surrounded by capsule (CA) and zona pellucid (ZP) (A); Zona-free embryos (CA) (B); the embryo without Zona and capsule (C).

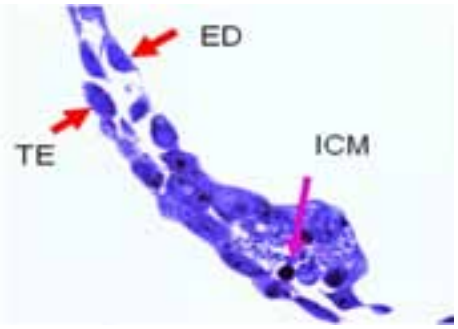

Fig. 3. Image of Electron Microscopy for day 7.5 horse blastocyst. Two layers of endoderm (ED) and trophectoderm (TE) was denoted by red arrows; the inner cell mass (ICM) was shown by the pink arrow.

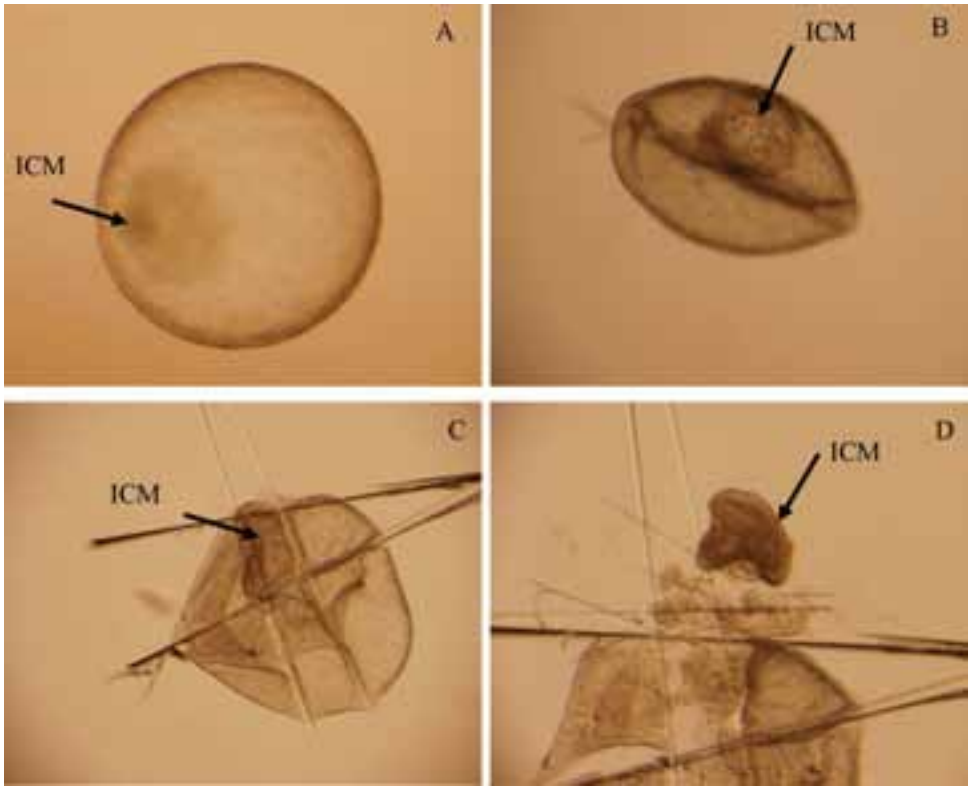

Fig. 4. ICM isolation by blastocyst cutting. The expanded blastocyst (A); ICM was isolated by cutting (B, C and D).

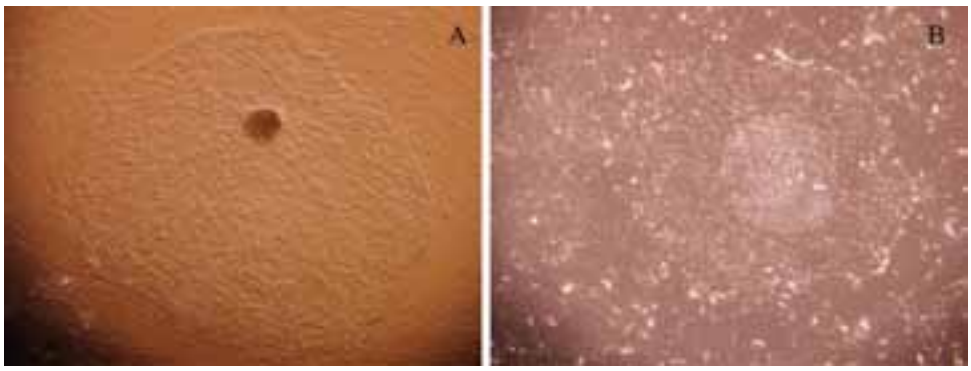

Fig. 5. Primary culture of ICM isolated by blastocyst cutting and cultured on MEF feeder cells. Different morphology of ESCs was shown in A and B.

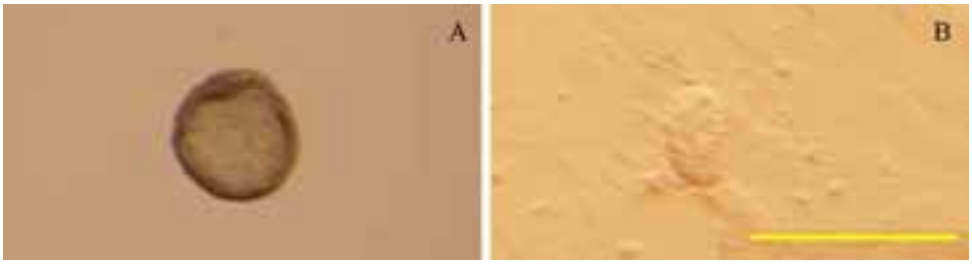

Fig. 6. ICM isolation by pipetting. Embryo was treated with TrypLE for 5 minutes (A); the ICM cells were cultured at day 1 on MEF feeder cells (B).

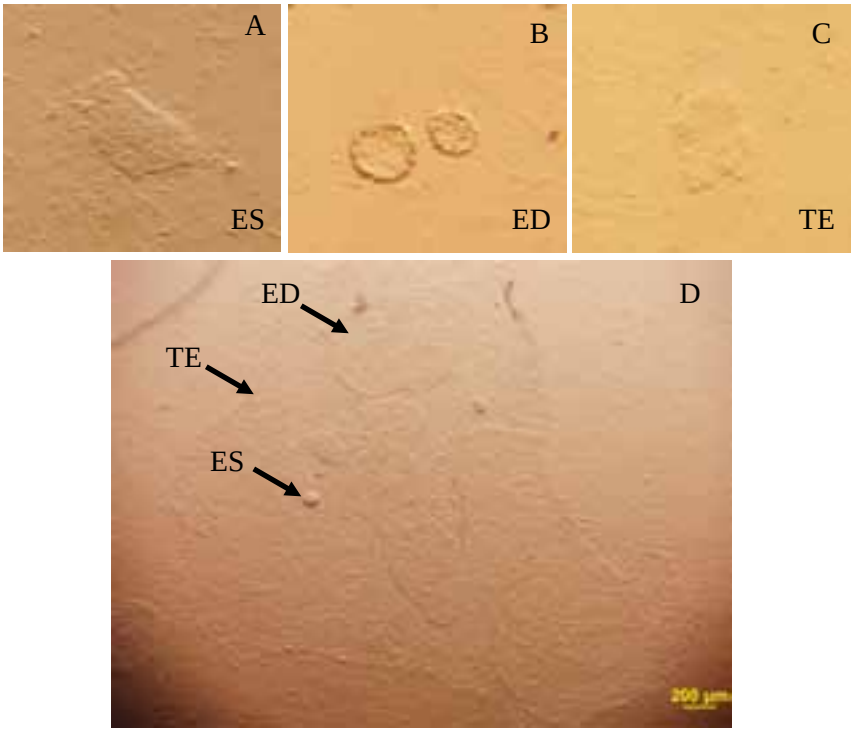

Fig. 7. Different morphologies of cells cultured from embryo pipetting. ESC-like colony (ES) at day 2 of culture (A); vesicles from endoderm cell-like cells (ED) at day 2 of culture (B) and trophectoderm cells (TE) at day 1 of culture (C); three types of cells were cultured in one colony (D).

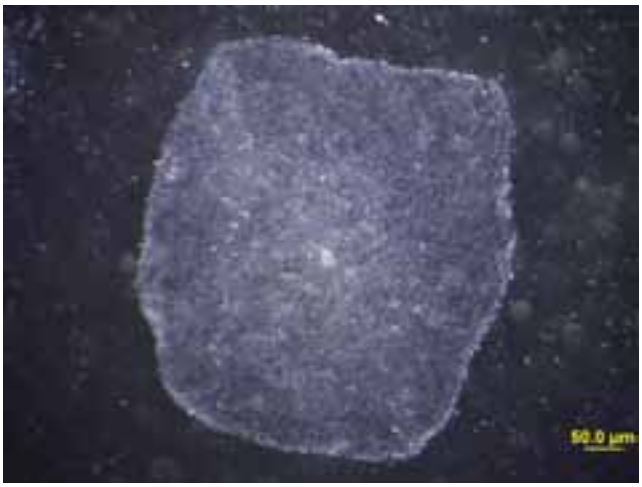

Fig. 8. Equine ESC-like colony cultured in at day 6 of passage 3.

Equine embryonic-derived stem cells from iSCNT embryos

Approximately 91% (1743/1917) of oocytes survived after bisection and the fusion rate between equine–bovine and bovine–bovine NT was 86.1% (396/460) and 90.1% (209/232), respectively. The development of parthenogenetic activation (PA), SCNT and iSCNT efficiency is shown diagrammatically in Figure 9. The blastocysts were obtained from both single and aggregated iSCNT embryo culture (Fig. 10). Three of the iSCNT embryos were bisected to isolate ICM and

trophectoderm parts (Fig. 11). One putative embryonic stem cell colony and one trophectoderm stem cell line was established (Fig. 12).

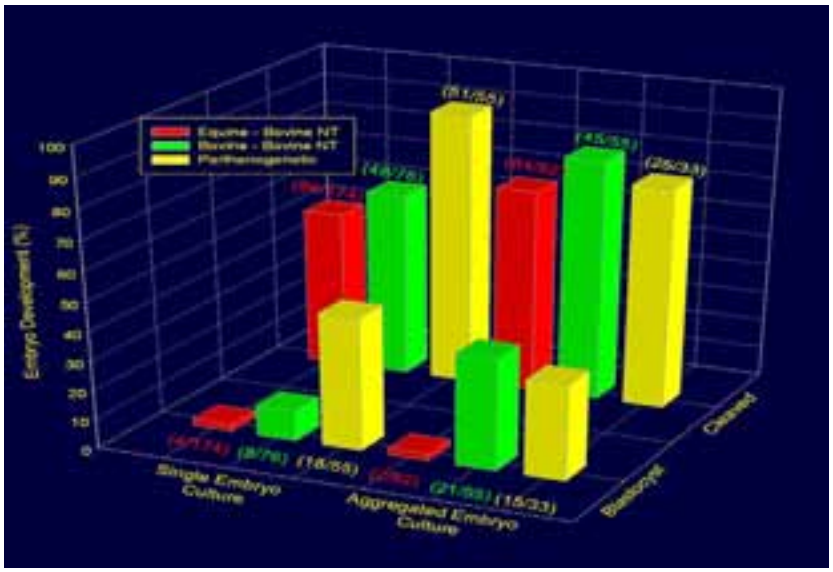

Fig. 9. The efficiency of nuclear transfer from equine-bovine iSCNT, bovine-bovine SCNT. Parthenogenetic activation was used as the control.

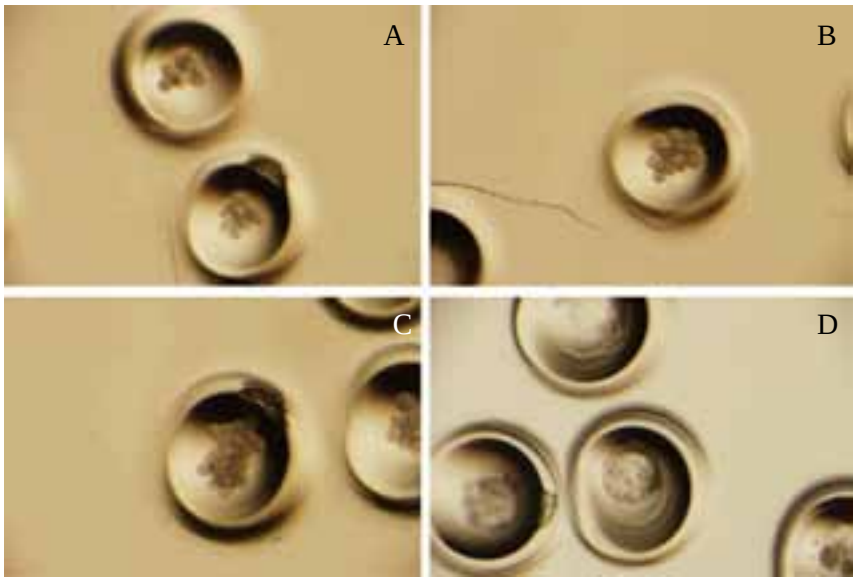

Fig. 10. In vitro development of aggregated iSCNT embryos. 4-cell (A), 8-cell embryo (B), early morula (C) and blastocyst (D).

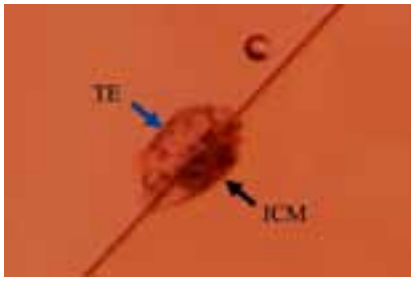

Fig. 11. Isolation of ICM and trophectoderm parts by blastocyst cutting. ICM (black arrow) and TE (blue arrow) was

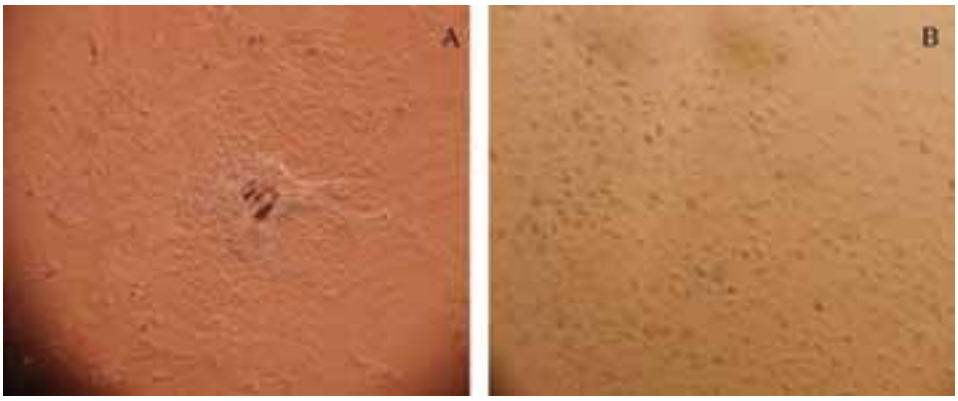

Fig. 12. Cell lines isolated from iSCNT embryos. Putative ntESCs were cultured on feeder cells (A); trophectoderm cell line was cultured without feeder cells.

Immunocytochemistry of equine putative ESCs from fertilised and iSCNT embryos

Immunocytochemistry of putative eESCs showed that the cells were partially positive for Oct-4 (Fig. 13), SSEA-1 (Fig. 14), TRA-1-60 (Fig. 15), SSEA-4 (Fig. 16), TRA-1-81(Fig. 17), CD 9 (Fig. 18), transforming growth factor-β receptor (TGFβ-R) (Fig. 19) and Alkaline phosphatase (AP) (Fig. 20), but were negative for SSEA-3; Two types of cells were generated from ntESCs. The large cells were positive for Oct4 (Fig. 21), SSEA-3 (Fig. 22), SSEA-4 (Fig. 23), Tra-1-60 (Fig. 24) and Tra-1-81 (Fig. 25), but negative for SSEA-1; the small cells were partially positive for Oct4 (Fig. 26), SSEA-1 (Fig. 27), SSEA-3 (Fig. 28), SSEA-4 (Fig. 29), Tra-1-60 (Fig. 30) and Tra-1-81 (Fig. 31). The AP staining was positive as well (Fig. 32). However, both of the cell lines from ntESCs could not be maintained after passage 12.

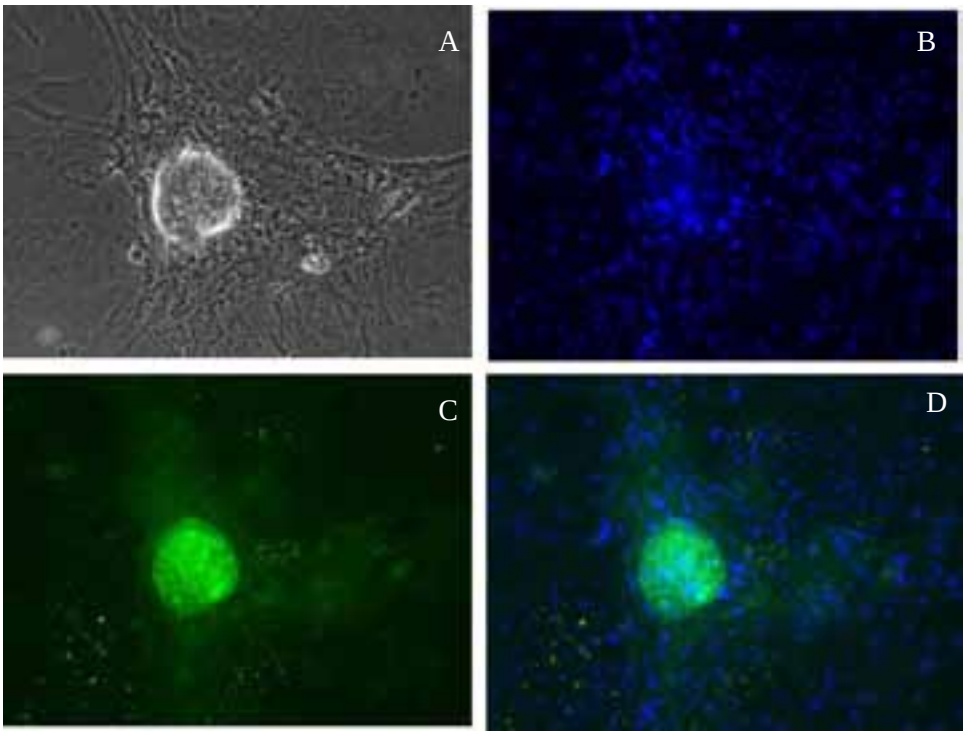

Fig. 13. Immunostaining of Oct4 for equine putative ESCs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

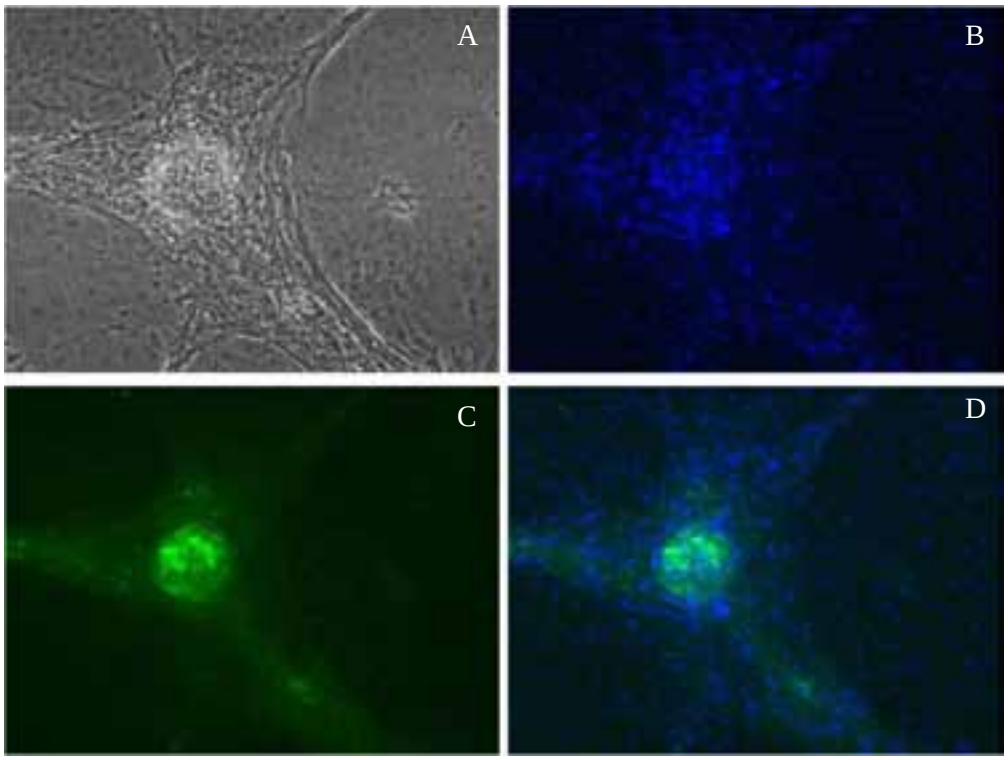

Fig. 14. Immunostaining of SSEA-1 for equine putative ESCs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

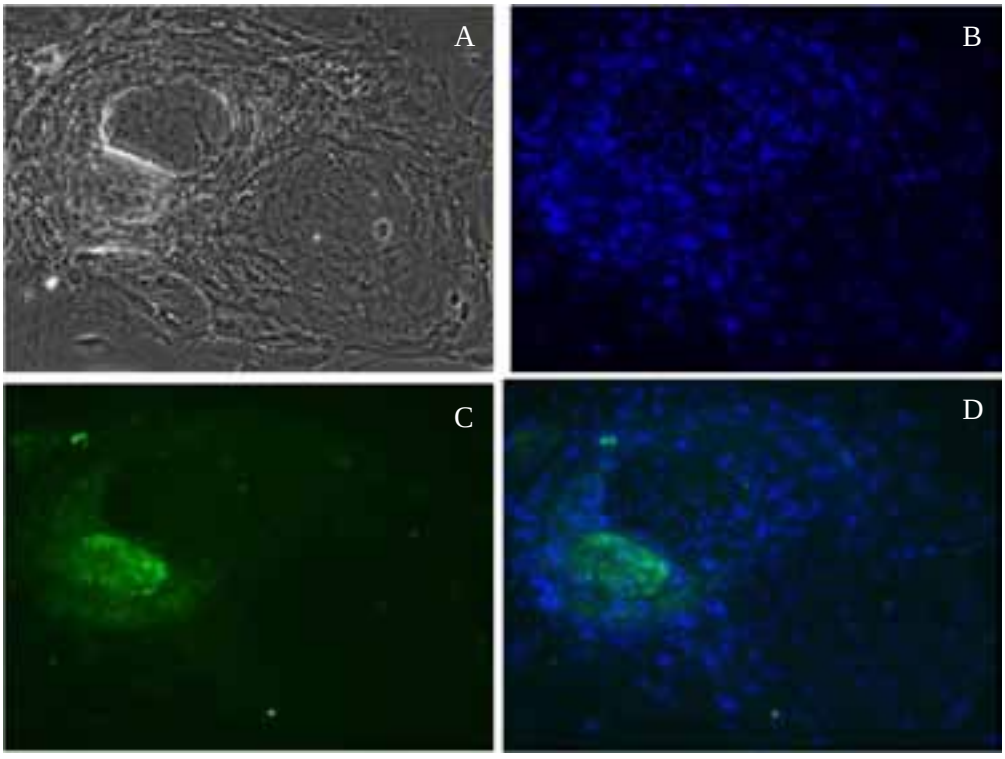

Fig. 15. Immunostaining of Tra-1-60 for equine putative ESCs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

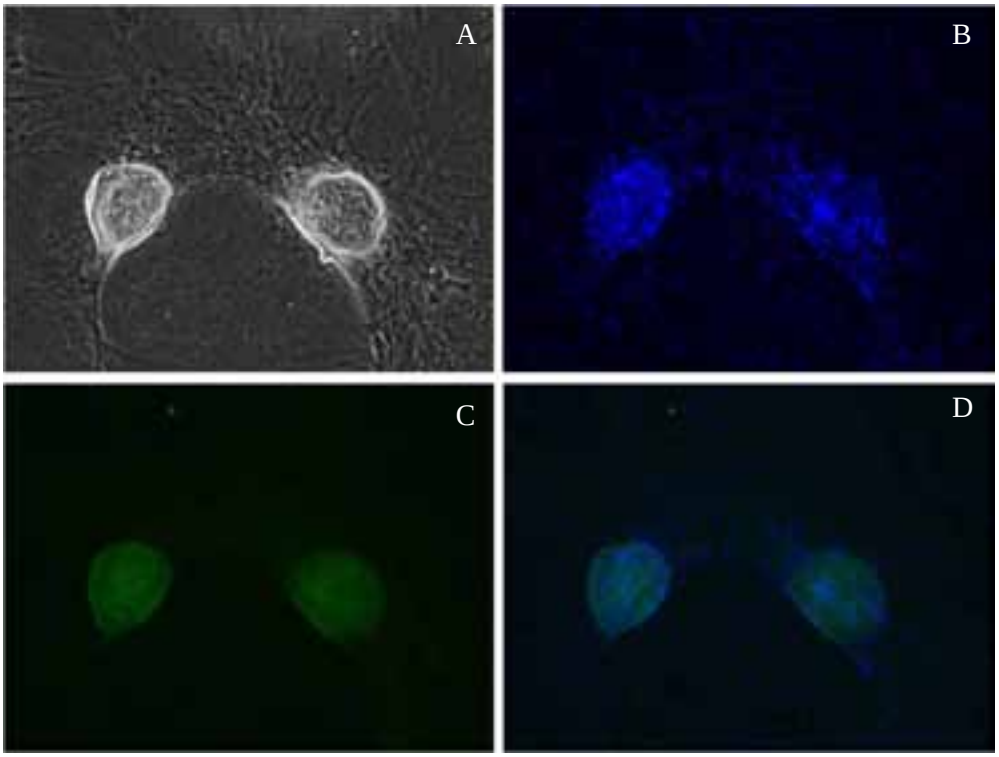

Fig. 16. Immunostaining of SSEA-4 for equine putative ESCs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

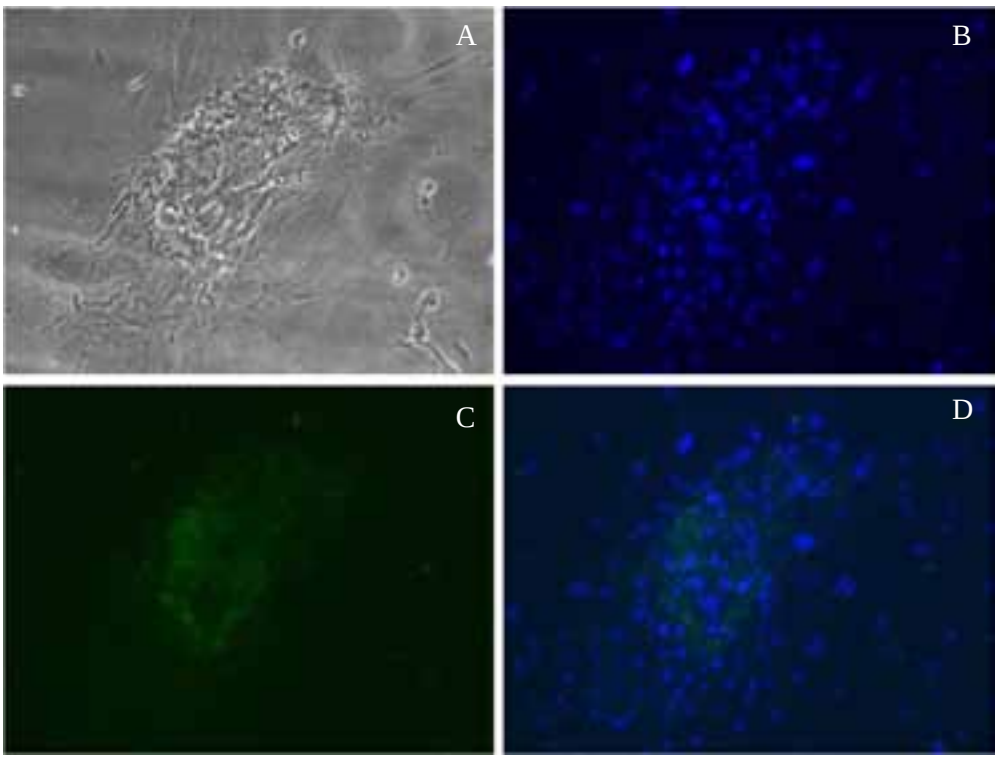

Fig. 17. Immunostaining of Tra-1-81 for equine putative ESCs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

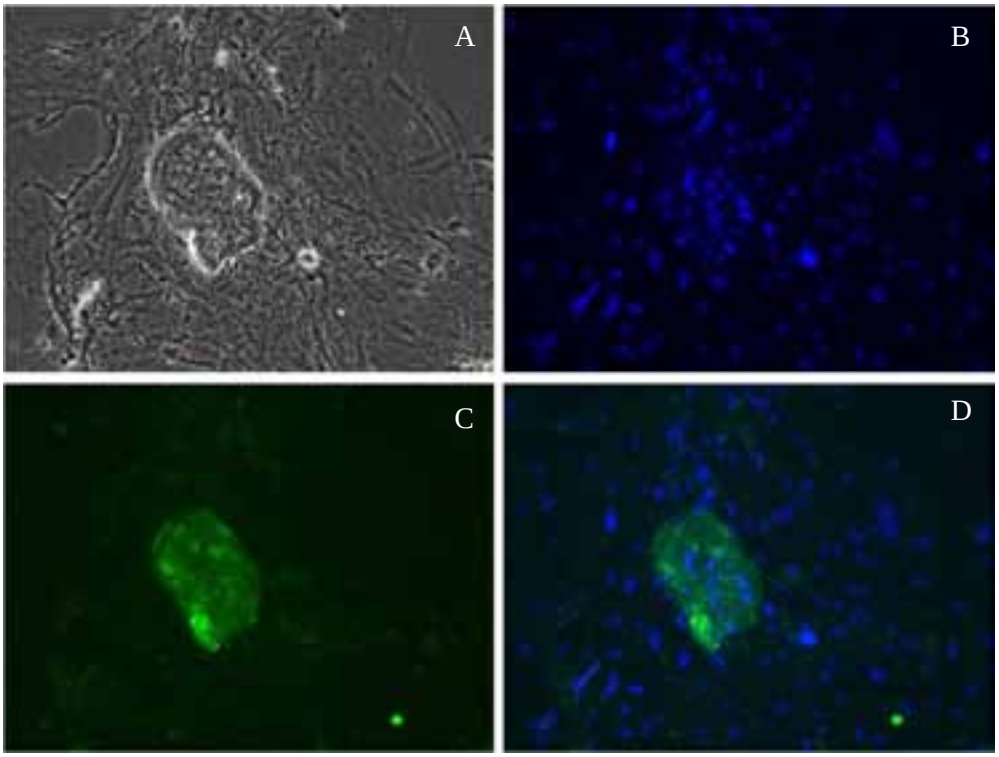

Fig. 18. Immunostaining of CD 9 for equine putative ESCs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

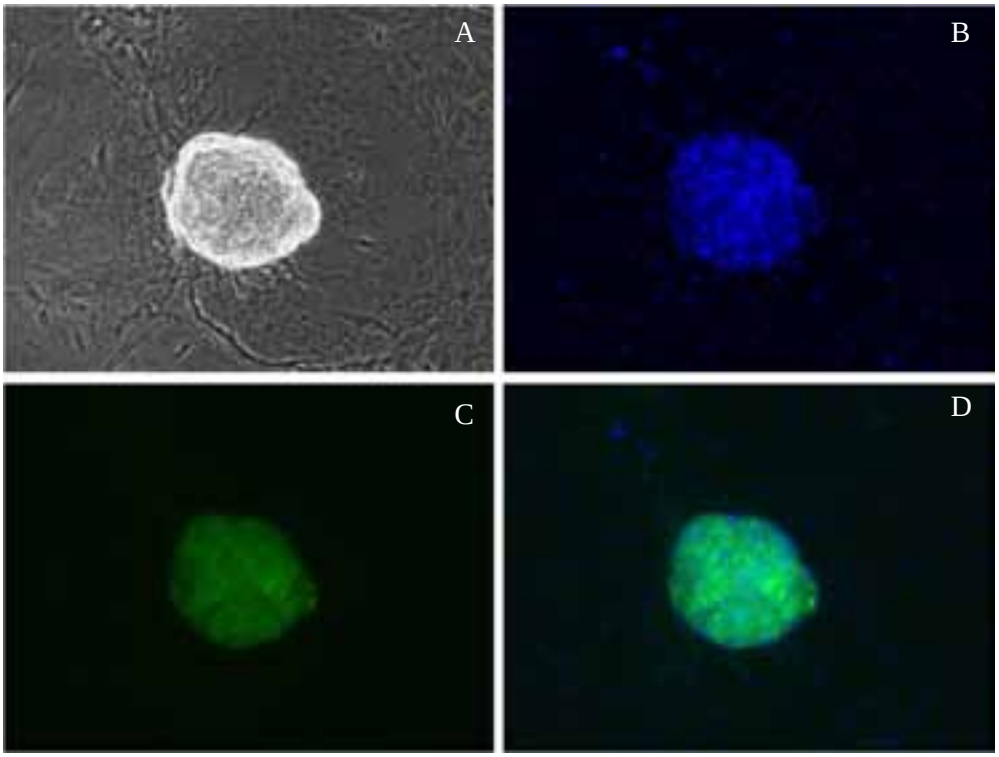

Fig. 19. Immunostaining of TGFβ-R for equine putative ESCs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

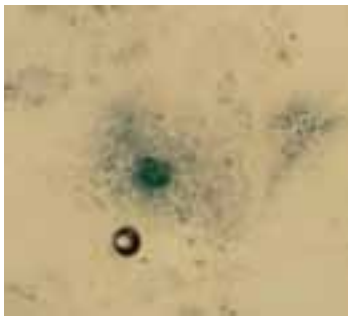

Fig. 20. Alkaline phosphatase staining for equine putative ESCs.

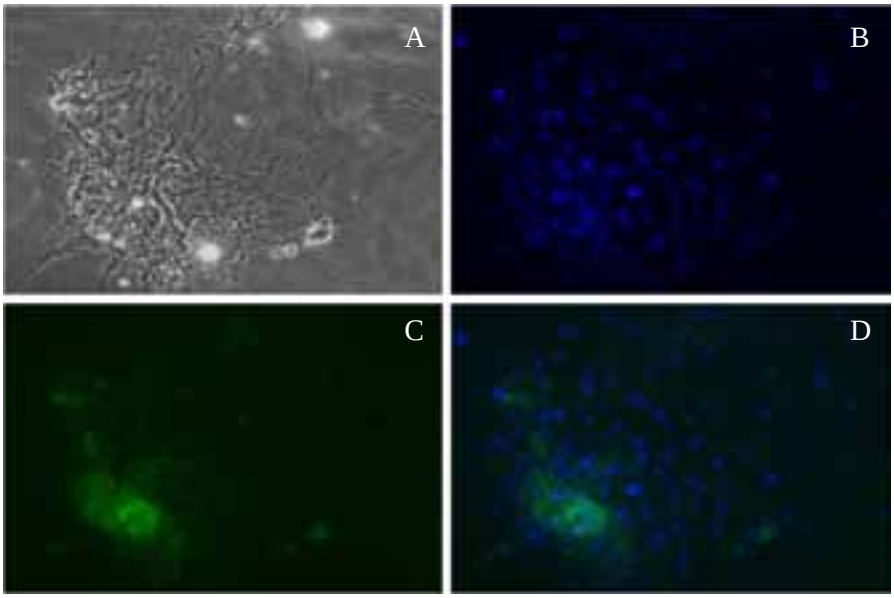

Fig. 21. Immunostaining of Oct4 for putative ntESCs (Large cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

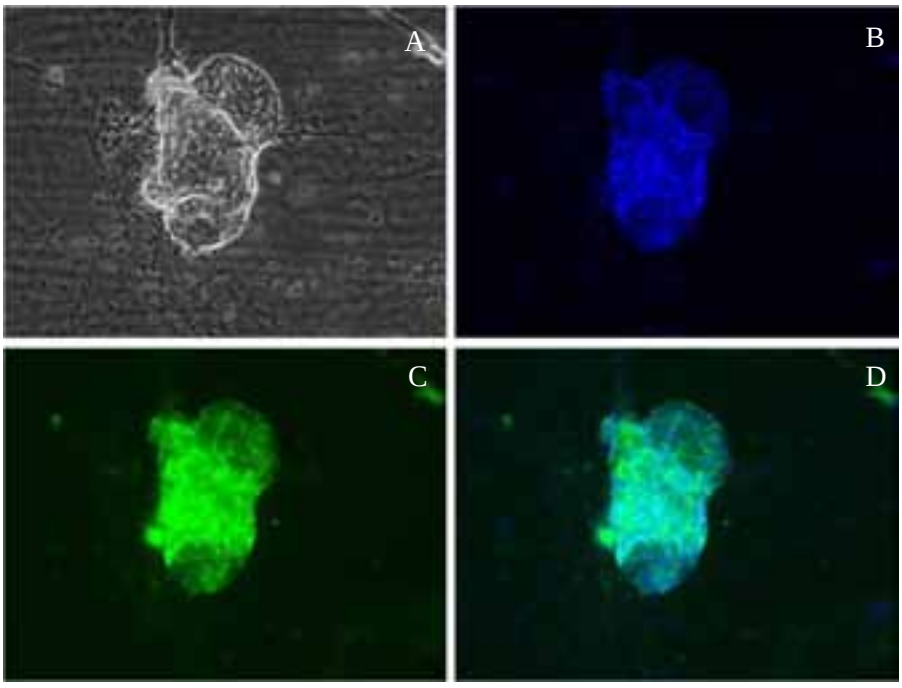

Fig. 22. Immunostaining of SSEA-3 for putative ntESCs (Large cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

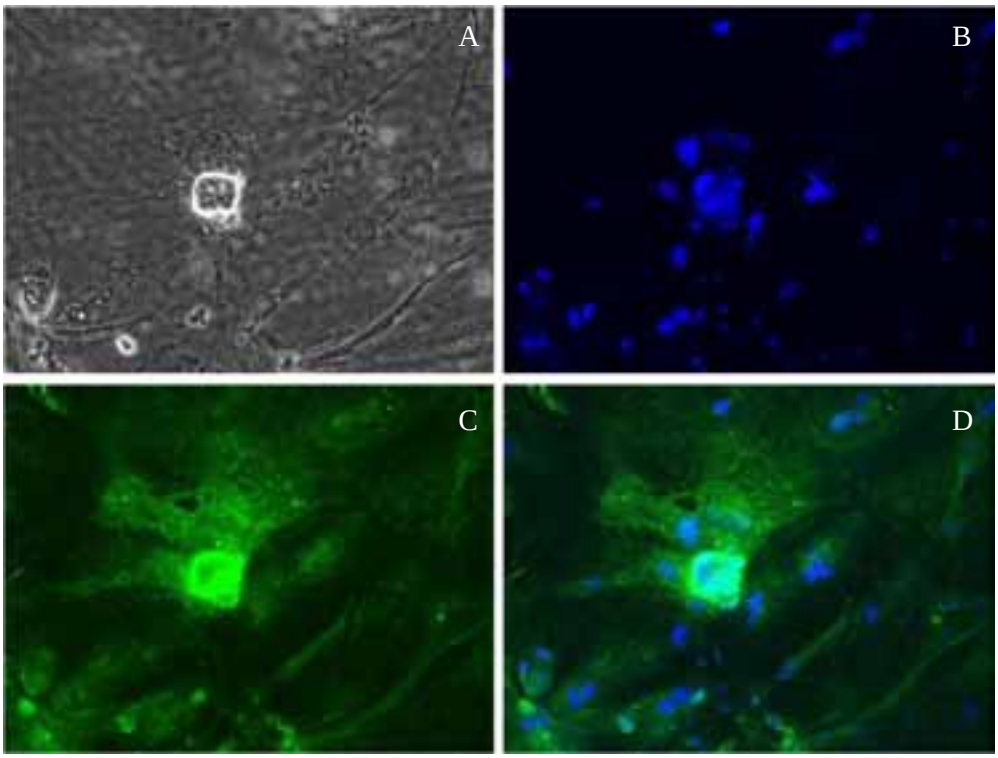

Fig. 23. Immunostaining of SSEA-4 for putative ntESCs (Large cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

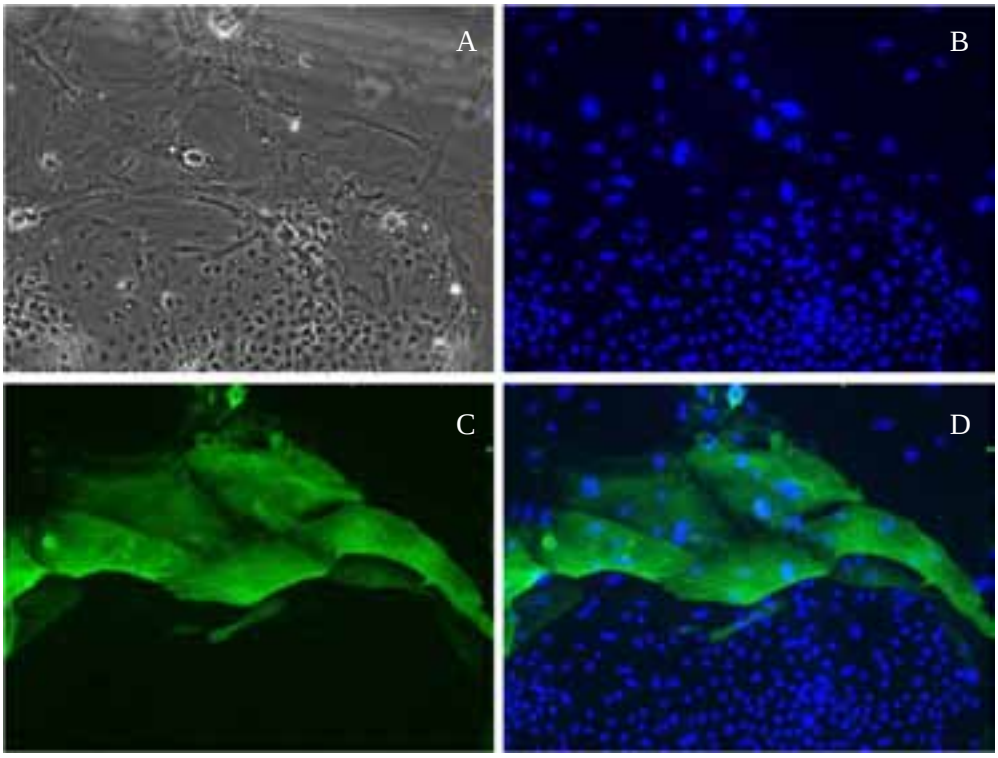

Fig. 24. Immunostaining of Tra-1-60 for putative ntESCs (Large cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

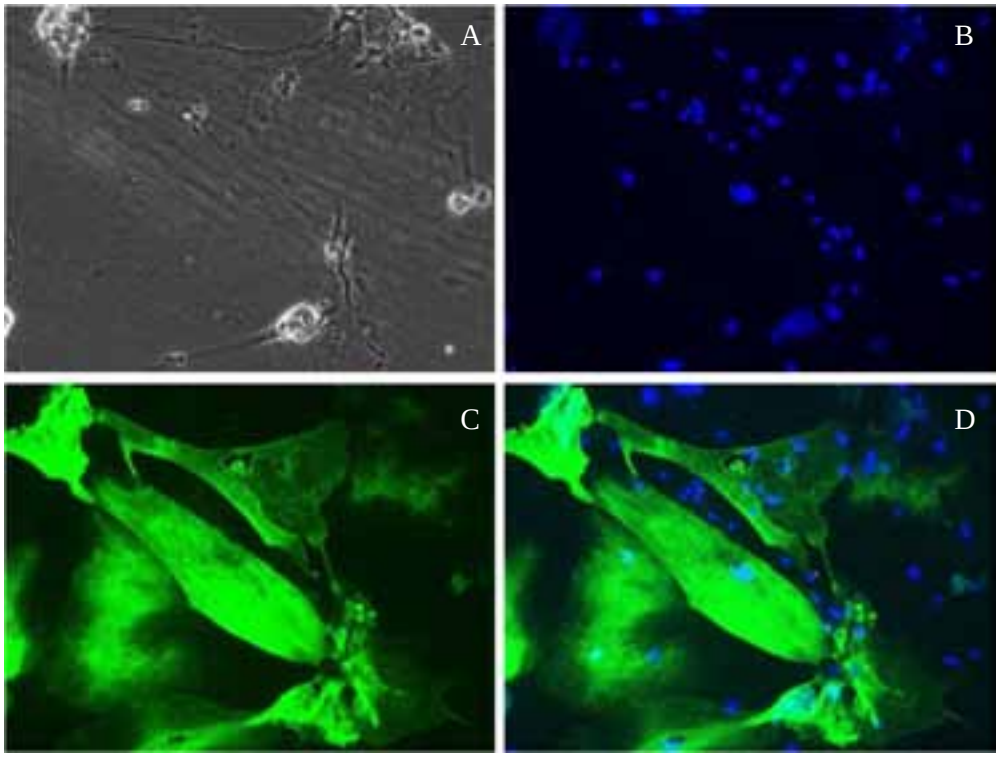

Fig. 25. Immunostaining of Tra-1-81 for putative ntESCs (Large cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

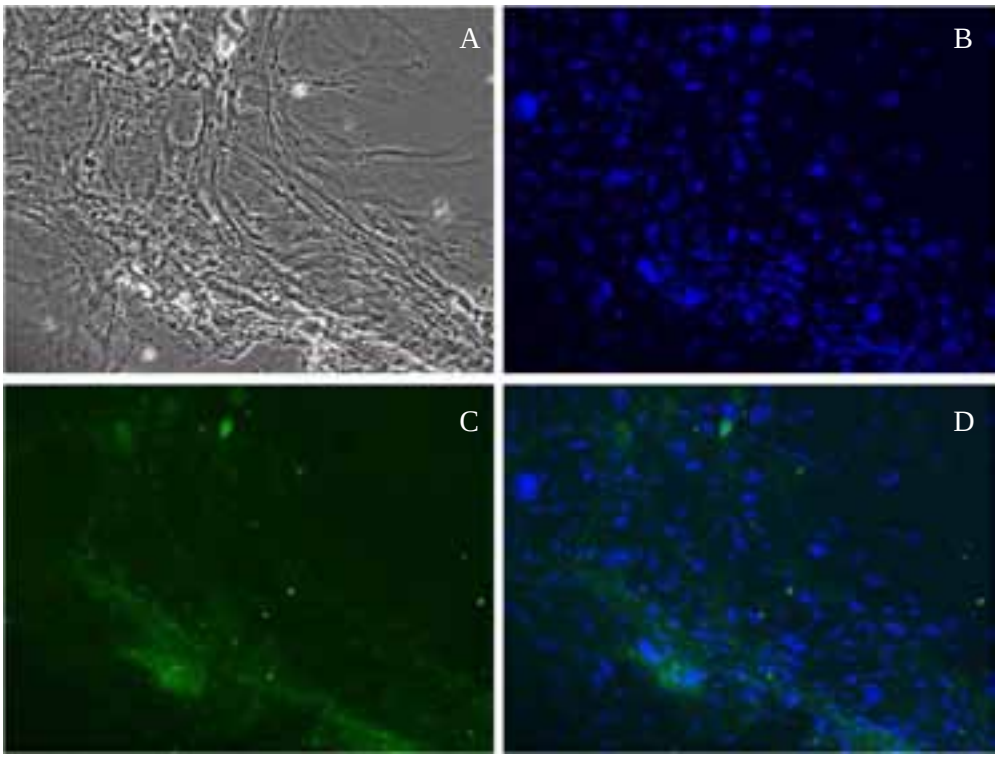

Fig. 26. Immunostaining of Oct4 for putative ntESCs (Small cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

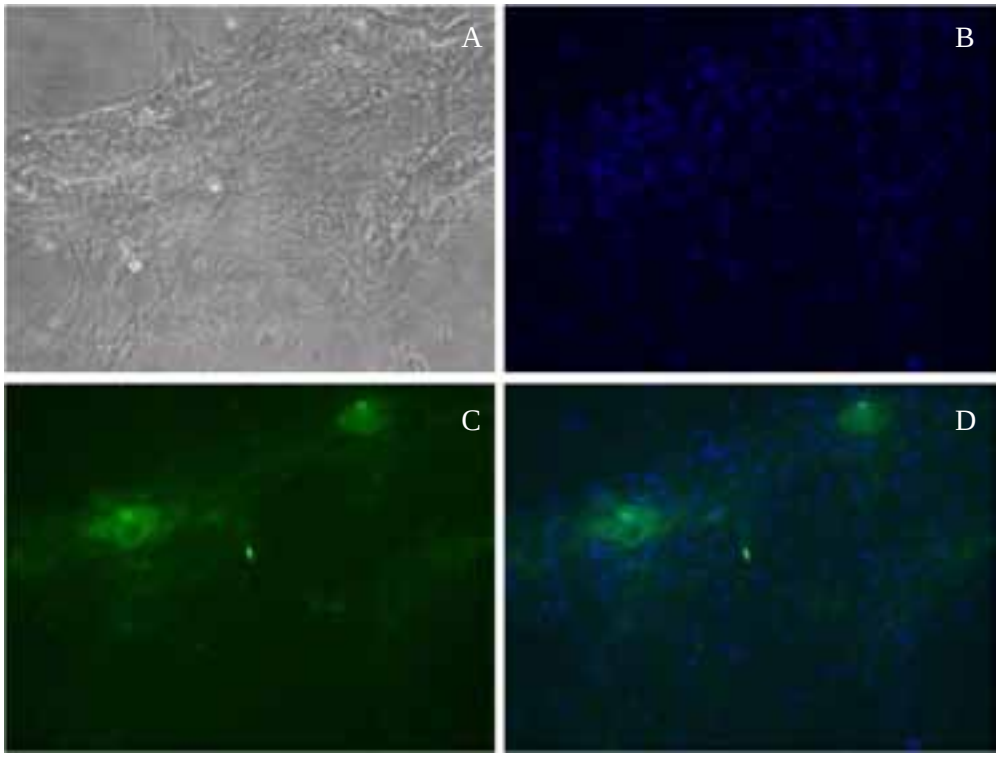

Fig. 27. Immunostaining of SSEA-1 for putative ntESCs (Small cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

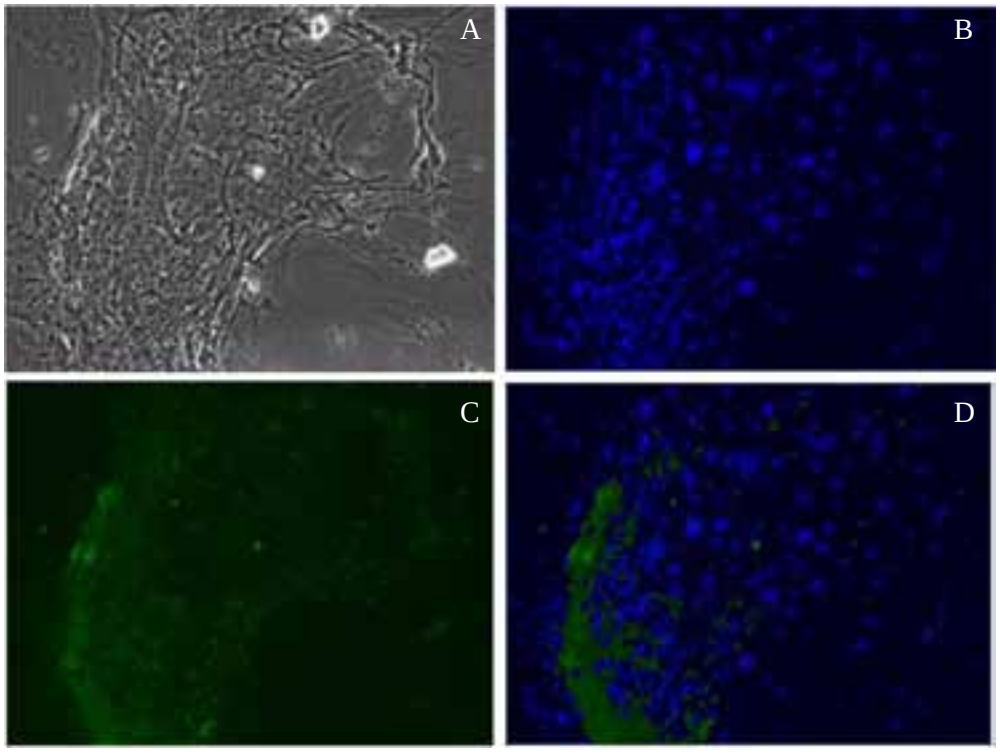

Fig. 28. Immunostaining of SSEA-3 for putative ntESCs (Small cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

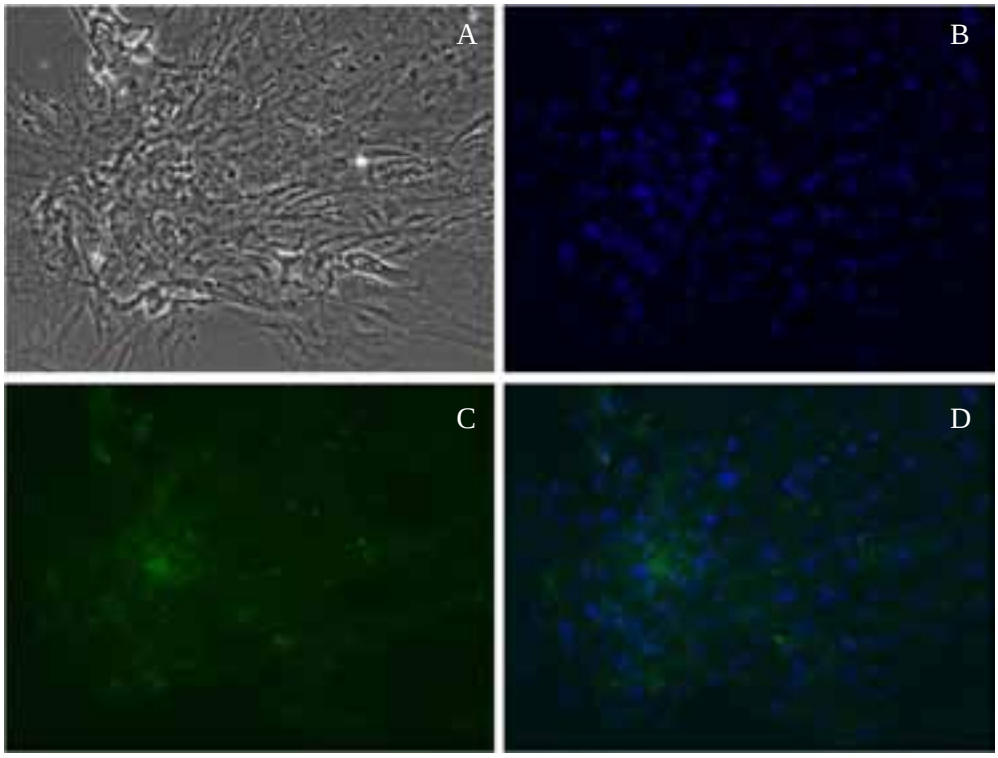

Fig. 29. Immunostaining of SSEA-4 for putative ntESCs (Small cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

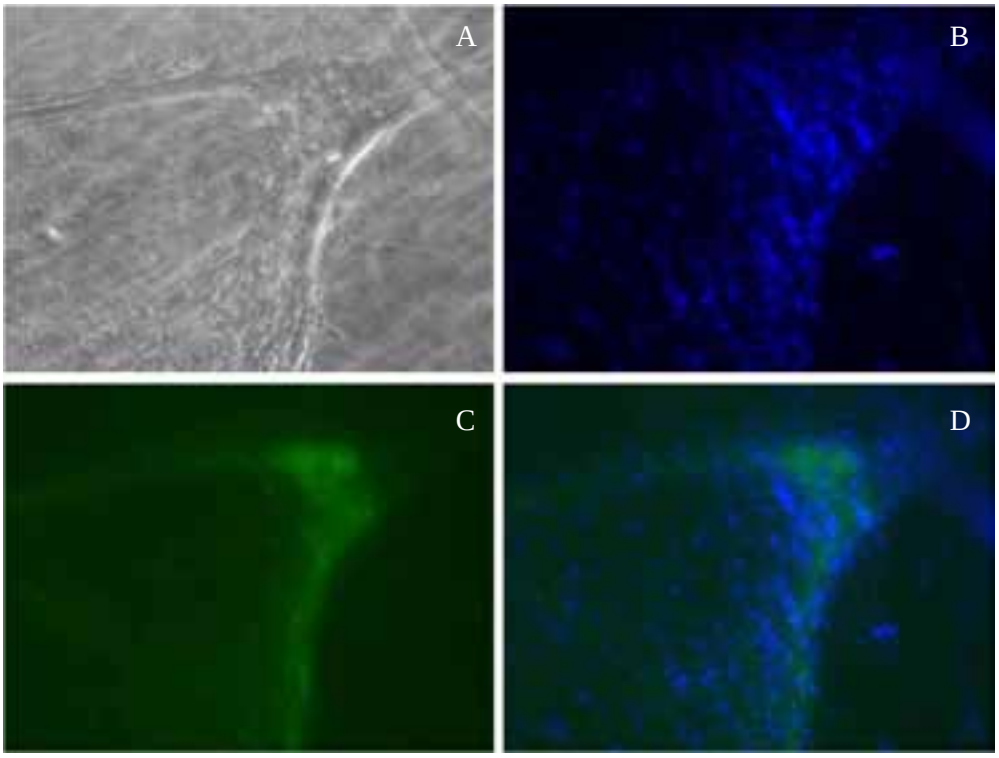

Fig. 30. Immunostaining of Tra-1-60 for putative ntESCs (Small cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

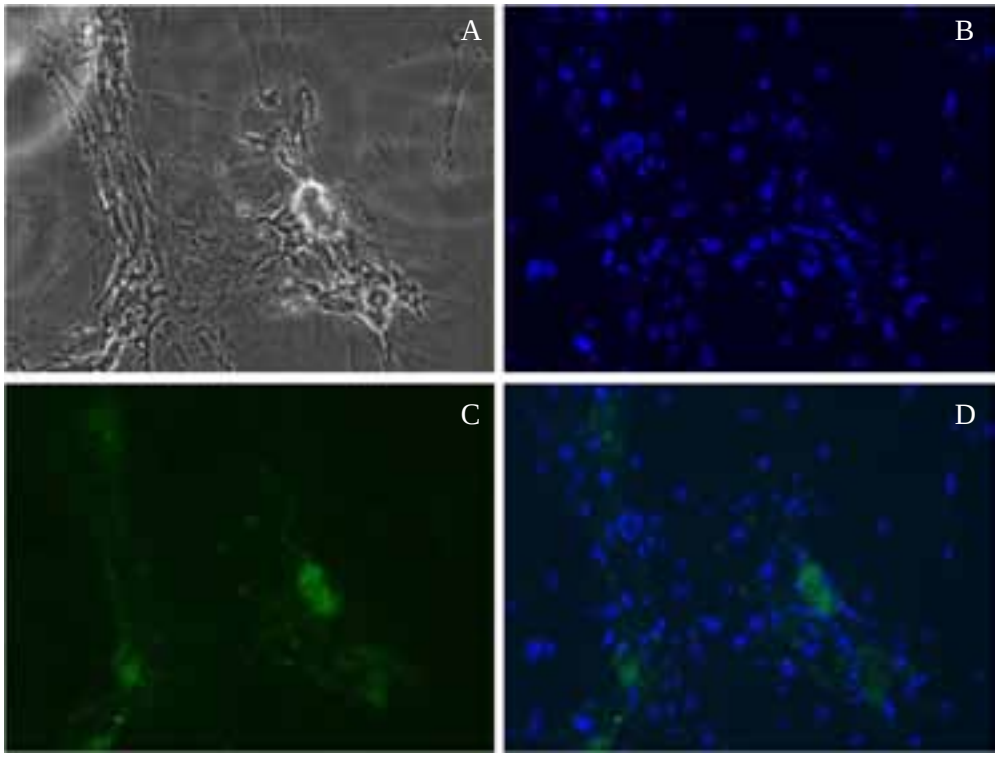

Fig. 31. Immunostaining of Tra-1-81 for putative ntESCs (Small cells). Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

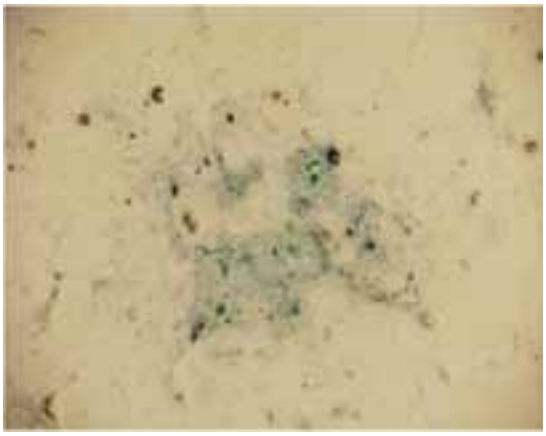

Fig. 32. Alkaline phosphatase staining for putative ntESCs (Small cells)

Optimisation of equine putative ESC culture conditions

The horse putative ESCs basically were able to be cultured in the medium with hLIF until passage 15 (Fig. 33 A and B). However, hLIF was not absolutely necessary and hbFGF appeared negative for maintaining horse putative ESCs (Fig. 33 C, D, E and F). Such eESCs were difficult to maintain colony-like cells without feeder cells up to passage 4 (Fig. 33 G and H). The horse putative ESCs had the capacity of proliferation after freezing preservation in liquid nitrogen (Fig. 34).

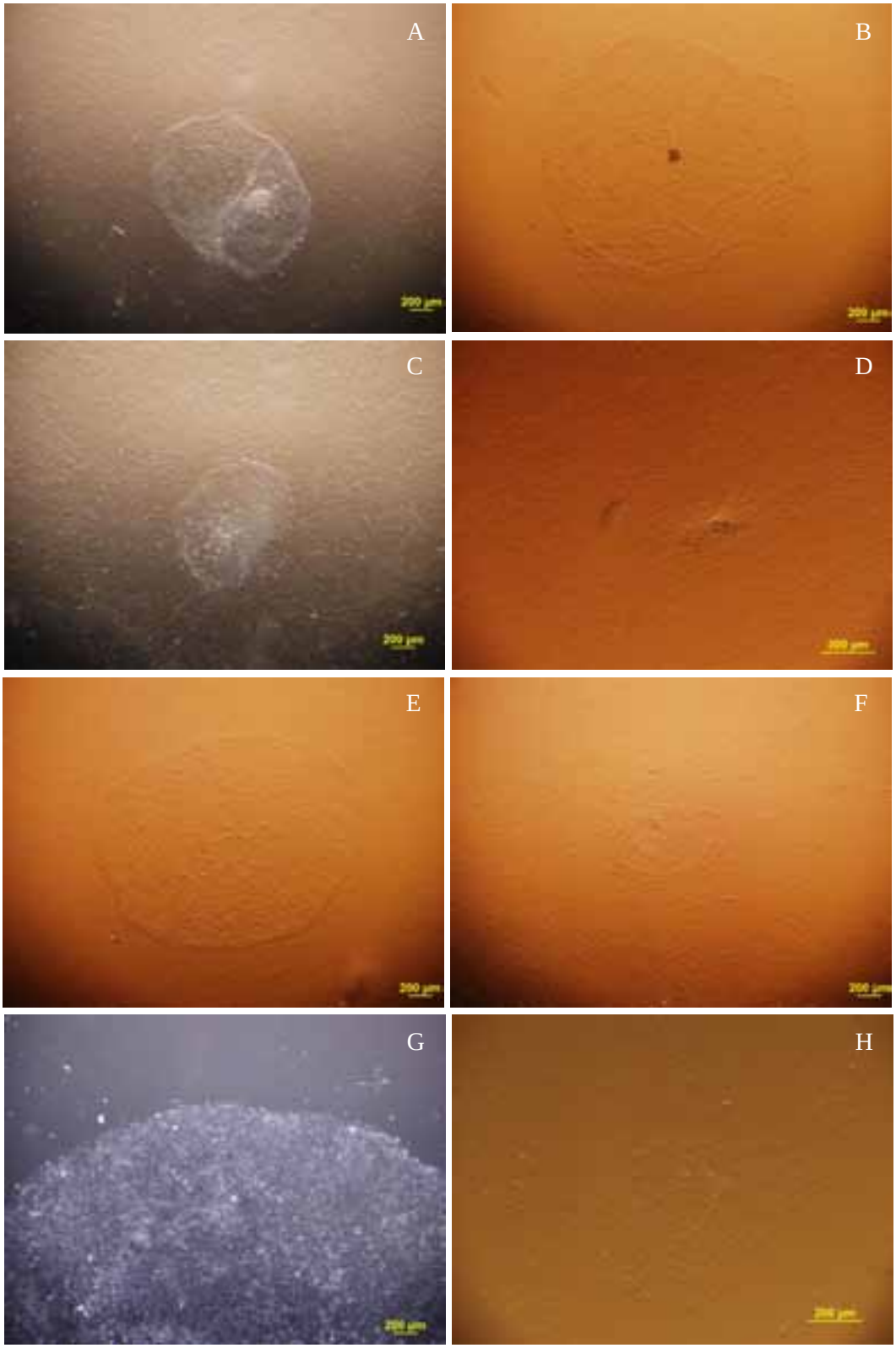

Fig. 33. Culture of putative eESCs under different conditions. eESCs at passage 6 (A) and passage 15 (B) on MEF feeder cells with hLIF in the culture medium; eESCs at passage 6 (C) and 10 (D) on MEF feeder with hLIF and hbFGF; eESCs at passage 12 on MEF feeder without hLIF(E); eESCs at passage 12 on MEF feeder with hbFGF (F); eESCs at passage 2 (G) and passage 4 (H) without MEF feeder with hLIF in the culture medium.

Fig. 34. Frozen-thawed eESCs cultured on MEF feeder cells.

Primary differentiation of equine putative ESCs

The horse putative ESCs had the potential to differentiate into embryoid body (EB)-like cells in vitro (Fig. 35).

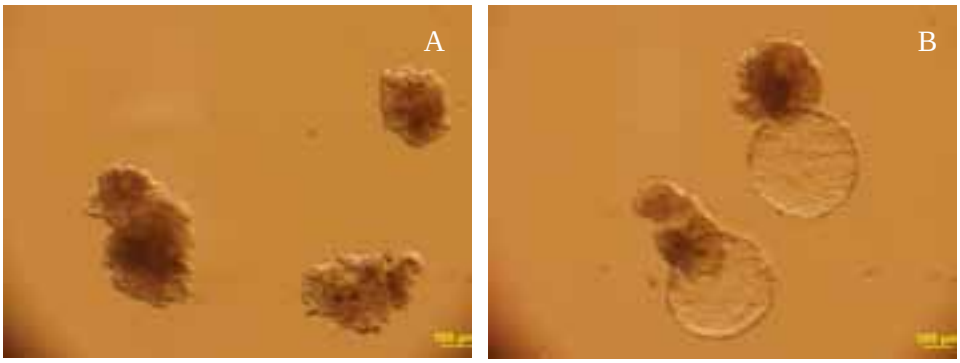

Fig. 35. Differentiation of horse putative ESCs. Embryoid body-like cells (A) and vesicles (B) formed after differentiation culture in vitro.

Isolation of ovine and equine AECS

Amnion epithelial cells were successfully isolated and cultured from ovine and bovine amnions. Primary cultures of the epithelial cells reached 90% confluence within 2-3 days of culture for ovine AECs (Fig. 36), and after 7-10 days for equine AECS (Fig. 37). Ovine AECs had the capacity to proliferate and to be passaged up to six times. Thereafter, the morphology of the cells began to change and cell proliferation was slowed-down, the cell size increased and the nucleus decreased in size relatively (Fig. 36).

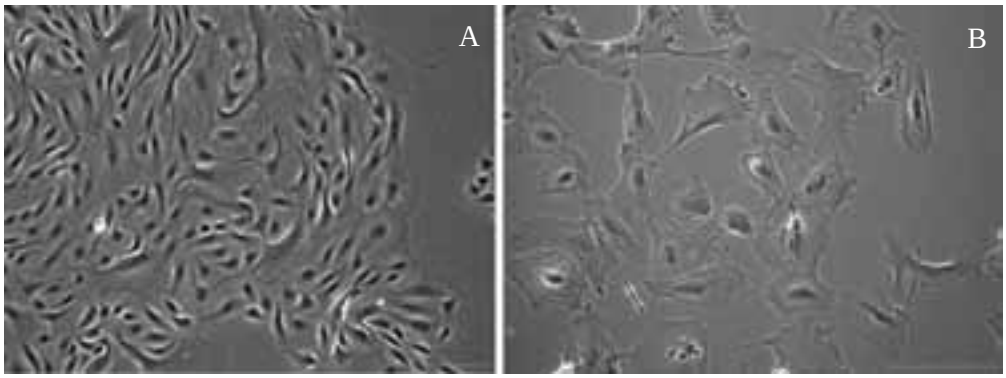

Fig. 36. Morphology of oAECs cultured at passage 1 (A) and passage 6

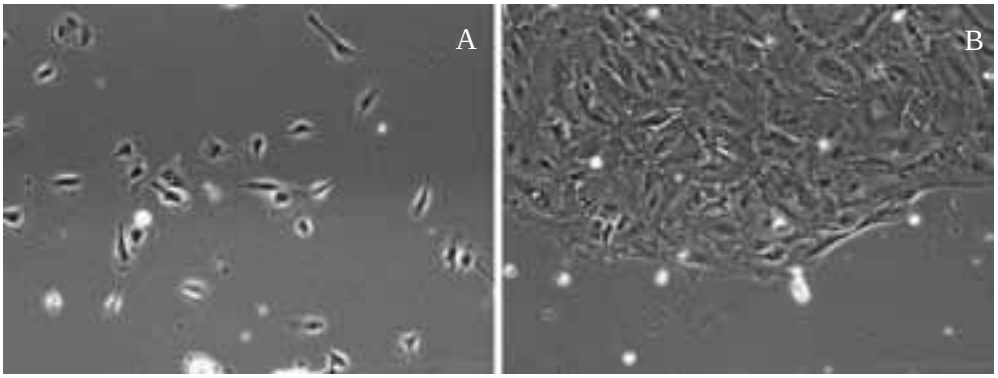

Fig. 37. Morphology of oAECs cultured at passage 1. Single cells (A) and colony–like cells (B) were shown.

Immunocytochemistry of ovine and equine AECs

At passage 1, both enzymatic digestion or untreated amniotic membrane-derived oAECs were found to be positive for Oct-4 (Fig. 38, 39), SSEA-1 (Fig. 40, 41) and SSEA-4 (Fig. 42, 43), and untreated amniotic membrane-derived oAECs were also weakly positive for Tra-1-60 (Fig. 44) and Tra-1-80 (Fig. 45). However, SSEA-3 was undetectable on oAECs derived from either method.

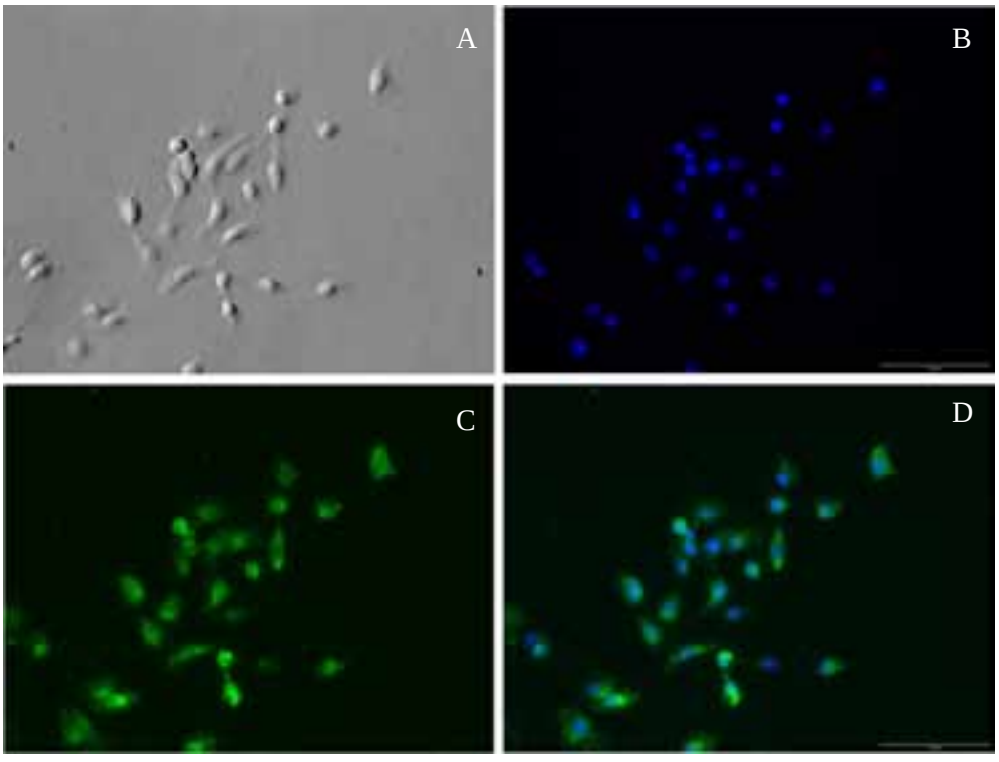

Fig. 38. Immunostaining of Oct4 for enzymatic digestion-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

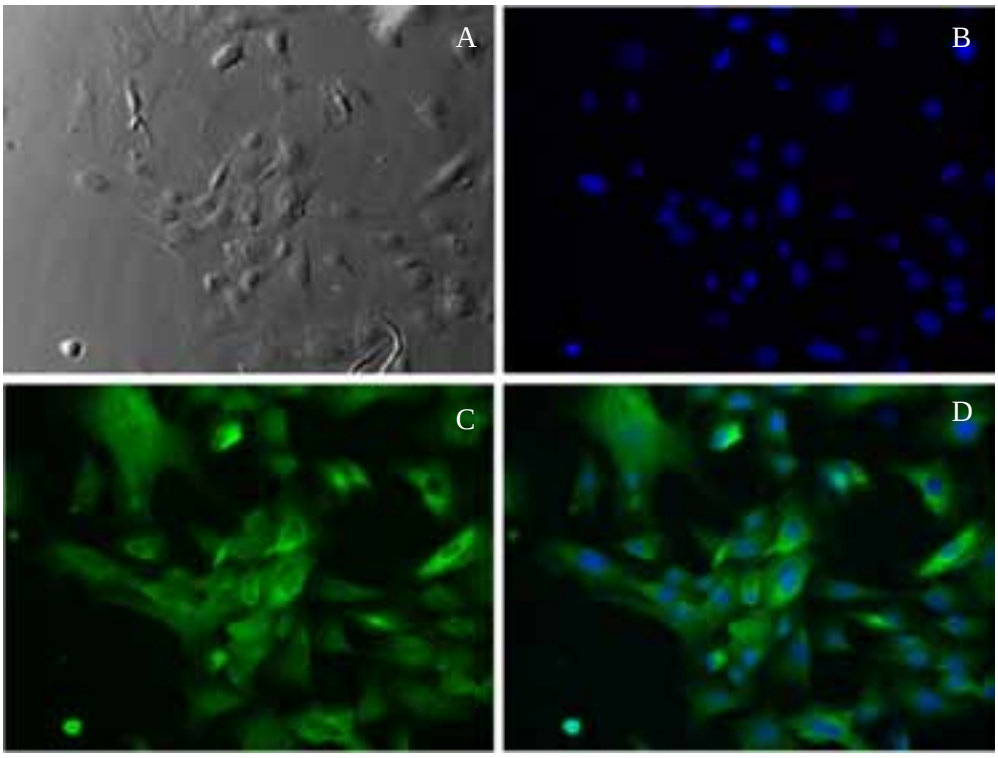

Fig. 39. Immunostaining of Oct4 for untreated amniotic membrane-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

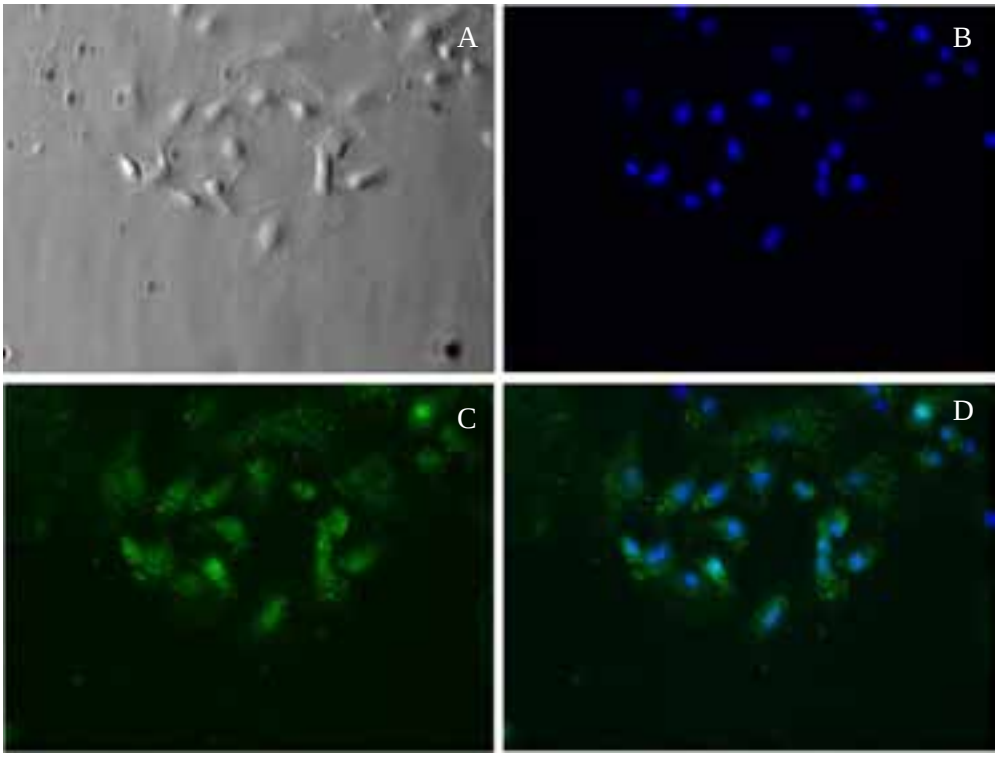

Fig. 40. Immunostaining of SSEA-1for enzymatic digestion-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

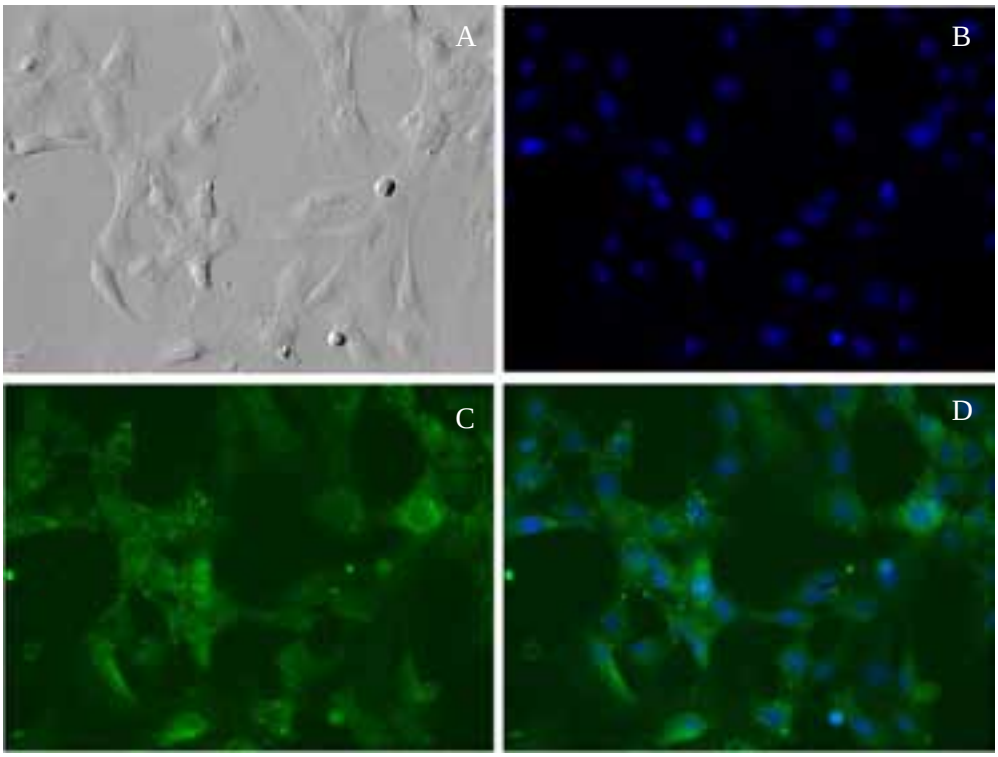

Fig. 41. Immunostaining of SSEA-1for untreated amniotic membrane-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

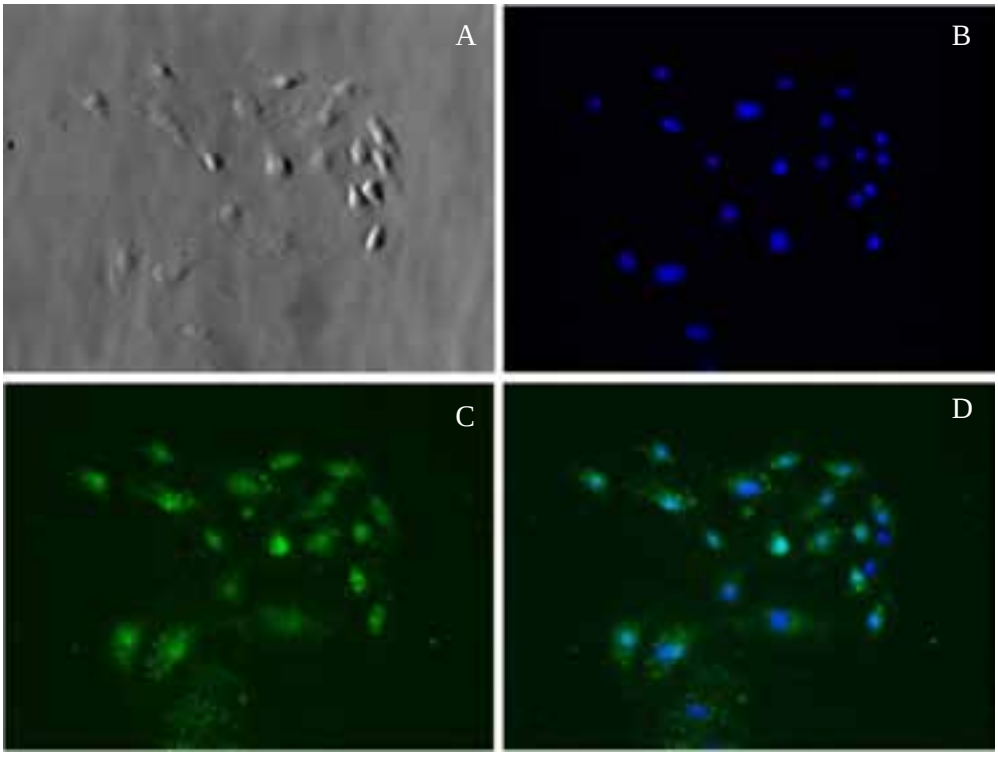

Fig. 42. Immunostaining of SSEA-4 for enzymatic digestion-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

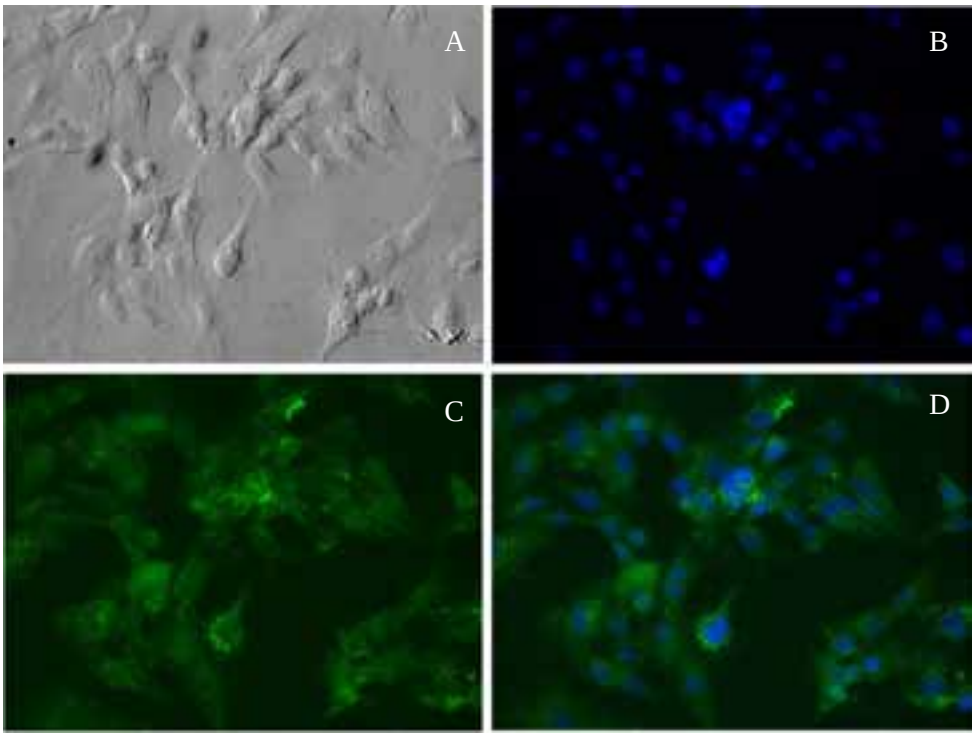

Fig. 43. Immunostaining of SSEA-4 for untreated amniotic membrane-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

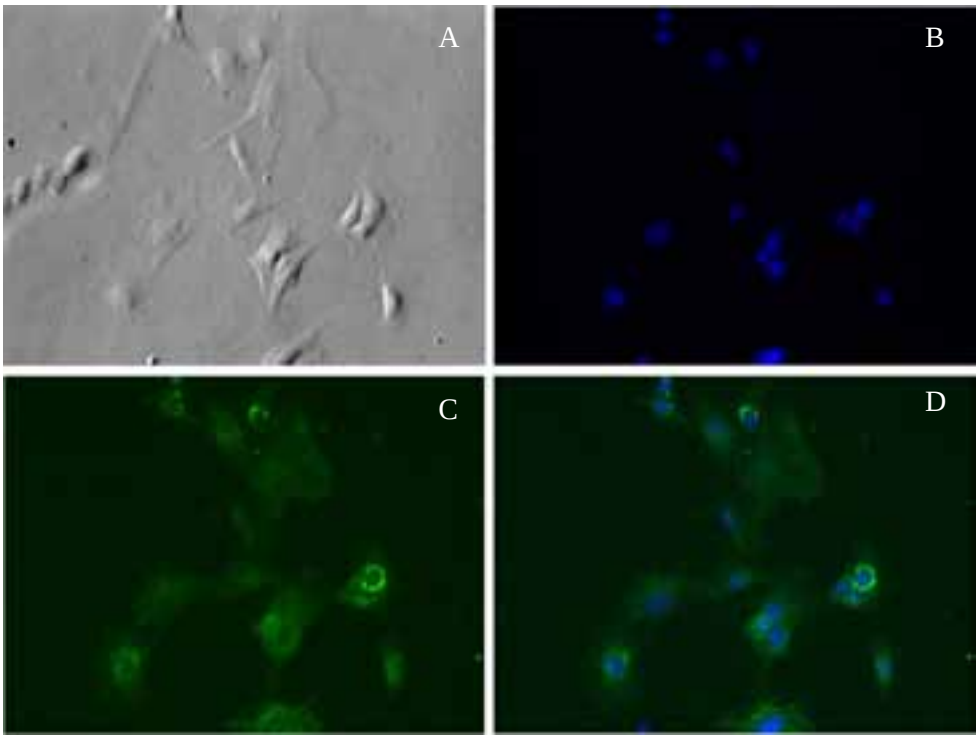

Fig. 44. Immunostaining of Tra-1-60 for untreated amniotic membrane-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

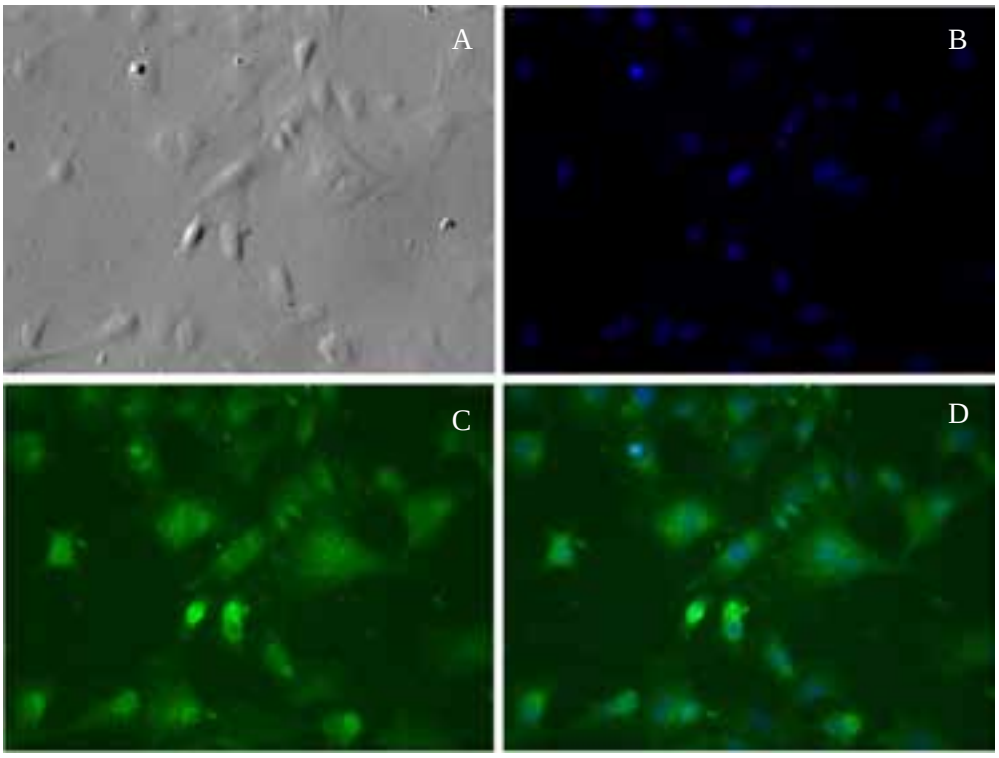

Fig. 45. Immunostaining of Tra-1-81 for untreated amniotic membrane-derived ovine AECs. Images of bright field (A), DAPI (B), FITC (C) and merged image of DAPI and FITC (D).

Preliminary differentiation of oAECs

Ovine AECs were spontaneously differentiated into vesicle-like cells when the cells were unattached in cultuue (Fig. 46 A and B). Some other cell types were also differentiated from ovine AEC culture (Fig.46 C, D and E).

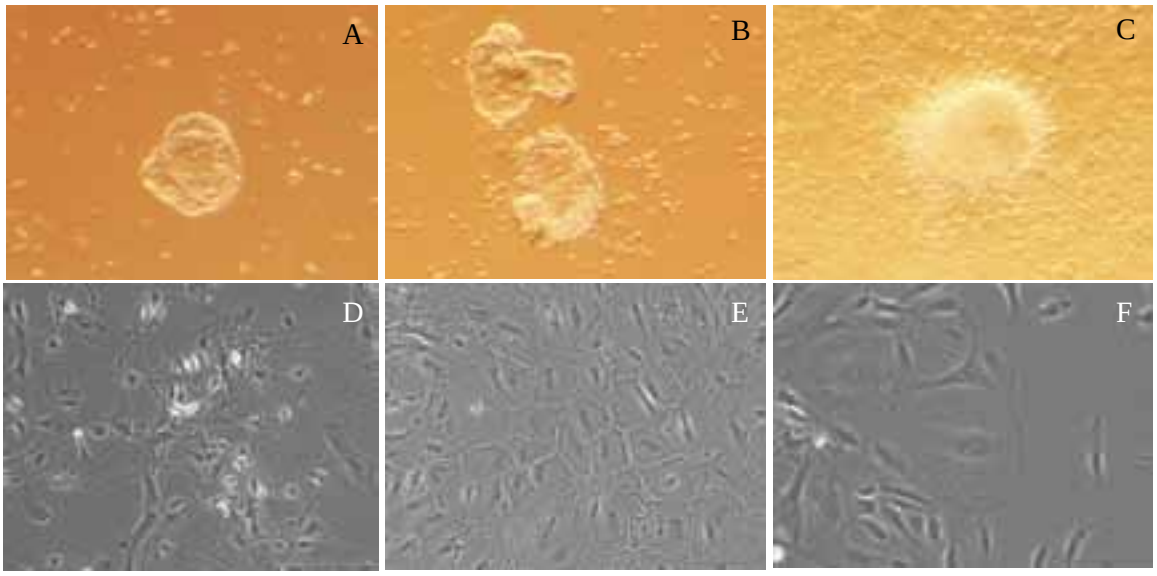

Fig. 46. Preliminary differentiation of ovine AECs in vitro. vesicle-like cells (A and B); neural cell-like cells (C and D); and epithelium-like cells (E and F) were shown.

Chondrogenic and osteogenic differentiation of oAECs

The results of the preliminary differentiation of ovine AECs showed that the cells had potential to differentiate into chondrogenic and osteogenic lineage cells. Chondrogenic lineage cells were visualised by staining of Alcian Blue (Fig. 47 A), and the differentiated cells containing mineral deposits were stained bright red by the Alizarin Red Solution for osteogenic lineage cells (Fig. 47 B).

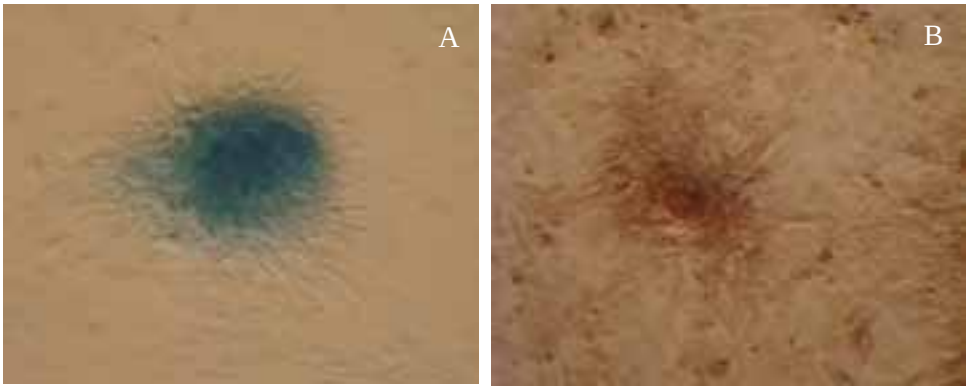

Fig. 47. Chondrogenic and osteogenic differentiation of oAECs. The chondrogenic lineage cells were visualised by staining of Alcian Blue (A); osteogenic lineage cells were stained with Alizarin Red Solution (B).

Discussion

Isolation of equine embryonic stem cells

To date, ES cell lines have been isolated from rodents (Martin et al., 1981), rabbits (Graves and Moreadith, 1993), pigs (Li et al., 2003) and primates (Thomson et al., 1995). However there are only two publications on isolation of ES-like cells in the equine (Saito et al., 2002, Li et al., 2006). In this study, equine ES-like cells appeared to express marker characteristics of ESCs for human and mouse, namely alkaline phosphatase, Oct-4, SSEA1, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81. Our result was similar to previous studies on the horse (Saito et al., 2002, Li et al., 2006). Despite derivation of these ESCs from in vivo fertilised or iSCNT embryos, ESC markers only were detected on some of the cells even within the same colony. Interestingly, the TE cell lines derived from both in vivo fertilised and iSCNT embryos were positive for Oct4, SSEA-1 and SSEA-4, negative for SSEA-3 (data not shown). Thus, it appears that established equine ESCs actually are embryonic-derived stem cells or embryonic stem cell-like cells for all these published results.

This means that there are different pathways to regulate embryonic development for the horse and even the ungulate animals. New growth factors different from human and mouse need to be screened further for maintaining an undifferentiated state of ungulate animal ESCs. Although these embryonic-derived cells cannot strictly be called embryonic stem cells, they are still pluripotent since they are able to differentiate to some somatic cell types. Further research is needed to investigate the differentiation potential to all types of somatic cells in vitro and in vivo. Due to the failure of teratoma formation, which is a required characteristic of embryonic stem cells, such equine ESCs should be named as ESC-like cells (Li et al., 2006). However, they may be used as the new stem cell resources for cell therapy.

Several studies have shown that oocyte cytoplasm from the bovine can support early development of embryos produced by iSCNT with the nucleus from sheep (Dominko et al., 1999), pig (Dominko et al., 1999; Yoon et al., 2001), buffalo (Kitiyanat et al., 2001; Saikhun et al., 2002), rat (Dominko et al., 1999), monkey (Dominko et al., 1999), horse (Li et al., 2002; Sansinena et al., 2002) and human (Chang et al., 2003), the rabbit oocyte supports the nucleus from panda (Chen et al., 2002), monkey (Yang et al., 2004) and cat (Wen et al., 2003), and the ovine oocyte supports the nucleus from argali (White et al., 1999), gaur (Lanza et al., 2000) and mouflon (Loi et al., 2001). However, horse embryos failed to develop to the blastocyst stage when bovine eggs were used as a recipient. Up to now, only ntESCs derived from iSCNT by transferring human cell nucleus into enucleated rabbit oocyte has been reported (Chen et al. 2003). No report on interspecies ntESCs using oocytes from other species in the literature has been found. In this present study, equine ESCs were generated from equine fibroblast-bovine oocyte nuclear transfer. This is the first report on the isolation of equine ESCs by iSCNT, although the cell line was lost after 12 passages. The resulting eESCs are perfectly matched to the donor immune system, thus no immunosuppressants would be required to prevent rejection if they were used therapeutically. These autologous stem cells have the potential to become any types of cells in the body and thus would be useful in regenerative medicine. Except for bovine oocytes, mouse and rabbit oocytes were also assessed for generation of ntESCs but failed to produce blastocysts by those iSCNT embryos (data not shown).

Differentiation potential of equine embryonic stem cells

Different transcription factors and their specific interactions are involved in the specific signalling pathways. Nanog, Oct4, STAT3, Smad1, Sox2, Zfx, c-Myc, n-Myc, Klf4, Esrrb, Tcfcp2l1, E2f1, and CTCF, are known to play different roles in ESC self-renewal regulation and specifying gene expression ESCs (Sato et al., 2003; Chambers, 2004; Liu et al., 2006), and the specific genomic

regions extensively targeted by different transcription factors (Chen et al., 2006). Bovine embryos and embryonic cells have not shown to be influenced by LIF (Rexroad and Powell 1997; Rodriguez et al., 2007), which is necessary for proliferation of mouse ES cells (Evans and Kaufman 1981). bFGF is a critical component of human embryonic stem cell culture medium (Amit et al., 2000,) and also supports culture of bovine (Gjorret and Maddox-Hyttel 2005) and porcine ESCs (Li et al., 2004), but is unable to support mouse ESCs (Ginis et al., 2004). Thus, we believe that the ungulate animal has different regulators to regulate and specify gene expression, which will maintain the ESCs undifferentiated and pluripotent state. Because the growth factor, hLIF or hbFGF is specifically for mouse or human ESC culture, it is unsuitable for equine ESC culture under our conditions. Further research is required to focus on the growth factor screening for maintenance of ungulate animal ESCs. Without considering the derivation of the putative equine ESCs from fertilised or iSCNT embryos, we found it is difficult to distinguish embryonic stem cells and other embryonic-derived stem cells from the coexistent cell population because both express stem cell markers. This suggests that conventional stem cell markers are unspecific for equine ESCs and is consistent with those of findings from bovine ESCs (Muñoz et al., 2008). However, such cells express some of the stem cell markers, and are able to differentiate into some somatic cell types. This is the possible reason why such ESCs cannot form teratoma after transplantation into SCID mouse (Li et al., 2006). The differentiation ability of ESC in vitro is evaluated by EB formation through cell aggregation. A wide variety of somatic cell types were differentiated from EBs, including derivatives of the three germ layers with bovine (Saito et al. 2003; Wang et al. 2005) and equine ESCs (Li et al., 2006). Therefore, EB formation is proof of their pluripotent character in vitro. Our preliminary differentiation also results in EB formation from putative eESCs. This result indicates that the putative ESCs isolated in our laboratory are at least partially pluripotent.

Isolation of ovine and equine embryonic stem cells

Human amniotic-derived stem cells have been successfully isolated from term delivered gestational tissue, and hAECs can express the transcription factors Oct-4, Sox-2 and Nanog that maintain the undifferentiated state of pluripotent stem cells and also several multipotent stem cell markers (Miki et al., 2005; Ilancheran et al., 2007; Kim et al., 2007a, 2007b). Due to the restriction of seasonal breeding of the horse, ovine amnion was used for derivation of epithelial stem cells to develop the protocols used in this study. Ovine and equine AECs have been isolated from sheep and horse amnion with the method used for human AEC isolation, and ovine AECs also showed positive for Oct-4, SSEA-1 and SSEA-4 and weakly positive for Tra-1-60 and Tra-1-80. These results agree with the findings from hAECs (Carraro et al., 2008). Our result showed that SSEA-3 was undetectable on ovine AECs. We found that two types of cells were isolated from ovine amnion. One type of cell easily became attached to the culture flask, but the other type preferred to stay semi-suspended in the culture media. Unlike ESCs, the ovine AECs only had the capacity to proliferate up to six passages. Thereafter, the cell morphology began to change and cell proliferation was slowed down. Long-term culture of ovine AECs remains a problem, and further research on this topic is required. Interestingly, cell morphology and behaviour of equine AECs isolated by the same protocol differed from ovine and human AECs; they appeared to be larger in cell size and cell proliferation was slower. Further characterisation of stem cell markers for equine AECs will need to be performed in order that they can be appropriately characterised.

Differentiation potential of ovine and amniotic-derived stem cells

It has been reported by our group and by others, that human AECs retain considerable plasticity, differentiating into lineages derived from each of the three primary germ layers in vitro. Neurones, astroglia, osteocytes, adipocytes, myocytes, cardiomyocytes, hepatocytes and pancreatic cells have been differentiated from hAECs (Miki et al., 2005; Ilancheran et al., 2007, Kim et al., 2007a, 2007b). In this present study, ovine AECs were isolated and spontaneously differentiated for the first time into several cell types with obviously different morphology. The preliminary differentiation of

chondrogenic and osteogenic lineage cells from ovine AECs were also confirmed through the staining of proteoglycans synthesized by chondrocytes with Alcian Blue and the staining of mineralised matrix produced by osteocytes with Alizarin Red S, respectively. The differentiated chondrocytes and osteocytes were, however, atypical of classical chondrocytes and osteocyte in morphology. Because this is the first experiment of differentiation of chondrogenesis and osteogenesis for ovine AECs, optimal conditions for their differentiation remain to be developed further. The capacity of AECs to engraft and differentiate into functional, tissue specific cells in vivo has not been adequately tested so far. Preliminary evidence has been obtained from our group that hEACs are able to specifically home to damaged tissue (lung) and differentiate into lung epithelium. Furthermore, hEACs have been shown to have anti-inflammatory and anti-fibrotic effects in damaged lung in a SCID mouse model (unpublished data). Our group has also successfully used hAECs for sheep spinal disk repair following xeno-transplantation, indicating their immune tolerance capacity as well as their osteogenic potential. Since our results indicated that ovine AECs have similar properties to those of the human, we are very excited at the concept that it will be possible to exploit the plasticity, anti-tumor, trophic, anti-inflammatory and immune-privileged features of domestic and companion animal derived AECs to develop novel and effective veterinary stem cell based therapies in a world first application of AECs.

Appendix

Chondrogenic differentiation medium

Component	Source	Volume	Concentration
DMEM/F12	Invitrogen	100 ml	—
Insulin-Transferrin-Selenium (100X)	Invitrogen	1 ml	1X
50 mM L-ascorbic acid	Sigma	100 µl	50 µM
40 mg/ml L-proline	Sigma	100 µl	40 µg/ml
100 µM dexamethasone	Sigma	100 µl	0.1 µM
10 µg/ml rhTGF-beta 3	Invitrogen	100 µl	10 ng/ml
Penicillin-Streptomycin (100X)	Invitrogen	1 ml	1X
FBS	Invitrogen	1 ml	1%

Store in the dark at 4°C up to two weeks.

Osteogenic differentiation medium

Component	Source	Volume	Concentration
DMEM/F12	Invitrogen	100 ml	—
Insulin-Transferrin-Selenium (100X)	Invitrogen	1 ml	1x
50 mM L-ascorbic acid	Sigma	100 µl	50 µM
100 µM dexamethasone	Sigma	100 µl	100 µM
1M β-glycerophosphate	Sigma	1 ml	10 mM
Penicillin-Streptomycin (100X)	Invitrogen	1ml	1x
FBS	Invitrogen	10 ml	10%

Store in the dark at 4°C up to two weeks.

Amniotic epithelial cell culture medium

Component	Source	Volume	Concentration
DMEM/F12	Invitrogen	410 ml	—
Penicillin-Streptomycin (100X)	Invitrogen	5 ml	1x
Insulin-Transferrin-Selenium (100X)	Invitrogen	5 ml	1x
MEM NEAA (100X)	Invitrogen	5 ml	1x
2-Mercaptoethanol (55 mM)	Invitrogen	900 µl	0.1 mM
FBS	Invitrogen	75 ml	15%

Store in the dark at 4°C up to two weeks.

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The Development of Horse Embryonic and Amnion Derived Stem Cells

By Jitong Guo, R. Tayfur Tecirlioglu, Angus McKinnon, Alan O. Trounson and Graham Jenkin

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Joint injuries are a major cause of economic loss in the horse industry. Even a small cartilage injury in a critical spot can end a successful career of a race horse.

This report focuses on the new stem cell technology for producing equine stem cells to treat horse cartilage injury and many other diseases. The protocols for isolation of equine embryonic stem cells, ovine and equine amnion epithelium stem cells are described in the report.

Cover photo courtesy Bruno Cannatelli

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