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Maintenance of horse embryonic stem cells in different conditions

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Summary

Embryonic stem cells (ESCs) are originally derived from the ICM of blastocysts and are characterized by their ability to self-renew and their pluripotencies. Only a few reports have been published on ESC isolations and line establishment in animals, even fewer in horses. However, it is still important to isolate equine ESCs for animal biotechnology and therapeutic applications. In the present study, we tried to derive horse ESC lines from the ICM of blastocysts fertilized *in vivo* and maintain their pluripotencies in different conditions. The primary horse ESCs were able to self-renew when they were cultured in basic medium on γ-irradiated MEFs. After 15 passages, immunohistochemistry of the putative horse ESCs showed that some cells in the colonies were positive for Oct-4, SSEA-1, GCTM-2, TRA-1-60 and TRA-1-81. Moreover, to optimize the culture conditions, these putative horse ESCs were cultured in basic medium supplemented with human leukemia inhibitory factor (hLIF) only, human basic fibroblastic growth factor (hbFGF) only, or hbFGF plus hLIF with or without heterologous (MEF) feeder cells. Based on our results, the heterologous feeder (MEF) cells are necessary to maintain the undifferentiated state for horse ESCs, and ESC-like cell morphology of horse ESCs were well maintained in the basic medium supplemented with or without hLIF. This result suggested that hLIF was neither prerequisite nor negative for maintenance of horse ESCs; bFGF seemed to be negative for maintenance of horse ECSs and the combination of hLIF and bFGF was unable to improve the culture condition.

Key words: Embryonic stem cells, Horse, hLIF, hbFGF, MEF

Introduction

Stem cells are characterized by their ability to self-renew and by their ability to give rise to a more or less spectrum of differentiated cell types. Embryonic stem cells (ESCs) are widely used for gene transfer and producing clonal offspring with desired traits (Hodges and Stice, 2003; Strulovici *et al*., 2007), studying cell development and differentiation (Nishikawa *et al*., 2007), drug study (Colin and John, 2007) and especially, cell therapy (Lerou and Daley, 2005; Mountford, 2008).

At the blastocyst stage of development, the preimplantation mammalian embryo separates into the inner cell mass (ICM) cells of the putative embryo and the trophectoderm cells that will eventually form the outermost layer of the fetal placenta. Pluripotent ESC lines were derived originally from the ICM of preimplantation embryos with retention of the stable developmental capacity to form derivatives of all three germ layers: ectoderm, endoderm, and mesoderm as well as the germ line (Evans and Kaufman, 1981; Thomson *et al*., 1998). Subsequently, it has become apparent that the culture requirements for proliferation and maintenance of ESCs in an undifferentiated state, and the nature of the stimuli required to induce ESCs to transform into specialized end-stage cells of specific phenotype, vary

between the human and various animal species (Evans and Kaufman, 1981; Thomson *et al*., 1998; Choi *et al*., 2002; Ginis *et al*., 2004; Liu *et al*., 2006; Rodríguez *et al*., 2007).

ESC lines have been well established in mouse (Evans and Kaufman, 1981) and human (Thomson *et al*., 1998). However, in spite of certain similarities between man and mouse with respect to the molecular regulation of pluripotency, there is a need for developing large animal models for safety testing of such procedures. Only a few of the reports were published on ESC isolations in animals, even fewer in horses. In recent years, attempts at derivation and/or establishment of ES lines has been reported for the chicken (Pain *et al*., 1996), hamster (Doetschman *et al*., 1988), sheep (Notarianni *et al*., 1991; Dattena *et al*., 2006), mink (Sukoyan *et al*., 1992), rabbit (Wang *et al*., 2006), pig (Notarianni *et al*., 1991; Li *et al*., 2003), miniature pig (Li *et al*., 2004), rat (Li *et al*., 2009), cow (Stice *et al*., 1996), and horse (Saito *et al*., 2002; Li *et al*., 2006; Saito *et al*., 2006). Attempts at the primary culture of cells of the inner cell mass of various species on a feeder layer of a primary culture of mouse embryonic fibroblasts (MEF) in the presence of leukemia inhibitory factor (LIF) and other growth factors have seldom been successful. The supplements of ESC culture medium are dependent on animal species. Different growth factors or even the same factors play different roles in ESC culture in different species. Determining the correct factors becomes more important for maintaining ESC. Although an attempt was made to isolate equine ESCs, (Saito *et al*., 2002; Li *et al*., 2006; Saito *et al*., 2006), maintenance of equine embryonic stem cells (eESCs) still remains a problem. Isolation and the establishment of equine ESCs would put horses on the front line of animal biotechnology and the modification of animal genomes as well as their therapeutic application.

The aim of the present study was to derivate horse ESC lines by repeated passage of ICM cells recovered mechanically from day-7 blastocysts as well as to optimize their culture conditions. We evaluated the growth capabilities of the putative eESCs when grown *in vitro* with human leukemia inhibitory factor (hLIF) only, human basic fibroblastic growth factor (hbFGF) only, or hbFGF plus hLIF with or without mouse fibroblast feeder cells.

Materials and Methods

Different media formula

α-minimum essential medium (α-MEM, Gibco), supplemented with 10% FBS (Gibco), penicillin (100 IU/ml, Invitrogen) and streptomycin (50 µg/ml, Invitrogen) was used for feeder cell culture. Basic medium was prepared for the culture of ICMs and horse embryonic stem cells using knock out Dulbecco's modified eagle's medium (DMEM, Gibco) containing 15% fetal bovine serum (FBS), 0.1 mM non-essential amino acids (Gibco), 2 mM L-glutamine (Invitrogen), 1% insulin-transferrin-selenium (Gibco), 100 µg/ml of streptomycin, 100 IU/ml of penicillin and 0.1 mM 2β-mercaptoethanol (Gibco). In addition, the media was supplemented with different growth factors including recombinant human leukemia inhibitory factor (hLIF, Chemicon) only, human basic growth factor (hbFGF, Sigma) only or hbFGF plus hLIF in order to culture putative horse embryonic stem cells in different conditions.

Preparation of feeder cells as co-culture

Mouse embryonic fibroblasts were cultured in supplemented α-MEM till passage 4. Confluent monolayer of cells in passage 4 were treated by triple^{TM} (Gibco), then were irradiated with γ-ray and were frozen in 1.5 cryovials using a freezing solution including 90% FBS plus 10% dimethyle sulfoxide (DMSO, Sigma). Each cryovial was thawed 1 day before its use as feeder layer for ICM or embryonic stem cells.

Embryo recovery, isolation of ICMs and their proliferation *in vitro*

Six horse day-7 embryos were collected. To determine embryonic age, mares were monitored daily by rectal examination and ultrasonography in order to detect follicles,

and finally, ovulation day. Then, embryos were collected non-surgically by uteri flushing after 7 days, as previously described (Allen, 1982). The collected embryos were transported from Shepparton Equine Hospital to the lab by a small transportable incubator. The ICM of blastocysts was isolated mechanically by pulled pasture pipette. Briefly, the blastocyst was treated by tryple^{TM} for 2-3 min to facilitate zona lysis. Then, blastocysts were transferred to basic medium and broken by pipetting. The obtained ICM pieces were moved onto a monolayer of MEF in basic medium containing hLIF (40 ng/ml). After 2-3 days, the medium was changed every day until days 8-10. Following this passage (P0), the culture medium was changed every day after first passage (P1) and the passages were performed by mechanical dissociation into cellular clumps through manual scraping with a needle every 6-8 days until passage 5 onwards. Besides, after passage 5, ICM-derived colonies were subjected to different culture conditions with or without MEF feeder cells for the next 10 passages (until passage 15): (1) basic medium supplemented with hLIF (40 ng/ml) only; (2) basic medium supplemented with hbFGF (4 ng/ml) only; (3) basic medium supplemented with hbFGF (4 ng/ml) plus hLIF (40 ng/ml); and (4) basic medium without growth factors.

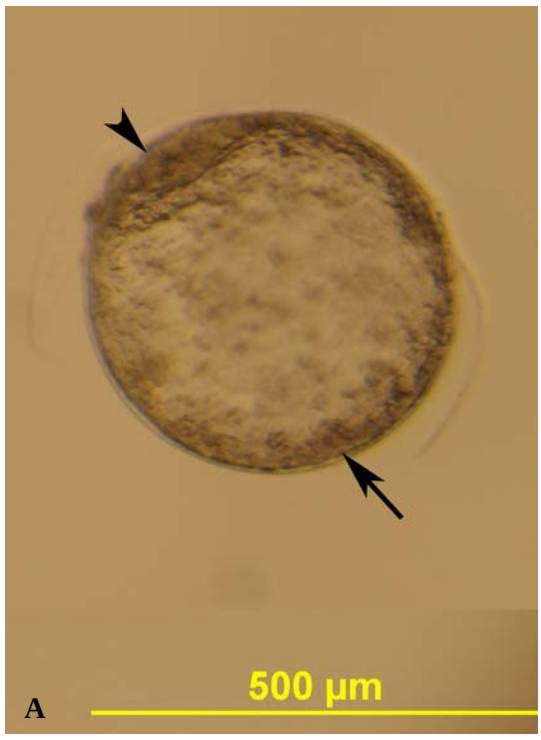
Immunostaining of cells for ES marker expression

Six ES cell markers including Oct-4, SSEA-1, SSEA-3, TRA-1-60, TRA-1-81, and GCTM-2 were searched for immunocytochemical staining, all of which have been identified previously on human and/or mouse ESCs. Briefly, the colonies of cultured cells on blue slides were fixed with pure cold ethanol, and then incubated for 30 min in a monoclonal antibody generated against one of the above mentioned 6 ES cell markers (Oct-4 was from Santa Cruz biotechnology and all other antibodies were from Chemicon) along with one negative control (Dakocytomation). After washing with PBS, the cells were incubated for 30 min with polyclonal rabbit anti-mouse (Dakocytomation) as the second antibody. Finally, the cells were stained with Hoechst in order to stain the nuclei, and then incubated for 5 min. Staining positive control (human embryonic stem cell-2) and negative control (lack of the primary antibody) were run under the same conditions.

Results

Recovery of horse embryos, isolation of ICMs and their proliferation and passages *in vitro*

All 6 ICMs were isolated successfully from day-7 blastocysts with distinct ICMs (Fig. 1A) that were fertilized *in vivo*. The isolated ICMs were mechanically disaggregated and seeded on fresh MEF with basic medium containing hLIF and, after 2-3 days of culture during passage 0 (P0), the cells from the ICMs attached to the feeder layer and formed primary expanded colonies. Morphological properties in the different areas of the colonies were different, that is, some parts of the colony were multilayer with compact cells and some other parts were almost monolayer. This characteristic could show the presence of cells with different growth capabilities (Figs. 1B and C).



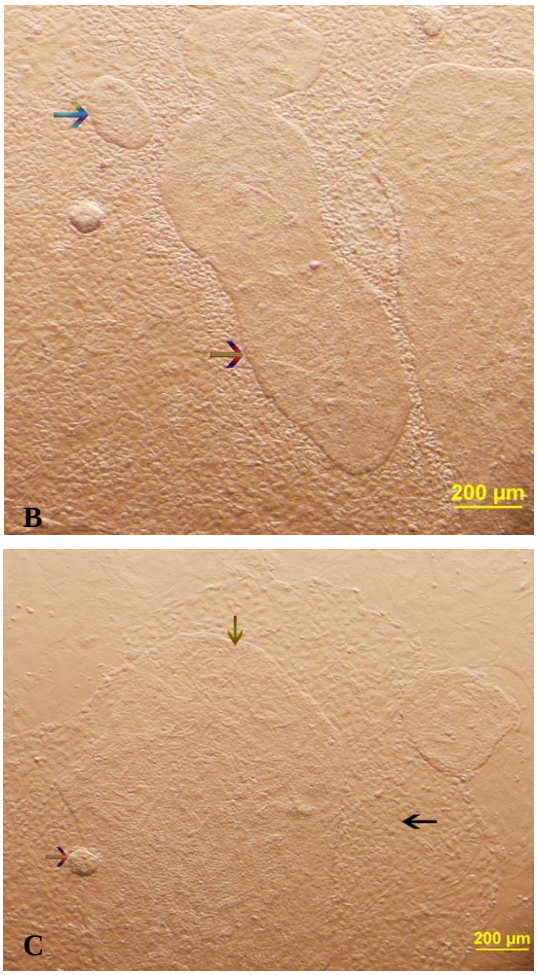


Fig. 1: A: a representative 7-day horse blastocyst with distinct inner cell mass (ICM). ICM indicated by arrowhead, trophoblastic cells indicated by arrow. B and C: Morphology of primary horse ICM outgrowth on MEF feeder cells after 4 days of culture. Different colonies have different sizes, thickness, and number of cell layers. Arrows point to different sized colonies. Note: there appear to be elongated cells within the centre of the flattened colonies (Fig. 1C)

The primary colonies were then disaggregated and cells from the monolayer or multilayer parts of the colonies seeded for the next passages separately to form new colonies called passage 1 colonies (P1). The cells removed from the multilayer parts of the colonies could better proliferate and form colonies. Then, they were disaggregated and seeded until passage 5 in the same conditions (P5). The putative eESCs were comprised of cells with a high nucleus/cytoplasm ratio and distinct cell borders, which formed flat cell colonies with clear borders (Fig. 1 and Fig. 2). The eESCs grown on the MEF feeder cells maintained colony form and continued to proliferate. eESCs that retained a predominate colony form on MEF cells were successfully cryopreserved, thawed, and recovered.

Maintenance of putative eESc in different culture conditions

The putative horse ESCs grown on the feeders with leukemia inhibitory factor (hLIF) or without growth factor conditions maintained colony form and continued to proliferate. Colonies were round and well-delineated with a few layers and clear borders (Figs. 2A1-A2, and Figs. 3A1-A2). The eESCs, on condition supplemented with hbFGF, grew nearly similar to conditions with hLIF or without growth factors in primary passages (Figs. 4A1 and A2), but this condition could not support eESCs in the next passages, and the cells started to differentiate and transform as elongated cells

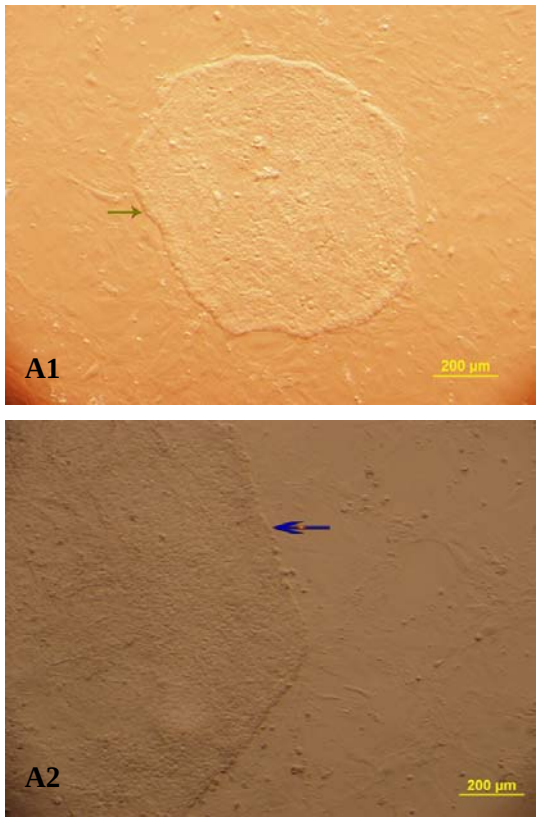


Fig. 2: A1-A2: Morphological characteristics of eESC colonies at passage 15 when maintained in the presence of hLIF on MEF feeder cells. Round, well-delineated and thin colonies with a few layers and clear border (arrows) can be seen. Moreover, cell borders are visible in A2

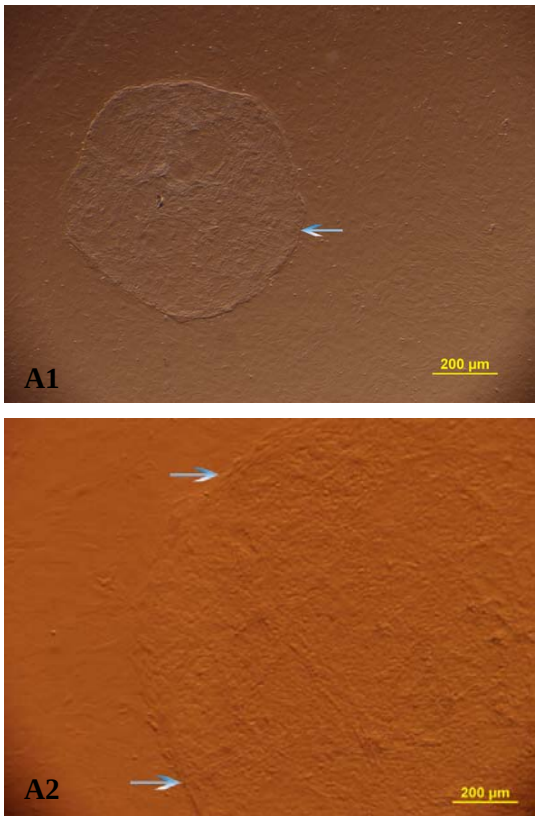


Fig. 3: A1-A2: Morphology of round and well-delineated eESC colonies at passage 15 on MEF cells without hLIF and bFGF. The clear border of colonies is characterized by arrows. Thinness and few layers of colony and cell borders can be visualized, too

(Fig. 4A3). Besides, growth rate slowed down and colony formation took more time. When eESCs were grown in the presence of both hbFGF and hLIF, they almost formed colonies in passage 6 (Fig. 5A1) and started differentiation in passage 7 (Fig. 5A2). However, their growth was stopped at passage 10 (Fig. 5A3). When eESCs were cultured without feeder layer, regardless of the presence or absence of exogenous growth factors, they could no longer keep colony form (Figures not shown).

Characterization of proliferated cells for ES marker expression

Expression of the stem cell markers in the eESCs was verified using immunocytochemistry. Marker analysis was carried out on eESCs at passage 15. All tested markers including OCT-4, SSEA-1, GCTM-2, TRA-1-60, and TRA-1-81 (Fig. 6) were detected in some parts of the colonies except SSEA-3, which could not be

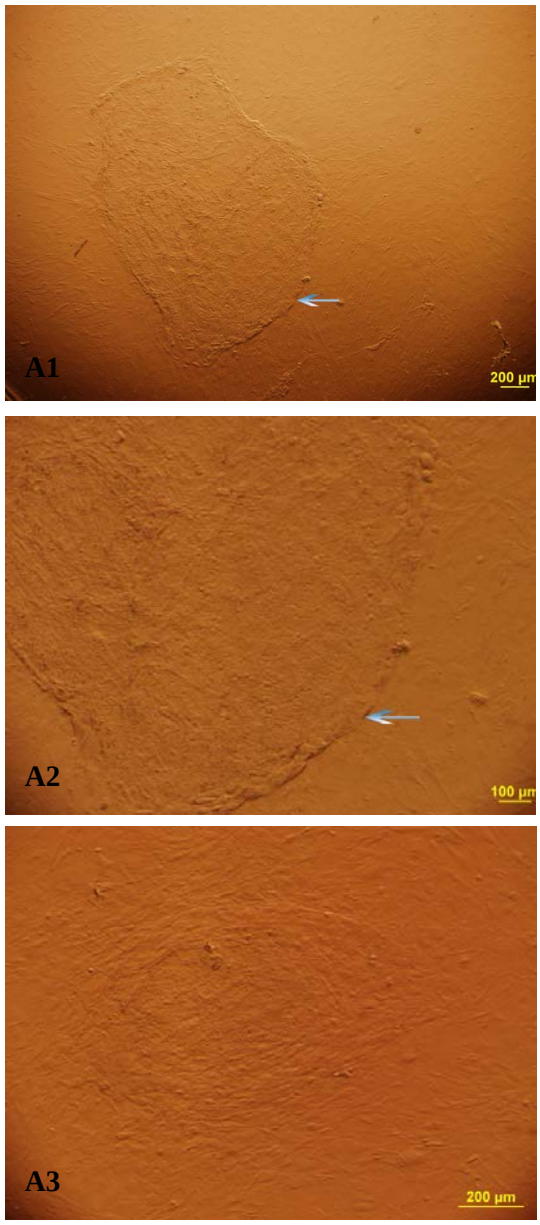


Fig. 4: Appearance of eESC colonies proliferated on MEF feeders with bFGF at passage 9 (A1 and A2) and passage 15 (A3). From passages 6 to 9, cells formed round and thin colonies with a clear border (arrows in A1 and A2), whereas they gradually lost colony form and cells started to differentiae and grew diffusely without any defined border after passage 9, as shown at passage 15 here. Differentiated cells can be seen as elongated cells in Fig. A3

detected, however, it is present in both mouse and human ESCs.

Discussion

In the present study, we found that

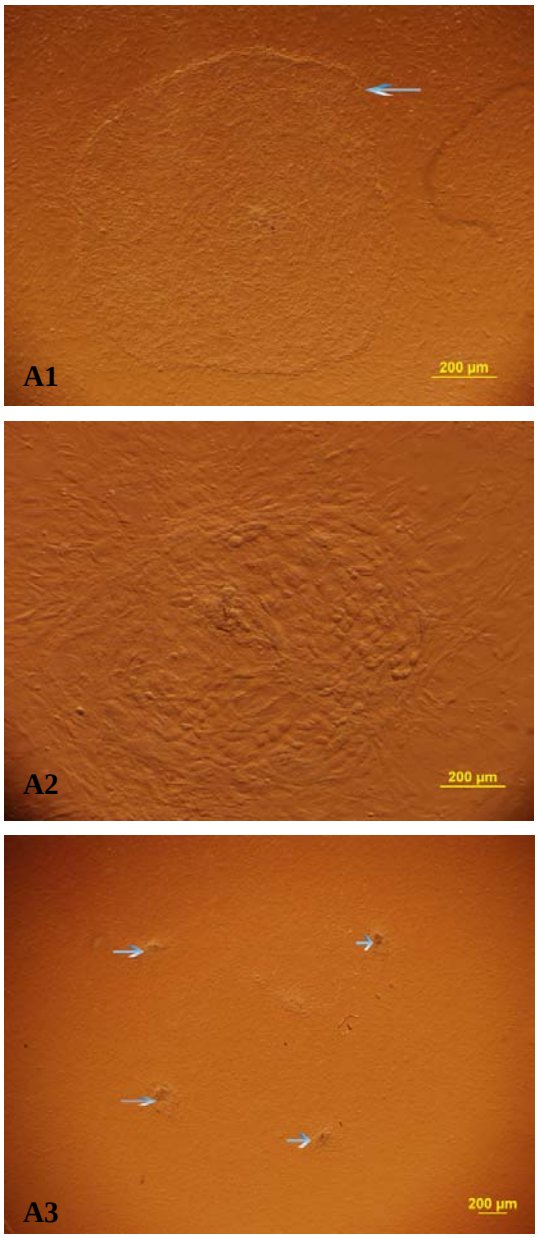


Fig. 5: Colony morphology of proliferated eESCs at passages 6 (A1), 7 (A2) and 10 (A3) being maintained in the presence of both bFGF and LIF. Although colonies almost maintained their morphology at first passage (6) as shown in A1, they start differentiation (elongated cells) at passage 7 (A2) and their growth was stopped at passage 10 (A3). Arrows in Fig. A3 demonstrate passaged cell pieces on MEF cells without any growth after a few days following passage 10

removing ICMs with a mechanical method was suitable in deriving the equine ESCs. We succeeded in derivating putative eESCs from blastocysts fertilized *in vivo*. Moreover, we compared the abilities of hLIF and hbFGF in proliferation of horse ICM cells and maintenance of their pluripotencies *in vitro*. Study of colony morphology and gene expression profile of eESCs showed some similarities and differences in comparison with both primate and murine ESCs.

In the previous studies, the isolation of putative equine ES-like cells or ESCs has been reported (Saito *et al*., 2002; Li *et al*., 2006; Saito *et al*., 2006), but an absolute result about the appropriate growth factor(s) which enable support proliferation of eESCs and that keeps pluripotency has not been mentioned. Evidence shows that several signaling pathways, such as LIF-STAT3, MAPK-ERK, PI3K, TGF-β, and bFGF signaling pathways as well as some transcription factors such as Oct4/Sox2 and Nanog, play key roles in maintaining growth and self-renewal of human and mouse ESCs (Sato *et al*., 2003; Liu *et al*., 2006; Chambers and Tomlinson, 2009). The use of feeder layers, media and methods of handling are the main themes in the culture conditions. Growth factors and other unknown substances play important roles in the maintenance and differentiation of the ESCs (Evans and Kaufman, 1981; Thomson *et al*., 1998; Familari and Selwood, 2006; Keefer *et al*., 2007). The decision to add LIF was based on culture conditions reported in other species such as pigs, rabbits, and ovine. The role of hLIF in different mammalian embryo culture has been controversial. For instance, hLIF did not influence bovine embryos and bovine ESCs (Rexroad and Powell, 1997; Rodríguez *et al*., 2007), whereas it is the best choice for proliferation of mouse ESCs (Evans and Kaufman, 1981). bFGF supports maintenance of human ESCs without feeders or serum (Klimanskaya *et al*., 2005). It also provides an important medium component for culture of cow (Gjorret and Maddox-Hyttel, 2005), and pig (Li *et al*., 2004), but not mouse ESCs (Ginis *et al*., 2004). Therefore, FGF may have variable success as a media supplement for other species. Thus, we added it as another main growth factor.

Murine ESCs typically grow in multilayer and compact piled-up colonial groups of cells. Besides, murine ESCs will begin to spontaneously differentiate at the

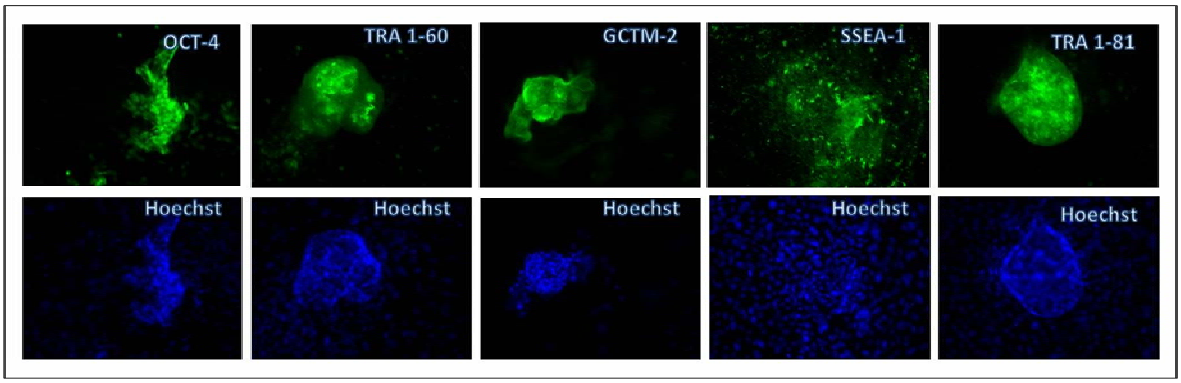


Fig. 6: Expression of ES cell markers on eESCs at passage 15. Oct-4, TRA-1-60, GCTM-2, SSEA-1 and TRA-1-81 were shown positive. Comparison of Hoechst staining and marker staining images for each marker show that only some partial cells of colony are positive

periphery of the colony (Evans and Kaufman, 1981; Ginis *et al*., 2004; Keefer *et al*., 2007). However, primate ESC colony morphology is generally flatter in appearance, and fewer cell layers and spontaneous differentiation tend to begin in the center of colonies (Thomson *et al*., 1998; Ginis *et al*., 2004; Keefer *et al*., 2007). For many animals – cow (Gjorret and Maddox-Hyttel, 2005), hamster (Doetschman *et al*., 1988), mink (Sukoyan *et al*., 1992), rabbit (Wang *et al*., 2006) and sheep (Notarianni *et al*., 1991; Dattena *et al*., 2006) - ESCs propagate as flattened colonies, almost a monolayer with individually distinct cells that have been described as epithelial-like or epithelioid (Familari and Selwood, 2006). Putative eESCs had a large nucleus-to-cytoplasm ratio similar to human and mouse ESCs (Ginis *et al*., 2004). The colony morphology in the presence or the absence of LIF on feeder cells was as thin and flattened colonies, almost a monolayer, with individually distinct cells like other ungulate animals (Keefer *et al*., 2007). Spontaneous differentiation tend to begin in the center of colonies, similar to human ESCs, if they are left undisturbed for a week or more without passage. Although Saito *et al*. (2002, 2006) and Li *et al*. (2006) mentioned horse ES-like cells more closely resembled those of mouse ES, the fewer cell layers along with the central differentiation pattern imply that our colonies were more similar to those of the primate ESCs.

The colonies grown in the presence of bFGF kept colony morphology in early passages, but start differentiation and lose colony morphology after a few passages. However, the colonies grown in the presence of bFGF plus LIF could not keep the morphology from the early passages. Thus, first, undifferentiated proliferation of horse ICM cells *in vitro* appears to be dependent upon the presence of appropriate feeder cells in agreement with the Saito *et al*. (2002, 2006) and Li *et al*. (2006) findings. Although human ESCs have been derived in feeder-free conditions for future clinical applications (Klimanskaya *et al*., 2005), most species, including horse, require a feeder-layer for ESC derivation (Familari and Selwood, 2006). Second, hLIF effects are not dominant on eESCs, indicating perhaps that hLIF might not have a dominant role in the maintenance of the undifferentiated stage of horse stem cell cultures, although Li *et al*. (2006) mentioned LIF is necessary for maintenance of the undifferentiated state of eESCs. Third, hbFGF cannot support proliferation of eESCs as it had been found for mouse ES cell cultures (Evans and Kaufman, 1981). This result also supports the Saito *et al*. (2002, 2006) findings, in which bFGF along with epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) were found to induce differentiation of eES-like cells. Fourth, the combination of hLIF and hbFGF is not a proper signal for maintenance of eESCs. Our findings implied that, in eESCs, the main signaling pathways for self-renewal are not similar to those of human and mouse ESCs, at least regarding hbFGF and hLIF signaling pathways.

ES and ICM cells of human and mouse

blastocysts as well as horse ICM cells express a panel of cell-surface markers that characterize their undifferentiated and pluripotent status (Evans and Kaufman, 1981; Thomson *et al*., 1998; Guest and Allen, 2007). Almost all publications in which ES cell markers have been tested have noted strong Oct-4 expression and alkaline phosphatase expression in derived ESC colonies, but the expression of other markers in use (SSEA-1, SSEA-3, and SSEA-4) differs considerably (Familari and Selwood, 2006). In this study, although cell surface markers only in some parts of colonies indicated the ability of ESCs to maintain undifferentiation and pluripotency, expression of cell surface markers in putative eESCs showed species-related differences. Putative horse ESCs expressed Oct-4, which had been found in both primates and mouse ESCs, and SSEA-1, which expresses in mouse ESCs, and GCTM-2, TRA-1-60, and TRA-1-81, which express in human ESCs (Evans and Kaufman, 1981; Thomson *et al*., 1998; Ginis *et al*., 2004). Although SSEA-3 was not detected, it is also absent in buffalo ESCs (Sritnaudomchai *et al*., 2007) and variable in bovine ESCs (Gjorret and Maddox-Hyttel, 2005), and is present in both mouse and human ESCs (Ginis *et al*., 2004). Saito *et al*. (2002, 2006) detected expression of alkaline phosphatase, Oct-4, STAT3 and SSEA-1, but not SSEA-3 and SSEA-4 in derived horse ES-like cells. Moreover, Li *et al*. (2006) also showed Oct-4, SSEA-1, TRA-1-60, TRA-1-81 and SSEA-4 expression in horse ESCs, though the intensity of staining for SSEA-4 was much lower than the other markers. In horse blastocysts Oct-4, TRA-1-60 and TRA-1-81 are predominantly localized to the ICM, while SSEA-1, SSEA-4 and even SSEA-3, which is absent in horse ESCs, are expressed in whole blastocyst (Guest and Allen, 2007). These differences might operate differently during embryo and ESC development.

In conclusion, our results showed that putative eESCs are able to self-renew when they are cultured in the basic medium on γ-irradiated MEFs. Moreover, the feeder cells are necessary to maintain the undifferentiated state for eESCs. The pluripotent stem cell markers only showed a part of the cell colonies, thus the results indicated that cell colonies were not completely purified embryonic stem cells. ESC-like cell morphology of putative eESCs could be maintained in the basic medium supplemented with or without hLIF. The result suggests that hLIF is not a prerequisite for maintenance of eESCs; bFGF seems to be negative for maintenance of horse ECSs and the combination of LIF and bFGF is unable to improve the culture condition. The new factors need to be investigated further to maintain eESCs in purified population, and horse LIF-receptor needs to be identified as to whether they are compatible with human LIF to find out horse right signal transduction pathways.

The isolation of equine ESs opens up the possibility of stem cell therapy in the horse and the study of early development and differentiation. Moreover, they can be a more appropriate model than mouse for human ESCs studies.

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