Trophoblast stem cell marker gene expression in inner cell mass-derived cells from parthenogenetic equine embryos

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Abstract

Although putative horse embryonic stem (ES)-like cell lines have been obtained recently from *in vivo*-derived embryos, it is currently not known whether it is possible to obtain ES cell (ESC) lines from somatic cell nuclear transfer (SCNT) and parthenogenetic (PA) embryos. Our aim is to establish culture conditions for the derivation of autologous ESC lines for cell therapy studies in an equine model. Our results indicate that both the use of early-stage blastocysts with a clearly visible inner cell mass (ICM) and the use of pronase to dissect the ICM allow the derivation of a higher proportion of primary ICM outgrowths from PA and SCNT embryos. Primary ICM outgrowths express the molecular markers of pluripotency POU class 5 homeobox 1 (*POU5F1*) and (sex determining region-Y)-box2 (*SOX2*), and in some cases, *NANOG*. Cells obtained after the passages of PA primary ICM outgrowths display alkaline phosphatase (AP) activity and *POU5F1*, *SOX2*, caudal-related homeobox-2 (*CDX2*) and eomesodermin (*EOMES*) expression, but may lose *NANOG*. Cystic embryoid body-like structures expressing *POU5F1*, *CDX2* and *EOMES* were produced from these cells. Immunohistochemical analysis of equine embryos reveals the presence of *POU5F1* in trophoblast, primitive endoderm and ICM. These results suggest that cells obtained after passages of primary ICM outgrowths are positive for trophoblast stem cell markers while expressing *POU5F1* and displaying AP activity. Therefore, these cells most likely represent trophoblast cells rather than true ESCs. This study represents an important first step towards the production of autologous equine ESCs for pre-clinical cell therapy studies on large animal models.

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Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the blastocyst and are pluripotent, i.e. have the capacity to contribute to all three germ layers, including the germline. Isolation, expansion and differentiation of ESCs *in vitro* are technologies that can lead to the use of ESCs in therapeutic treatments, thus raising hopes for new protocols for treating previously incurable diseases such as arthritis and Parkinson's disease. Immunocompatible ESCs that could be tolerated by the immune system would alleviate the need for administration of immunosuppressor drugs to the host following therapy. Immunocompatible ESCs can be generated using somatic cell nuclear transfer (SCNT) to introduce a somatic cell nucleus into an enucleated oocyte (Hochedlinger & Jaenisch 2003). Cells from embryos generated by SCNT are genetically identical to the donor cell, except for the mitochondrial DNA, which can be inherited from the recipient oocyte. Histocompatibility of SCNT-derived tissues and cells was nevertheless

demonstrated in studies using bovine species (Lanza *et al.* 2002, Theoret *et al.* 2006). Alternatively, fully major histocompatibility complex (MHC)-matched ESCs can be derived from parthenogenetic (PA) embryos, if they are heterozygous for MHC loci (Kim *et al.* 2007a). PA embryos develop from an unfertilised oocyte that is rendered diploid either naturally or experimentally (Balakier & Tarkowski 1976).

ESC lines from PA embryos have been derived from mouse, non-human primate and human species (Kaufman *et al.* 1983, Allen *et al.* 1994, Cibelli *et al.* 2002, Lin *et al.* 2003, Kim *et al.* 2007b). Such ESC lines have also been derived from mouse SCNT embryos (Kawase *et al.* 2000, Munsie *et al.* 2000, Wakayama *et al.* 2001). Derivation of putative ESC lines from bovine NT embryos was also reported (Cibelli *et al.* 1998, Wang *et al.* 2005), but not from equine PA nor from SCNT embryos. Recent evidence suggests that SCNT-derived ESCs are functionally equivalent to IVF-derived ESCs and have the same differentiation potential (Surani & Barton 1983, Allen *et al.* 1994, Munsie *et al.* 2000,

Wakayama *et al.* 2001, 2006, Hochedlinger & Jaenisch 2002, Rideout *et al.* 2002, Lin *et al.* 2003, Brambrink *et al.* 2006, Kim *et al.* 2007b).

The horse is an economically important domestic species. ESCs are needed in horses to provide an abundant and high-quality source of cells for use in stem cell-based therapies and to develop these therapies for human disease.

Putative ESC lines have been derived from farm animals including cows (Evans *et al.* 1990, Sims & First 1994, Cibelli *et al.* 1998, Iwasaki *et al.* 2000, Mitalipova *et al.* 2001, Saito *et al.* 2003, Wang *et al.* 2005), pig (Notarianni *et al.* 1990, Piedrahita *et al.* 1990, Hochereau-de Reviers & Perreau 1993, Wheeler 1994, Chen *et al.* 1999, Li *et al.* 2003, 2004a, 2004b) and horses (Saito *et al.* 2002, Li *et al.* 2006). However, few have demonstrated pluripotency *in vivo*, and none have contributed to the germline. ESC derivation protocols and culture conditions need to be optimised in species other than mice, non-human primates and humans. In the horse, it is currently not known whether it is possible to obtain autologous ESC lines from SCNT and PA embryos. In this study, we examine different protocols to obtain primary equine ICM outgrowths from PA and SCNT embryos and evaluate the expression and significance of different pluripotency-associated markers for the characterisation of equine cells derived from primary ICM outgrowths.

Results

Conditions affecting the derivation of primary equine ICM outgrowths from *in vivo*, PA and SCNT embryos

Upon plating, isolated ICM cells grew either as a flattened sheet of morphologically differentiated cells or as a domed mound – or primary ICM outgrowth – sitting on the flattened sheet of cells. Different culture conditions were tested to derive primary ICM outgrowths from *in vivo* (Fig. 1A), PA (Fig. 1B) and SCNT (Fig. 1C) blastocysts. Table 1 shows the proportion of primary ICM outgrowths obtained, in relation to embryo origin. Although high derivation efficiency was found in all three groups (66% for *in vivo*, 48% for PA and SCNT embryos), the efficiency was significantly lower when using PA compared with *in vivo* embryos (Table 1). This suggests that origin of the embryo influences derivation of primary ICM outgrowths.

Feeder cells from different origins were used and compared to evaluate the influence of feeder cells on the derivation of primary ICM outgrowths in each experimental group. Bovine umbilical cord fibroblast (BUCF), equine umbilical cord fibroblast (EUCF) and equine embryonic fibroblast (EEF) cell lines were generated and used 24–48 h after mitotic inactivation for derivation. No significant difference was found in the derivation efficiency with any of the feeder cells used, for any of the groups (*in vivo*, PA or SCNT),

Figure 1 Characteristics of *in vivo*, PA and SCNT equine embryos. Morphology of 7.0 dpc *in vivo* blastocyst (A), 7.0 days post-activation PA blastocyst (B) and SCNT blastocyst (C). Isolated ICM from *in vivo* (D) and PA (E) blastocysts dissected with trypsin, and ICM from a PA blastocyst dissected with pronase (F). An early PA blastocyst (G), an early-expanded PA blastocyst without its zona pellucida (H) and a hatching PA blastocyst (I). A PA embryo without a detectable ICM (J). Bars = 100 μ m.

Table 1 Total number of primary equine inner cell mass (ICM) outgrowths is influenced by blastocyst origin.

Blastocyst origin	Number of attached blastocysts	Number of primary ICM outgrowths/number of attached blastocysts (%)
<i>In vivo</i>	38	25 (66%) ^a
PA	172	82 (48%) ^b
SCNT	27	13 (48%) ^{ab}

^{a,b}Values with different superscripts in the same column are significantly different ($P=0.04$).

suggesting that the origin of the feeder cells is not a primary factor to consider when deriving primary ICM outgrowths (Table 2).

To isolate the ICM, equine embryos were treated enzymatically either with trypsin (Fig. 1D and E) or with pronase (Fig. 1F). Pronase was found to be ineffective for the dissociation of trophoblast cells from *in vivo* embryos, while trypsin dispersed trophoblast cells effectively, allowing ICM isolation. In the *in vivo* group, 72% of the ICMs isolated with trypsin yielded primary ICM outgrowths (Table 3). Both trypsin and pronase were used with PA and SCNT embryos. Although a higher ratio of primary ICM outgrowths was obtained with pronase (41% for PA and 47% for SCNT groups), there was no significant difference with respect to the enzyme used to isolate the ICM (Table 3).

Embryos from *in vivo*, PA and SCNT groups were classified according to their age and developmental stage before they were used for derivation of primary ICM outgrowths. *In vivo* embryos aged between 6.0 and 9.5 days post-coitum (dpc) were used, and no statistical difference was found regarding the efficiency of primary ICM outgrowth formation. Similarly, PA and SCNT embryos aged between 6.0 and over 10.0 days post-activation were used. It was found that in these groups, formation of primary ICM outgrowth was significantly less efficient when using older embryos (10.0 days post-activation and over), compared with the use of embryos aged between 6.0 and 9.5 days post-activation (Table 4). Likewise, no difference was observed in *in vivo* embryos from different developmental stages, whereas PA and SCNT late blastocysts, including late-expanded, hatching (Fig. 1I) and hatched blastocysts, gave a significantly lower number of primary ICM outgrowths compared with early (Fig. 1G) and early-expanded (Fig. 1H) blastocysts (Table 5).

The parameter of ICM identification in PA and SCNT embryos was evaluated to detect possible influence on the derivation of primary ICM outgrowths. Blastocysts with a detectable ICM and those with a non-detectable ICM (Fig. 1J) were classified, and their ability to form a primary ICM outgrowth was evaluated. PA and SCNT blastocysts presenting a clearly visible ICM were more prone to develop into a primary ICM outgrowth than embryos that were not showing a clearly identifiable

ICM (Table 6). Together, these results suggest that prior morphological evaluation of embryos is beneficial for the improvement of derivation efficiency of primary ICM outgrowths from PA and SCNT embryos.

Morphological and molecular evaluation of primary equine ICM outgrowths

Between 1 and 5 days post-plating, primary ICM outgrowths formed in culture. Differences were observed between primary outgrowths from *in vivo*, PA and SCNT blastocysts. In the *in vivo* group, primary ICM outgrowths emerged on a sheet of flattened cells (Fig. 2A and B). These outgrowths consisted of a rounded mass composed of tightly compacted cells, where individual cells were not discernable (Fig. 2A). After a few days in culture, a layer of cells displaying a different morphology formed as an outer layer around the tightly compacted cell mass (Fig. 2B). When isolated and plated onto a new feeder layer for 2–3 days, the ICM outgrowth-derived cells appeared to have readily undergone morphological differentiation into a flattened sheet of cells. In contrast, tightly compacted cells of the ICM outgrowths from PA and SCNT embryos were surrounded by cells that had a darker and a more rounded and dispersed appearance than the cells composing the outer layer of *in vivo* ICM outgrowths (Fig. 2C–F). Notably, in two cases, primary ICM outgrowths from PA embryos displayed a similar morphology to that observed with *in vivo* embryos.

Cells composing the primary ICM explant – the primary ICM outgrowth together with the flattened sheet of cells – were characterised by analysing marker gene expression by reverse transcriptase-PCR (RT-PCR; Fig. 3). POU class 5 homeobox 1 (*POU5F1*) and (sex determining region-Y)-box2 (*SOX2*) transcripts were expressed in 7/8 and 6/8 ICM explants respectively. Notably, *SOX2* expression was weaker in one *in vivo* ICM explant. *NANOG* was clearly expressed in three ICM explants analysed, from SCNT and *in vivo* explants groups only. Trophoblast stem (TS) cell marker genes caudal-related homeobox-2 (*CDX2*) and eomesodermin (*EOMES*) were expressed in 6/8 and 8/8 ICM explants respectively, with representatives in all groups. These results suggest that the ICM explants derived from *in vivo*, PA and SCNT embryos may display pluripotency as well as TS cell marker genes expression, as shown by RT-PCR.

Table 2 The relative number of primary equine inner cell mass (ICM) outgrowths is not influenced by the feeder cells used.

Feeder layer	Number of primary ICM outgrowths		
	<i>In vivo</i> blastocysts (%)	PA blastocysts (%)	SCNT blastocysts (%)
EEF	6/10 (60%)	51/111 (46%)	4/11 (36%)
EUCF	14/20 (70%)	16/32 (50%)	9/13 (69%)
BUFC	5/6 (83%)	14/26 (54%)	N/A

N/A, not analysed.

Table 3 Relative number of primary equine inner cell mass (ICM) outgrowths is not influenced by the method of ICM isolation.

Method of ICM isolation	Number of primary ICM outgrowths		
	<i>In vivo</i> blastocysts (%)	PA blastocysts (%)	SCNT blastocysts (%)
Trypsin	23/32 (72%) ^a	20/52 (38%) ^b	2/9 (22%) ^b
Pronase	N/A	54/131 (41%) ^b	9/19 (47%) ^b

^{a,b}Values with different superscripts in the same row are significantly different ($P=0.006$).

Morphological, molecular and biochemical evaluation of equine ES-like cells obtained after passages of PA primary ICM outgrowths

The cells obtained after the first passage of PA primary ICM outgrowths displayed morphological characteristics of ESCs (Fig. 4). Individual cells showed a high nucleus to cytoplasm ratio, and colonies had defined borders (Fig. 4A and B). Compact colonies of densely packed cells were observed 1 day after passage (Fig. 4C). After 5 days in culture, colonies became flattened, and two characteristic morphologies were observed; either a monolayer of cells with dispersed structures composed of tightly packed cells (Fig. 4D) or flattened colonies with a defined border (Fig. 4E). As shown in Fig. 4F and G, colonies were still displaying alkaline phosphatase (AP) activity after five passages. Cells were cultured in suspension for 7 days without leukaemia inhibitory factor (LIF) or feeder cells to obtain cystic embryoid body (EB)-like structures (Fig. 4H).

The pluripotent identity of PA cells obtained after passages of primary ICM outgrowths was assessed by RT-PCR to evaluate marker gene expression (Fig. 5). *POU5F1* was expressed in all cells derived from three distinct primary ICM outgrowths. *SOX2* was expressed in two out of three groups, and *NANOG* was either expressed at very low levels or could not be detected. On the other hand, *CDX2* and *EOMES* were present in all cells derived from primary ICM outgrowths. These results suggest that the cells obtained show expression of markers by RT-PCR of both mouse pluripotent ES and TS cells, although *NANOG* is not clearly expressed in these cells. The cystic EB-like structures, produced by culturing the PA cells obtained after passaging ICM outgrowths in suspension without LIF or feeder cells, expressed *POU5F1*, *CDX2* and *EOMES*, but the expression of both *SOX2* and *NANOG* was absent, suggesting that the EB-like structures were composed of TS cells.

***POU5F1* and *GATA6* expression in *in vivo*, PA and SCNT equine embryos**

The expression and localisation of *POU5F1* and *GATA6*-binding protein-6 (*GATA6*), markers of pluripotent cells and of primitive endoderm (PE) respectively, were

examined by immunofluorescence and confocal microscopy. A *POU5F1* antibody detecting specifically the isoform associated with pluripotency (*POU5F1A*) was used. In *in vivo* embryos (Fig. 6A), *POU5F1* was expressed in trophectoderm (TE), PE and ICM cells of the blastocyst at 7.0 dpc, whereas *GATA6* was restricted to PE cells. In 6.0 days post-activation PA embryos (Fig. 6B), both *POU5F1* and *GATA6* were expressed distinctly in TE and ICM cells. In 8.0 days post-activation PA non-expanded (Fig. 6C) or expanded blastocysts (Fig. 6D) and in 7.5 days post-activation SCNT blastocysts (Fig. 6E), *POU5F1* and *GATA6* were expressed in both TE and PE cells, whereas a few ICM cells expressed exclusively *POU5F1*, and not *GATA6*. Together, these results suggest that *POU5F1* is not restricted to the ICM in the equine embryo, as it also localised to the extraembryonic compartments of TE and PE.

Discussion

This study compares the efficiency of primary ICM outgrowth derivation from equine *in vivo*, PA and SCNT embryos. To our knowledge, this is the first report of *in vitro* proliferation of cells derived from primary ICM outgrowths from *in vivo*, PA and SCNT equine embryos positive for AP activity, as well as for *POU5F1* and *SOX2* expression. The impact of different derivation parameters on growth of primary ICM outgrowths was compared. Furthermore, this study characterises the expression of *POU5F1* and *GATA6* proteins in *in vivo*, PA and SCNT equine blastocysts, and shows the expression of *POU5F1* in the ICM as well as in the TE and the PE. *POU5F1*, *CDX2* and *EOMES* expression was also demonstrated in AP-positive cells derived from PA primary ICM outgrowths.

PA and SCNT embryos may have placentation defects, and this has been attributed to abnormal imprinting of paternally expressed genes (Surani & Barton 1983, Ohgane *et al.* 2001, Singh *et al.* 2004). However, similarity of transcriptional profiles of ESC lines derived from *in vitro* fertilised and SCNT mouse embryos suggests that these embryos are suitable for production of functional ESC lines (Brambrink *et al.* 2006,

Table 4 Relative number of primary equine inner cell mass (ICM) outgrowths is influenced by the age of the blastocyst.

Age of blastocyst (days*)	Number of primary ICM outgrowths		
	<i>In vivo</i> blastocysts (%)	PA blastocysts (%)	SCNT blastocysts (%)
6.0–7.5	16/23 (70%)	59/108 (55%) ^a	6/8 (75%) ^a
8.0–9.5	9/15 (60%)	16/40 (40%) ^{ab}	4/6 (67%) ^{ab}
10.0 and over	N/A	7/24 (29%) ^b	3/13 (23%) ^b

^{a,b}Values with different superscripts in the same column varies significantly ($P=0.02$).

*Days post-coitum for *in vivo*, days post-activation for PA and SCNT blastocysts.

Table 5 Relative number of primary equine inner cell mass (ICM) outgrowths depends on the developmental stage of the blastocyst.

Developmental stage	Number of primary ICM outgrowths		
	<i>In vivo</i> blastocysts (%)	PA blastocysts (%)	SCNT blastocysts (%)
Early blastocyst	9/10 (90%)	49/99 (49%) ^a	5/7 (71%) ^a
Expanded blastocyst	13/22 (59%)	19/34 (56%) ^a	4/6 (67%) ^{ab}
Late blastocyst*	3/6 (50%)	2/14 (14%) ^b	4/14 (29%) ^b

^{a,b}Values with different superscripts in the same column are significantly different ($P=0.008$ for PA and $P=0.05$ for SCNT).

*Includes late, expanded blastocysts, hatching and hatched blastocysts.

Wakayama *et al.* 2006). Moreover, studies in the mouse suggest that SCNT and PA ESC lines have the same differentiation potential as IVF- or *in vivo*-derived ESC lines (Surani & Barton 1983, Allen *et al.* 1994, Munsie *et al.* 2000, Wakayama *et al.* 2001, 2006, Hochedlinger & Jaenisch 2002, Rideout *et al.* 2002, Lin *et al.* 2003, Kim *et al.* 2007a). In the present investigation, almost half of the embryos from the PA and SCNT groups yielded primary ICM outgrowths, which indicates high derivation efficiency. These data support that PA and SCNT embryos give rise efficiently to primary ICM outgrowths, despite the likelihood of abnormal placentation later on in development. However, our data suggest that the *in vivo* embryos give a statistically higher number of primary ICM outgrowths, indicating a possible difference in the phenotype of *in vitro* and *in vivo* grown ICMs from blastocysts.

In previous reports describing the derivation of putative ESC lines from horse embryos, immunosurgery (Li *et al.* 2006) and microsurgery (Saito *et al.* 2002) were used to isolate the ICM. In the rabbit, immunosurgery was found to be less effective than trypsin digestion of PA embryos, as immunosurgery leaves extraembryonic tissues attached to the ICM (Wang *et al.* 2007). In accordance with these data, immunosurgery of *in vivo* bovine embryos was shown to leave the PE and sometimes the TE cells attached to the ICM (Talbot *et al.* 1995, 2005). Therefore, we did not make use of immunosurgery to isolate the ICM from *in vivo* embryos in this study. In preliminary attempts, mechanical isolation of the ICM with a scalpel blade did not allow a complete removal of extraembryonic tissue, while plating of the whole embryo did not allow attachment within 48 h of culture in most cases (data not shown) in this study. Enzymatic isolation of ICM was found to be the most effective for ICM isolation from PA and NT embryos and allowed rapid attachment of the ICM to the feeder layer within 24 h.

Earlier attempts to maintain equine ESCs in culture have made use of BUCFs (Saito *et al.* 2002), mouse embryonic fibroblasts and EEFs (Li *et al.* 2006), but no study has yet compared the effect of using feeders from different sources on the derivation of primary ICM

outgrowths. In this study, we have derived and compared the potential of feeder cells of different sources – BUCFs, EUCFs and EEFs – for the derivation of primary ICM outgrowths. No significant differences were observed when using different feeders. Similarly, Li *et al.* (2006) reported no difference between feeders from mouse or equine origins for culture and maintenance in undifferentiated state of established putative equine ESCs. The use of EEFs would be preferable as they are homologous, secrete species-specific hormones and factors, and prevent cross-species contamination and transmission of xenopathogens. Nevertheless, all the feeders used in this study failed to maintain NANOG-positive ESCs after passaging. In our experience, the absence of LIF was not detrimental to the maintenance of *POU5F1* and AP-positive cells after passaging of PA primary ICM outgrowth, although the absence of feeders was detrimental (data not shown). This dependence on feeder cells, but not on LIF, is consistent with the data reported by Saito *et al.* (2002), but in contradiction with Li *et al.* (2006), who observed that both LIF and feeder cells were required for the maintenance of undifferentiated ES-like cell state, although all three studies made the use of human recombinant LIF at a concentration ranging from 10 to 40 ng/ml. The significance of LIF in the maintenance of the undifferentiated state of equine ESCs merits further investigation.

Embryos at different developmental stages were used to assess primary ICM outgrowth formation. In this study, a larger proportion of primary ICM outgrowths was obtained if early-stage blastocysts were used, in comparison with late-stage blastocysts. This could be explained by the prolonged length of exposure time to *in vitro* culture, which may be detrimental to the viability and integrity of the blastocysts. Bovine *in vivo* embryos from 16-cell stage to hatched blastocyst aged 10–11 dpc are believed to be the most permissive stages for bovine ES-like cell derivation (Sims & First 1994, Strelchenko 1996, Cibelli *et al.* 1998, Iwasaki *et al.* 2000). Our results suggest that, for equine *in vitro* embryos produced by parthenogenesis or cloning, early blastocysts aged <9.5 days post-activation should be the permissive condition for the derivation of primary ICM outgrowths. Whether the use of earlier stage embryos would have resulted in a better outcome is not known, but according to experiments carried out with bovine embryos, little

Table 6 Relative number of primary equine inner cell mass (ICM) outgrowths depends on ICM identification prior to isolation.

ICM identification	Number of primary ICM outgrowths	
	PA blastocysts (%)	SCNT blastocysts (%)
Detectable	61/117 (52%) ^a	11/16 (69%) ^a
Not detectable	12/37 (32%) ^b	2/11 (18%) ^b

^{a,b}Values with different superscripts in the same column are significantly different ($P=0.03$ for PA and $P=0.007$ for SCNT).

Figure 2 Formation of primary ICM outgrowths *in vitro*. Primary ICM outgrowths at day 1 (A, C and E) or day 5 (B, D and F) of culture, derived from *in vivo* (A and B), PA (C and D) or SCNT (E and F) blastocysts. Bars=200 μ m.

success has been achieved in the derivation of ESC lines from morula and 8-cell stages (Strelchenko 1996, Mitalipova *et al.* 2001).

Primary ICM outgrowths derived from *in vivo* equine embryos are composed of a compact, tightly packed mass of small cells surrounded by another cell type, presumably PE cells. This mass grows on the top of a monolayer of epithelioid cells. This morphology has been described as well for bovine primary ICM outgrowths (Talbot *et al.* 1995, Keefer *et al.* 2007), suggesting a close resemblance between equine and bovine ICM cells organisation, although the presence of an outer layer of PE is not described in other reports on equine ES-like cells (Saito *et al.* 2002, Li *et al.* 2006). In our study, the primary ICM outgrowths derived from PA and SCNT blastocysts had a different morphology than that observed for *in vivo*-derived primary ICM outgrowths. The PE cells covering the ICM outgrowth were more abundant, and growing as rounded and loosely attached cells on top of the ICM outgrowth. This morphology has also been described in ICM primary outgrowths from bovine PA and NT blastocysts (Kwon *et al.* 2009). The surrounding PE cells may be protecting the undifferentiated ICM cells from differentiation-inducing factors present in the culture medium, because isolation of ICM outgrowth-derived cells for passaging and plating resulted in rapid cell differentiation. It is not yet clear in equine species whether LIF is required to

maintain the undifferentiated state, as reports are not unanimous on this matter (Saito *et al.* 2002, Li *et al.* 2006). In human ESCs, LIF induces differentiation and may activate differentiation pathways such as MAPK/ERK (Humphrey *et al.* 2004). In large animals, it is not clear whether inhibition of MAPK/ERK pathway by PD98059 does result in maintenance of the undifferentiated state of ES-like cells (Cao *et al.* 2009).

The presence of the pluripotency marker genes *POU5F1*, *SOX2* and *NANOG* by RT-PCR in one primary ICM outgrowth from *in vivo* embryo and in two from NT embryos strongly suggests the presence of undifferentiated ICM cells in these initial cultures. In addition, the high expression of *NANOG* in *in vivo* and SCNT, but not in PA, ICM outgrowths suggests that SCNT embryos have a behaviour that is similar to *in vivo* embryos after they have been placed in culture. The presence of TS marker genes *EOMES* and *CDX2* suggests that trophoblast is also a component of these heterogeneous cultures, even if the ICM was initially isolated. The heterogeneity of the cultures might be explained by the incomplete removal of extraembryonic cells upon ICM isolation or by the spontaneous differentiation of ICM outgrowth-derived cells into extraembryonic cells. It could also be explained by a dysregulated expression of genes as an artifact of *in vitro* culture, as suggested by Choi *et al.* (2009).

The cells that have been derived in this study from PA and SCNT primary ICM outgrowths display a high nucleus to cytoplasm ratio, and the colonies have defined borders, which are characteristics of mouse and human ESCs (Robertson 1987, Thomson *et al.* 1998). These cells grow in tightly packed colonies initially, as described in other reports of equine ES-like cells (Saito *et al.* 2002, Li *et al.* 2006). However, their morphology changes a few days after passage, all the while retaining AP activity. They grow either as multilayered cell clumps in the middle of flattened epithelioid cells or as flattened colonies, as it was described for bovine PA and NT-derived ES-like cells (Wang *et al.* 2005).

It was reported in the literature that colony morphology and AP activity are not reliable markers for identification of ES cells in large animals such as equine,

Figure 3 Molecular characterisation of primary colonies derived from equine *in vivo*, PA and SCNT embryos by RT-PCR. *POU5F1*, *NANOG* and *SOX2* are molecular markers for pluripotent ESCs. *CDX2* and *EOMES* are molecular markers for TS cells. *GAPDH* is a constitutively expressed gene and serves as a control for gene expression.

Figure 4 Characterisation of cells derived from equine PA and SCNT primary ICM outgrowths. High nucleus to cytoplasm ratio and defined borders in a cell colony derived from PA (A) and SCNT (B) blastocysts. Morphology of a PA-derived cell colony 1 day (C) or 5 days after passage (D and E). AP-positive colonies in purple (F and G). EBs at day 7 (H). Bars=200 µm.

bovine and porcine species (reviewed in Keefer *et al.* (2007)). In bovine species, AP is expressed in the trophoblast and the epiblast of the embryo (Talbot *et al.* 1995, Vejlsted *et al.* 2005), as well as in TS cells in culture (Cao *et al.* 2009). In equine species, AP was reported to be almost exclusively active in the ICM of the blastocyst (Guest & Allen 2007). This specificity of AP activity to the equine ICM may only apply to embryos *in situ*, since our results demonstrate the presence of AP activity in differentiated cells. Hence, together, these results suggest that the presence of AP activity and distinct morphology are not sufficient to claim obtention of ESCs.

Molecular profiling of pluripotency and extraembryonic markers as well as *in vitro* differentiation were therefore conducted to confirm the identity of the PA cells obtained after passaging of primary ICM outgrowths. *POU5F1*, *SOX2*, *CDX2* and *EOMES* expression was observed in most of the cells, but *NANOG* was either not detected or very weak. Differentiation was induced and EB-like structures showed a cystic morphology after 7 days of culture. Appearance of cystic EB-like structures following suspension culture in differentiation-inducing medium was also reported by Saito *et al.* (2002) and Li *et al.* (2006). We conducted molecular characterisation of these cystic EB-like structures to obtain further information on their phenotype, as human EBs derived from ESCs of cystic morphology have been shown to be able to differentiate into all three germ layers as well as into extraembryonic tissues (Gerami-Naini *et al.* 2004). Following induction of differentiation, *POU5F1*, *CDX2* and *EOMES* expression was maintained, but *SOX2* expression was lost. These results suggest that differentiation is directed preferentially into extraembryonic cells, positive for

POU5F1 expression. *POU5F1* was also detected in EB-like aggregates from other equine ES-like cell lines (Li *et al.* 2006), suggesting that ES-like cells from this study are of a similar cell type to the ones described in this study. In our study, a further indication of the differentiation of cells from the primary ICM outgrowths into TS cells upon passages is their short lifespan in culture; none of the PA cells obtained after passages of primary ICM outgrowths could be maintained beyond seven passages. Altogether, these results suggest that the PA cells obtained in this study have the hallmarks of TS cells positive for the expression of *POU5F1* mRNA and AP activity. To ascertain whether *POU5F1* protein is expressed in equine extraembryonic tissues, immunolocalisation was conducted in *in vivo*, PA and SCNT embryos.

Figure 5 Molecular characterisation of equine PA cells derived from primary ICM outgrowths and of EBs by RT-PCR. *POU5F1*, *NANOG* and *SOX2* are molecular markers for pluripotent ESCs. *CDX2* and *EOMES* are molecular markers for TS cells. *GAPDH* is a constitutively expressed gene and serves as a control for gene expression.

Figure 6 Immunofluorescent localisation of POU5F1 and GATA6 proteins in equine *in vivo* (A), PA (B, C and D) and SCNT (E) embryos. In 7.5 dpc *in vivo* embryos (A), POU5F1 localises to the TE, PE and ICM, whereas GATA6 is specific to the PE. In 6.0 days post-activation PA early blastocysts (B), cells from the TE and PE are positive for both POU5F1 and GATA6. In 8.0 days post-activation PA blastocysts at early phase of expansion (C), the same pattern is observed for POU5F1 and GATA6, but a few cells are exclusively positive for POU5F1 in the ICM. In 8.0 days post-activation PA expanded blastocysts (D), POU5F1 localises to the TE, the PE as well as to the ICM, whereas GATA6 is expressed in the PE, but not in the ICM cells. In 7.5 days post-activation SCNT blastocysts (E), POU5F1 and GATA6 proteins are observed in the TE as well as in the PE, whereas the ICM cells express exclusively POU5F1. No signal is observed in PA embryos incubated with both negative control IgGs (F). Bars = 100 μ m.

This study highlights the expression of both POU5F1 and GATA6 proteins in *in vivo*, PA and SCNT blastocysts. POU5F1 was found to be localised in the TE, PE as well as in the ICM of equine *in vivo* blastocysts. The distribution of POU5F1 was similar in equine PA and SCNT embryos. In two previous studies of the localisation of POU5F1 in equine 7 and 11 dpc *in vivo* blastocysts, POU5F1 was shown to be very weak in the TE and mostly specific to the ICM (Guest & Allen 2007, Choi *et al.* 2009). In our study, although POU5F1 immunofluorescence signal appears to be brighter in the ICM than in the TE in the three 7 dpc equine blastocysts examined, we are not confident that the technique used is allowing precise quantification of the signal. The immunolocalisation studies conducted in the equine blastocyst by Choi *et al.* (2009) suggest that POU5F1 signal in the TE would be an artifact of culture, due to a lack of repression of its expression by the uterine environment. Our results tend to agree with these findings, since the POU5F1 signal of the TE of *in vitro*-derived embryos seems to be brighter relatively to the

signal of the ICM, in comparison with what is observed with the *in vivo* embryos.

In this study, GATA6 was predominantly found in the PE, and already expressed in 7 dpc *in vivo* embryos, showing that the ICM had undergone differentiation into epiblast and hypoblast at this stage. In PA and SCNT embryos, the distinction between GATA6-negative epiblast and GATA6-positive hypoblast was not clear at 7 days post-activation. This could reflect that PA and SCNT embryos develop at a slower pace *in vitro*, and that the signaling for pluripotency genes might be dysregulated in *in vitro* embryos (Choi *et al.* 2009). In sharp contrast in the mouse, *Gata6* and *Pou5f1* expression is segregated when epiblast appears in early post-implantation embryos (Chazaud *et al.* 2006). Interestingly, the presence of only a few ICM cells exclusively POU5F1-positive in equine PA and SCNT embryos might explain in part why the primary ICM outgrowths obtained in this study were much smaller in size. On the other hand, the high number of GATA6–POU5F1 double positive cells in PA and SCNT equine embryos might explain why the

primary ICM outgrowths derived from these embryos had a tendency to differentiate more readily into PE *in vitro*. The expression of POU5F1 protein in equine TE and PE indicates that POU5F1 is not a sufficient marker to identify pluripotent ESCs in this species. The presence of *POU5F1*, *CDX2* and *EOMES*, but not *NANOG*, in the cell lines derived in this study from PA primary ICM outgrowths rather suggests that TS cell lines were derived.

Overall, this study shows that it is possible to derive primary ICM outgrowths from PA and SCNT equine embryos. Both the use of early-stage blastocysts with a clearly visible ICM and the use of pronase to dissect the ICM allow the derivation of a higher proportion of primary ICM outgrowths from these embryos. This study further indicates that markers currently used to characterise mouse and human ESCs may not be suitable to characterise equine ESCs, and that more fundamental knowledge of ICM cell biology in the equine species is required to determine the culture conditions that will allow proliferation of equine ESCs in an undifferentiated state in culture. The results presented in this study illustrate an advance in the determination of protocols for the derivation of primary equine ICM outgrowths and the characterisation of equine ICM-derived cells. It also represents an important step forward for the development of autologous equine ESC therapies in an important pre-clinical species, the horse.

Materials and Methods

Animals and embryo recovery

Procedures involving the recovery of *in vivo* embryos from mares were carried out following standard protocols accepted by the International Embryo Transfer Society, which are in compliance with the guidelines of the Canadian Council on Animal Care. Ovulation in mares was synchronised by injection of cloprostenol sodium (125 µg i.m., Intervet, Montreal, QC, Canada) at day 10 of the estrus cycle (day 0 is taken as the day of ovulation), followed 36 h later by an injection of human chorionic gonadotropin (2000 IU i.v., Intervet) and artificial insemination 12 h later with 500×10^6 progressively motile spermatozoa. Blastocysts were recovered by repeated uterine flushing between days 6 and 8 following artificial insemination, using a medium composed of tissue culture medium-199 (TCM-199), 25 mmol/l HEPES (Invitrogen) and 10% (v/v) fetal bovine serum (FBS, Invitrogen), and were transported to the laboratory in a portable incubator within 1–3 h from recovery.

Oocyte recovery for *in vitro* production of embryos

Equine ovaries were obtained from the local slaughterhouse and transported to the laboratory in sterile saline within 2 h. Cumulus–oocyte complexes (COCs) were recovered by aspirating follicles measuring between 5 and 30 mm in diameter with a 18 G needle. COCs were washed in

TCM-199 supplemented with 1 mg/ml fatty acid-free BSA (Sigma) and 5 µl/ml gentamicin (Sigma). Oocytes were matured for 20 h *in vitro* in a medium composed of TCM-199 supplemented with 10% FBS, 5 µl/ml gentamicin, 5 µg/ml LH (Bioniche, Belleville, ON, Canada), 1 µg/ml FSH (Folltropin-V, Bioniche), 50 ng/ml epidermal growth factor (Sigma) and 100 ng/ml insulin-like growth factor 1 (Invitrogen). To remove cumulus cells, COCs were pipetted in TCM-199 containing 25 mmol/l HEPES, 10% FBS and 400 U/ml hyaluronidase (Sigma) for 2–5 min. Oocytes displaying a homogeneous cytoplasm were selected for NT.

Nuclear transfer

Fibroblast donor cells were isolated from skin biopsies taken from an adult horse. Isolation and culture of fibroblasts was carried out using 0.01% (w/v) collagenase I (Sigma), as described in the 'Derivation of feeder cell lines' section. Fibroblasts were cultured for 3–5 passages and frozen. Thawed fibroblasts were used for NT between passages 3 and 5. For NT, a single fibroblast donor cell was placed into the perivitelline space of an enucleated oocyte using a micromanipulator. The ooplasm–fibroblast couplets were transferred in fusion buffer (0.3 mol/l mannitol, 50 µmol/l CaCl_2 and 100 µmol/l MgCl_2 , all from Sigma) to a fusion chamber and exposed to a double direct current pulse of 1.5 KV/cm for 30 µs. Activation of fused couplets was induced by injection of stallion sperm extract prepared according to Choi *et al.* (2002), followed by 4 min treatment with 8.5 µmol/l ionomycin (Sigma). Reconstructed embryos were then incubated for 3 h in 2 mM 6-dimethylaminopurine (6-DMAP, Sigma), washed and cultured in drops of DMEM:nutrient mixture F-12 (DMEM/F-12) containing 10% FBS under mineral oil at 38.6 °C in an atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 for 6–11 days. At day 5 of culture, fresh medium was added to each drop.

Parthenogenetic activation

To produce PA equine embryos, denuded oocytes were selected for the presence of a polar body and activated with 5 µM ionomycin for 4 min, then incubated 3 h in 2 mmol/l 6-DMAP. Activated oocytes were cultured in DMEM/F-12 containing 10% FBS for 6–11 days as described for the NT embryos.

Derivation of feeder cell lines

An equine foetus from the first trimester of gestation was collected at the local slaughterhouse and carried to the laboratory in sterile saline. Fragments of skin and muscle were dissected into small pieces using a scalpel blade, and fibroblasts were isolated according to the method described in Yoo *et al.* (2007).

Equine and bovine umbilical cords were collected from newborns at the time of parturition. The Wharton's jelly was dissected and washed in PBS with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin, Invitrogen). The Wharton's jelly was dissociated into small pieces using scissors and digested with 0.01% (w/v) collagenase I at 37 °C for 3 h.

Dislodged cells were resuspended by pipetting and placed in a 15 ml tube. Cell suspension was centrifuged at 200 *g* for 10 min. The pellet was resuspended in feeder cell medium, filtered to eliminate cell clumps and plated into T25 culture flasks. Feeder cells were mitotically inactivated with mitomycin C (10 µg/ml, Sigma) for 3 h, dissociated with 0.25% (w/v) trypsin–0.01% (w/v) EDTA (Invitrogen) and plated at a density of 100 000 cells/ml in four-well plates (Nalge Nunc International, Naperville, IL, USA), pre-treated with a solution of 0.1% (w/v) gelatin (Sigma). Fibroblasts were maintained in an atmosphere of 5% CO₂, at 38.6 °C.

Embryo culture and formation of primary ICM outgrowths

Zonae pellucidae were removed using 10 mg/ml pronase (Sigma). Embryos were classified according to their age, developmental stage and appearance of their ICM, and were subjected to different protocols for ICM isolation. In the trypsin protocol, *in vivo*, PA and SCNT embryos were placed in 0.25% trypsin–EDTA for about 15 min, until trophoblast cells began to dissociate. Embryos were pipetted several times to dislodge trophoblast cells from the blastocyst. In the pronase protocol, PA and SCNT blastocysts were incubated with 10 mg/ml pronase for 5–30 min, until trophoblast cells began to dissociate. Isolated ICMs were placed in culture on BUCF, EUCF or EEF feeder cells, 24–48 h after mitotic inactivation. Equine ICMs were cultured in equine ESC medium consisting of 85% DMEM-knockout/F-12 (Invitrogen), 15% FBS (HyClone, Logan, UT, USA), 0.1 mmol/l 2-mercaptoethanol (Sigma), 1% (w/v) non-essential amino acids, 200 µmol/l L-glutamine, 1 mmol/l sodium pyruvate (Invitrogen) and 0.04 µg/ml human LIF (Chemicon International, Temecula, CA USA). Medium was changed every day, except for the first 48 h of culture. Equine PA and SCNT cell lines derived from primary ICM outgrowths were maintained in an atmosphere of 5% CO₂, at 38.6 °C.

To passage cells, the primary ICM outgrowth was cut using a scalpel under a stereomicroscope. Collagenase IV (1 mg/ml in DMEM-KO/F-12, Invitrogen) was used to dislodge the selected part of the colony, and the cell clump was placed in 0.25% trypsin–EDTA a few seconds to allow partial dissociation into smaller clumps. Clumps were washed in equine ESC culture medium when possible or placed directly in a well with a new feeder layer. EEFs were used to culture PA cell lines. To induce differentiation, dissociated cells were cultured for 7 days in suspension without feeder cells or LIF.

Molecular and biochemical characterisation

AP activity was detected using the AP detection kit (Chemicon International) according to the manufacturer's instructions. Total RNA was purified using the Qiagen micro RNA isolation kit (Qiagen), according to the manufacturer's instructions. DNase I (Invitrogen) treatment was performed once during the purification and once during the RT protocol. RT was performed using Superscript II (Invitrogen) as recommended by the manufacturer. For all samples, a negative RT was used as a control, consisting of an RT reaction omitting the reverse

transcriptase. In addition, PCR omitting the cDNA was used as a negative control. All primers were designed to span at least two exons. The following primers and annealing temperatures (Ta) were used: *POU5F1*: forward 5'-TCCCAGGACATCAAAGCTCTGCAGA-3', reverse 5'-TCAGTTGAATGCATGGGAGAAGCCCAGA-3', Ta=57 °C; *NANOG*: forward 5'-GACAGCCCCGATTCATCCACCAG-3', reverse 5'-GCACAGGTCTGACTGTTCCAGG-3', Ta=57 °C; *SOX2*: forward 5'-GGC GGC AAC CAG AAG AAC AG-3', reverse 5'-AGA AGA GGT AAC CAC GGG GG-3', Ta=57 °C; glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*): forward 5'-GTCCATGCCATCACTGCCAC-3', reverse 5'-CCTGCTTCACCACCTTCTTG-3', Ta=55 °C; *EOMES*: forward 5'-CCACCGCCACCAAACTGAGATG-3', reverse 5'-CAGTATTAGGAGACTCTGGGTGAA-3', Ta=57 °C; and *CDX2*: forward 5'-CAGCCAAGTGAAAACCAGGACGA-3', reverse 5'-CCTCTCCTTTGCTCTGCGGTTC-3', Ta=57 °C.

Immunofluorescence

Three *in vivo* embryos, ten PA embryos and three SCNT embryos without zonae pellucidae were fixed with –20 °C methanol and then stored at –20 °C until immunofluorescence staining. Embryos were washed once in 50% (v/v) methanol/PBS and three times for 5 min in PBS. Blocking was performed by incubating the embryos for 15 min in blocking solution (10% PBS–Tween-20 (PBST) and 10% normal goat serum). Double-labelling immunofluorescence was carried out overnight at 4 °C in a humid chamber using a cocktail of POU5F1 (C-10) mouse MAB (Santa Cruz, Santa Cruz, CA, USA, sc-5279) specific to isoform POU5F1A 1:50 (4 µg/ml) and GATA6 rabbit polyclonal antibody (Santa Cruz, H-92) 1:50 (4 µg/ml). As a control, a cocktail of mouse IgG_{2b} antibody 1:25 (4 µg/ml) and rabbit IgG antibody 1:500 (4 µg/ml, R&D systems, Minneapolis, MN, USA) was used. Primary antibodies were washed three times for 5 min in PBST. This was followed by a 1 h incubation with a cocktail of secondary antibody prepared with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (Invitrogen) both at 1:1000 concentration. All antibodies and control isotypes were diluted in the blocking solution. Blastocysts and outgrowths were washed three times in PBST and placed in the SlowFade Gold mounting medium containing 4',6-diamidino-2-phenylindole (Invitrogen). Representative embryos of each group are shown in Fig. 6.

Statistical analyses

The χ^2 test was used to determine statistical differences between experimental groups. Statistical analyses were performed with the JMP program (SAS Institute, Cary, NJ, USA), and a probability level of $P < 0.05$ was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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