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# Equine embryos and embryonic stem cells: Defining reliable markers of pluripotency

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***Abstract***

Cartilage and tendon injuries are a significant source of animal wastage and financial loss within the horse racing industry. Moreover, both cartilage and tendon have limited intrinsic capacity for self-repair, and the functionally inferior tissue produced within a lesion may reduce performance and increase the risk of re-injury. Stem cells offer tremendous potential for accelerating and improving tissue healing, and adult mesenchymal stem cells (MSC) are already used to treat cartilage and tendon injuries in horses. However, MSCs are scarce in the bone marrow isolates used, have limited potential for proliferation and differentiation *in vitro* and do not appear to noticeably improve long-term functional repair. Embryonic stem cells (ESC) or induced pluripotent stem (iPS) cells could overcome many of the limitations and be used to generate tissues of value for equine regenerative medicine. To date, six lines of putative ESCs have been described in the horse. All expressed stem cell-associated markers and exhibited longevity and pluripotency *in vitro*, but none have been proven to exhibit pluripotency *in vivo*. Moreover, it is becoming clear that the markers used to characterise the putative ESCs were inadequate, primarily because studies in domestic species have revealed that they are not specific to ESCs or the pluripotent inner cell mass, but also because the function of most in the maintenance of pluripotency is not known. Future derivation and validation of equine embryonic or other pluripotent stem cells would benefit greatly from a reliable panel of molecular markers specific to pluripotent cells of the developing horse embryo.

***Keywords***

stem cell; pluripotency; cell differentiation; embryo; inner cell mass; horse

## *1. Potential for stem cell therapy in the horse*

Injury-induced failure of horses to compete in the races for which they were bred and trained costs the racehorse industry an estimated US$6.5 billion per year [1]. Moreover, many of the more common equine athletic injuries affect musculoskeletal tissues with a limited capacity for complete functional repair (e.g. cartilage and tendon). In this respect, osteoarthritis is a frequent cause of equine lameness [2] with joint problems accounting for 15 to 25% of horses lost from training [3,4], while injuries to the superficial digital flexor tendon account for up to 46% of limb injuries, which are in turn responsible for 76% of all injuries sustained during Thoroughbred horse races [5]. The limited intrinsic capacity for self-repair, particularly in adults, makes treatment of cartilage defects challenging [6], and while tendons will eventually heal given careful management, the repaired tissue is functionally inferior and predisposes to reduced performance and high re-injury rates [7]. The economic and welfare costs of these performance-related injuries in horses have stimulated interest in stem cell-based regenerative medical techniques to accelerate and improve healing. Indeed, mesenchymal stem cells (MSC) are already used clinically in horses in an attempt to improve both cartilage and tendon repair following injury [8,9]. In addition, because of the similarities in size, load and types of injury suffered to joints in horses and humans, a recent FDA report concluded that the horse was the most appropriate model animal for testing and monitoring clinical effects of new therapies for certain types of joint injury in man [10]. This paper briefly reviews the current state of stem cell therapies in the horse, and the potential role for and limitations associated with the isolation and characterization of equine embryonic stem cells.

## *2. Current equine stem cell therapies*

Mesenchymal stem cells (MSC) are the local, multipotent precursors of connective tissues such as muscle, fat, tendon, ligament, bone and cartilage [11]. Their ability to self-renew and to differentiate into the respective tissues of interest means that they offer tremendous potential for equine regenerative medicine. Bone marrow-derived MSCs are currently the cell of choice for

67 treating cartilage or tendon injuries in horses [12]. In general, MSCs are recovered from bone
68 marrow aspirates and part-purified by density gradient centrifugation and selective adhesion onto
69 tissue culture plastic, and then expanded to confluent monolayers *in vitro* [12]. As conditions
70 required for *in vitro* tenogenesis are poorly described, undifferentiated MSCs are currently injected
71 directly into a tendon deficit [8,13]. For the treatment of cartilage injuries, MSCs can either be
72 transplanted directly or differentiated into chondrocytes by pellet culture in chondrogenic medium
73 containing serum, a mixture of growth factors (including TGFβ1, TGFβ3, BMP2, BMP4, BMP6,
74 BMP7 or FGF2) and dexamethasone [4,9,14,15].
75 Preliminary studies in laboratory animals using MSCs for the treatment of tendon injuries
76 have shown an improvement in collagen-fibre regeneration, histological appearance and mechanical
77 function of tendon-like tissue [reviewed in 16]. By contrast, controlled scientific studies in horses,
78 although limited in number, have shown no noticeable improvement in tendon structure or function
79 compared to controls [8,13]. Studies using MSCs to treat articular cartilage injuries in rabbits, dogs
80 and goats also report improvements in the quality of cartilage repair [reviewed in 9,12]. However,
81 while treatment with autologous MSCs also appeared to improve cartilage repair in osteoarthritic
82 knees of human patients, it did not yield significant clinical improvement [17]. Similarly in the
83 horse, while MSCs improved the early healing response in articular cartilage lesions, it did not
84 enhance long-term tissue repair over controls [9]. The latter authors concluded that the
85 disappointing long-term results may be because MSCs are lost from the lesion over time, and they
86 recommend that future efforts should be directed towards inducing differentiation into chondrocytes
87 before implantation [9].
88 There are also technical limitations to the use of MSCs for the repair of cartilage and tendon.
89 MSCs are scarce in bone marrow aspirates, require considerable time (~32 d) for expansion
90 between isolation and implantation, and have a more limited potential for *in vitro* proliferation and
91 targeted differentiation than cultured chondrocytes. In addition, bone marrow-derived adult MSCs
92 are more difficult to isolate with increasing donor age and show reduced plasticity and growth as a

factor of both increasing donor age and number of *in vitro* passages [18-20]. On the other hand, the harvesting and auto-transplantation of expanded chondrocytes or tenocytes is not a viable option because two rounds of surgery would be required, and isolation of the cells required for expansion in culture may damage existing functional tissue.

Embryonic stem cells (ESC) may offer a solution to the drawbacks encountered when using MSCs for tissue repair, since they offer virtually unlimited proliferation potential and longevity [21]. Isolated from the inner cell mass of blastocyst stage embryos, ESCs are truly pluripotent and, thus, able to give rise to all three germ layers of a developing embryo (endoderm, ectoderm and mesoderm) [22,23]. In addition, ESCs are amenable to genetic modification and differentiation into specific cell types or incorporation into chimeric animals, and thereby offer the potential for generating reporter cell lines or gain/loss of function disease models [24,25]. Such models would be invaluable to studies of the more than 189 genetic diseases described in the horse, of which approximately 95 have high homology with human genetic defects [1]; or to elucidate the underlying genetic causes of early embryonic death which is a source of considerable economic loss to the equine breeding industry [26]. Unlike MSCs, however, undifferentiated ESCs can not be used for direct transplantation because of the risk of teratoma formation [23]. Nevertheless, culture conditions have been described for the directed differentiation of human and mouse ESCs into both cartilage and, recently, tendon [27,28].

## *3. Progress in equine ESC isolation*

To date, the isolation and partial characterization of six putative equine ESC lines has been reported [29-31], although attempts to derive equine ESCs from *in vitro* produced embryos, or using interspecies somatic cell nuclear transfer, have so far been unsuccessful [32,33].

Saito *et al.* [29] microsurgically dissected the ICM from Day 6 to 7 *in vivo* blastocysts and then cultured the resulting cells for 3 to 5 d on bovine umbilical cord fibroblasts in αMEM

supplemented with fetal calf serum and human LIF. ES-like cells were selected and passaged on fresh feeder layers every 6 to 7 d. Two out of the three ICMs (67%) cultured yielded ES-like cell lines which had a stable karyotype and maintained *in vitro* proliferation potential for more than 56 passages (392 cell divisions; Table 1). The cells could be maintained without LIF and were positive for several stem-cell associated markers including octamer binding protein 4 (*OCT4*; also known as *POU5F1*), signal transducer and activator of transcription 3 (*STAT3*), alkaline phosphatase (*AP*), and stage-specific embryonic antigen 1 (*SSEA1*). These ES-like cells could also be induced to differentiate into neural precursors by culture in the presence of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF); and into haematopoietic and endothelial precursors using bFGF, stem cell factor (SCF) and oncostatin M (OSM).

In the study by Li *et al.* [30], the ICMs of Day 8 *in vivo* expanded blastocysts were dissected immunosurgically and then cultured for 7 d on mouse embryonic fibroblasts in DMEM/nut mix F12 supplemented with fetal calf serum and human LIF. ES-like cells were then mechanically passaged on fresh mouse embryonic fibroblasts or horse fetal fibroblasts every 4 to 7 d. Approximately 33% of ICMs cultured in the presence of feeders and LIF yielded ES-like cell lines (n = 3) and a fourth cell line was derived in a follow-up study [31] (Table 1). In contrast to the ES-like cells produced by Saito *et al.* [29], however, both feeder cells and LIF were required to maintain cells in an undifferentiated state. Karyotype stability was not reported in this study but, after more than 26 passages, cells were still positive for *OCT4*, *STAT3*, *AP*, *SSEA1*, *SSEA4*, Trafalgar-1-60 (*TRA-1-60*) and *TRA-1-81*. Removal of LIF and feeder layers from the *in vitro* culture medium resulted in the differentiation of the ES-like cells into neural ectoderm, visceral endoderm and cardiac and haematopoietic mesoderm. In contrast to human ESC controls, however, the four lines of equine ES-like cells did not form teratomas when injected into the testes of immunocompromised mice.

Compared to most other large domestic species [34], isolation of ES-like cells from *in vivo* horse blastocysts therefore appears to be relatively straightforward. All six cell lines described

above exhibited ESC-like characteristics such as longevity, expression of stem cell-associated markers and *in vitro* pluripotency (Table 1). On the other hand, the absence of any data verifying *in vivo* pluripotency of the cells (e.g. teratoma or chimera formation) means that the cells cannot yet be definitively classified as ESCs. Li *et al.* [30] suggested that the failure of their equine ES-like cells to generate teratomas in an immunoprivileged site may be a peculiarity of equine ESCs. Certainly, the failure of teratoma formation is difficult to explain given that murine and primate ESCs, porcine ES-like cells, and bovine and porcine pluripotent ICM all yield teratomas under similar conditions [22,23,35-37]. Equine ICM transplantation experiments may help to determine whether this anomaly really is due to a peculiarity of equine pluripotent cells, or because the ES-like cells tested were not true ESCs. In this latter respect, it is possible that sub-optimal culture conditions for the equine ES-like cells led to a loss of pluripotent potential over time. Indeed, murine ESCs have been reported to lose their ability to generate pure ESC-derived offspring, or contribute to the germ line in chimeras, with increasing passage number [reviewed in 38]. The equine cells used in Li *et al.*'s teratoma trials were between passage 14 and 26 [30], by which time culture-induced changes could have reduced their *in vivo* pluripotent potential. It has also been reported that even recently isolated murine epiblast SCs, which closely resemble human ESCs, are unable to form chimeras, or do so with poor efficiency, just like ES-like cells from large domestic species [39-41]. In short, it appears that ES-like cells derived from the epiblast of a range of domestic species, which may include the horse, have more restricted pluripotent potential than true ESCs, and the current putative equine ESCs could quite conceivably actually be epiblast SCs.

Sub-optimal culture conditions can also inadvertently select and enrich an alternative SC line. In ungulates, trophectoderm and primitive endoderm can outgrow cultured ICM even after careful dissection or immunosurgery, while these cells can still form embryoid-like bodies reminiscent of those formed by ESCs [34]. In addition, epithelial cells grown on fibroblast feeder-layers can both look ES-like and be *AP* positive [34], while even multipotent (murine) adult progenitor cells have been shown to be able to differentiate into endoderm, ectoderm and mesoderm

[42]. Currently, our understanding of the processes controlling pluripotency in equine cells is both rudimentary, and based on the assumption that they will be comparable to those described for mice or man. Reliable markers or cell behaviour tests for the pluripotent state are clearly required both to definitively characterise existing lines of equine ESCs and to advance our understanding of how to manipulate cellular differentiation in equine cells. Moreover, without these markers, attempts to derive new cell lines or improve culture conditions will be impaired because any culture-induced changes that alter their pluripotent potential will go undetected.

### *4. Problems with existing pluripotency markers in domestic species*

A golden rule in the characterisation of ESCs is that their behaviour should recapitulate what occurs naturally *in vivo* [21]. Likewise, markers for pluripotency in ESCs should only be expressed in cells destined to form the embryonic ICM. SSEA1, SSEA3 and SSEA4 are all cell surface antigens associated with pluripotent stem cells, but their precise functions in the maintenance of pluripotency is not known. Furthermore, none of these markers can be used in isolation as a cross-species definitive indicator of pluripotency because there are considerable between species differences in their expression in both ESCs and embryos. For example, *SSEA1* is expressed in mouse but not human ESCs [22,23], *SSEA3* is expressed in human ESCs and horse embryos but not horse ES-like cells [23,31], and the expression of *SSEA4* differs between horse ES-like cell lines [29,31] (Table 1). Moreover, SSEA1 and SSEA4 proteins are not confined to the ICM of caprine embryos but are also expressed in the trophectoderm [43]. TRA-1-60 and -81 are also cell surface antigens that recognize different epitopes of a common glycoprotein of which the exact function is not yet known [44]. Furthermore, while AP staining is also widely used to characterise ESCs it is neither ESC nor ICM-specific, since it also labels other stem cell types and is expressed at different times by either the epiblast or trophectoderm [34,45,46].

Even genes for which a function in the maintenance of pluripotency has been validated using knockout mouse models often have different expression patterns in other species. In the mouse, *Oct4* is known to specify the ICM while *Nanog* specifies the pluripotent epiblast; both are therefore restricted to the ICM in expanded murine blastocysts [47-49]. By contrast, OCT4 protein is strongly expressed in both the ICM and trophectoderm of human, primate, bovine, caprine and porcine blastocysts [43,48,50-52], but also primitive endoderm cell lines [34]. Moreover, both *NANOG* mRNA and protein appear to be completely absent during the period of ICM and epiblast formation in the pig [51], while in primate blastocysts, *NANOG* rather than *OCT4* has been proposed to specify the ICM [52].

The different expression of *OCT4* and *NANOG* in species other than the mouse, suggests that there are species-specific differences in genes regulating pluripotency and early lineage segregation which may reflect differences in embryonic development. In the pig and cow, for example, prolonged *OCT4* expression in the trophectoderm has been proposed to be necessary for rapid proliferation of the trophectoderm during embryonic elongation on Days 11 to 12 after fertilization [48,51]. Differences in the form of, and mechanisms underlying embryogenesis and placental development may also partly explain why the derivation of embryonic stem cells has proven so difficult in many large domestic species [34]. In short, accepted murine ESC markers may not be appropriate to other species, and a species-specific approach is likely to be necessary to validate adequate markers of pluripotency.

## *5. Pluripotency markers in equine embryos*

Since early equine embryogenesis differs significantly to that of the mouse, pig, cow and human [34,53], it is reasonable to anticipate different temporal and spatial expression patterns for putative pluripotency genes in the horse. The genome of the early equine embryo is thought to activate at the 4 to 8 cell stage, i.e. during the prolonged 6 d migration through the oviduct [54].

When the embryo enters the uterus on Day 6, it is at the compacted morula stage, but within a few hours has developed into an early blastocyst with the first clearly visible signs of trophectoderm and ICM differentiation [53,55]. At around the same time, a unique glycoprotein tertiary embryonic coat (the 'blastocyst capsule') forms between the trophectoderm and zona pellucida; it remains in place until around Day 21 and is essential for the continuation of pregnancy [56]. After segregation of the ICM into epiblast and hypoblast on around Day 8 primitive endoderm forms as separate colonies distributed sporadically over the interior of the trophoblast, which then expand and coalesce [57]. The next microscopically visible evidence of a major cell differentiation event, gastrulation, commences on Day 11 to 12 with the appearance of the primitive streak and the first discernable mesoderm cells [57,58]. Unlike porcine and bovine conceptuses, the pre-implantation equine conceptus remains spherical (i.e. it does not elongate) and highly mobile, migrating continuously throughout the uterus until approximately Day 16, a process that is essential to maternal recognition of pregnancy [59]. The trophectoderm first makes direct physical contact with the endometrium as late as Day 22 to 23, following dissolution of the blastocyst capsule and, at around Day 35, a specialized band of trophectoderm cells invades the endometrium to establish the temporary, eCG-secreting endocrine organs, the endometrial cups. True placental formation (i.e. trophectoderm-endometrium interdigitation) does not start until as late as Day 40 of gestation, and is then of the non-invasive epitheliochorial type [60].

It is quite conceivable that the various idiosyncrasies of early equine conceptus development profoundly affect the pattern of gene expression within the various cell lineages of the conceptus. However, the genes regulating pluripotency and early lineage segregation in the horse have yet to be fully described. To date, just three studies have reported the (changes in) expression or ICM-specificity of putative pluripotency markers in early horse embryos [31,33,61] (Table 2). Hinrichs *et al.* [33] reported that OCT4 protein was expressed in the cytoplasm and nucleus of immature oocytes and that the level of expression decreased gradually during the first 4 d of *in vitro* culture in embryos produced by ICSI, before increasing again in the cell nuclei during Days 5 to 7,

presumably as a result of embryonic genome activation. Interestingly, OCT4 protein was found in both the ICM and trophectoderm of Day 7 to 10 blastocysts and lost from the trophectoderm of *in vitro* embryos larger than 1 000 μm in diameter only if they had been exposed to the uterine environment for 2 to 3 d by embryo transfer [33]. For *in vivo* Day 7 to 10 blastocysts, OCT4 protein expression was strong in the ICM and weak in the trophectoderm, and completely confined to the ICM of embryos exceeding 600 μm in diameter. AP staining was similarly stronger in the ICM than in the trophectoderm [31,33]. Our own studies suggest that *OCT4* mRNA expression decreases successively during the morula to early blastocyst and the early to expanded blastocyst transitions, a pattern consistent with a role in ICM specification. We also recorded down-regulation of *NANOG* mRNA, but only at the early to expanded blastocyst transition, a pattern that fits a role in specification of the epiblast [61]. These preliminary findings need however to be confirmed at the level of protein expression and distribution. Similar to the situation for OCT4, proteins of the pluripotency-associated cell surface markers SSEA1, SSEA3, SSEA4, TRA-1-60 and TRA-1-81, were found to be expressed throughout both the ICM and trophectoderm of Day 7 blastocysts [31] (Table 2), raising similar questions as to their suitability as pluripotency markers.

During morula to expanded blastocyst development in the horse, there is a rapid increase in cell number from 160 to over 2 900 that occurs predominantly in the trophectoderm [62]. As in the pig and cow, the dramatic proliferation of trophectoderm cells may explain the prolonged expression of *OCT4* in this tissue. The even more prolonged expression of *OCT4* by trophectoderm of *in vitro* horse embryos may reflect the significant retardation of development that appears to occur under these conditions [63], and may partially explain why deriving ESCs from this source has proven difficult [33]. It is possible that a similar delayed down-regulation in trophectoderm cells until the horse embryo exceeds a threshold size or developmental stage also applies to other pluripotency markers, although this remains to be tested. Certainly, it appears that the panel of markers used to characterise pluripotency and ESCs in the horse to date are inadequate.

## *6. Where to from here?*

Identification and validation of embryonic stem cells and indeed understanding of the molecular conditions required for pluripotency in domestic species such as the horse requires a reliable set of molecular markers proven to be both specific and exclusive to the pluripotent cells of the developing embryo. Since the details of embryogenesis differ markedly between species, the exact determinants of pluripotency may also vary; such studies should therefore be carried out on a per species basis. Nevertheless, recent work to define the interaction networks controlling pluripotency in mouse and human ESCs has provided a useful list of candidate genes to evaluate in other species [64,65]. We have been using just this type of information to examine the expression of potential pluripotency-associated genes in equine embryos and have found that factors such as growth and differentiation factor 3 (*GDF3*), developmental pluripotency-associated 4 (*DPPA4*) and telomerase reverse transcriptase (*TERT*) are down-regulated during the morula to early blastocyst transition [61]. The time-course of mRNA expression for these genes therefore suggest that they may be useful markers for pluripotency, and whole mount immunofluorescent studies are ongoing to examine the ICM-specificity of these, and other candidate, genes at the protein level. In addition to this candidate-gene 'bottom-up' approach, newly emerging molecular tools such as equine-specific microarrays will enable a 'top-down' approach to examine comparative global gene expression profiles in equine embryos and putative ESCs and thereby identify a wide array of genes with potential roles in sustaining or terminating pluripotency.

Once defined, a suitable panel of pluripotency markers could be used to further characterise existing equine ES-like cells [29-31], and to assist in the establishment and validation of newly derived pluripotent cell lines. Moreover, understanding the molecular mechanisms regulating pluripotency in the horse will permit more detailed classification of the diverse range of stem cells currently used in equine regenerative medicine; an analogous process was recently initiated for human regenerative medicine [65].

For some domestic species, optimizing specific culture conditions that maintain pluripotency is likely to be critical to successfully establishing ESCs. In the pig and cow, for example, primary epiblast cells can only be cultured on STO feeder cells, are highly sensitive to lysis and rapidly differentiate within 48 to 96 h [38]. By contrast, the isolation of ES-like cells from cultured ICM appears to be more robust and efficient in the horse [29,30]. There is, however, still a need to optimize culture conditions for equine ES-like cells (by determining optimal media, feeder cells and cytokine requirements) to avoid culture-induced changes that may compromise indices of cellular pluripotency, such as teratoma forming ability. In this respect, reliable markers would permit the monitoring of pluripotency state during culture. Although beyond the scope of this review, alternative cell types, such as embryonic germ cells (EGCs) may overcome problems of limited *in vivo* pluripotency in equine ES-like cells, as has been demonstrated for porcine and bovine EGCs [reviewed in 41].

A potential advantage of ESCs is 'off the shelf' use for allogenic transplantation. However, if they are to be used in this way, it is important to examine the likely immunogenicity of these cells and their derivatives. Transplantation rejection is governed primarily by MHC antigens and there are indications that some ESC derivatives do not express some MHC antigens and/or have other characteristics that help them avoid rejection [66,67]. If equine ESCs do not show reduced MHC expression, alternative technologies such as somatic cell nuclear transfer-derived ESCs and induced pluripotent stem (iPS) cells may offer ways of generating 'disease-specific' or 'patient-specific' pluripotent cells. Indeed, patient-specific genetic defects have already been treated by a combination of iPS, cell and gene therapy technologies in the mouse [68], while the recent report of iPS generation from porcine cells [69] suggests that derivation of similar pluripotent cells may soon be possible for the horse.

## *7. Conclusion*

Due to their unlimited potential for proliferation and ability to differentiate into all three germ layers, embryonic stem cells offer tremendous potential for regenerative therapy of tissues with a limited capacity for self-repair. However, it is clear that (i) existing equine ES-like cells are not yet fully characterised, and (ii) current markers used to characterise pluripotency in equine cells are inadequate. Establishing a panel of validated pluripotency markers specific to the horse should simplify future attempts to identify, isolate, characterise, and classify equine pluripotent stem cells derived either from embryos or from other tissues, e.g. by iPS technology.

## *8. Author contributions*

DP was the primary author. TS read and critically revised the manuscript. Both authors approved the final manuscript.

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## *Tables*

**Table 1: Characteristics of the six reported equine embryonic stem cell lines.**

| | Saito *et al.* [29] | Li *et al.* [30]; Guest and Allen [31] |
|---|---|---|
| origin | Day 6 to 7 *in vivo* blastocysts | Day 8 *in vivo* expanded blastocysts |
| number of cell lines | 2 | 4 |
| feeder layer | bovine umbilical cord fibroblasts | mouse embryonic or horse fetal fibroblasts |
| maintenance | feeders only | feeders + LIF |
| karyotype | normal (at passage 20) | not reported |
| longevity | >56 passages (392 cell divisions) | >26 passages |
| *OCT4* | + | + |
| *STAT3* | + | + |
| *AP* | + | + |
| *SSEA1* | + | + |
| *SSEA3* | - | - |
| *SSEA4* | - | + |
| *TRA-1-60* | not reported | + |
| *TRA-1-81* | not reported | + |
| *in vitro* pluripotency | ectoderm, mesoderm | ectoderm, endoderm and mesoderm |
| *in vivo* pluripotency | not reported | - |

**Table 2: Localized protein expression for pluripotency-associated markers in Day 7 to 10 equine blastocysts [31,33].**

| | inner cell mass | trophectoderm |
|---|---|---|
| OCT4 | ++ | +/- |
| STAT3 | not reported | not reported |
| AP | +++ | + |
| SSEA1 | ++ | ++ |
| SSEA3 | ++ | ++ |
| SSEA4 | ++ | ++ |
| TRA-1-60 | +++ | + |
| TRA-1-81 | +++ | + |

+++, ++, + and -: strong, intermediate, weak and no expression respectively