Characterization of Fetal Gonad-Derived Cells by Stem Cell Markers

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줄기세포 Marker를 이용한 돼지 태아 생식선 유래 세포의 특성화

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ABSTRACT

In mammals, male and female germline stem cells are derived from primodial germ cells. Despite many efforts to identify stem cells from gonads, there has been little successe to identify germline stem cells yet. In this study, we isolate and characterized porcine germline stem cells using only stem cell markers that are prevalently expressed in various tissues. Gonadal cells derived from both male and female formed colonies and showed AP activities and different lectin binding properties. Pluripotency of germline stem cells was also identified by positive signals against putative stem cells markers such as SSEA-1 and SSEA-3. In addition, nestin was also found in primary gonad cells that have a similar morphology to the AP-positive cells. The nestin expression suggests that the germline stem cells may have similar expression of the prevalent stem cell markers found in other tissues. The demonstration of nestin expression together with pluripotent cell markers calls further investigation of the possible differentiation of nestin-positive cells into neurons.

(Key words : Porcine, Germline stem cells, SSEA-1, SSEA-3, Nestin)

Ⅰ. INTRODUCTION

In mammals, male and female germline stem cells are derived from primodial germ cells (PGCs) (Feng et al., 2002). Male germline stem cells are able to produce mature spermatozoa while conserving the self-renewal potential of stem cells. Female germline stem cells are controlled by the surrounding somatic stem cells (Surani, 2004). The PGCs differentiate very early stage and often migrate to gonads in developing embryo and destined to ultimately give rise to gametes. The mechanisms for the PGC migration may be surface molecules or exogenous chemo-attractants or physical properties surrounding extracellular matrix and adjacent cells as well as PGC themselves (Doitsidou et al., 2002; Kuwana et al., 1999; Shim et al., 1997).

Embryonic germ cells are derived from primodial germ cells (PGCs) of the developing fetus (Bendel-Stenzel et al., 1998) and share several morphological characteristics such as high level of intracellular alkaline phosphatase (AP), specific cell surface glycolipid and glycoproteins (Shamblott et al., 1998). These properties are not specific for all pluripotent stem cells. However, only a few specific markers such as AP, SSEAs have

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being used to identify stem cell properties.

In particular, spermatogonial stem cells are difficult to be identified by morphological, antigenic or biochemical criteria. Therefore, many researchers have tried to identify specific markers with no great success. So far, there are no unique phenotypic markers to identify spermatogonial stem cells yet (Kubota et al., 2003).

In this study, to isolate and characterize porcine germline stem cells, we cultured primary porcine germline cells in the presence of LIF, FSH and estradiol benzoate for 16 days *in vitro*. Pluripotency of the acquired germline stem cells were analyzed by AP staining, lectin binding assay and immunocytochemical methods using putative stem cells markers such as stage-specific embryonic antigen-1 (SSEA-1), SSEA-3 and nestin.

Ⅱ. MATERIALS AND METHODS

1. Animals and primary cell culture

The experimental fetuses were obtained from pregnant Landrace pigs. Fetuses were immediately removed by dissecting the uterus showing the evidence of pregnancy. The length of crown-rump in the fetuses analyzed was about 6.3 cm. Isolated male or female gonads were washed with PBS and then the tissues were dissociated with 0.025% (v/v) trypsin and 1mM EDTA (Sigma Chemical Co., St. Louis, MO, USA) was added to dissociate cells. Following incubation at 37℃ for 10min, the cells were dispersed into single cells and then cultured in DMEM (GibcoBRL, Grand Island, NY, USA) with 10% FBS and 10 g/l ITS (insulin, transferrin, selenious acid), 10 ng/ml hFSH, 10^{-8} M estradiol benzoate, 5 ng/ml LIF and 25 ng/ml bFGF (Sigma) at 37℃ in 5% CO_2. The cells were cultured for 16 days *in vitro* and then analyzed.

2. Immunocytochemical analysis and Lectin binding

The cells on the coverslips were fixed in 2% (w/v) paraformaldehyde (Sigma) for 15 min at room temperature. To visualize intracellular distribution of stem cell markers, the cells were washed with PBS (pH 7.4) containing 0.1% (w/v) Tween 20 (PBT) and preincubated for 1 h with 10% normal goat serum (GibcoBRL) to eliminate non-specific binding. To detect nestin peptide, the cells were incubated for 90 min with anti-nestin antibody (BD Biosciences, San Doego, CA, USA), SSEA-1 or SSEA-3 on the surface were detected by specific antibodies (BD Biosciences) under non-permeable condition. After washing in PBT three times, cells were labeled with nestin, SSEA-1 and SSEA-3 antibodies, followed by goat anti-mouse Ig antibody to conjugated to FITC (Sigma). To detect UEA-1- or WGA-binding sites on the surface of colonies from germline cells, the fixed cells were stained with 10g /ml FITC conjugated UEA-1 or WGA (Sigma) for 20 min. The cells were incubated in 4g/ml of 4',6-diamidino-2-phenylindole dihydrochloride, hydrate (DAPI, Sigma) for 15 min for nucleus staining and analyzed under a Zeiss Axoplan Fluorescence Microscope (Zeiss, Oberkochen, Germany) and recorded by a Nikon digital camera (Nikon Co, Japan).

3. Alkaline phosphatase staining

Colonies formed cells were fixed with 4% (w/v) paraformaldehyde for 20 min and then washed with Tris-maleate buffer and developed in AP staining solution (0.4 mg/ml naphtol AS-MX phosphate, 1mg/ml Fast Red, 0.08% $MgCl_2$ in Tris-maleate buffer) for 20 min.

Ⅲ. RESULTS

1. General appearance of E45 fetuses from pregnant pigs

Fetuses had 6.3cm of crown length, and they were considered as E45 fetuses (Robinsin, 1973) (Fig. 1a-c). The sexes are classified by the appearance of the external genitalia in individual fetuses. Isolated male or female gonads showed oval and no turing shape and sunken kidney bean like shape, respectively. The sexes were verified by reexamining the external genitalia under a stereomicroscope. The external and gonadal sexes were consistent in all sex-identified fetuses. With experiences, examining the gonadal morphology was sufficient to sex the fetuses

2. Colonies of germline stem cells showed activity of alkakine phosphatase

After 2 days of *in vitro* culture most of cells in the gonads

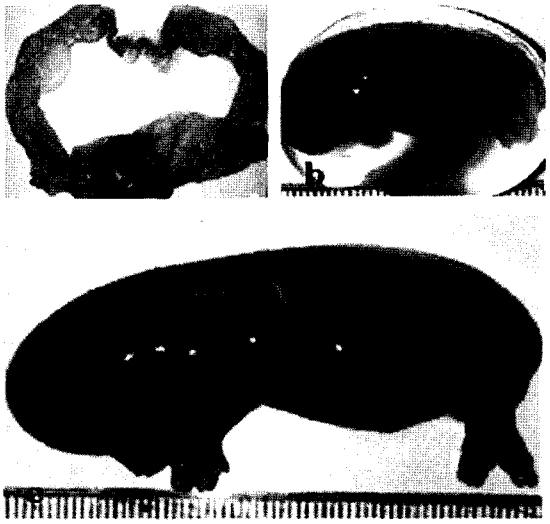

Fig. 1. Isolation of porcine E45 embryos from pregnant pigs. The embryos from pregnant Landrace pigs were immediately removed by flushing on day 45. Individual conceptus (a) was isolated from pregnant uterus, and fetal membranes (b) were subsequently removed to isolate E45 embryos (c). The length of the embryos was 6.3 cm.

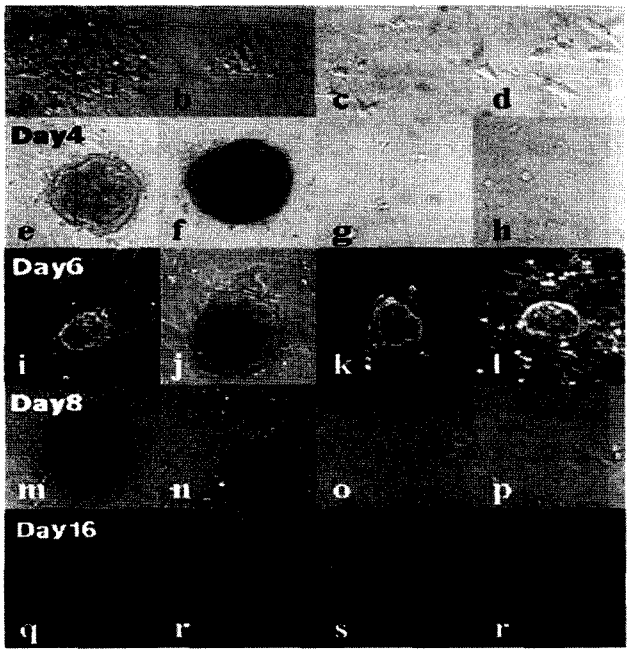

Fig. 2. Formation of islands of germline stem cells. Male or female gonads were isolated and cultured. The gonads dispersed and formed monolayer at day 2 (a-d). Among the culture, distinct colonies of cells were found as islands at day 4 (e, f). After 16 days, the cells formed colonies (q-r).

Table 1. Formation of germline stem cell colonies showing positive AP staining[1]

Sex	No. of gonad used	No. of colonies	No. of AP (+) Colonies (%)
Male	23	81	17 (20.9)
Female	20	104	12 (11.5)

[1]AP: alkaline phosphatase.

dispersed and formed monolayer (Fig. 2a-d). Among the cultures, distinct colonies of cells were found as islands at day 4. Either male or female gonad cells formed colonies of cells although the formation was more frequent in male gonad cells. To determine pluripotency of cells forming colonies, we analyzed activities of alkaline phosphatase by substrate reaction. 20.9% of colonies from male gonad and 11.5% of female gonads showed strong positive signals (Fig. 3, Table 1). Interestingly, female germline stem cells showed slightly lower activity of alkaline phosphatase than male counterparts (Fig. 3). To classify specific AP-stained colonies, we exclude partially AP-stained colonies (arrows, Fig. 4a) or AP staining-negative colonies from group of AP-positive colonies (Table 1). Some of scattered cells also presented positive AP staining (Fig. 4c). These cells also showed positive SSEA-3 signals on their surface (Fig. 4b).

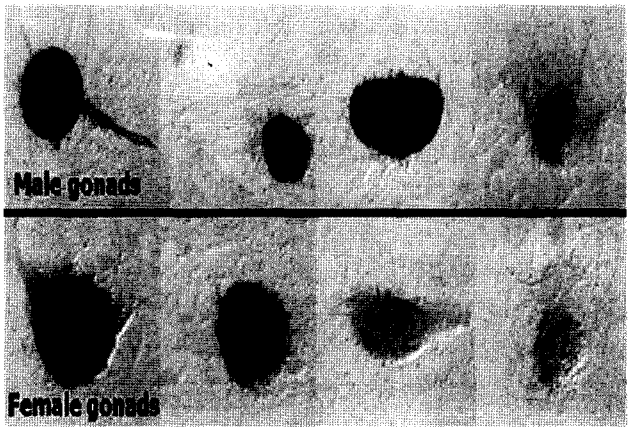

Fig. 3. Alkaline phosphatase-positive colonies of germline stem cells. At DIV 16, cultured colonies were analyzed by AP staining. 20.9% of colonies from male gonad and 11.5% of female gonads showed strong positive signals.

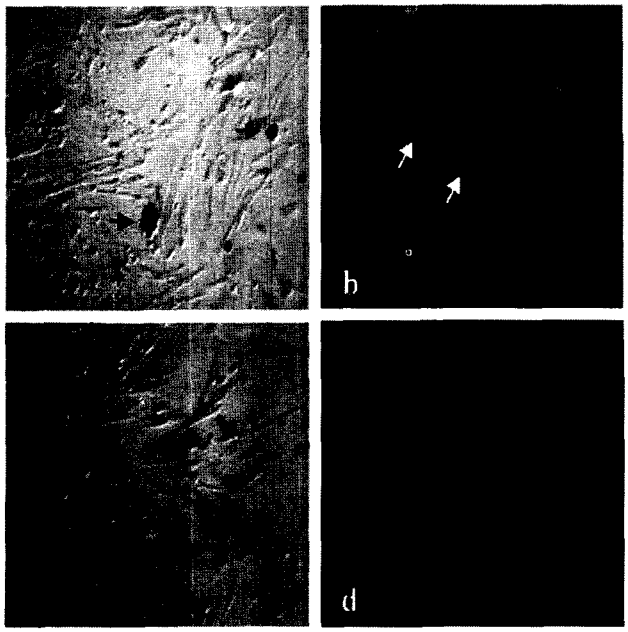

Fig. 4. Various types of gonadal cells showing AP positive signals. At 16 days in culture, some cells showed partially AP staining positive signals in the colonies and these cells were excluded from group of AP-positive colonies (arrow, a). In addition, AP staining negative cells also detected (a, c). The cells that did not consist of colonies showed positive AP staining (c) as well as positive SSEA-3 signals on their surface (b and d; SSEA-3 (green) and nuclei (blue)).

3. Specific stem cell marker-positive germline stem cells

To characterize property of colonies, we investigated the distribution of specific antigens known to be expressed in various stem cells of other tissues. Porcine germline stem cells were characterized with SSEA-1 and SSEA-3, which are highly expressed on pluripotent stem cells (Capela and Temple, 2002). Colonies showed specific positive spots on the surface of the colonies for SSEA-3 (Fig. 4e-h), whereas colonies stained with the secondary antibody alone gave no signal (data not shown). The antibody recognizing SSEA-1 stained the cells inconsistently and weakly (Fig. 4a-d). We also investigated expression of nestin in colonies of germline stem cells by immunocytochemistry. Nestin is an intermediate filament (IF) often used as a marker for CNS progenitor cells and neuroepithelial stem cells (Lendahl et al., 1990). The colonies showed weekly expressed nestin along the intermediated filaments (Fig. 4i-m).

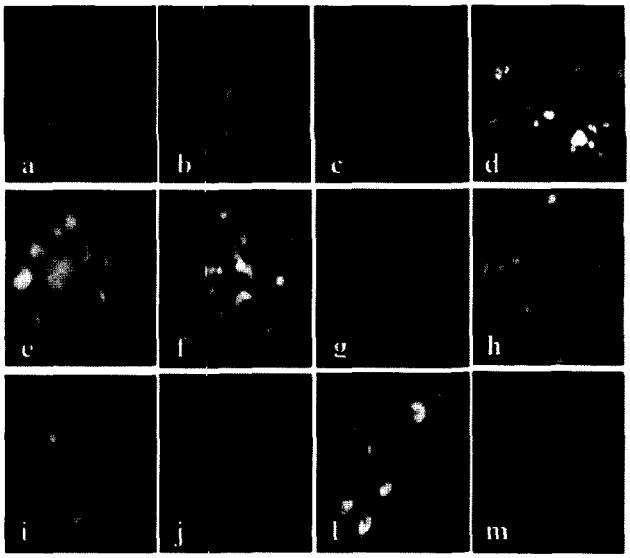

Fig. 5. SSEA-1, SSEA-3 and nestin positive germline stem cells. Porcine colonies were characterized with a SSEA-1, SSEA-3, which are strongly expressed on pluripotent stem cells. The antibody recognizing SSEA-1 stained the cells inconsistently and weakly (a, c). Colonies showed specific positive spots on the surface of the colonies for SSEA-3 (e, g). These colonies also stained with anti-nestin antibody, as a marker for CNS progenitor cells and neuroepithelial stem cells. Nestin weakly expressed along the intermediated filaments (i, l).

4. Highly expressed glycoproteins on the germline stem cells

Pluripotent stem cells often share common properties such as high level of specific cell surface glycolipid and glycoproteins (Shamblott et al., 1998). To verify an expression of such glycoproteins on the surface of colonies derived from germline stem cells, we investigated a distribution of glycoproteins using lectins such as UEA-1 and WGA. FITC-conjugated UEA-1 or WGA were tested for binding to the cells consisting colonies. UEA-1 and WGA strongly detected on the surface of colonies (Fig. 5), whereas no signals on the surface of colonies incubated with secondary antibodies only (data not shown).

Ⅳ. DISCUSSION

The aim of this study was to characterize biological

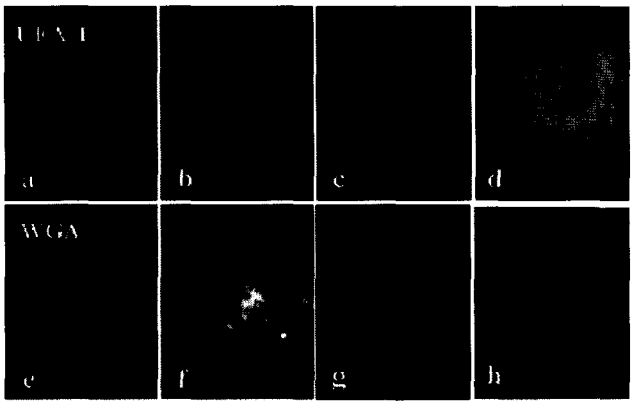

Fig. 6. UEA-1 and WGA positive germline stem cells forming colonies. The colonies were stained with FITC conjugated UEA-1 or WGA. UEA-1 was detected on the surface of colonies (a, c). WGA also strongly detected on the surface of the colonies (e, g).

properties of porcine germline stem cells. We first established a porcine primary germline cell culture system using LIF, FSH, estradiol benzoate and ITS (insuline, transferrin, selenious acid) supplementation. Porcine germline stem cells formed colonies at DIV 4. At DIV 15, the cells gave rise to highly compacted colonies suggesting that strong cell-cell adhesion was maintained. The morphology of colonies was similar among the colonies regardless of the sexes and resembles mouse ES and EG cells (Gallagher et al., 2003).

We examined an activity of alkaline phosphatase as a marker of proliferating undifferentiated cells. The cultured germline stem cells showed strong activity of alkaline phosphatase. 20.9% of male derived germline stem cells showed strong positive alkaline phosphatase staining. However, female germline stem cells showed lower activity (11.5%). Lower activity of alkaline phosphates may be due to early cellular differentiation and subsequent loss of pluripotency in female germline stem cells. We suspect that this might reflect the fact that many researchers failed to establish female germline stem cells.

It was another interesting observation that not only colonies but also cells in the monolayer showed positive AP staining. Some cells with similar morphology to AP positive cells in monolayer also stained with anti-SSEA-3 antibodies. The results suggest that germline stem cells might have various types of proliferation in culture. However further morphological studies are still reamined. In these results, our established culture system was enough to maintain proliferation of stem cells, and acquired male or female germline stem cells had pluripotency as other stem cells.

A few cells leave the developmental continual maintenance and repair of the tissues and organs throughout the life span of the individual. Some of the precursor cells were known as pluripotent stem cells (Young et al., 2004). Pluripotent stem cells have some properties such as high level of specific cellular surface molecules and glycolipid and glycoproteins. To characterize property of stem cells, many specific surface molecules have been studied by immunochemical or molecular or biochemical analysis.

We also identified pluripotency of acquired germline stem cells with monoclonal antibodies against specific antigens which are known to be expressed in various stem cells of other tissues.

In human or procine PGCs and ES cells, expression of SSEA-1 and SSEA-3 were reported by Takagi et al. (1997) and Shamblot et al. (1998). Thus, we tested pluripotency of germline stem cells with SSEA-1 and SSEA-3. Colonies showed specific positive spots on their surface for SSEA-3. However, antibody recognizing SSEA-1 resulted in inconsistent and weak staining. Obtained germline cells in our study were believed to be pluripotent stem cells sharing common stem cell markers on their surface.

The presence of nestin-positive cells in colonies suggests that these germline stem cells may play a role as CNS progenitor cells and neuroepithelial stem cells. Stem cells in the nervous system have been studied their potency using anti-nestin antibodies (Lendahl et al., 1990; Pevny and Rao, 2003). In addition, these germline stem cells had an ability to form neurosphere-like structures suggesting a potential capability of self-renewal and differentiation to nerons. Therefore, nestin-expressing germline stem cells may retain a potency of neural populations raising a possibility that stem cells from the gonad could differentiate into neurons, astrocytes, etc.

UEA-1 and WGA also strongly detected on the surface of the colonies. This result also support that the colonies were composed of pluripotent stem cells. Stem cells in other species including mice can be identified morphologically and with presence of specific markers (Young et al., 2004). Other

techniques such as, scanning electron microscopy, glycoconjugate histochemistry also have been applied (Bez et al., 2003; Lee et al., 2000; Takagi et al., 1997). The results of these lectin binding studies were consistent with the reports that stem cells derived from porcine or mouse embryos had an affinity to lectins, such as UEA-1, WGA, Con A, DBA and PSA, etc. (Takagi et al., 1997).

The present study demonstrates that male and female germline cells from porcine E44 embryos had a pluripotency as stem cells. These germline stem cells expressed SSEA-1, SSEA-3 and nestin. Interestingly, results of nestin expression suggest that the germline stem cells can differentiate neurons. Additionally, positive signals of alkaine phosphatase staining and lectins (UEA-1, WGA) binding also confirm the presence of germline stem cells. At present, little is known regarding stem cells in pigs. Further studies of germline stem cells may contribute to our understanding of pluripotent stem cells. Moreover, genetic modification of these pluripotent stem cells may allow more efficient and precise way of generating transgenic animals that will contribute in curing many human diseases.

V. REFERENCES

1. Bendel-Stenzel, M., Anderson, R., Heasman, J. and Wylie, C. 1998. The origin and migration of primordial germ cells in the mouse. Sem. Cell Dev. Biol. 9:393-400.
2. Bez, A., Corsini, E., Curti, D., Biggiogera, M., Colombo, A., Nicosia, R. F., Pagano, S. F. and Parati, E. A. 2003. Neurosphere and neurosphere-forming cells: morphological and ultrastructural characterization. Brain Res. 993:18-29.
3. Capela, A. and Temple, S. 2002. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron 35:865-875.
4. Doitsidou, M., Reichman-Fried, M., Stebler, J., Koprunner, M., Dorries, J., Meyer, D., Esguerra, C. V., Leung, T. and Raz, E. 2002. Guidance of primordial germ cell migration by the chemokine SDF-1. Cell 111:647-659.
5. Gallagher, E. J., Lodge, P., Ansell, R. and McWhir, J. 2003. Isolation of murine embryonic stem and embryonic germ cells by selective ablation. Transgenic Res. 12:451-460.
6. Kuwana, T. and Rogulska, T. 1999. Migratory mechanisms of chick primordial germ cells toward gonadal anlage. Cell Mol. Biol. 45:725-736.
7. Lee, C. K., Weaks, R. L., Johnson, G. A., Bazer, F. W. and Piedrahita, J. A. 2000. Effects of protease inhibitors and antioxidants on *In vitro* survival of porcine primordial germ cells. Biol. Reprod. 63:887-897.
8. Lendahl, U., Zimmerman, L. B. and McKay, R. D. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60:585-595.
9. Matsui, Y., Toksoz, D., Nishikawa, S., Nishikawa, S., Williams, D., Zsebo, K. and Hogan, B. L. 1991. Effect of steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. Nature 353:750-752.
10. Pevny, L. and Rao, M. S. 2003. The stem-cell menagerie. Trends Neurosci. 26:351-359.
11. Resnick, J. L., Bixler, L. S., Cheng, L. and Donovan, P. J. 1992. Long-term proliferation of mouse primordial germ cells in culture. Nature 359:550-551.
12. Robinson, H. P. 1973. Sonar measurement of fetal crown-rump length as means of assessing maturity in first trimester of pregnancy. Br. Med. J. 4:28-31.
13. Shamblott, M. J., Axelman, J., Wang, S., Bugg, E. M., Littlefield, J. W., Donovan, P. J., Blumenthal, P. D., Huggins, G. R. and Gearhart, J. D. 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc. Natl. Acad. Sci. USA. 95:13726-13731.
14. Shim, H. and Anderson, G.. B. 1998. *In vitro* survival and proliferation of porcine primordial germ cells. Theriogenology 49:521-528.
15. Shim, H., Gutierrez-Adan, A., Chen, L. R., BonDurant, R. H., Behboodi, E. and Anderson, G. B. 1997. Isolation of pluripotent stem cells from cultured porcine primordial germ cells. Biol. Reprod. 57:1089-1095.
16. Wheeler, M. B. 1994. Development and validation of swine embryonic stem cells: a review. Reprod. Fertil. Dev. 6:563-568.
17. Young, H. E. and Black, A. C. Jr. 2004. Adult stem cells. Anat. Rec. 276:75-102.

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