Research Article

Pluripotency Markers in Stem Cells of Bats

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Abstract

Stem cells, a special kind of undifferentiated cells, are able indefinitely to go through mitotic cell division without losing their properties and produce differentiated cell lines. Due to their potential use in medicine, there is considerable research interest focusing in the study of embryonic stem cells and adult stem cells. Special emphasis has been placed on the study of embryonic germ cells, which characterized by expressing stem cell markers SSEA-1, SSEA-3, SSEA-4, TRA1-60, TRA1-8, alkaline phosphatase, periodic acid-Schiff and others. Stem cell research in bats could be the guide for further embryologic studies in other wild mammal species. We used SSEA-1, Oct-4, Stella, Sox-9 and Nanog stem cells markers (immunohistochemistry and RT-PCR) and observed the mRNA expression in bat embryos of A. jamaicensis and adult tissues of A. jamaicensis and B. plicata bats. An AP+ cell population and SSEA-1+ (primitive intestine) population were identified using markers during stages, 9, 10, 11 and 12 -the beginning of development. Other positive cell populations were identified (stages 11 and 12) using two other markers: PAS and Oct-4. On the other hand, we observed higher levels of mRNA expression of Oct-4 and Nanog in the first stages but not in more advances stages of development, and the expression of Stella and Sox-9 showed levels of expression alternating times along of stages. This is the first report of pluripotency markers of stem cells in A. jamaicensis, Oct-4 presence in functional gametes of B. plicata adults and the presence of Stella, Nanog and Sox-9 in bat embryonic and adult tissues.

Keywords: germinal cells, alkaline phosphatase, periodic acid-schiff, Stage-Specific Embryonic Antigen-1, Octamer-4, bats
Abbreviations: GCs: Germinial Cells; AP: Alkaline Phosphatase; PAS: Periodic Acid-Schiff; SSEA-1: Stage-Specific Embryonic Antigen-1; Oct-4: Octamer-4; Dppa3: Developmental Pluripotency-Associated Protein 3; Sox-9: SRY-box containing gene 9; Nanog: Homeobox protein-nanog

Introduction

In mammals, germinal cells (GCs) are segregated in an asymmetric form during each cell division of the embryo, and in each case a different cell line is generated. There is evidence that GC inherently have all of the machinery needed to determine their germinal-cell status. Their expression is influenced by extracellular factors and cell-cell interactions, which induce these precursors of cell to divide [1-5].

GCs have been studied and identified during the first embryogenesis stages in mice [1,2,4], rats [6] and chickens [7]. These cells migrate through the hindgut to the gonadal ridges, where they differentiate in spermatogonia in the testes and oocytes in the ovaries, eventually becoming gametes [8]. Given that GCs show significant alkaline phosphatase (AP) activity it has been possible to use markers for this enzyme to identify GCs during early stages of embryogenesis (chicken and mice) near the yolk sac in the extraembryonic region [9,10]. Other markers used to characterize GCs in chicken embryos are Periodic Acid-Schiff (PAS) [7,11,12] and the stage-specific embryonic antigen SSEA-1 which has also been used in turkey embryos [13].

SSEA-1 is considered one of the best antibodies used to identify GCs in embryos of mice [14-16], chickens [17,18] and pigs [19]. It also has been used as a marker for other germinal cell lines during embryonic development of the rat [20]. SSEA-1 positive (SSEA-1+) cells have been identified in the dorsal spine of chicken embryos [21], in mouse and chicken embryonic stem cells, and in embryonic carcinoma cells of mice and chickens [22-24]. For these reasons, SSEA-1 has been considered as a pluripotency marker [25]. Oct-4 (a transcription factor also known as Pou5f1), as with SSEA-1, is an excellent pluripotency marker, and these two immunohistochemical markers are the most frequently used in the characterization of germinal cell lines, such as embryonic stem cells. Oct-4 has been found in mouse oocytes, the ectoderm of recently implanted mouse embryos, GCs, and embryonic stem cells. Oct-4 has similar functions to other pluripotency genes like Sox-9, Nanog and Esg-1 [26]. Stella, also known as PGC7 or Dppa3, is another gene that is expressed in GCs and their descendants, including oocytes [27-29]. Given that Oct-4 and Stella are specifically expressed in germinal cell lines, they have been considered as key molecules in the differentiation and development of germinal cells [30,31]. Oct-4 is a transcription factor necessary for the survival and migration of GCs during the last stages of embryo development [32]. This is contrary to the Stella gene, which is responsible for the specification of germinal cells when the embryo is in the epiblast phase or during its first stages of development [33].

Nanog is another transcription factor, overall with Sox2, factors critical for the pluripotency and self-renewal of embryonic stem cells. Their down regulations lead to differentiation, accompanied with changes in cell motility [34]. SOX-9 recognizes the sequence CCTTGAG along with other members of the HMG-box class DNA-binding proteins. It acts during chondrocyte differentiation and, with steroidogenic factor 1, regulates transcription of the anti-Müllerian hormone (AMH) gene. SOX-9 also plays a pivotal role in male sexual development; by working with Sf1, SOX-9 can produce AMH in Sertoli cells to inhibit the creation of a female reproductive system [35,36].

Even though germinal cell line studies are numerous, most research to date has been limited to laboratory animals (birds, mice, rats, pigs). On occasion, we work has been done on human cells due to the clinical importance of such research [37].

However, similar research projects on wild animals such as bats (which are the second most diverse mammalian order and represent more than 20% of known mammalian species [38] are practically non-existent, with
exception of recent projects for example, identification of cortical germ cells in adult ovaries from three phyllostomid bats [39], the generation and characterization of bat-induced pluripotent stem cells [40] and the characterization of gametes in two phyllostomid bat species [41]. But not emphasis in pluripotency markers.

The purpose of the current work was to identify pluripotency markers and characterize germinal cells in embryos of the bat Artibeus jamaicensis and in testes and tissues of adult Artibeus jamaicensis and Balantiopteryx plicata using AP, PAS, SSEA-1 immunohistochemical and genetic expression markers like, Oct-4, Stella, Sox-9 and Nanog.

Materials and Methods

Due to the difficulty of breeding and keeping bats in captivity, embryos were taken from pregnant females collected in the wild from the state of Colima and Jalisco, Mexico. In total, 18 A. jamaicensis embryos and 1 male adult as well as 6 B. plicata adult males were available; all of which were preserved in 70 % ethanol soon after capture. All animal maintenance and handling were carried out in accordance with the Guidelines of the Mexican Law of Animal Protection (NOM-062-ZOO-1999), and under the Institutional Committee for the Care and Handling of Laboratory Animals from the Faculty of Chemistry (CICUAL programme), from the National Autonomous University of México. Appropriate permits were obtained from the Instituto Nacional de Ecología, Dirección General de Vida Silvestre FAUT.0103 to C. Sánchez-Hernández.

Embryo size for A. jamaicensis varied from stages 9 to 20 (2 × 2 to 30 × 15 mm) and were homologized with the defined stages for Carollia perspicillata [42] based mainly on embryo size and organ histological development. One embryo corresponded to stage 9 (2 × 2 mm), stage 10 (3 × 4 mm), stage 11 (9 × 7 mm), another to stage 12 (10 × 12 mm) and 14 embryos to stages 17 to 20 (14 × 12 to 30 × 15 mm).

Artibeus jamaicensis embryo histology

In order to describe histologically and determine the location of developing organs in A. jamaicensis embryos, the two specimens of stages 11 and 12 (9 × 7 and 10 × 12 mm) were selected and used as a point of comparison with older and larger embryos. Embryos were fixed using buffered formalin for 60 min or Bouin’s for 120 min. They were dehydrated in an alcohol gradual series of 80, 90, 96, and 100 %, with a 40-min period at each step. Xylene was used as a clearing agent for another 40 min, and embryos were embedded in Paraplast overnight. The next day they were included in histology cassettes, and 5-µm sagittal and horizontal histological sections were prepared using a Leica RM2125R microtome. The sections were stained with haematoxylin and eosin (H&E). For further morphological analyses, sections were microphotographed using a Leica DMLB microscope in conjunction with the Pixe Link software.

SSEA-1, Oct-4 and Stella immunohistochemistry

All slides of the 18 A. jamaicensis embryos and 6 B. plicata adults were incubated with primary antibodies for SSEA-1, Oct-4 or Stella (Santa Cruz Biotechnology), in a 1:200 dilution at 4°C overnight. The following day the samples were incubated with anti-goat, anti-mice or anti-rabbit secondary antibodies, respectively at 4°C overnight; all secondary antibodies were horseradish peroxidase (HRP) conjugates. The next day the signal was developed using 3,3'-diaminobenzidine (DAB, Vector).

Alkaline phosphatase and periodic acid-Schiff treatment

For AP treatment, the 18 embryo slides were immersed in acetone for 5 min at 4°C, after which they were incubated in a 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium (NBT-BCIP) solution for 1 hour at 37°C. The samples were fixed in sucrose-buffered formalin for 5 min, washed in running water and counterstained with eosin for 5 min. After washing the samples, a second time in running water, they were embedded in resin. As a
positive control to corroborate the presence of GCs, three mouse embryos with a 12-day gestation period were used for the AP reaction, and three chicken embryos in primitive streak stage were used for the PAS reaction. For PAS reactions the paraffin was removed from the sections and they were rehydrated. They then were submerged for 5 min in periodic acid, washed for 3 min in running water, and placed in Schiff reagent for 15 min. Samples were washed, dehydrated and embedded for further observation and microscopic analysis.

**RNA preparation, DNA synthesis and reverse transcription-polymerase chain reaction**

RNA was extracted from adult tissue of *Artibeus* and *Balantiopteryx*, and embryo tissue of *Artibeus* samples previously stored in AM7020 RNAlater® (Applied Biosystems) via the Tripure® method (11667165001, Roche Applied Science). Prepared RNA sample quality was analyzed with NanoDrop® and Bioanalyzer® (Agilent Technologies). Total RNA was transcribed to complementary DNA (cDNA) according to the SuperScript First-Strand Synthesis System for RT-PCR protocol (Catalog No. 11904-018, Invitrogen). Briefly, 1 μg of total RNA, 1 μl dNTPs mix, 1 μl Oligo(dT)12-18 (0.5 μg/μl) were brought to a final volume of 10 μl with DEPC-treated water. The mixture was pre-incubated for 5 min at 65°C, and chilled on ice for 5 min. We added 2 μl 10x RT buffer, 4 μl 25 mM MgCl₂, 2 μl 0.1 M DTT, 1 μl RNase inhibitor, and incubated at 42°C for 2 min. Then we added 1 μl SuperScript® II RT to each tube, mixed and incubated the mixture at 42°C for 50 min, completing the reaction at 70°C for 15 min. We collected reaction material after brief centrifugation at 4°C. Finally, we added 1 μl of RNase H to each tube and incubated for 20 min at 37°C before proceeding to blank DNA amplification, five microliters of cDNA were amplified with Oct-4, Stella, Sox9, Nanog and β-actin oligonucleotides in a Palm-Cycler® thermal cycler (Corbett Life Science CG1-96) in a total volume of 50 μl containing 5 μl 10X PCR buffer, 1.5 μl 50 mM MgCl₂, 1 μl 10 mM dNTP mix, 1 μl sense oligonucleotide at 10 mM concentration, 1 μl antisense oligonucleotide at 10 mM concentration, 0.3 μl Taq DNA polymerase (5 units/μl), 5 μl complementary DNA (from the earlier reaction) and lastly 35.2 μl DEPC-treated water.

The Oct-4 oligonucleotide were the following: sense (5'-3') GAG GAG TCC CAG GAC ATG AA and antisense (5'-3') GTG GTC TGG CTG AAC ACC TT, Stella 5' TAG CCT GGG GGT AGA CTC GGT 3' and 5' AAC GAG AGA AGG GAG GGC TTC 3'. Sox-9 oligonucleotide were the following: sense (5'-3') AAT CTC CTG GAC CCC TTC AT and antisense (5'-3') GTC CTC CTC GCT CTC CTT CT, Nanog oligonucleotide were the following: sense (5'-3') TTC CCAAAG CTT TTG and antisense (5'-3') GGC CAG TTG TTT TTC TGC C.

There were amplification cycles with the following characteristics: 94°C for 2 min, (94°C for 30 s, 57°C for 1 min, 68°C for 1 min), 68°C for 7 min, and the pairs of bases results were 151, 30, 264 and 458 respectively. The following oligonucleotides were utilized for β-actin: sense CCAAGGCCAACCGAGAAGACTG and antisense AGGTACATGTTGGTCCGCCAGAC. There were 35 amplification cycles for β-actin with the following characteristics: 94°C for 5 min, (94°C for 30 sec, 66°C for 30 sec, 68°C for 1 min), 68°C for 7 min. Is 587 pb the result fragment by these gene. The amplified samples were run through electrophoresis in 2 % agarose gel; the PCR products were stained with 2.5 μl GelRed® (41002, Biotium). Gel photographs were taken with an ultraviolet light transilluminator coupled to a computer running Dolphin-1D® software (Wealtec). Densitometry was performed according to the parameters established by the software and t student test was applicable.

**Results**

**Histology and stage assignation of *Artibeus jamaicensis* embryos**

Stage 11 and 12 embryos (9 × 7 and 10 × 12 mm) of *A. jamaicensis* were stained with haematoxylin and eosin (H&E). This allowed for ready identification of the fore (FB), middle (MB) and hind brain (HB), pharyngeal arches (PA), ear (Ea), heart (H), lung (L), bladder (B), liver (Li), stomach (S), intestines (I), vertebrate column (VC), forelimb
(FL) buds and hind limbs (HL) (Figures 1A-1C). These organs show a degree of development similar to C. perspicillata embryos of stage 11 and 12.

**Figure 1:** *Artibeus jamaicensis* embryo histology. (A) Longitudinal section of one embryo length 9 × 7 mm (stage 11) stained with H&E; this allowed to easily identify the fore (FB), middle (MB) and hind brain (HB), pharyngeal arches (PA), ear (Ea), heart (H), bud lung (BL), liver (Li), intestines (I), vertebral column with 24 to 25 somite's (SC), hind limbs (hl). (B) Longitudinal section of one embryo length 12 × 10 mm (stage 12) shows the eye (Ey), stomach (S), lower and upper jawbone (J), umbilical vein (UV), bladder (B), lung (L), and sacrum (Sa). (C) Increased magnification (20x) of the same longitudinal section of embryo (12 × 10 mm, stage 12), making visible the primitive gut zone (GZ).

**SSEA-1 antibody immunohistochemistry**

For the same sections (stage 11 and 12 embryos), SSEA-1 immunolabel was found in the germinal centers of heart (H), liver (Li), and forelimb buds (fb), hind limbs (hl) and chondrogenesis sites (C) localized in vertebral column (SC). (Figure 2A). At greater resolution (40x, Figure 2B), the germinal cells are identifiable in the primitive intestine zone (PI), along the germinal line, where germinal cells have been observed migrating towards the gonadal ridges in mice. Thus, the presence of germinal cells at this location has been corroborated for bats. Figure 2C shows a negative control for the primitive intestine zone for bat embryos.

**Figure 2:** *Artibeus jamaicensis* embryo SSEA-1 immunohistochemistry. (A) Longitudinal section of *A. jamaicensis* embryo of 9 × 7 mm (stage 11) shows immunolabel in the germinal centers of heart (H), liver (Li), forelimb buds (fb), hind limbs (hl) and chondrogenesis sites (C) localized in vertebral column (SC). Bar 20 µm. (B) At greater resolution (40x), embryo of length 9 × 7 mm (stage 11) depicts germinal cells (GC) located in the primitive intestine (PI) zone, along the germinal line, where they have been observed migrating towards the gonadal ridges in bats. (C) SSEA-1 negative control in longitudinal section for the primitive intestine zone (PI) for bat embryo (stage 12).
Oct-4 antibody immunohistochemistry

The adult sections of testes of *B. plicata* (Figure 3A) show the seminiferous tubules surrounded by interstitial cells (IC). In the interior of the tubules, Sertoli (SeC) and spermatogenic cells (SC) can be identified. Some cells in the series are Oct-4 positive (Figure 3B). In contrast, the Oct-4 label in *A. jamaicensis* embryos was observed in chondrogenesis sites in the vertebral column (SC), as well as liver (Li) and lung (L) germinal centers (Figure 3C). Figure 3D depicts an Oct-4 negative control for an *A. jamaicensis* embryo.

![Figure 3: Localization of embryo Oct-4 immunohistochemistry.](image)

Stella antibody immunohistochemistry

In *A. jamaicensis* embryo sections (stages 9 and 10), no tissue was found to be positive for Stella (Figure 4A). In contrast, Stella-positive cells, which probably belong to a germinal line given their location, were present in the primitive intestine of mouse embryo positive control tissue (Figure 4B). Figure 4C shows a negative control that consists of a 12-day gestation of mouse embryo.
Figure 4: Localization in embryo Stella immunohistochemistry. (A) *A. jamaicensis* bat embryo in which no tissue is positive for Stella. (B) In contrast, mouse embryo 12-day gestation (positive control tissue), exhibits Stella-positive cells. (C) Negative control (40x) of 12-days of gestation mouse embryo.

**Alkaline phosphatase treatment**

The adult sections of testes of *B. plicata* were treated so as to make AP presence evident. Germinal cells can be clearly distinguished in the seminiferous tubules (ST), due to their ovoid shape and clear cytoplasm. Furthermore, their size is significantly larger than that of other cell types (Figure 5A). As a positive control, sections of a 12-day gestation mouse embryo were used. In the control, AP activity can be observed in the dorsal mesentery (Figure 5B). The AP label is evident in chondrogenesis (C) sites along the vertebral column in *A. jamaicensis* embryos (Figures 5C & 5D).

Figure 5: Alkaline phosphatase treatment (AP). (A) Adult testes sections of *B. plicata* were treated in order to make evident AP presence. Germinal cells can be distinguished clearly in the seminiferous tubules (ST) due to their ovoid shape and clear cytoplasm. (B) As a positive control, sections of a 12-days gestation mouse embryo were used. AP activity is seen in the dorsal mesentery (DM). (C, D) AP label is evident at chondrogenesis sites (C) along the vertebral column in *A. jamaicensis* embryos.

**Periodic acid-Schiff treatment**

The periodic acid-Schiff treatment showed positive cells in the same chondrogenesis sites as the AP reaction in vertebral column and zones with hyaline cartilage (hC) (Figures 6A & 6B). In addition, other PAS+ cells were found in lung tissue (L) belonging to larger-size embryos (30 × 15 mm; Figure 6C).
Figure 6: Periodic acid-Schiff treatment (PAS). (A, B) A. jamaicensis embryos (stages 17-20) showed positive cells in the same chondrogenesis sites as did the AP reaction in vertebral column (arrow heads) and zones with hyaline cartilage (C). (C) PAS+ cells were found in lung tissue (L) belonging to larger-sized embryos (30 × 15 mm, stage 20).

mRNA expression

The analysis of mRNA expression levels by semi-quantitative RT-PCR of Oct-4, Nanog, Sox9, Stella and β-actin in bats embryos of A. jamaicensis and skin, testicle, liver and lung adult tissue of the same species, reveals significant differences in embryos tissues for Oct4 (p ≤ 0.001), and Nanog (p ≤ 0.04) with relation to the adult tissue (Figures 7A & 7B), as well as lower levels of mRNA expression for Sox-9 (p ≤ 0.54) and Stella (p ≤ 0.4) with no significant differences for these genes. Significantly, the gene expression pattern Stella has an on and off in stages analyzed. It is on in stages 12 and 14 and off at 9 and 10. On the other hand, it is observed that in the case of control tissue expression of genes Oct4, Nanog and Stella remains constant at 0.5 optical density units (Figure 8).

Figure 7: Reverse transcription-polymerase chain reaction in adult tissue of Artibeus and Balantiopteryx. (A) Germ cell specific gene primers were used. (B) Densitometry analysis of expression of Oct-4, Nanog, Stella and SOX-9 (relative to a beta-actin internal control) in testicle, skin, liver and lung adult tissue. Data are presented as mean ± S.D. of three independent experiments.
Discussion

Morphological, physiological and molecular comparisons among species have provided additional insight with respect to embryo development in mammals. A particular stage of development can be assigned to *A. jamaicensis* embryos by comparing them with *C. perspicillata* embryos (a partially sympatric species of the same family and similar size) given morphological and histological similarities of the two species. However, studies of germinal cell lines, allow for a better understanding of embryonic development in this mammal group.

In our project, SSEA-1+ and Oct-4+ cells belonging to germinal lines were identified in tissue buds of the liver, lung and vertebral column of *A. jamaicensis* embryos. The fact that these are specific markers for pluripotent cells and were AP+ and PAS+ for the same regions, may indicate that these cells belong to the embryonic stem-cell (ESC) line. However, in order to be confirm this statement, other specific genes were used Oct-4, Nanog, Sox-9 and Stella to differentiate them from other positive cells as well as other adult bats tissues (testes, skin, liver and lung).

In order to be sure of the pluripotency of Oct-4+ cells, testes from adult bats were used as a positive control. In this case, the label indicated that cells belonged to a GCs line due to the fact that this tissue was Oct-4+ (immunohistochemistry) for a few cells inside the seminiferous tubules. This finding and the gene expression we found in all embryos stages further indicates that these cells are potentially pluripotent, information that agrees with Okamura et al. [43]. Okamura et al. [43] indicated that stem cells in spermatogonia express Oct-4, although the exact function of Oct-4 in spermatogenic germinal cells is still unknown. Besides the expression of Nanog, Stella and Sox-9 confirms the pluripotency of these cells.
Mise et al. [44] and collaborators proposed that there is a difference in target genes expression located downstream of the Oct-4 transcription factor. In these target genes are activated by this transcription factor, the cells are related to pluripotency, if they are negatively regulated by Oct-4, a differentiation to a specific cell line takes place.

In the case for Stella, its expression was verified in GCs belonging to mouse embryos (12-day gestation period; these cells were located in the intermediate mesoderm) due to their analogy with AP+ embryos, where GCs are clearly visible and identifiable because of their morphology. In addition, we found the mRNA expression of Stella in stages 12, 14 as well as in the testes and somatic tissues of the adult bat. Stella formerly considered as a marker of lineage in chick and mouse GCs (8,16), but is now established as a marker of pluripotency for any germ lineage [45]. The increased expression of Nanog gene and Oct-4 in the embryonic stages 9 to 12, consistent with the evidence that Nanog is a transcription factor than supports the embryonic stem (ES) cell self-renewal, as Oct-4 with a similar expression (stages 9-12), maintaining both proliferation and are essential for pluripotency [25].

Ours is the first report of immunohistochemistry of pluripotency markers in embryonic tissues of A. jamaicensis and the Oct-4 presence in functional gametes of B. plicata adults. Also, we were able to demonstrate by RT-PCR, the presence of Oct-4, Stella, Nanog and Sox-9 in bat embryonic and adult somatic tissues is reported by first time.

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