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Research Article

Characterization of Cell Lineages Derived From Tactile Hair Follicles of Dog Fetuses

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Abstract

Skin problems and wounds seriously affect human health, as well as dogs and other animals. New applications aim at finding appropriate progenitor cells to establish cell therapies. In order to test their potential value for such purposes, we isolated, cultivated and characterized cells from follicles of the tactile hairs in dog embryos and fetuses. We investigated a total of 16 samples of dog fetuses of 30 to 40 days of gestation, obtained from castration procedures undertaken by the public health programs of the State of São Paulo - Brazil. We divided the samples in three groups representing the transition from embryo to fetus, through cell cycle analysis, cell culture, as well as flow cytometry, immunocytochemistry, immunohistochemistry and qPCR-RT analysis for 20 relevant markers. We successfully maintained cells for 10 to 30 days in cell culture. Data showed the characteristic expression of markers for pluripotent and/or mesenchymal stem cells, i.e. OCT 3/4, Nanog, CD-105, CD-90, SSEA-4, STRO-1, VEGFR-1, and Ki-67 as well as S-100, which is specific for the identification of developing hair cells and multipotent dermal cells. Samples from the older fetuses (40 days) showed a comprehensive development of hair cells, but also contained an appropriate differentiation potential that is useful for therapeutic applications. In conclusion, data indicated that cells derived from hair follicles of dog fetuses, especially around day 40, resulted in multipotent, mesenchymal stem cells that may be of value for stem cell therapies in order to improve regeneration of the skin.

INTRODUCTION

The complex structure of the skin and its physicochemical properties provide an efficient barrier against exogenous factors [1]. Traumatic skin losses through injuries, acute or chronic wounds as well as skin problems caused by allergic and autoimmune diseases seriously diminish the health status in humans [2,3] but also affect dogs and other animals [4]. In addition to classical, tissue-engineered skin substitute therapies [5,6] recent therapies aim at finding pools of progenitor cells for stem cell applications [7-11]. Skin tissues and their derivatives are well established sources for cell based therapy [12]. Various populations of stem cells have been derived from dermal and epidermal skin tissues, including pluripotent and committed cells, and are not only regarded as adequate for skin cell therapies, but also as a model for epithelial-mesenchymal transition compared to other organs [13-17]. In particular, outer root sheat are important in that regard, because they contain stem cells throughout their life cycle [12,16,18,19]. Human hair bulbs have been successfully used for the isolation and establishment of stem cell lineages derived from both the whole bulb or from the partial bulb in hair samples from the posterior neck [16,19,20,21]. For instance, such cell lineages have been applied to prevent tumorigenesis during wound repair or to enhance wound healing [16,21-23]. Results in animal models mainly focus on rodents and have likewise established the cultivation, characterization and application of stem cell lineages derived from the follicles of tactile hairs or vibrissae as well as dermal papillae cells [21,24,25]. A study has been done on the dog [26]. Further studies would be helpful, because dogs suffer from similar disorders as humans [27]. However, investigation should include fetal stages that are regarded to have a greater proliferation potential than cells derived from adults. In addition, canine fetuses and embryos are easily available in castration campaigns and clinics, causing less bioethical considerations than tissue samples from living adult individuals. Therefore, we proposed to isolate and to culture cells derived from tactile hair follicles of dog fetuses and to characterize their potential stem cell nature by means of cell cycle, cell culture, as well as flow cytometry, immunocytochemistry, immunohistochemistry and qPCR-RT analysis for relevant markers as previously used in similar contents [26,28-35].

MATERIALS AND METHODS

Sampling

Material was obtained from castration programs of Public Health Clinics in Sao Paulo. The experiment was approved by the School of Veterinary Medicine of the University of Sao Paulo, under the protocol number 3041/2013. The Committee follows the Basel Declaration.

To establish cell culture, we used tissue samples of dog fetuses of three gestational periods, respectively, 30 days (group I, N = 6), 35 days (group II, N = 6) and 40 days (group III, N = 4). The gestational age of fetuses was estimated according to the crow-rump length of the fetuses according to Evans & Sack (1973) [36]. Upon collection, fetuses were maintained in sterilized, refrigerated boxes and processed two hours after. The tissue samples were obtained with sterilized scissors and pincers by making a V-shaped incision in the rostral region and submitted to cell culture procedures.

Analysis of the cell cycle

The tissue samples digested by trypsin were centrifuged for 10 min at 1500 rpm. The residual liquid was discarded and the cells were resuspended in 100 µL of the neutralization solution (0.5 trypsin inhibitor, 0.1 g/l RNase A and 1.2 g/l spermine). The cell pellet was fixed in ice-cold ethanol 70% and maintained overnight at -20°C. For initial analysis, we used PBS containing 1.8µg/ml propidium iodide (Sigma Chemical Co, St.Louis, MO, USA), added it to the cell pellet and incubated the pellet in the dark for 20 min at room temperature. As a result, at least 10,000 events were established, using CellQuest software (Becton Dickinson, San Jose, CA, USA) to perform the analysis. Flow citometry analysis was performed using a BD Biosciences FacsCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The DNA content in the cell cycle phases (sub-G1, G0/G1, S and G2/M) was analyzed by the WinMDI 2.8 software (Becton Dickson, San Jose, CA, USA). Events were measured in the FL2-H fluorescence channel with excitation at ƛ= 488nm.

Immunohistochemistry

Samples were fixed in 4% paraformaldehyde for 48 hours. All samples were submitted to dehydration procedures by series of ethanol with increasing concentration from 70 to 100%; then cleared in xylene and included in Paraplast Plus® resin (cod.125387-89-5, Sigma- Aldrich®, St. Louis, Missouri, USA) and cut into 5µm slices that were adhered to microscopy blades. Immunohistochemistry was performed for S-100 (1:200, Mouse Monoclonal Antibody, Abbiotec™, cod. 92126, San Diego, CA, USA). Cell count was performed using light microscopy in ten fields randomly selected, approximately 200 cells/field, and captured for marker expression rate (%) detection.

RNA extraction

The samples were fragmented in a tissue pulverizer (Mikro-Dismembrator U, B. Braun Biotech International, Melsungen, Hesse, Germany). Total RNA was extracted from approximately 100 mg tissue after homogenization in 1 mL of Tri-Reagent (Life Technologies Rockville, MD, USA) according to the manufacturer's protocol. The extracted RNA was stored at -80°C. Complementary DNA (cDNA) was synthesized from the total RNA of each sample. First-strand cDNA synthesis used 1 µg of total RNA, 1 µL of oligo (dT) primer (0.5 µg/µL), 1 µL of a solution of all four deoxyribonucleoside triphosphates (each at 10 mM), and 10× Superscript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). The Quantitative Real-Time Polymerase Chain Reactions after reverse transcription (qRT-PCR) were conducted by using the Platinum SYBR Green q-PCR supermix UDG kit (Invitrogen). The qPCR was done in a final volume of 25 µL with 3.0 µL of cDNA (20 ng/ µL), 0.5 Platinum Taq Mix, 12.5 µL 2 X SYBR Green Reaction Mix (containing 3 mmol/L MgSO4), and 0.2 µM sense/antisense primers. Negative samples were run for each qPCR-RT assay consisting of no RNA in the reverse transcriptase reaction and no cDNA in the PCR. For qPCR-RT, fifty cycles of amplification were performed at 95°C (15 sec) and 60°C (1 min). All assay plates were run on an Applied Biosystems Step One Plus (Applied Biosystems, Foster City, CA, USA). Data values (Cycle Threshold [Ct] values) were extracted from each assay with the SDS v2.0 software tool (Applied Biosystems). Primers were designed by using the Primer3_cgi v0.2 program. *S-100 [NM_002531] sense, 5" GGT GCC TAT GCT GTT CAC C 3" and anti-sense 5" ACC ATG ACG GTC AGC TTG TT 3";* and housekeeping gene ribosomal protein *S5 (RPS5) mRNA RPS5 5" TCACTGGTGAG/ AACCCCCT 3"/CCTGATTCACACGGCGTAG*.

Obtaining cells and primary culture

Samples of all vibrissae were obtained by a V-shaped incision with sterilized scissors in the snout region, focusing on the bulb and the outer root sheath. The samples were transferred to a sterilized Petri disk of 75 cm^2, washed with phosphate buffer solution (PBS) (Sigma Aldrich® 73173-1L, Sigma- Aldrich®, St. Louis, Missouri, USA,) with 2mL of penicillin–streptomycin (Sigma Aldrich®), and then collagenases solution was added for enzymatic separation of cells. After that, the cells were transferred to another sterilized Petri disk, filled with 5mL of Dulbecco Modified Essential Medium (DMEM) High Glucose culture medium supplemented with 10% of FBS (Fetal Bovine Serum) (Sigma Aldrich®) and 1% of penicillin and streptomycin. The samples were debrided with 2 sterilized scalpels and were dissociated through gentle trituration in pestle (Bel-Art™ cod. F199230001, Fisherscientific by Thermo Fisher Scientific, Waltham, Massachusetts, USA) in 3ml of Dulbecco Modified Essential Medium (DMEM) High Glucose culture medium supplemented with 10% of FBS (Fetal Bovine Serum) (Sigma-Aldrich®, St. Louis, Missouri, USA). The resulting material was distributed in two 50mL sterilized tubes, with (a) 3mL of trypsin 0.25% (Sigma- Aldrich®, St. Louis, Missouri, USA) and (b) 2mL of collagenase type IA-S (Sigma- Aldrich®, St. Louis, Missouri, USA), respectively. The samples were submitted to digestion during 20 min and were homogenized every 10 min. For cell culture, the samples were divided onto 2 cm^2 sterilized Petri disks containing 2mL of DMEM High Glucose with 10% FBS and 1% of penicillin and streptomycin, and incubated at 37ºC and 5% of CO2. Finally, we added 1mL of DMEM High Glucose complete medium in order to enhance cell growth.

Secondary cell culture and expansion

Freezing assay was done with 10% DMSO medium plus 90% Fetal Bovine Serum (FBS). After the freezing period, the thawed cells were placed in flasks with DMEM High Glucose supplemented with 10% FBS, 1% penicillin and streptomycin, and kept in an incubator at 37ºC and 5% CO_2. As the cells grew, 1mL of trypsin was added and the content of the flasks was transferred to a 15mL tube with 2mL DMEM High Glucose and centrifuged for 5 min at 1000 rpm. Part of the resulting pellet was suspended in DMEM High Glucose with 10% FBS and 1% penicillin/ streptomycin, and placed in new culture flasks with DMEM High Glucose with 10% FBS and 1% penicillin /

streptomycin antibiotics. The remaining portion was suspended in DMEM High Glucose with defined KSFM (Keratinocyte Serum Free Medium, Life Technologies™, Gibco®, catalog number 10785-012, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 1% penicillin/ streptomycin and put in a new culture flask with KSFM and incubated at 37ºC with 5% CO_2.

Immunophenotyping by Fluorescence

Cells, after expansion in the second passage with DMEM High Glucose medium, were fixed in 4% paraformaldehyde. Cells were washed with PBS and Tween solution 3 times for 5 min each time. Next, we added 10µL of 1% Triton for permeation of the cell membranes. We added these antibodies (1:100): STRO-1 (Abcam®.ab102969, Cambridge, UK), Nanog (Abcam®.ab21624 Cambridge, UK), and CD-117- FITC conjugate mice monoclonal antibody (Sigma Aldrich® SAB4700711). Finally, 1µL of Alexafluor® 568 goat anti-rabbit IgG (H+L) (Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added to the samples, which were kept away from light at room temperature for 45min. Samples were washed with PBS and distilled water. The samples were fixed with DAB (Diaminobenzidine, Sigma Aldrich®, St. Louis, Missouri, USA) and placed on microscopy blades.

Flow cytometry

The following antibodies with proven affinity to hair follicle were used for analysis and added to the cell cultures established in DMEM High Glucose. STRO-1mouse monoclonal (Santa Cruz®, Biotechonogy, Inc. sc4773, Dallas, Texas, USA) and SSEA-4 mouse monoclonal (Abcam®.ab16287, Cambridge, UK), were used to mark mesenchymal cells isolated from gengival tissues [37] ; CD-117 rabbit polyclonal (Abcam®.ab5616, Cambridge, UK), used to mark interfolicular epidermis stem cells [38]; OCT3/4 mouse monoclonal IgG (Santa Cruz®.sc-5279, Dallas, Texas, USA) and Nanog rabbit polyclonal (Abcam®.ab21624, Cambridge, UK), both used to identify mesenchymal stem cells in the dermis [39], CD-90 mouse monoclonal (Abcam®.ab23894, Cambridge, UK), which marked dermal sheaths of hair follicles [40]; CD-34 rabbit monoclonal (Abcam® .ab81289, Cambridge, UK), to identify hair follicle bulge stem cells [41]; CD-105 mouse monoclonal (Abcam®.ab11414) , observed in human hair follicle mesenchymal stem cells [42] ; MCP-1 mouse IgG (Santa Cruz®. sc32771, Dallas, Texas, USA), related to inflammatory processes of regulation of leukocytes in skin tissues [43]; HSP-47 mouse monoclonal (Abcam®.ab54874) and CD-1A mouse monoclonal (Santa Cruz sc5265), expressed in fibroblasts of the dermis [44]; VEGFR-1 rabbit monoclonal (Abcam®.ab32152), demonstrated in human outer root sheath cells [31] ; tumor necrosis factor (TNF) Death receptor (DR)-4 mouse anti TNF (Abcam®.ab9809), expressed in non melanoma skin tumor cells [45]; IL-1β receptor Rabbit polyclonal (Abcam®.ab2105), related to a growth inhibitor of the hair follicle [46]; caspase-3 active mouse IgG (Santa Cruz®.sc7272), which induces apoptosis of the hair follicle with the TNF-ą [47]; Ki-67 Rabbit polyclonal (Abcam®.ab15580), expressed in the outer layer of hair follicles [48]; and CD-45 RO monoclonal mouse Anti-CD45-FITC (Sigma® F4149, St. Louis, Missouri, USA), present in the hematopoietic cells of the dermal papilla [49]. To ensure the permeation of the antibodies STRO-1, OCT3/4, HSP-47, caspase-3 active and Ki-67, Triton-x 100 was added to the cell cultures. After this preparation, the cell culture for each studied group was distributed in tubes and each tube supplemented by 2µL of one of the antibodies. The secondary antibody Alexa Fluor® 546 goat anti mouse IgG (H+L) (Life Technologies™, Thermo Fisher Scientific, Waltham, Mass., USA) was added for better cell control. The samples were analyzed using FacsCalibur flow cytometer (Becton Dickson, San Jose, CA, USA). Cell Quest and Win Midi 2.9 software were used to create histograms.

Statistical analysis

Statistical analyses for qRT-PCR, for the S-100 gene and flow cytometry were conducted by variable analysis (ANOVA), followed by the TUKEY- KRAMER test. P values lower than 0.05 were regarded as significantly different.

RESULTS AND DISCUSSION

Cell cycle phases

The cell cycle analysis showed that G0/G1 and S phases were similar among the groups, but group III had a significantly higher percentage of cells in the G2/M phase. Group I had a slightly higher amount of fragmented DNA or cells at the stage of apoptosis than the others (Figure 1).

Immunohistochemistry

In group I, the S-100 marker showed weak staining indicating low expression of the marker (Figure 2A). Group II showed an increase in staining intensity when compared to Group I, in particular in the outer root sheath of the hair follicle, indicating a slight development in cell maturation (Figure 2B). Group III had the most pronounced staining intensity, distributed along the whole hair follicle and in the outer root sheath, indicating more mature cells in comparison to Group I and Group II (Figure 2C).

qPCR-RT for the S-100 gene

After analyzing the expression of S100 gene in all three groups, we observed that both group II and III had a significantly higher expression of the gene compared to group I, with Group III demonstrating the highest values, indicating that the hair follicle cells were in a phase of maturation (Figure 3).

Cell culture

The DMEM High Glucose medium successfully maintained cell growth, proliferation and development of our samples. The cells grew around a hair shaft on the culture plate (Figure 4A-B). During the cell expansion phase, after the thawing procedure, cell growth occurred in a similar growth pattern as observed in the primary cultures, despite a slower development (Figure 4C). A portion of the cells were cultured in KSFM medium and cell growth occurred at a faster rate than with DMEM-High medium, and the cells had a fibroblast-like appearance (Figure 4D). In both procedures, cells were cultivated for 10 to 30 days. After enzymatic separation by collagenases, two cell populations, R1, with round shape, and R2, with fibroblast-like appearance, occurred in the flow cytometric analysis. In all groups, R2 was the more numerous populations. However, in group III, the R2 population was significantly more sizable than R1 (Figure 5).

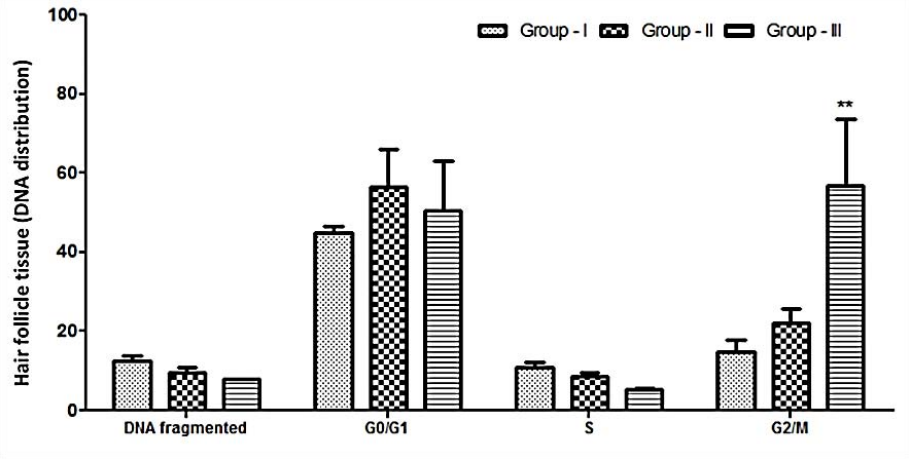

Figure 1 Cell cycles analysis. Cells with fragmented DNA indicating apoptosis; G0/G1 indicating maintaining or growing cells: S = Synthesis or DNA replication; G2/M = cells before or at mitosis. **Significant differences.

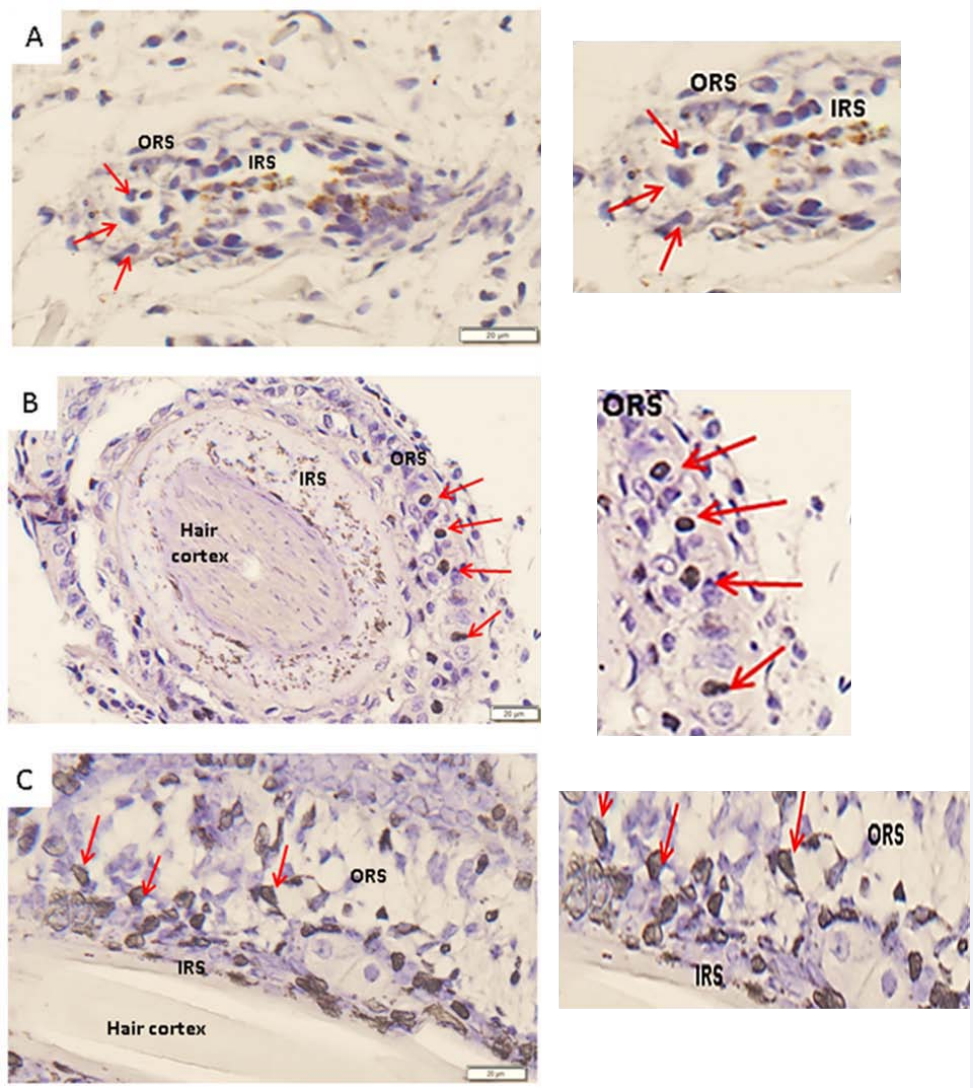

Figure 2 Immunohistochemistry of the hair follicle. A. group I, B. group II and C. group III. IRS: Inner root sheath. ORS: Outer root sheath. Arrows indicate the expression of the S-100 marker.

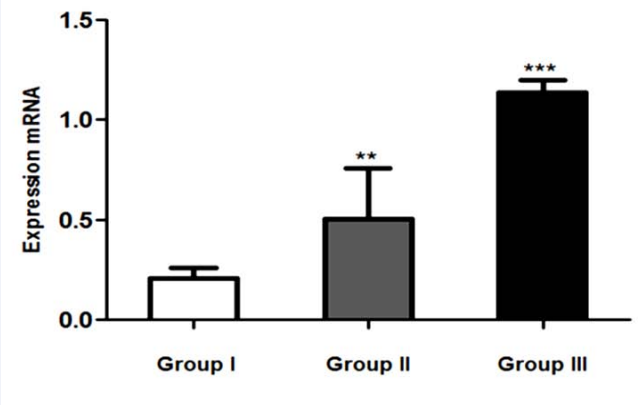

Figure 3 Expression of S-100 gene. Groups II and III had significantly higher expression than group I, with the latter resulting as highest expression. Vertical axes expressed the different statics of the expression mRNa-qrt analysis of the S-100 gene.

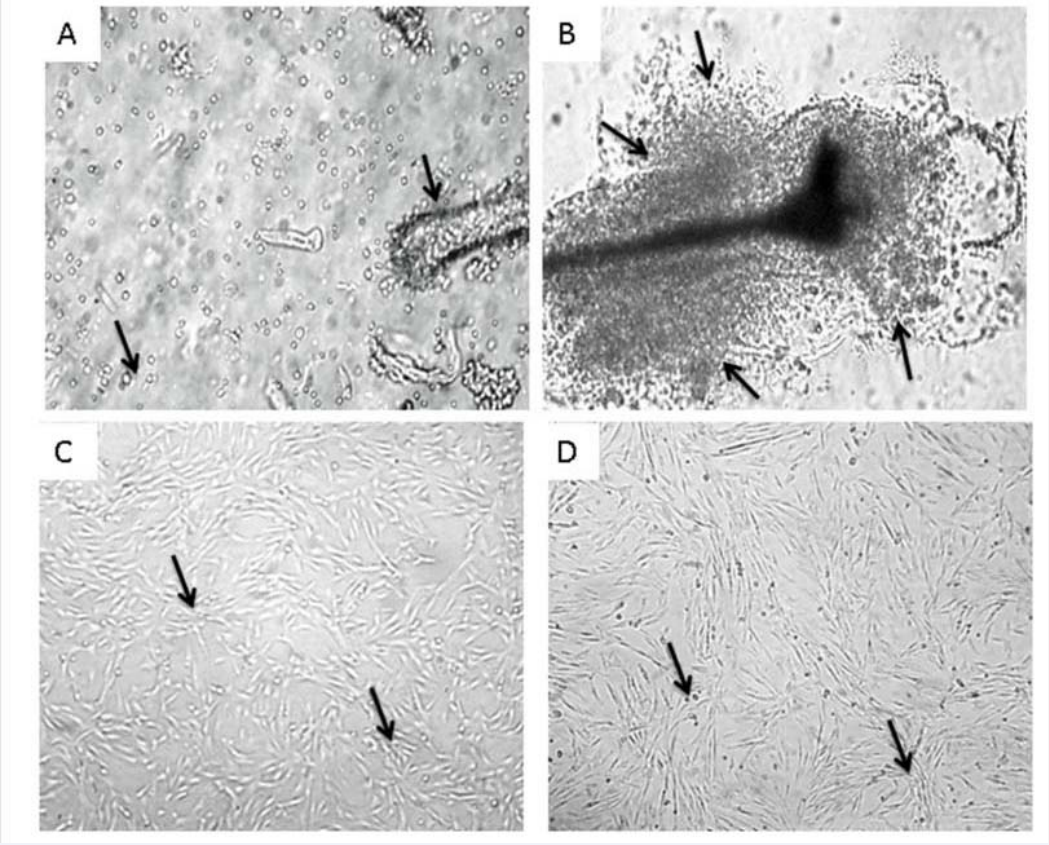

Figure 4 Cell culture of hair follicle cells. A/B. Primary culture. Group I at 1st and 5th day of culture. Magnification 20x. C. Expansion cell culture. Group I at 7th day, 20x. D. Medium supplemented by KSFM. Group I at 10th day. Magnification 20x. Growing cells are marked by arrows.

Immunophenotyping by Fluorescence

All markers, i.e., CD-117, STRO-1, and Nanog showed positive responses in all groups. CD-117 and Nanog had low intensities in group I, whereas STRO-1 was low in group II (Figure 6).

Flow cytometry

Flow cytometry analysis was performed after cell growth, expansion and freezing, in order to measure the expression of stem cell markers. In order to measure stem cell pluripotency, the following markers were used: OCT3/4, Nanog, CD105, CD90, SSEA-4 and STRO-1 in all analyzed groups. All markers had a significantly positive expression response (Figure 7). Nanog and CD-90 expression increased in intensity from group I to III: although Nanog and CD-90 were expressed in all three groups, they achieved their highest expression in group III, indicating a higher pluripotency factor in more mature cells (Figure 7A-C). Likewise, STRO-1 expression presented significantly diverse

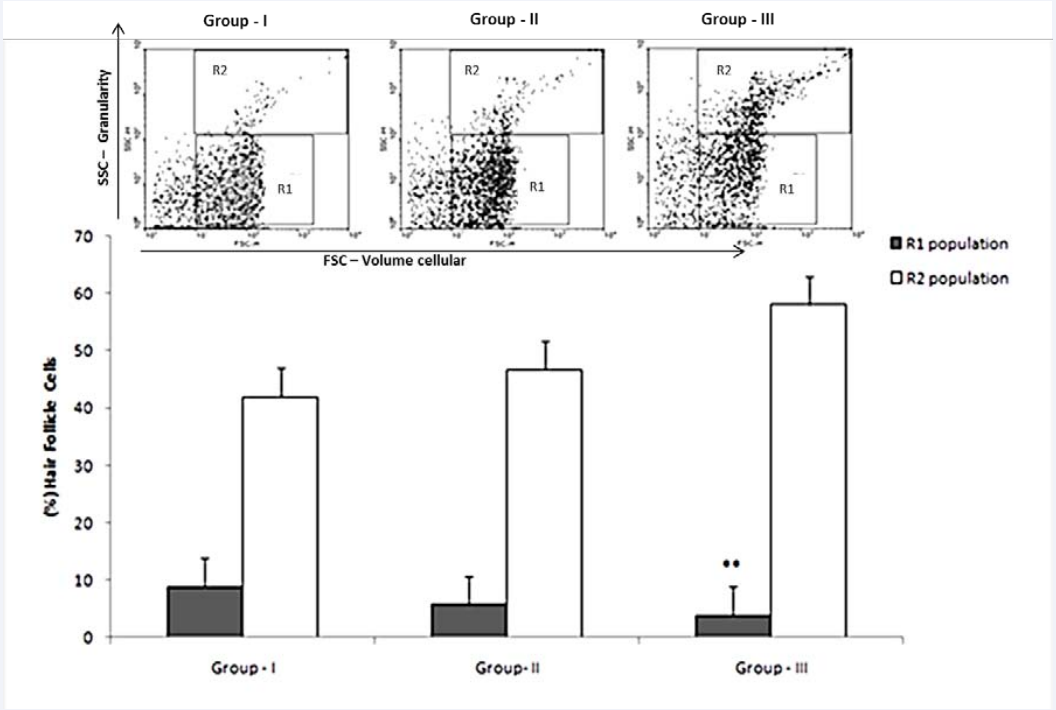

Figure 5 Flow cytometry. Graphic of bars, showing the medium of the populations of hair follicle cells of canine fetuses R1 and R2 in period of 10, 20 and 30 days.** Difference between R1 and R2.

intensities among the groups, but was at its lowest in group II (Figure 7B). CD-105 was significantly higher in group II than in group III (Figure 7B-C). SSEA-4 showed a significant difference in its expression, since it was most intense in group I (Figure 7A) and OCT3/4 was highest in group III (Figure 7 A- B-C). The markers used to verify cell hematopoietic expression were CD-117, CD-34, CD-1A, MCP-1, IL1-β and CD45 RO. CD-117 and MCP-1 had a constant expression in all three groups. CD-117 was significantly lower in group I than in the others (Figure 7D-F). CD-34 had the significantly highest values in group I, but also group II was significantly higher than group III (Figure 7D-E). The IL1-β receptor was highest in group II and III (Figure 7E-F). Finally, CD-1A, CD45 RO and MCP-1 showed little variation in intensity throughout the observed period (Figure 7D-F). Markers used to verify stem cell proliferation and cell death were HSP-47, TNF-DR4, VEGFR-1, Ki-67 and caspase-3. The proliferation and angiogenesis markers HSP-47 and VEGFR-1 were significantly higher in group I than in the groups containing older fetuses; whereas the apoptosis marker TNF-DR4 was significantly higher in group I compared to group III (Figure 7 G-I). Ki-67 had the significantly highest expression in group III. The cell death marker caspase-3 expression did not show a significant difference in the three groups analyzed, indicating its proliferation maintenance capacity (Figure 7 G-I).

Stem cells are located in restrictive environments called 'niches' that play an integral part in stem cell survival and function. According to the "stem cell niche" hypothesis, interactions with the niche cells are crucial to the self-renewal process of stem cells suggesting that they may be regulated by their environment, rather than by intrinsic programming [50]. In our study, cells from tactile hair follicles of dog fetuses were successfully maintained in cell culture and the expression of relevant markers suggests that they represent mesenchymal cell lineages. We used several different markers because the hair follicle cells showed a variety of cell types, such as hematopoietic cells and keratinocytes.

In tissue samples from the snout area all phases of a cell cycle were present with a significant increase of cells before or at the stage of mitosis in samples of day 40 of gestation. In addition, we found increasing intensities of immunostaining for the S-100 protein in both the inner and outer root sheaths and along the bulb. Also, we observed a significantly increasing expression of the S-100 gene. Data indicate that the hair follicle cells were in a phase of maturation, starting from only slight development in group I, which represents the embryo-fetal transition, toward the true fetal phase at the end of the observed period of gestation. The pronounced expression of the S-100 gene and protein, which is characteristic for developing hair cells and multipotent dermal cells, in association with the onset of differentiation or regeneration of hair follicle cells [33,34,51,52] indicates that older fetuses of group III are appropriate choices for further studies involving models of clinical evaluation in dogs as well as tissue engineering, for example.

Maintenance of the cells was successful in the primary and secondary passage, similar to examples with rats, where the hair follicle cells formed layers within 2 to 4 weeks [23,53]. To establish steps of analysis, e.g. flow cytometry, there was no need in our samples to add complements such as DSFK or fibroblast growth factor-2 (FGF-2) as was done in other experiments [54-56]. Hair follicles in general present a variety of cell types. However, in cell culture, only two distinct populations survived, and the fibroblast-like cell type appeared as the most numerous

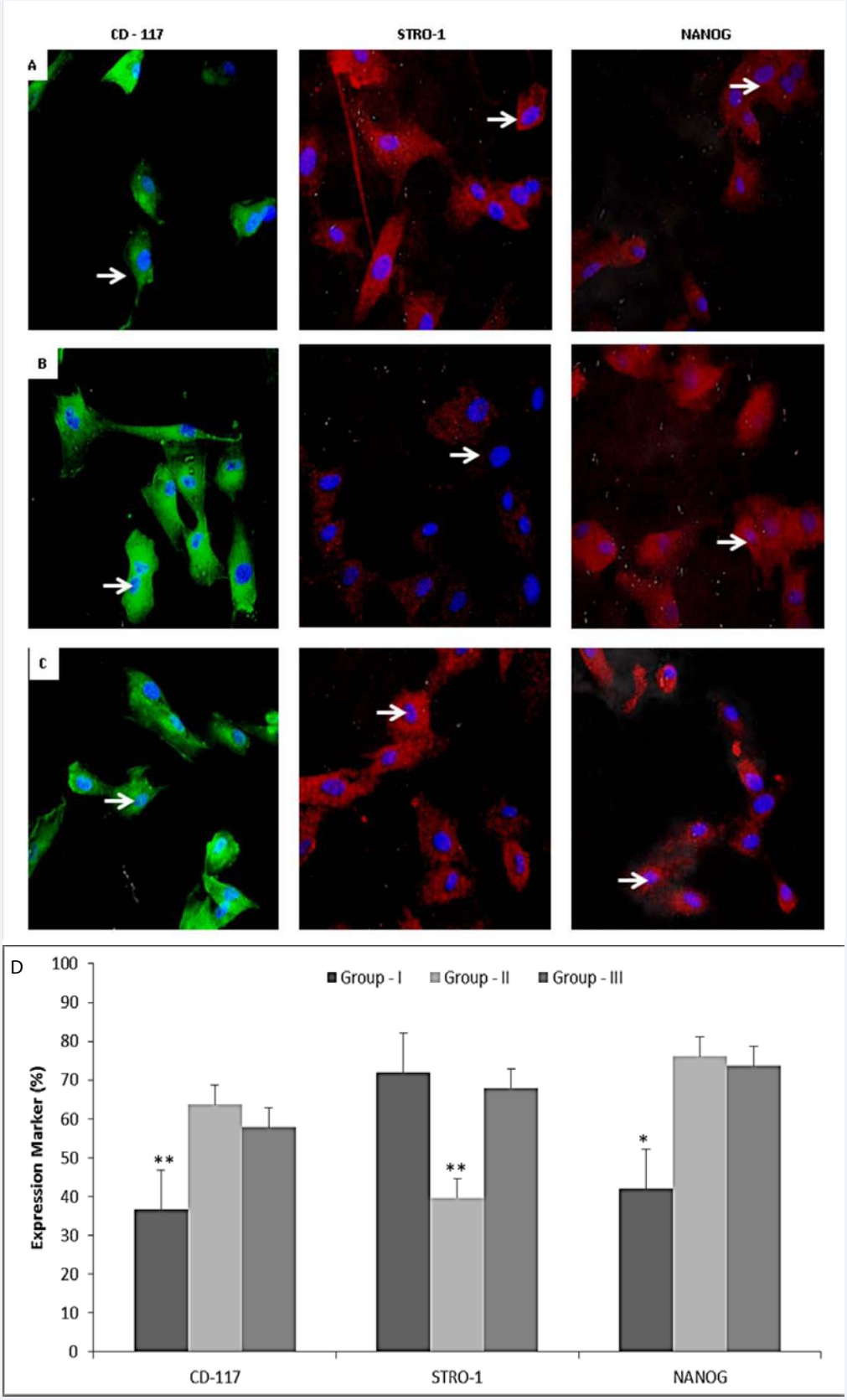

Figure 6 Immunocytochemistry: CD-117, STRO-1, Nanog. A. group I, B. group II and C. group III. CD-117 and Nanog had low staining in group I and STRO-1 was lowest in group II. Nuclei, in blue, marked by arrows. D. Marker medium expression. ANOVA variance statistics test, *p<0.05 and **P<0.01.

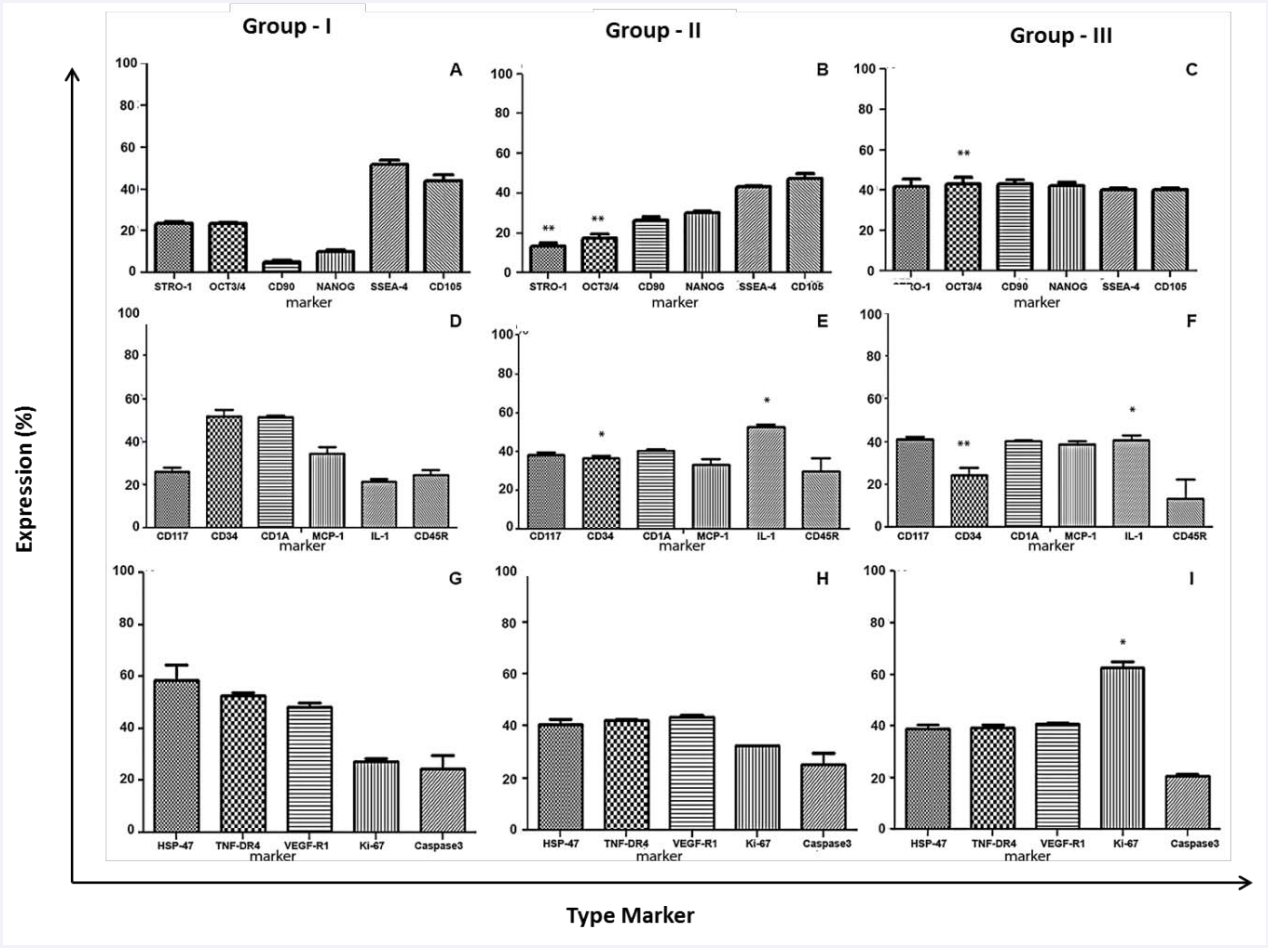

Figure 7 Immunophenotyping by flow cytometry. A-C. Pluripotency markers. D-F. Hematopoietic progenitor cell markers. G-I. Proliferation and cell death markers. Group I (day 30) in left row, group II (day 35) in the middle and group III (day 40) right row. **Significant differences.

populations by far. Fibroblast-like appearance is typical of mesenchymal stem cells in cell culture [22,37,57,58].

In addition, samples showed high amounts of mesenchymal cells with multi- or pluripotency characteristics [16,22,29,38,39,51,59]. All applied markers representative for such cells had positive response in fluorescence and flow cytometry, but the peaks of intensity varied. Results for selected markers used for the immunophenotyping analyses by fluorescence were consistent with the outcome of the more sensitive flow cytometry. Immunophenotyping analysis of cultivated hair follicle stem cells of fetuses was performed, and the results demonstrated that the cells expressed characteristics of mesenchymal stem cells on surface molecules, whereas the hematopoietic marker ($CD117^{+}$) had only a relative expression.

Flow cytometry analyses in the observed phases of gestation resulted in maintenance of expression of the marker CD-105, which is associated with the reprogramming of hair follicle cells [38], the proliferation marker MCP-1, the hematopoietic progenitor cell marker CD-1A and CD45 RO [60], as well as the cell death indicator caspase-3 active, suggesting great activity regarding cell origin differentiation and regeneration in the developing hair follicles. CD105 (endoglin), also identified in canine hair follicle cells, is a component of the receptor complex of transforming growth factor-beta (TGF-β), which is involved in cell proliferation, differentiation and migrant mesenchymal stem cells [61]. In the transition from embryo to fetus, represented by our group I of day 30 of gestation, we found significantly high values for SSEA-4 as a widespread pluripotency marker [37], CD-34, the proliferation marker VEGFR-1(which suggested angiogenetic processes) and HSP-47. Likewise, other studies identified hematopoietic progenitor cells and angiogenetic factors in hair follicles [19,22,29,32,34,38,39,41,42,49,60,62-64]. Also, TNF DR-4 as marker for apoptosis was significantly higher during the embryo to fetal transition phase similar to other studies [45,65,66], suggesting an especially high turn-over of cells before reaching the true fetal phase. Finally, the following markers were more specific for the true fetal phase: Oct3/4, CD-90, Nanog and STRO-1, which are also indicators for pluripotency to human and rodent hair follicle stem cells [19,22,29, 32,33,38,39,41,42,45,49,63-65]. CD117 was first differentially expressed on the surface of hematopoietic stem cells (HSCs), multipotent progenitors and myeloid progenitors. CD117-expression appears to be in hair matrix keratinocytes of melanocyte-deficient hair follicles in mice treated with an anti-Kit antibody, and was found to be hair-cycle-dependent. Involvement in hair growth control was also indicated by the observation that anagen development in c-Kit-deficient mice is

significantly retarded [67]. The observed expression of CD-117, as a stem cell growth factor and surface marker, particularly in hematopoietic progenitor cells; IL1-β receptor, which is suggested to be a growth inhibitor of hair follicles [46,68], and also Ki-67 as a proliferation marker [35] were more specific for the older group. Furthermore and in conclusion, there seemed to be also an intense cell trafficking during the fetal phases associated with slightly different differentiation potential in canine hair follicle mesenchymal stem cells.

CONCLUSION

Results from the various fields of analysis showed that hair follicle cells of dog fetuses differentiated in culture. The cells had satisfactory characteristics of *in vitro* growth, confirmed by cell cycle analysis, and the expression of relevant markers, especially of pluripotency markers such as OCT3/4, Nanog, CD-105, CD-90, SSEA-4 and STRO-1, suggesting that they had characteristics of mesenchymal stem cell lineages. In particular, samples from the older fetuses demonstrated a comprehensive development of hair cells, showed by a higher expression of the S-100 marker, but also contained an appropriate differentiation potential that is necessary for therapies. Thus, cells derived from tactile hair follicles derived from true fetuses (40 days) were multipotent, mesenchymal stem cells and so may be of value for stem cell therapies to aid in management of chronic wounds in dogs.

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