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Neural differentiation of canine mesenchymal stem cells/multipotent mesenchymal stromal cells

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Abstract

Background: Ability of adipose tissue-derived multipotent mesenchymal stromal cells/mesenchymal stem cells (ASCs) to differentiate in neural lineages promises progress in the field of regenerative medicine especially for replacing damaged neuronal tissue in different neurological disorders. Reprogramming of ASCs can be induced by supplying growth medium with chemical neurogenic inductors and/or specific growth factors. We investigated the neural differentiation potential of canine ASCs using several growth media (KEM, NIMa, NIMb, NIMc) containing various combinations of neurogenic inductors: B27 supplement, valproic acid, forskolin, N2-supplement and retinoic acid. First the cells were preconditioned in proliferation medium, followed by induction of neuronal differentiation. Six canine ASCs cell lines were assessed, half from female and half from male donors. The cell morphology, growth dynamics, viability were observed along with expression of neuron and astrocyte specific markers, which were assessed by immunocytochemistry and flow cytometry.

Results: After 3, 6 and 9 days, elongated neural-like cells with bipolar elongations were observed and some oval cells with light nuclei appeared. After three and nine days of neural induction, differentiation into neurons and glial cells was observed. Expression of neuronal markers tubulin beta III (TUBB3), neurofilament H (NF-H) and glial fibrillary acidic protein (GFAP) was observed by immunocytochemistry. High GFAP expression (between 70 and 90% of all cells) was detected after three days of growth in neural induction medium a (NIMa) by flow cytometry, and expression of adult neuronal markers NF-H and microtubule associated protein-2 (MAP2) was detected in around 25% of cells. After nine days of ASCs differentiation a drop in expression rates of all markers was detected. There were no differences between neural differentiation of ASCs isolated from female or male dogs.

Conclusions: The differentiation repertoire of canine ASCs extends beyond mesodermal lineages. Using a defined neural induction medium the canine ASCs were able to transform to neural lineages, bearing markers of neuronal and glial cells and also displayed the typical neuronal morphology. Differentiated ASCs can be a source of neural cellular lineages for regenerative therapy of nerve damage and also could be applicable for modeling of neurodegenerative diseases.

Background

Multipotent mesenchymal stromal cells (MSCs), also commonly referred to as mesenchymal stem cells are self-renewing, multipotent, adult stem cells that have a mesodermal and neuroectodermal origin (1,2). They are found in many tissues such as adipose tissue, bone marrow, cord blood, chorionic folds of the placenta, amniotic fluid, blood, lungs, etc., most of which are easily accessible and represent a potentially important source of cells. The ability of MSCs to transdifferentiate into osteoblasts, chondroblasts and adipocytes in *in vitro* conditions has been demonstrated in numerous studies (3,4). Until the year 2000, there was a widely accepted hypothesis that MSCs are capable to differentiate only into the mesodermal lineages. However, this was challenged when rat MSCs isolated from the bone marrow exposed to butyl

hydroxyanisole, β-mercaptoethanol and dimethylsulfoxide started to express proteins specific for the nervous system (5).

Most studies on neural differentiation of MSCs were carried out with human and rodent cells (2,5–13). In veterinary medicine, dogs are interesting for development of novel regenerative treatments, and in addition to benefiting canine patients, these therapies might show translational potential as dogs could be very interesting model of human neurological disorders. Few studies already reported the induction of canine MSCs into neural lineages (14–17), but there is no standard and optimized protocol for the neuronal induction of canine MSCs. GFAP, MAP2, A2B5, S100, TUBB3, nestin and NEUN are markers of neural cells and can be used as markers of cellular differentiation *in vitro*. Canine adipose tissue-derived multipotent mesenchymal stromal cells (ASCs) could be induced to express some of these neuronal genes after growing in the presence of neurogenic inductors valproic acid and forskolin (14,16), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (15), in commercial neurogenic differentiation medium (18) and in medium containing N2 supplement, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (19). Neurospheres generated from the canine ASCs were used to enrich for neural lineages expanded enough to transplant (19) and when grown in hypoxia showed higher expression of neuronal marker nestin (20). Recently, neural differentiation of human ASCs was evoked by conditioned medium obtained from glial cells (21).

In the present study four different cell growth media were tested to determine their ability to induce differentiation of canine ASCs into neural lineages. The media components included B27 supplement, valproic acid, forskolin, N2-supplement and retinoic acid. One of the tested media enabled prominent neural differentiation, observed by morphological changes at the cellular level, as well as by expression of glial marker GFAP and neuronal markers NF-H and MAP2, latter detected by immunocytochemical stainings and flow cytometry.

Results

Morphology

Morphological characterization of ASCs was assessed at 6 time points: untreated cells at 80% confluence, 24 and 48 hours after addition of pre-differentiation medium and 3, 6 and 9 days after induction of differentiation of ASCs. Canine ASCs cultured in basal growth medium displayed typical fibroblast-like morphology (Fig. 1 A and B).

Pre-differentiation medium STIM2 was highly toxic to the cells, while medium STIM1 was suitable for cell culture. STIM1 contained L-glutamine, B27 supplement and two growth factors, EGF and bFGF. The pre-differentiation was introduced in order to enhance ASCs proliferation (by EGF and bFGF) and to slowly direct the lineage commitment to neural cell types. Neural cells were also maintained in this medium by B27 supplement, which includes the retinoic acid. The first morphological changes were noticed after 24 hours of treating ASCs with the pre-differentiation medium STIM1 when neuronal-like cells appeared, identified by their elongated shape with bipolar elongation (Figure 1 C and D). Additionally, some oval

cells appeared with light nuclei (Figure 1C). The same cellular morphology was noticed after 48 hours of growth in STIM1 medium, but more dead cells were detected (Figure 1 E and F). ASCs seeding density affected the efficiency of differentiation, which suggests that the cell communication is essential for inducing ASCs to differentiate into neural phenotypes.

After pre-differentiation, cells were grown in four differentiation media. KEM medium was highly toxic to cells, which died already after 3 days in this medium (Figure 2). Its toxic effects were probably due to the abundance of neural differentiation inducing factors and therefore we further tested only three differentiation media with different compositions.

NIMa, NIMb and NIMc all contained L-glutamine, B27 and N2 supplements and varying concentrations of retinoic acid. During growth in NIM differentiation media (Figure 3), the number of cells with neuronal phenotype increased and they appeared more branched. Already at 3 days after the onset of differentiation, some cells obtained a neuron- or glial-like morphology with tree-like processes (Figure 3A, D and G). The concentration of retinoic acid in NIMa, NIMb and NIMc media was 10nM, 100nM and 10μM, respectively. The most prominent neural like morphologic changes were observed after 6 days of cultivation in the NIMa medium (Figure 3B), although they were also observed in the other two media. The concentration of retinoic acid in NIMb and NIMc might have been too high, since many apoptotic cells were also observed. Therefore, NIMa medium was determined to be the most suitable neural induction medium and all subsequent experiments were conducted with NIMa medium. Following 9 days of incubation in NIMa medium neural-like cells appeared. These cells were more elongated and branched and their cytoplasmic elongations resembled dendrites (Figure 3C).

Viability

Cell numbers were determined at three time points. 10^5 cells were seeded per ml of growth medium and cells were counted after 24 hours of pre-differentiation in STIM1 medium and at 3 and 9 days after induction of differentiation by NIMa medium (Figure 4A). No differences in growth dynamics between cells derived from male and female dogs were observed at 24 hours and 72 hours after seeding (Figure 4A). However, the viability of cells was reduced on the ninth day of differentiation and this reduction was statistically significant ($p < 0.001$). Interestingly, the viability on the ninth day of cell culture was significantly lower with cells from female donors in comparison to cells from male dogs, *i.e.* 83.4 ± 1.0% vs. 93.5 ± 2.3%, respectively ($p < 0.01$) Figure 4B).

Immunofluorescence

Canine brain tissue sections were stained with antibodies directed against neural markers to determine the reactivity of these antibodies against canine epitopes and thus the usefulness for their further use on

cellular cultures. Neurons labelled positively with antibodies directed against NF-H, MAP2 and TUBB3 and astrocytes labelled positively with antibodies against GFAP (Figure 5). Antibodies directed against nestin did not detect nestin in the canine brain.

Differentiation of canine ASCs into neural lineages was determined by expression of TUBB3, NF-H and GFAP. Immunocytofluorescence analysis revealed the presence of neuronal cytoskeleton proteins TUBB3, NF-H and GFAP, a marker of glia cells, after 3 and 9 days of growth in the differentiation medium NIMa (Figure 6 and Figure 7).

Flow cytometry

Expression of neural markers NF-H, MAP2 and GFAP was further characterized by flow cytometry in ASCs grown in differentiation media for 3 and 9 days. As the size of multipotent mesenchymal stromal cells is similar to the size of lymphocytes, the gating of cells was done similarly. The expression of markers was comparable for differentiated cells derived from female and male dogs (Figure 8). Average expression of NF-H in undifferentiated cells from male donors was 1.4 ± 0.8%, MAP2 0.7 ± 0.4 % and GFAP 2.3 ± 1.4% and from female donors NF-H 0.9 ± 0.3 %, MAP2 1.4 %, GFAP 5.3 ± 1.1 %. After 3 days of differentiation in NIMa media expression in cells derived from male dogs was: NF-H 32.6 ± 0.8 %, MAP2 33.6 ± 0.1 %, GFAP 90.4 ± 0.6 % and in ASCs from female dogs: NF-H 33.3 ± 1.4 %, MAP 22.8 ± 5.4 %, GFAP 73.5 ± 22.3 %. The expression of all markers decreased 6 days later (after 9 days of differentiation) with cells derived from male dogs to NF-H 8.7 ± 0.4 %, MAP2 9.0 ± 5 %, GFAP 17.8 ± 12.1 % and in cells derived from female dogs to NF-H 11.7 ± 11.0 %, MAP2 13.6 ± 10.7 %, GFAP 30.9 ± 14.3 % (Figure 8). Percentage of differentiated cells after 9 days of differentiation was lower in male dogs, but the difference was not statistically significant.

Discussion

The differentiation potential of MSCs into neural cells makes them interesting and potentially useful for neural reconstitution in neurodegenerative diseases, stroke and spinal cord injuries. Whether the transplantation of ASCs alone would be sufficient to treat spinal cord injuries and other neurological disorders, or *in vitro* differentiation would be needed prior to the transplantation, is still being studied and therefore, there is a need to develop optimal procedure for the induction of neuronal differentiation in MSCs.

In the present study we confirmed that canine adipose tissue derived MSCs are capable of neural differentiation *in vitro* and, furthermore, explored which kind of neural induction medium is the most suitable for neural differentiation of canine ASCs. In previous studies, rat and human multipotent mesenchymal stromal cells were shown to transdifferentiate into neural phenotypes by exposing these cells to a variety of neurogenic inductors, such as β-mercaptoethanol, butylated hydroxyanisole, potassium chloride (KCl), valproic acid and forskolin (2,5,8,11,12). Alternative methods of inducing

chemical differentiation of human and canine MSCs to a neural lineage involved the addition of growth factors such as bFGF, EGF, neuroblast factor (N2), B27 supplement and retinoic acid (7,13,15,22). One study also showed that canine adipose tissue derived stromal cells could be differentiated into neuronal cells by incubation with dibutyryl cyclic adenosine monophosphate (dbcAMP) and isobuthylmethylxanthine (IBMX) (17).

We tested two pre-differentiation media to condition the cells to neural differentiation. Serum-free medium (STIM1) with added growth factors EGF, bFGF and B27 supplement was suitable for cell culture whereas STIM2 turned out to be highly toxic to the cells, probably due to high concentration of β-mercaptoethanol. This pre-differentiation step was introduced due to previous studies showing that culturing ASCs under active proliferation conditions greatly improves their propensity to differentiate toward neurogenic lineages (23). The neurogenic inductors in differentiation media tested were B27 supplement, valproic acid, forskolin, KCl and butylated hydroxyanisole (BHA) in KEM medium and B27 supplement, N2 supplement and retinoic acid in NIM media. As KEM was toxic to the cells and cells died after only 3 days of incubation, the NIM media were further assessed for triggering the neural differentiation of canine ASCs. In NIMa medium, containing the lowest concentration of retinoic acid (10 nM), the most prominent neural-like cellular phenotypes were observed and thus this was the medium of choice for subsequent experiments. Retinoic acid, a metabolite of vitamin A, has roles in cell differentiation, neurite outgrowth and cell survival (24,25). It induced post-mitotic neuronal phenotypes in various cells *in vitro* (24,26,27) and might have been the crucial co-factor in NIMa medium for canine ASCs to switch from proliferation to differentiation. However, retinoic acid could also be highly toxic to cells both *in vitro* and *in vivo*, and higher concentrations of retinoic acid in media NIMb and NIMc was probably causing dying of cells. Although ASCs also seem to differentiate into neural phenotypes in media NIMb and NIMc, there were many apoptotic cells in the cell culture with these two media.

Cell numbers were the highest on the third day after the addition of the NIMa medium and cells from male and female dogs grew similarly during the neural induction. The viability of all cells was reduced at day 9 of differentiation and was statistically significantly lower in ASCs derived from female than male donors. Stressors such as neurogenic factors present in NIMa medium and/or low serum concentration and poor nutrition might have increased ASCs death rate. Why female cells were more sensitive to these effects remain unexplained and will have to be studied in the future. One option that might be interesting to test in the future studies would be the addition of estradiol to the differentiated cells. Estradiol has many important functions in various parts of the brain and perhaps adult female cells need estradiol for their optimal survival.

Cell population obtained by the cultivation of ASCs in neural-differentiation medium is often a mix of cells expressing one or more neural-specific markers. The most studied markers included nestin, TUBB3, S100 and GFAP (28). Differentiated rat and human multipotent mesenchymal stromal cells were shown to express mature neural markers such as GFAP, MAP2, TUBB3 and neuron specific enolase (NSE). Such cells also possess voltage-gated calcium channels and the ability to upregulate the glutamate receptor (2,5,8,11,12). In the present study, after 24-hour preconditioning in pre-differentiation medium STIM1 and

subsequent incubation in NIMa differentiation medium for 3 or 9 days, cells expressed proteins characteristic for mature neurons and astrocytes. Immunofluorescence analysis showed expression of neuronal markers NF-H and TUBB3 and glial marker GFAP. The neural phenotype was confirmed by flow cytometry showing very high expression of GFAP after 3 days of growth in the differentiation medium and increase of mature neuronal markers NF-H and MAP2 expressing cells in comparison to undifferentiated cells. In undifferentiated cells the basal expression of neural markers was already detected but at very low levels. This basal level of expression of neural markers in a cell culture of undifferentiated canine ASCs was reported in a recent study, where some of the neural markers and neurotrophic factors were expressed already in undifferentiated cells, albeit at low levels (14).

The expression of neural markers for all ASCs was the highest after three days of growth in the differentiation medium and after 9 days of cell culture the expression of all markers tested dropped. This reduction in immunopositive cells might have been due to de-differentiation of cells or apoptosis. To our best knowledge, none of the previous studies on neural differentiation of canine ASCs followed the expression of neural markers *in vitro* for more than 3 days, thus it remains unknown if the drop in the expression might have occurred in other studies if cells would remain in induction media for longer periods.

MSCs directed, at least partially to neuronal lineages, could provide an opportunity in developing novel therapeutic treatments for a variety of disorders of the central nervous system such as spinal cord injuries and neurologic conditions affecting the brain (Alzheimer's, Parkinson's, Huntington's disease, stroke, cerebral palsy, brain ischemia, traumatic brain injury, amyotrophic lateral sclerosis …). As dogs and humans share their environment and have similar lifestyles, some of their diseases are alike. For instance canine cognitive dysfunction, which is in many aspects similar to human Alzheimer's disease (29), could be potentially treated with cell replacement therapy by transplanting neurally differentiated ASCs in the canine brain. A previous study has shown that human umbilical cord derived MSCs, transplanted into canine brains, migrated and enhanced endogenous neural stem cell population in the subventricular zone (30). This indicates that MSCs secreted some factors to support neural differentiation of endogenous stem cells. This has been also corroborated in Phase 1 human clinical trial of AD, where repeated intracerebroventricular injections of adipose-derived stromal vascular fraction improved cognition in AD patients (31). The therapy with partially neurally differentiated autologous MSCs would exploit the neuroprotective and neurotrophic features of MSCs and the neurally differentiated cells would lend the supportive (glia) and functional (neurons) roles to the diseased/injured brain. In this way various neurological conditions could be potentially treated in dogs, since the autologous ASCs are easy accessible and their expansion, differentiation and subsequent transplantation could be strictly monitored. However, for potential future use in regenerative medicine, the timing of neuronal differentiation would have to be explored meticulously, as this could be one of the critical points to achieve the highest quality of cells used for treatments.

Conclusion

The differentiation repertoire of canine ASCs clearly extends beyond mesodermal lineages. Their *in vitro* induction to form neural lineages, although partial, might be enough in the future to treat neurological conditions as a combinatorial treatment, as MSCs already possess neuroprotective properties, such as anti-inflammation, anti-astrogliosis, neuronal extension and neuronal regeneration effects. However, as results of this study show, there are important differences in the ability of different media to induce neural differentiation, therefore, in the future studies, the optimal composition of growth media will have to be determined, optimized and synchronized to develop general guidelines for the induction of canine ASCs into neural cells.

Methods

Cell origin

Adipose tissue was collected by veterinarians at veterinary clinics. All dogs were privately owned patients and were under general anesthesia for other procedures, not purposefully to collect samples for this study. All dogs' owners gave their written consent to use the removed adipose tissue for research purposes and all the experiments were performed according to the procedures and guidelines approved by the National Health Service branch of the Slovenian Ministry of Health. Additional ethical permission was not needed according to Slovenian legislation and interpretation by Administration for safe food, veterinary and plant protection, which is responsible for issuing licenses for experiments with animals. Subcutaneous adipose tissue (<1 cm^3) was aseptically removed from the back area between the dog's scapulae. Samples were obtained from six different dogs aged 2 to 9 years (mean age 5.5 years) of different breeds and both sexes, three males and three females.

Isolation of canine ASCs

Canine ASCs were isolated from adipose tissue and characterized based on the routine protocols developed by the Animacel Ltd. Briefly, fat tissue was minced, washed three times in PBS buffer and digested over night with an equivalent amount of collagenase I solution containing 2 mg/ml collagenase I and 4 mg/ml BSA in HEPES buffer, pH 7.4. Collagenase I activity was stopped by a double volume of PBS and the resulting cell suspension was filtered through the falcon strainer (pore size 100 μm). Cells were grown in the cultivation medium, which was previously demonstrated to be specific for canine ASCs cultivation (32) and was developed by Animacel Ltd. After 24 hours non-adherent cells were washed off. ASCs were cultured until 80% confluence was reached, detached, counted with Bürker Turk Chamber hemocytometer and passaged. Again, cells were maintained until 80% confluence, detached and cryopreserved in a freezing medium consisting of DMEM Glutamax, FBS and DMSO, for later use.

Cultivation of ASCs

The high cell density is a key parameter for the successful differentiation of multipotent mesenchymal stromal cells into neural lineages. Isolated ASCs (first passage) were plated into different growth flasks depending on the designed experiment. During the optimization and monitoring of the morphology of undifferentiated and differentiated cells, two T25 flasks were used for cells from each dog, plated at a density of 10^6 cells/cm^2. Three additional T25 flasks were required for flow cytometry, for each point of differentiation and for each animal, with the cells at the same density. Furthermore, for the immunocytochemical characterization of differentiated ASCs, individual round dishes with 35 mm diameter were used with cells at a density of $8x10^4$ cells/cm^2 and 24-well plates with cells plated on glass cover slips coated with laminin at a density of $2x10^4$ cells/cm^2.

Neural differentiation

Canine ASCs in a second passage were plated in different flasks based on the type of the experiment. Cells were grown in our standard cultivation medium. After 24 hours, the cultivation medium was replaced by the pre-differentiation neural induction medium (mitogenically stimulated; STIM). Two pre-diffrentiation media were tested. STIM1 consisting of DMEM Glutamax, EGF (20 ng/ml), bFGF (20 ng/ml) and 1x B27 supplement (supplied as 50x stock solution, Gibco); and STIM2 consisting of DMEM Glutamax, β-mercaptoethanol (100 μM) and 20% FBS. After 24 hours, the pre-differentiation medium was replaced by the neural differentiation medium. Four different differentiation media were prepared:

1. KEM media consisting of DMEM Glutamax, 1% FBS, 2x B27 supplement, valproic acid (2mM), forskolin (10 μM), hydrocortisone (1 μM), KCl (5 mM), butylated hydroxyanisole (BHA; 200 μM), insulin (5 μg/ml) and 0.1% penicillin/streptomycin.
2. NIMa (neural induction medium a) composed of DMEM Glutamax, 2x B27 supplement, 1x N2-supplement, 1% FBS, 0.1% penicillin/streptomycin and 10 nM retinoic acid.
3. NIMb consisting of DMEM Glutamax, 2x B27 supplement, 1x N2-supplement, 1% FBS, 0.1% penicillin/streptomycin and 100 nM retinoic acid.
4. NIMc composed of DMEM Glutamax, 2x B27 supplement, 1x N2-supplement, 1% FBS, 0.1% penicillin/streptomycin and 10 μM retinoic acid.

The cells were further processed for morphological, immunocytochemical and flow cytometry analysis after 3, 6 and 9 days following neural induction.

Viability assay

To determine the cell numbers and the viability of canine ASCs cells after exposure to neural differential medium, dye exclusion assay was used. The viability was determined at three time points (after 24 hours of pre-differentiation in STIM1 medium and at 3 and 9 days after induction of differentiation) by adding

Trypan Blue dye into cell suspension and counting live/dead cells using a Bürker Turk Chamber under the light microscope. The viability of cells grown in control media was determined for comparison to neurally differentiated cells. All experiments were performed in triplicates.

Immunohistochemistry on dog brain

Dog brain tissue sections were used as control for the reactivity of antibodies. Dog brain was obtained at Veterinary faculty of University of Ljubljana following the dog's owner's approval. The dog was sixteen years old male, euthanized due to advanced cognitive dysfunction. The brain was surgically removed, cut in small cubes and fixed in 4% paraformaldehyde at 4 °C for several days. Pieces of the brain were embedded in paraffin using an automated tissue processor (Tissue processor Leica TP 1020). Tissue blocks containing the area of the frontal cortex were cut in 7 µm sections and further processed for immunohistochemistry. After dewaxing sections were subjected to antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0,05% Tween 20, pH 6) by boiling the slides for 10 minutes in a microwave oven. This was followed by blocking the unspecific epitopes in 1.5% BSA, 10% normal goat serum, 0.1% TritonX-100 in PBS for 60 minutes at room temperature. Sections were then incubated overnight at 4 °C with the following primary antibodies: mouse anti-GFAP (1:400, G3893, Sigma), mouse anti-NF-H (1:400, AB1989, Millipore), mouse anti-bIII tubulin (1:400, sc-80005, Santa Cruz Biotechnology), rabbit anti-MAP2 (1:400, AB5622, Millipore) and chicken anti-nestin (1:200, ABIN187958, Neuromics Antibodies). The next day slides were washed with PBS for 5 min and incubated with secondary antibodies (anti-rabbit Alexa Flour 555 or anti-mouse Alexa Flour 555 diluted 1:1000 in blocking buffer (both from Invitrogen)) for 1h at room temperature in the dark. The nuclei were counterstained with DAPI (Sigma). Slides were then washed with PBS thrice for 5 minutes and mounted in ProLong Gold Antifade Mountant (Molecular Probes). Stainings were visualized with a confocal microscope (Zeiss LSM 710) and ZEN software.

Immunofluorescence analysis of cells

Immunocytochemistry was performed on cells to evaluate the presence of the neural markers after the induction with differentiation medium. One 24-well plate was prepared per each time point with round glass cover slips coated with laminin (10 µg/ml). Next day, the second passage canine ASCs were plated at a density of $2x10^4$ cells/cm^2 in 1 ml of the basal growth medium. At 3 and 9 days of differentiation cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at room temperature, rinsed three times in PBS and permeabilised with TBST for 5 min. Blocking of unspecific epitopes was performed with blocking solution (10% FBS, 1% milk powder, 0.02% Na-azide, TBST, pH 7,2) for 1h. Cells were then incubated overnight at 4 °C with the primary antibodies: rabbit anti-GFAP (1:400, G3893, Sigma) conjugated with Alexa Flour 488, rabbit-anti-NF-H (1:400, AB1989, Millipore) and rabbit anti-bIII tubulin (1:1000, AB9354, Millipore). The next day cells were rinsed three times with PBS and one time with 2% FBS in PBS and incubated with secondary donkey anti-rabbit Cy2 conjugated IgG antibody or secondary

donkey anti-mouse Cy3 conjugated IgG antibody (both 1:500; Jackson Immunoresearch) for 2h at room temperature in dark. Anti-GFAP antibody was covalently labelled with mouse anti-rabbit Alexa Flour 488 IgG using Alexa Fluor® 488 Protein Labeling Kit (Molecular Probes). The nuclei were counterstained with DAPI. Stainings were visualized with a confocal microscope (Zeiss LSM 710). Neural differentiation was detected by observing random viewing fields under the microscope and by comparing cellular morphology. NF-H and TUBB3 positive cells were identified as neuronal lineage and GFAP-positive cells were identified as glial lineage (astrocytes).

Flow cytometry

ASCs cell cultures were detached for flow cytometry analyses at three time points (pre-differentiation, 3 and 9 days of differentiation). Confluent cells were harvested using trypsin (TrypLE™ Express, Gibco) digestion for 5 min at 37 °C, centrifuged for 5 min at 1400 rpm at 4 °C and resuspended at a concentration of 10^7 cells/ml in PBS. Five aliquots were prepared per time point (pre-differentiation, 3 and 9 days of differentiation) per animal each containing 100 µl (concentration of 10^6 cells/ml) and were transferred to flow cytometry facility at Medical Faculty, University of Ljubljana. The aliquots were centrifuged for 5 min at 1600 rpm at room temperature and pellet was fixed with 4% paraformaldehyde (pH 7.4) for 10 min at room temperature, rinsed three times in PBS, and in parallel permeabilized with 0.1% Triton X-100, and blocked with 2% FBS, 5% milk powder, 0.02% penicillin/streptomycin for 10 min. Cells were incubated for 30 min in the dark at 37 °C with following primary antibodies: rabbit anti-NF-H (1:400, AB1989, Millipore), rabbit anti-MAP2 (1:200, AB5622, Millipore) and mouse anti-GFAP (1:400, G3893, Sigma) conjugated with Alexa Flour 488 (Life Technologies). After incubation samples were centrifuged 5 min at 1600 rpm, rinsed two times with 2% FBS in PBS and the ones stained for NF-H and MAP2 incubated with secondary donkey anti-rabbit IgG Cy2 conjugated antibody (1:500, Jackson Immunoresearch). Anti-GFAP antibody was previously labelled as described above. All samples were analyzed by BD FACS Canto flow cytometer (BD Biosciences). Appropriate isotype matched controls conjugated to FITC were used to identify nonspecific staining for GFAP antibody. For unconjugated primary antibodies, controls included isotype matched unconjugated primary controls and incubation with the secondary antibody alone. Data was analyzed using FACSDiva™ version 6.1.2 (BD Biosciences) analysis software.

Statistical analysis

Immunocytochemical staining and cell morphology were evaluated only qualitatively by observing random viewing fields by observer blind to the differentiation conditions. Differences in cell numbers and cell viability between control and differentiated ASCs were analyzed by repeated measures ANOVA followed by Bonferroni post-hoc test with sex as independent variable and day of culture as within factor. Differences between groups in expression of neuronal markers detected by flow cytometry were analyzed

by ANOVA followed by Bonferroni post-hoc test. All differences were considered statistically significant at $P < 0.05$.

Declarations

Ethics approval and consent to participate

Since study was conducted on client-owned animals undergoing routine clinical procedure with owner's approval, no approval of ethical committee was needed according to Slovenian legislation and official opinion from The Administration of Republic of Slovenia for Food Safety, Veterinary and Plant protection, responsible for issuing ethical permits for animal experiments.

Consent for publication

'Not applicable'

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

ASCs: Adipose tissue-derived multipotent mesenchymal stromal cells/mesenchymal stem cells; BDNF: Brain-derived neurotrophic factor; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor; FBS: Fetal bovine serum; GFAP: Glial fibrillary acidic protein; MAP2: Microtubule associated protein 2; MSCs: Multipotent mesenchymal stromal cells; NGF: Nerve growth factor; NIMa: Neural induction medium a; NIMb: Neural induction medium b; NIMc: Neural induction medium c; NF-H: Neurofilament H; TUBB3: Tubulin beta III.

Competing interests

G.M. is partial owner of Animacel Ltd., L.M. is the CEO of Animacel Ltd.. Other authors have no

competing interests to declare.

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Authors' contributions

V.K.G. and G.M. designed the study. S.P.M., V.K.G., L.M., and A.N.K. performed the experiments. V.K.G. and S.P.M. analyzed the data and prepared Figures. S.P.M. and G.M. wrote the manuscript. All authors read

and approved the final manuscript.

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Figures

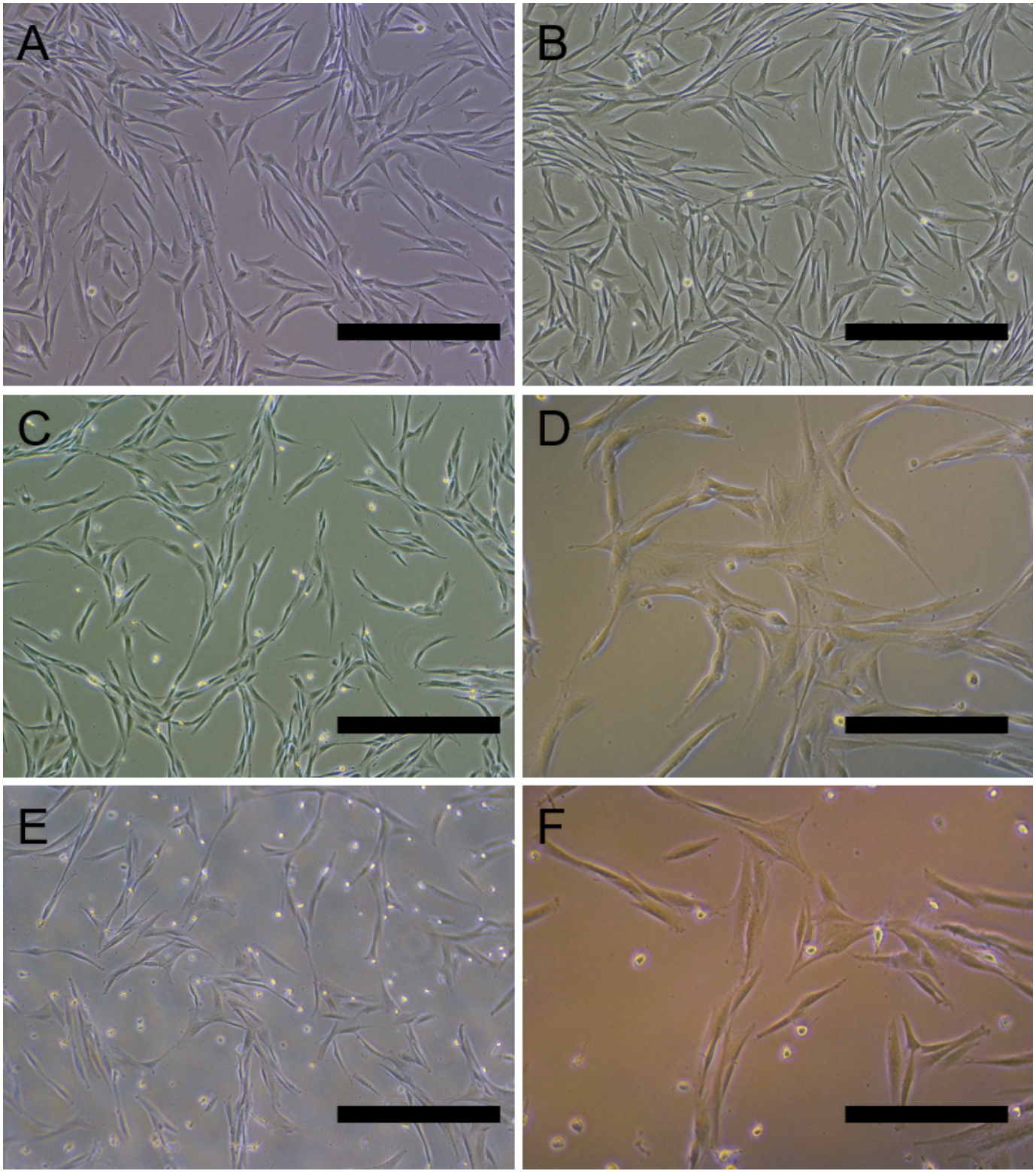

Figure 1

Canine ASCs typical growth pattern prior to differentiation (A, B) and their cellular morphology after 24 hours (C, D) and 48 hours (E, F) in pre-differentiation medium STIM1. Scale bars are 200 µm (A,B,C,E) and 100 µm (D,F).

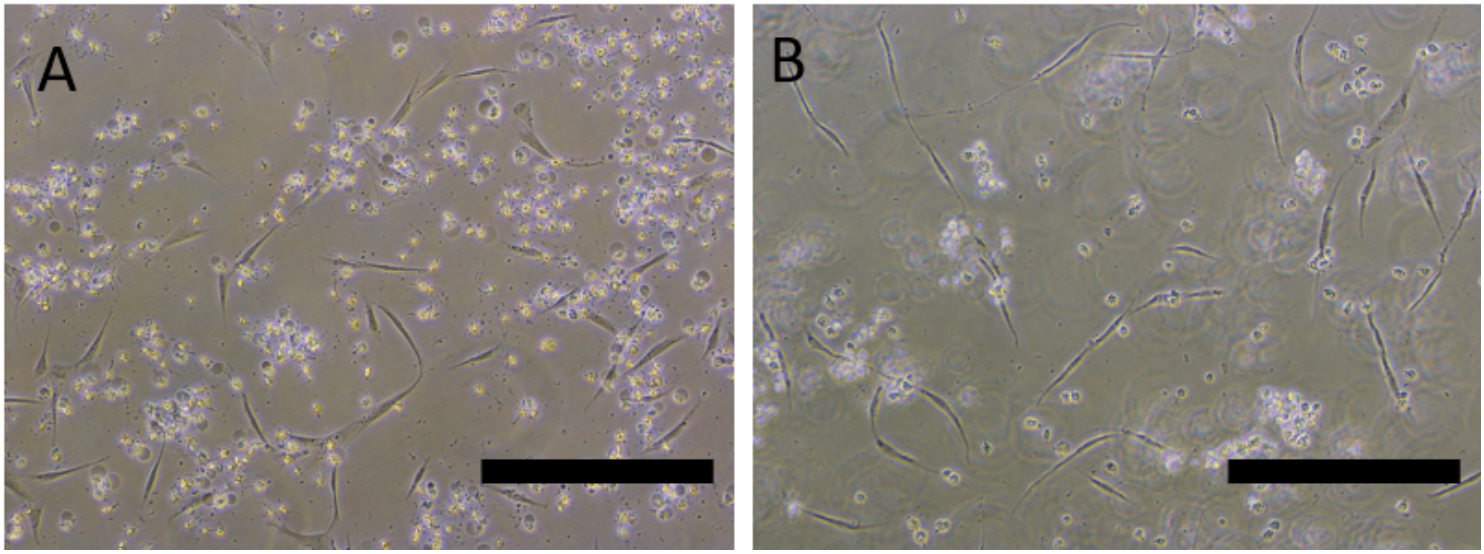

Figure 2

Canine ASCs grown in KEM differentiation medium for 1 (A) and 3 days (B). Prior to addition of KEM, cells were grown in pre-differentiation medium STIM1 for 24 hours.. Scale bars are 200 µm.

Figure 3

Morphology of canine ASCs grown in NIMa (A, B, C), NIMb (D, E, F) or NIMc (G, H, I) differentiation media for 3, 6 or 9 days. Cells were first exposed to pre-differentiation medium STIM1 for 24 hours. Numerous thin protrusions resembling axons or dendrites are clearly seen in figures B and C. Scale bars are 100 μm (A, C, H, I) and 200 μm in all other images.

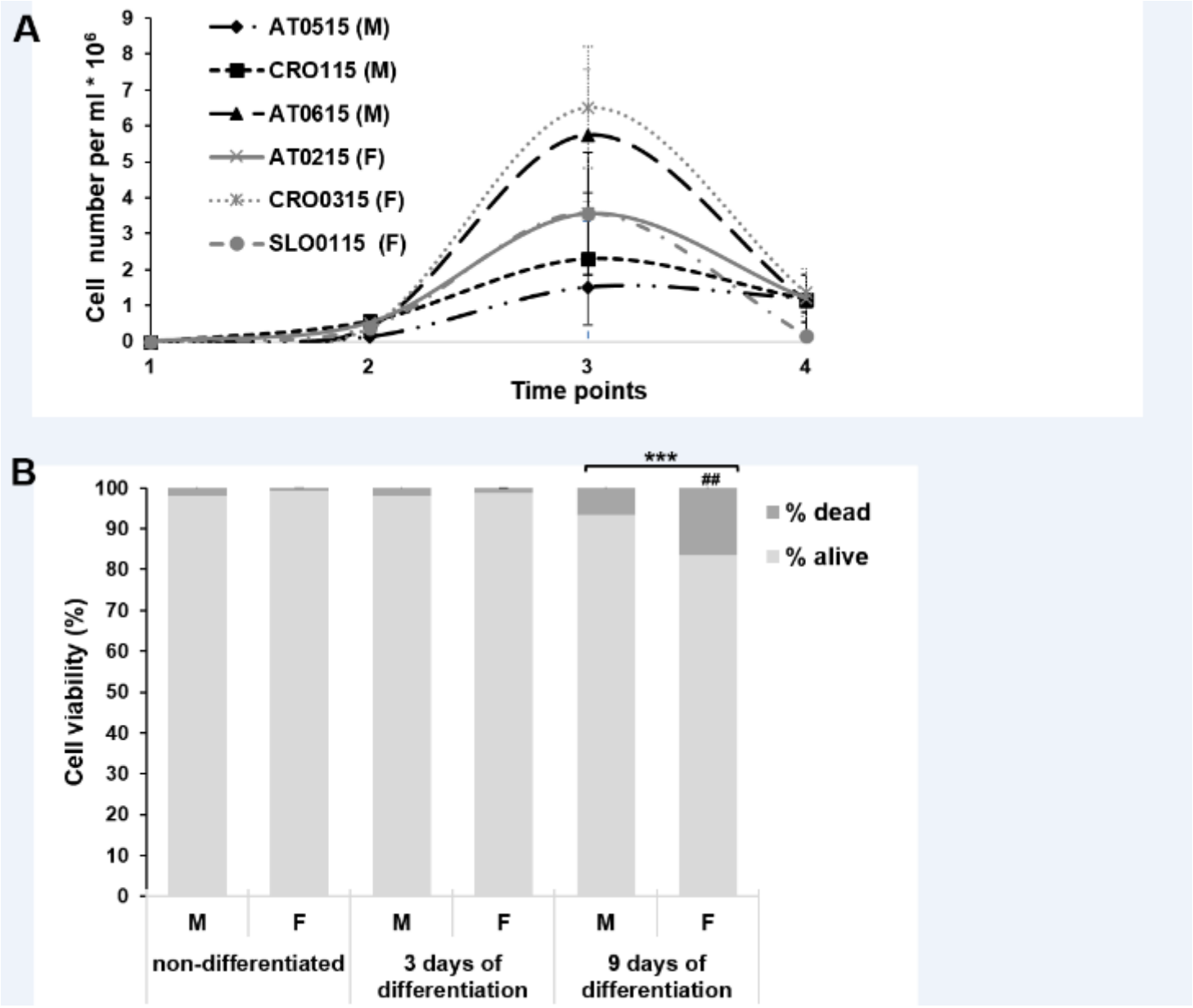

Figure 4

Growth curves (A) and viability plots (B) for ASCs. (A) Growth curves for six canine ASCs before and after the induction of differentiation. At time point 1 cells were seeded and time point 2 represents number of cells at the beginning of the treatment with pre-differentiation medium STIM1, which was applied for 24 hours. Cell numbers increased after 3 days of differentiation (time point 3) but decreased again after 9 days of differentiation (time point 4), although differences in cell numbers were not statistically significant. (B) Average viability of male and female dogs derived ASCs before and after the induction of

differentiation. The viability decreased in both male and female cells on day 9 of cell culture ($P < 0.001$) and there was statistically significant difference in viability between male and female cells ($P < 0.01$) at this time point.

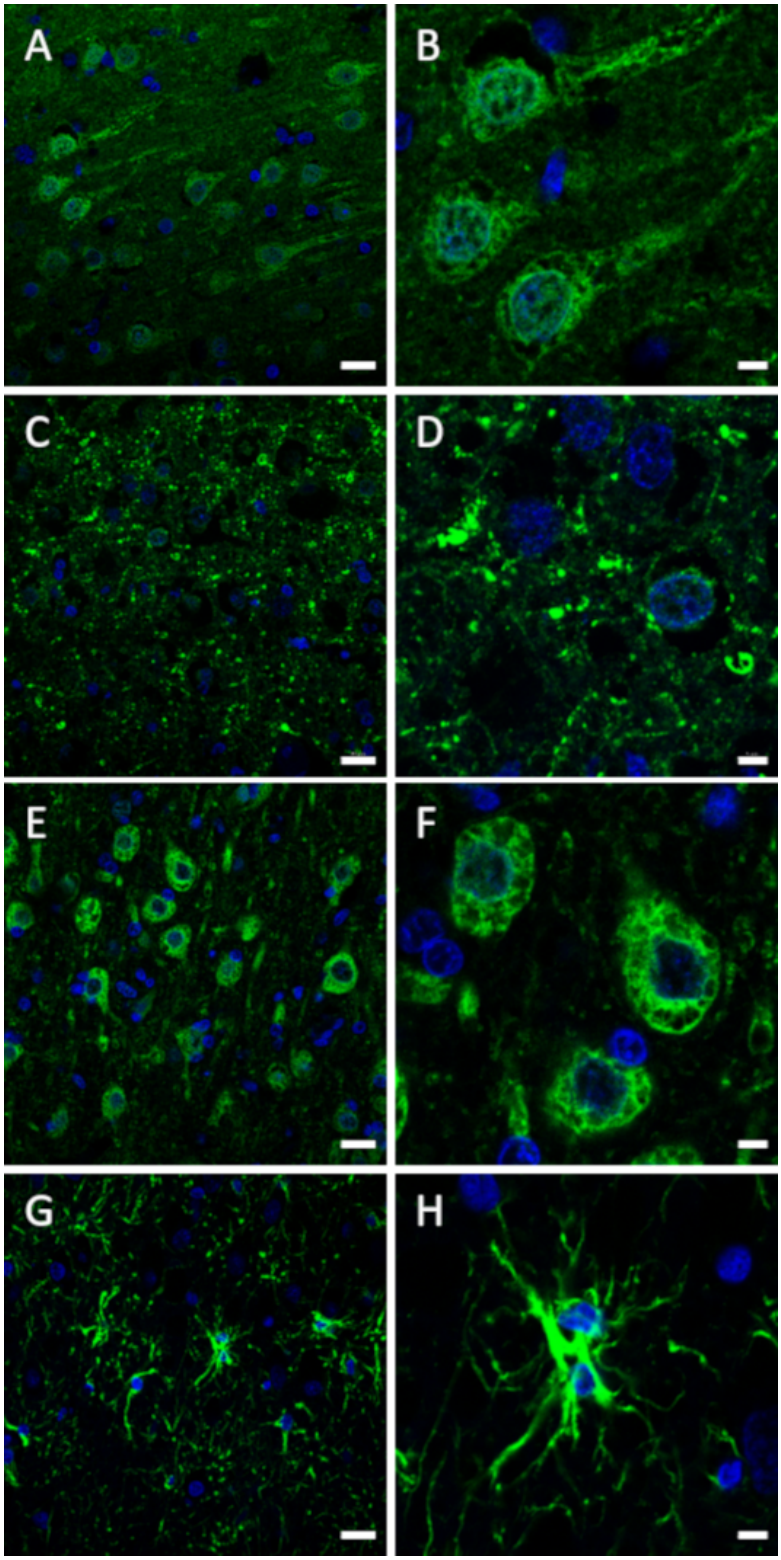

Figure 5

Canine brain immunofluorescence stainings. The frontal cortex of a 16-years-old dog was stained with antibodies against TUBB3 (A, B), NF-H (C,D), MAP2 (E,F) and GFAP (G,H). Nuclei were counterstained with

DAPI (blue). Scale bars are 20 μm in images in left column and 5 μm in images in right column.

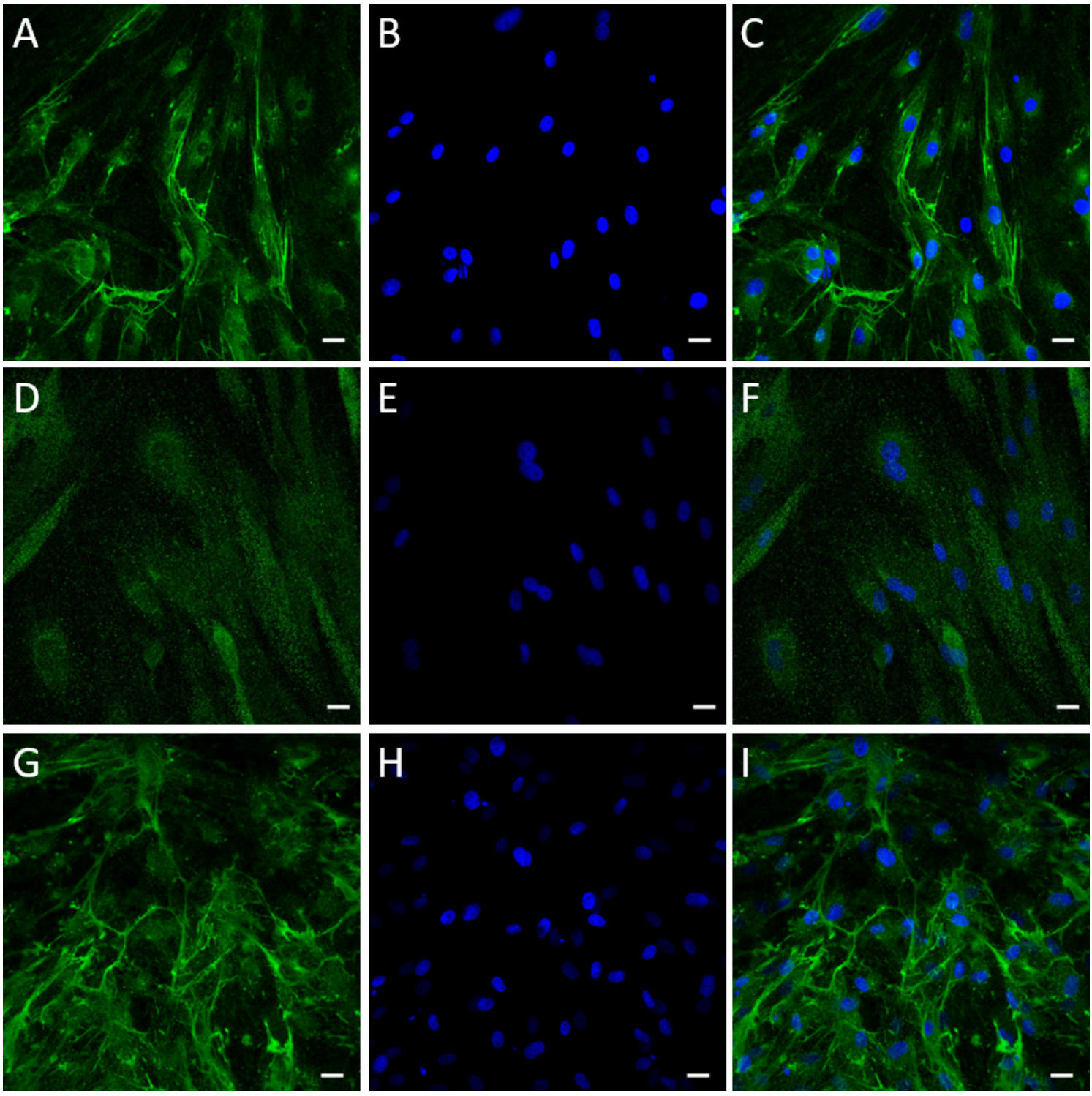

Figure 6

Expression of neural markers TUBB3 (A,C), NF-H (D,F) and GFAP (G,I) in canine ASCs exposed to differentiation medium NIMa for three days. Prior to addition of NIMa cells were grown in pre-differentiation medium STIM1 for 24 hours. Nuclei were counterstained with DAPI (blue). Scale bars are 20 μm.

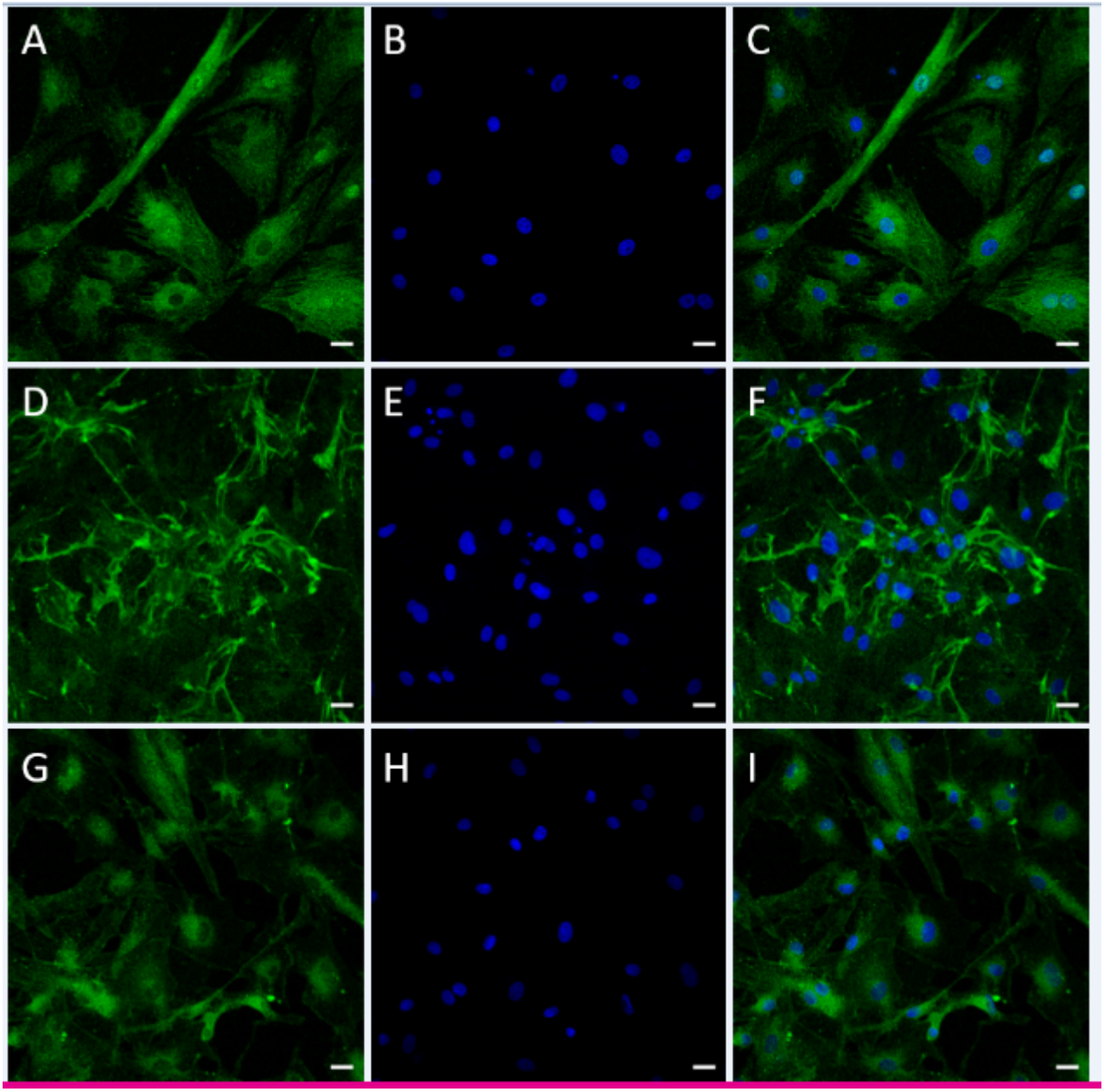

Figure 7

Expression of neural markers TUBB3 (A,C), NF-H (D,F) and GFAP (G,I) in canine ASCs exposed to differentiation medium NIMa for nine days. Prior to addition of NIMa cells were grown in pre-differentiation medium STIM1 for 24 hours. Nuclei were counterstained with DAPI (blue). Scale bars are 20 µm.

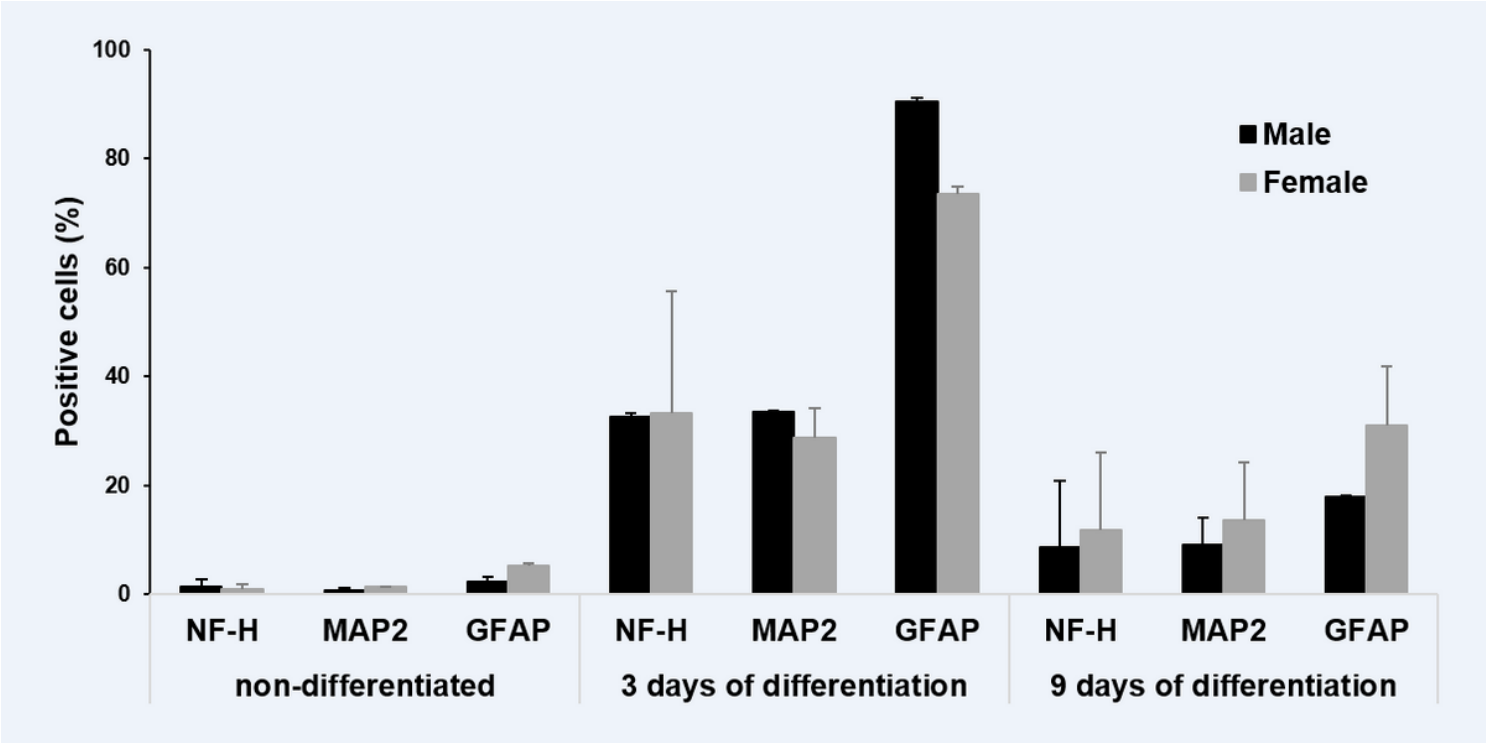

Figure 8

Percentage of NF-H, MAP2 and GFAP positive cells prior to differentiation and following 3 or 9 days differentiation in NIMa medium. Flow cytometry analysis was conducted. Data are presented per female and male derived ASCs as mean percentages ± SD. Differences between sexes were not statistically significant.