

Isolation and phenotyping of potential stem cells from the umbilical cord of the bottlenose dolphin (*Tursiops truncatus*)

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ABSTRACT We have successfully isolated cells with stem-like properties from bottlenose dolphin (*Tursiops truncatus*) umbilical cord. Our results show that this cetacean species has embryonic fetal and adult stem cells as do humans and other studied mammals. This accomplishment allows to eventually investigate whether dolphins, due to their unique adaptations to aquatic environments, have special stem cell lineages or distinctive mechanisms of cell programming. Further characterization of their potency to differentiate into multiple cell lineages would fulfill numerous applicative purposes. We characterized, developed and refined a new protocol for obtaining potential stem cells from umbilical cord tissues of the bottlenose dolphin. Tissue samples were taken from umbilical cords of successful deliveries immediately after placenta ejection and collection from the water. Umbilical cord samples (2-3 cm³) were excised and subjected to enzymatic digestion and mechanical dissociation. Viable cells from specimens resident in the Oceanografic Valencia were cultured and subsequently isolated and tested for pluripotent characteristics (cell morphology, phenotype and expression of surface markers). Cell viability was confirmed also after freezing/thawing. The established protocol is suitable for collection/isolation/culture of dolphin potential mesenchymal stem cells from dolphin umbilical cord, which can be deposited in cell banks for future research needs.

KEY WORDS: *marine mammal, injury, mesenchymal stem cell, cryopreservation, stem cell banking*


Introduction

Bottlenose dolphin (BD), *Tursiops truncatus*, is a species highly represented in zoological and marine parks worldwide. It has been thoroughly described in publications for scientific and non-scientific audience, reaching an enormous popularity even through movies and TV shows. BDs can experience skin injuries (*rakemarks*) and external soft tissue, up to bone damage, through their lives as a result of daily activity and/or social interactions. Nevertheless, the very efficient and fast wound healing mechanisms described in dolphins may limit infection processes associated with open wounds (Zasloff, 2011). Scientists have been trying to isolate dolphin cells for a long time (Cecil and Nigrelli, 1970) but, despite the great efforts, their availability is still limited, also considering that primary cells need to be immortalized in order to have a long-term system

for investigation (Yu *et al.*, 2005).

Mesenchymal stem cells (MSCs) are an attractive cell population for studies of musculoskeletal tissue regeneration and wound healing (Niemeyer *et al.*, 2010; De Almeida *et al.*, 2012; Wei *et al.*, 2013). Multiple sources of MSCs have been described in humans and animals (Hill *et al.*, 2019; Trindade *et al.*, 2017; Barberini *et al.*, 2014; Ullah *et al.*, 2015), including adipose tissue, endometrium, dental tissues, bone marrow, ligaments, and lung, where they express specific cell surface markers (e.g. clusters of differentiation) (Ullah *et al.*, 2015; Mushahary *et al.*, 2018). Our study aimed at

Abbreviations used in this paper: APC, allophycocyanin; BD, Bottlenose dolphin; DMEM, Dulbecco's modified eagle medium; DPBS, Dulbecco's phosphate-buffered saline; DUC, dolphin umbilical cord; FITC, fluorescein isothiocyanate; GM, growth medium; MSC, mesenchymal stem cell; PE, phycoerythrin; UC, umbilical cord.

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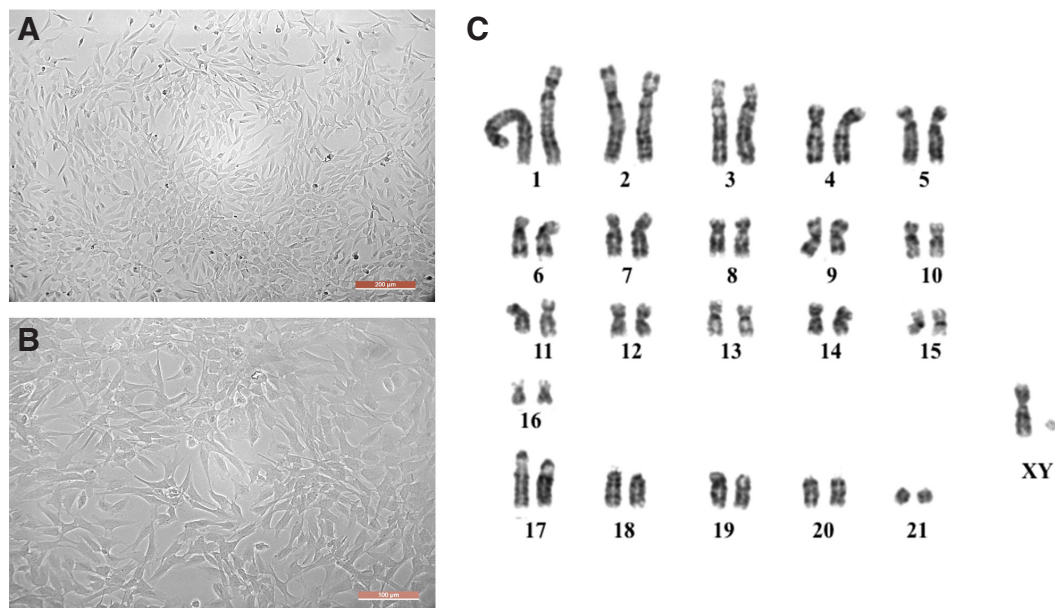


Fig. 1. Dolphin umbilical cord (DUC) mesenchymal stem cells (MSCs) with G-banded karyotype. (A-B) DUC-MSCs on tissue culture polystyrene imaged at passage 5 on bright-field microscopy. (C) G-banded karyotype from cells at passage 4.

isolating and characterizing cells with stemness properties from the BD umbilical cord.

Experimental Protocols

Tissue collection

Umbilical cord (UC) tissue samples were collected *post-partum* after placenta expulsion by an individual maintained in the Oceanográfico (Valencia, Spain). The placenta and the adjoining UC were ejected 5 hours 40 minutes after the calf delivery. The UC samples from Valencia were used in all the experiments described. UC tissue samples were also obtained from two additional female dolphins, maintained in the Acquario di Genova, and in Oltremare (Riccione), respectively 6 hours 30 minutes and 7 hours after calf delivery. These last samples were used to validate the protocol of cell isolation, culture properties and expansion procedure (described below).

MSC isolation and expansion

UC samples over a 2 x 2 cm surface section area were excised, placed in a sterile Petri dish, further reduced to 2–3 cm² fragments using sterile scalpel and forceps, then washed twice in 1x Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Ab) to clean the tissue and remove residual blood. The tissue was divided into smaller pieces in a laminar flow hood using sterile scalpel and forceps and washed three times in Dulbecco's Modified Eagle Medium, DMEM (Lonza; DMEM/Ab). Tissue was then dissociated enzymatically overnight at 37 °C in DPBS/Ab containing 0.07% collagenase type IA (Sigma Aldrich, Missouri, USA). The day after the digested tissue was collected and washed three times in DPBS/Ab, centrifuged (500 g, 3 min), and the pellet was resuspended in growth medium GM [DMEM/Ab supplemented with 10% heat inactivated fetal bovine serum (FBS), 2mM L-glutamine, 0.3% D-glucose] and plated in a 6 cm diameter Petri dish, incubated overnight at 37 °C. The day after the medium was removed and replaced with fresh GM. The adhering cells, classified as potential dolphin umbilical cord mesenchymal

stem cells (DUC-MSCs) were allowed to grow until nearly confluent for subsequent analytical steps.

The counting of disaggregated cells was performed using a hemocytometer on an inverted light microscope, using Trypan blue exclusion assay to check cell viability. Cells were plated on tissue culture plastic flasks in GM and incubated at 37 °C in 5% CO₂, 21% O₂ atmosphere. DUC-MSCs morphology was analyzed at passage 2 using ImageJ software (<http://imagej.nih.gov/ij/>) on images collected with a Leica DM 6000 light microscope supported by the Leica Application Suite (LAS) software v4.5 (Leica, Buffalo Grove, IL, USA). Cells were cultured for two passages in GM medium prior to plating and expansion in T25 followed by 3xT75 tissue culture polystyrene flasks. Cells were allowed to grow until nearly confluent for freezing procedure, preceded by cell trypsinization and counts. Vials of 10⁶ cells were subject to slow freezing (~1 °C/min) controlled by an isopropanol bath container (Nalgene/Thermo Scientific) in a –80 °C freezer. After a week cells were removed from the isopropanol bath container and stored in cryovials containers in the –80 °C freezer for a period ranging from 1 week to several months. Three cryovials of 10⁶ cells from passage 3 were shipped frozen from Valencia to Italy where they were used for karyotyping (University of Florence) and further expansion and RNA extraction (University of Ferrara). For thawing, cryovials were transferred from –80 °C freezer storage into a dry ice container, brought to a 37 °C water bath, and rapidly thawed with agitation at 37 °C.

RNA extraction and gene amplification

A total of 10⁶ cells from passage 4 were used for RNA extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. PCR were performed in 50 µl total reaction mix containing 10 ng cDNA, 19 µl nuclease-free water (Qiagen), 0.5 µM forward primers (*REX1F*, *NANOGF*, *SOX2F*, *OCT4F*), 0.5 µM reverse primers (*REX1R*, *NANOGR*, *SOX2R*, *OCT4R*) and 25 µl 2X DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, MA, USA) and run as follows: 1 cycle of 95 °C for 3 min, 30 cycles of 95 °C for 30 sec, 53 °C for 30 sec, 72 °C for 30 sec, 1 final cycle of 72 °C for 5 min. The primer sequences for *REX1*, *NANOG*, *SOX2*

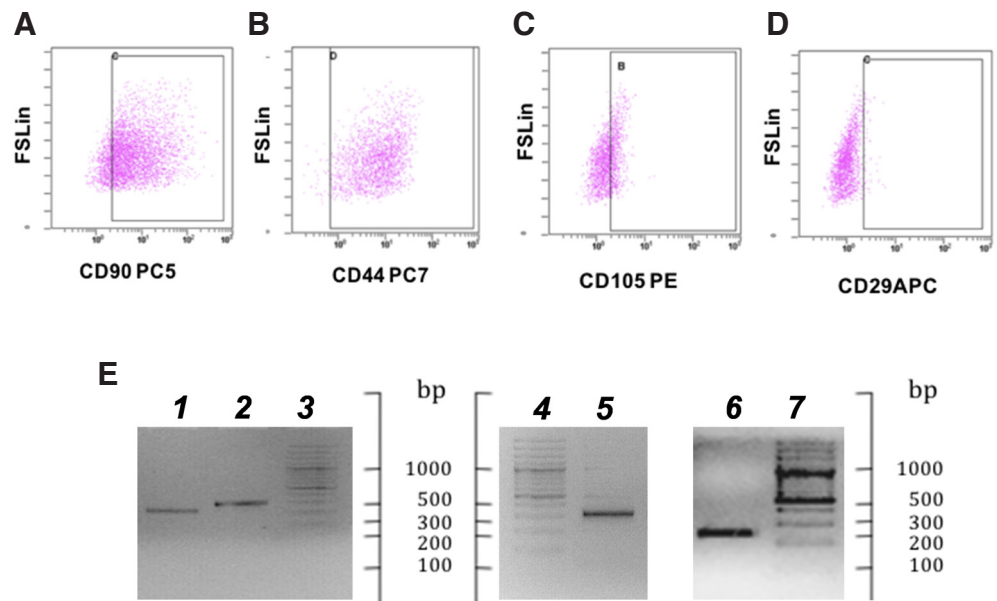


Fig. 2. Expression of stem cells markers in Dolphin umbilical cord mesenchymal stem cells (DUC-MSCs). FACS analysis of CD90-, CD44-, CD105-, and CD29-immunoreactive cells, boxed inside each plot (A-D). X axis, number of cells; Y axis, fluorescence measured by forward scatter FS light. (E) Agarose gel electrophoresis of amplification products of the gene markers NANOG (1) OCT4 (2), REX-1 (5) and SOX-2 (6) (lanes 3,4,7, DNA ladder, 100 bp).

and *OCT4* were designed on the gene-specific *T. truncatus* sequences obtained searching the dolphin genome database at www.ensembl.org [Acc.n. XM_004328739 (*REX1*), XM_004325830.1 (*NANOG*), XM_004311784.1 (*SOX2*), XM_004311235.1 (*OCT4*) at www.ncbi.nlm.nih.gov] (Table S1). PCR products were visualized by electrophoresis (120V for 25 min on 2% agarose gel), then quantified and Sanger sequenced (BMR Genomics, Padova, Italy). The sequences obtained were analyzed by MEGA6 software (<http://www.megasoftware.net/>) and BlastX (www.ncbi.nlm.nih.gov) to confirm gene identity.

Karyotyping

A total of 1.5×10^6 cells from passage 4 were subcultured from T25 flasks into three T75 flasks (Corning, Sigma-Aldrich Missouri, USA). Metaphases were harvested 48 hrs after dividing confluent flasks and used for karyotyping. The cells were processed as follows: 3 hrs treatment with 0.01 $\mu\text{g/ml}$ ColcemidTM, centrifuged (1200 rpm for 10 min) to remove supernatant, exposed for 20 min at 37 °C to a hypotonic treatment (10 ml, 1:1 mixture of 0.075M KC1 and 0.4% sodium citrate). The cells were prefixed by adding 500 μl fixing solution (3:1, methanol:acetic acid) and then pelleted by centrifugation and resuspended in 5 ml fixing solution. The fixing solution was changed two times by centrifugation and resuspension. Slides were prepared according to standard procedures. Briefly, after final centrifugation, cells were resuspended in small amount of fixative and dropped onto slides cleaned in ethanol with 1% HCl. The slides were aged by overnight incubation at 65 °C. Slides were GTG-banded followed Seabright (1971). Briefly, slides were incubated for 1.5 to 2 min in a 0.05% trypsin solution in PBS at pH 7.2. The slides were then rinsed in Hank's balanced salt solution (HBSS) for 15 sec and stained with 2% Giemsa solution in 10% Sorensen's buffer, pH 6.8, for 30 min.

Flow cytometry

A total of 3×10^5 cells from passage 4 was used for flow cytometry. Cells were pelleted and resuspended in warm medium minus non-essential amino acid, NEAA (Thermo Fisher Scientific, MA, USA)

to obtain three vials of 100 μl each. Five μl of FITC-labelled mouse monoclonal antibody anti-human CD45 (560976, BD Pharmingen Biosciences, San Josè, CA, USA) were added to vial 1; 5 μl of phycoerythrin (PE)-labelled mouse monoclonal antibody anti-recombinant vaccinia virus containing human CD105 (L-isoform) cDNA (ab53321, Abcam, Cambridge, UK), 5 μl of PE-Cy7 labelled mouse monoclonal antibody anti-human CD44 (560533, BD Pharmingen Biosciences), and 5 μl of allophycocyanin (APC)-labelled mouse monoclonal antibody anti-purified human $\alpha 5\beta 1$ fibronectin receptor (CD29) (561794, BD Pharmingen Biosciences) were added to vial 2; 5 μl of R- phycoerythrin covalently linked to cyanin 5.1 (PC5) mouse monoclonal antibody anti-CD90 (PN IM3703, Beckman Coulter) were added to vial 3.

All vials were incubated for 1 h at room temperature, in the dark. Then 1 ml DPBS was added to each vial and were centrifuged (300 x g for 5 min), the pellet was resuspended in 500 μl fresh DPBS and covered with aluminum foil prior analysis in Cytomics FC 500 (Beckman Coulter, Brea, CA, USA). The antibodies used in this experiment were previously tested and validated (Johnson *et al.*, 2012, Griffith *et al.*, 2014).

A total of 1.5×10^9 cells from passage 3 were resuspended in cryopreserving medium [CM: Dulbecco's Modified Eagle Medium (DMEM), Invitrogen], supplemented with 10% high quality dimethyl sulfoxide and 10% FBS (Sigma Aldrich, Missouri, USA) and slowly frozen (-1 °C/min) controlled by an isopropanol bath container (Nalgene/Thermo Scientific) in a -80 °C freezer.

Results and Discussion

Cells with stemness properties were successfully isolated and characterized from dolphin umbilical cord (DUC-MSCs). The cells grew well for up to 9-10 passages in culture before senescence (Fig. 1). They were adherent to plastic with a spindle morphology defined by their circularity and axial *ratio* (major/minor axis) (0.310 ± 0.096 and 2.93 ± 1.28 , respectively); average spread area was $1812 \pm 448 \mu\text{m}^2$ (Fig. 1). Throughout the culture process, the cells retained their characteristic spindle shape and reached 80–90%

confluence with a doubling time of seven days. The shape and size of DUC-MSCs were similar to the adipose tissue derived SCs from dolphin and other marine and terrestrial mammals (Johnson *et al.*, 2012; Hoogduijn *et al.*, 2013; Griffeth *et al.*, 2014). Differently from other MSCs described in the same or other species, DUC-MSCs grew very slowly, suggesting species-specific characteristics or, most likely, the need for optimization of media composition and/or culture parameters, which are currently standardized on cultured cells of terrestrial mammals.

DUC-MSCs were also characterized for number and morphology of chromosomes to exclude aberrations due to culture conditions. The G-banding pattern confirmed the normal karyotype ($2n = 44$) previously described in *T. truncatus* (Stephen *et al.*, 2015) (Fig. 1, C). Karyotyping also showed the male gender (XY) of the dolphin calf born in Valencia.

Typical SC-specific markers, such as Rex-1, Sox-2, Oct-4, and Nanog, were notably expressed. Subamniotic MSCs did not lose Oct-4 and Nanog expression after freeze-thawing. Moreover, significant expression of typical SC specific markers was observed. FACS analysis on DUC-MSCs for CD90, CD44 and CD105 are shown in Figure 2 (A-C). These surface protein markers were specifically expressed on 85% cells (CD90⁺), 99% (CD44⁺) and 35% (CD105⁺). Differently from what is described in human MSCc umbilical cord derived the fraction of CD29⁺ was much lower (3%) (Fig. 2, D) (Carrade and Borjesson, 2013; Tong *et al.*, 2011).

The expression of SC markers was also verified at transcriptomic level to investigate the gene expression mechanisms regulating the pluripotency property of the cells. Specifically, *REX1*, *OCT-4*, *NANOG* and *SOX2* were chosen from literature as marker of stemness (Loh *et al.*, 2006; Zhang and Cui, 2014; Lamoury *et al.*, 2016). *OCT4* (octamer-binding transcription factor 4 or *POU5F1* - POU domain, class 5, transcription factor 1) and *NANOG* are transcription factors required to maintain the pluripotency and self-renewal of embryonic SC through the control of a cascade of pathways that are intricately connected to govern pluripotency, self-renewal, genome surveillance and cell fate determination. *SOX2* (SRY sex determining region Y - box 2), a member of the SoxB1 transcription factor family, is an important transcriptional regulator in pluripotent SCs. Together with *OCT4* and *NANOG*, *SOX2* co-operatively controls gene expression in pluripotent SCs and maintain their pluripotency. Furthermore, *SOX2* plays an essential role in somatic cell reprogramming, reversing the epigenetic configuration of differentiated cells back to a pluripotent embryonic state (Zhang and Cui, 2014). Amplification products had the expected size for *REX1* (318 bp), *NANOG* (232 bp), *OCT4* (267 bp) and *SOX2* (244 bp) (Fig. 2, E). Sequencing confirmed the identity of amplified products. Following expansion at passage 4, DUC-MSCs were slowly frozen in CM as previously described (Mancía *et al.*, 2012) and subsequently thawed, ensuring the safety and efficacy of cryopreservation and establishing the prospect of SC banking. The use of SCs is a promising novel approach for the treatment of many diseases and injuries in veterinary care. UC membrane represents a valuable source for MSCs, because they are abundant sources and there are less ethical issues unlike embryonic SCs. Here we describe the first isolation of potential stem cells from BD-UC. These could provide a safe and efficient treatment for tissue injuries, and therapies offering a great alternative to more invasive, time-consuming isolation/proliferation of autologous MSCs from a live dolphin.

Conclusions

The protocol presented here is a practical alternative to more invasive and time-consuming methods for isolation/proliferation of autologous mesenchymal stem cells from live individuals. The availability of an easily renewable source of dolphin stem cells from marine parks could aid the investigation of the unique and specific properties of mesenchymal stem cells and all derived cell lines in dolphin vs human, whilst being novel *in vitro* models of cell and tissue differentiation, for morphogenesis and comparative developmental biology studies. These research avenues have potential biomedical applications in regenerative medicine and the treatment of immune disorders.

Competing interests

The authors declare no competing financial interest.

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Authors' contributions

AM was a major contributor in experimental design, data acquisition, statistical analysis and writing of the manuscript. CG, DGP and BB were major contributors to sample retrieval and collection. VMM, MM and RS were contributors for data acquisition and analysis. LA was contributor in data interpretation and critical analysis of the manuscript. All authors have read, reviewed and approved the final manuscript.

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Ethics approval

The umbilical cords were collected from three *Tursiops truncatus* females maintained in zoological institutions operating within the zoo directive framework (EU 22/1999) in Italy and Spain that regulates husbandry and management of zoo animals. The samples were collected without physical manipulation or disturbance of the females and their calves, and utilized for the routine veterinary checks of the placentas after birth.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. Samples (e.g. DUC-MSCs) are available from the corresponding author on reasonable request.

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