Research in Veterinary Science 182 (2025) 105471

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Establishment and characterization of the Cuvier's beaked whale (*Ziphius cavirostris*) myogenic cell line

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ARTICLE INFO

Keywords:
Ziphius cavirostris
Cetacean
Myogenic cells
In vitro model

ABSTRACT

In this study we present the first *in vitro* model based on a muscle-derived cell line from a male Cuvier's Beaked whale stranded along the Italian coastline.

In the myogenic cell line at T0 stage we performed the growth rate assay, the karyotyping, the CBA chromosome banding, G banding, nucleolar organizer regions and telomere analysis and immunocytochemical analysis. The cell characterization at the T2 and T4 stage included: assessment of fusion index, the ultrastructural analysis and immunocytochemical analysis.

The population doubling time was determined to be ~54 h. The cell fusion index at the T2 stage was 8.2 +/− 5 %, at the T4 stage increased at index 28 +/− 10 %. The karyotype analysis revealed a 2n = 42, XY, two pairs of nucleolus organizer regions (NORs), characteristic CBA-banding and PNA-telomeric regions by FISH-mapping.

The immunocytochemical results revealed that we generated a heterogeneous population of myogenic cells. A cell population express β-actin, myosin and vimentin and a sub-population of cells was desmin-positive.

In details, at the T0 stage, both cell populations were undifferentiated mononucleated myoblast. At the T2 and T4 stage, the cells were capable of fusion in elongated multinucleated myotubes, probably resulting from the fusion of the myoblasts.

This Cuvier's Beaked whale cell line, represents a new opportunity to better understand the physiological features of the cetacean *Ziphius cavirostris* myogenic cells. The possibility of setting up culture conditions that mimic the *in vivo* microenvironment, strengthen the importance of *in vitro* models for toxicological studies investigating water pollutants effects on cells.

1. Introduction

Cuvier's beaked whale (*Ziphius cavirostris*), the only member of the Ziphiidae family, is a deep diver marine mammal capable of reaching depths of more than 3000 m and hold their breath longer than any other cetacean (Shearer et al., 2019); observations by scientists suggest that the median duration of foraging dives is around 60 min before the animal returned to the surface to recover (Quick et al., 2020).

Cuvier's beaked whales, as top predators, are exposed constantly to widespread persistent organic pollutants (POPs), capable of long-range transport, persist and to bio-accumulate, thereby causing adverse effects to the marine environment and its living organisms (Green and Larson, 2016; Fair and Houde, 2018; Remili et al., 2020). Recently, the decreasing population trend has led to the International Union for Conservation of Nature (IUCN) categorizing the Mediterranean subpopulation of Cuvier's beaked whale as vulnerable on the red list of threatened species (IUCN, 2018).

Cuvier's beaked whale is one of the least known cetacean species worldwide due to its diving behavior, offshore distribution, and limited time spent at the surface remains relatively understudied (Shearer et al., 2019).

Marine mammals are a remarkable group that provide invaluable insights into the mechanisms of evolution, particularly through their unique physiological adaptations providing new insights into the

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https://doi.org/10.1016/j.rvsc.2024.105471
Received 1 October 2024; Received in revised form 20 November 2024; Accepted 21 November 2024
Available online 26 November 2024

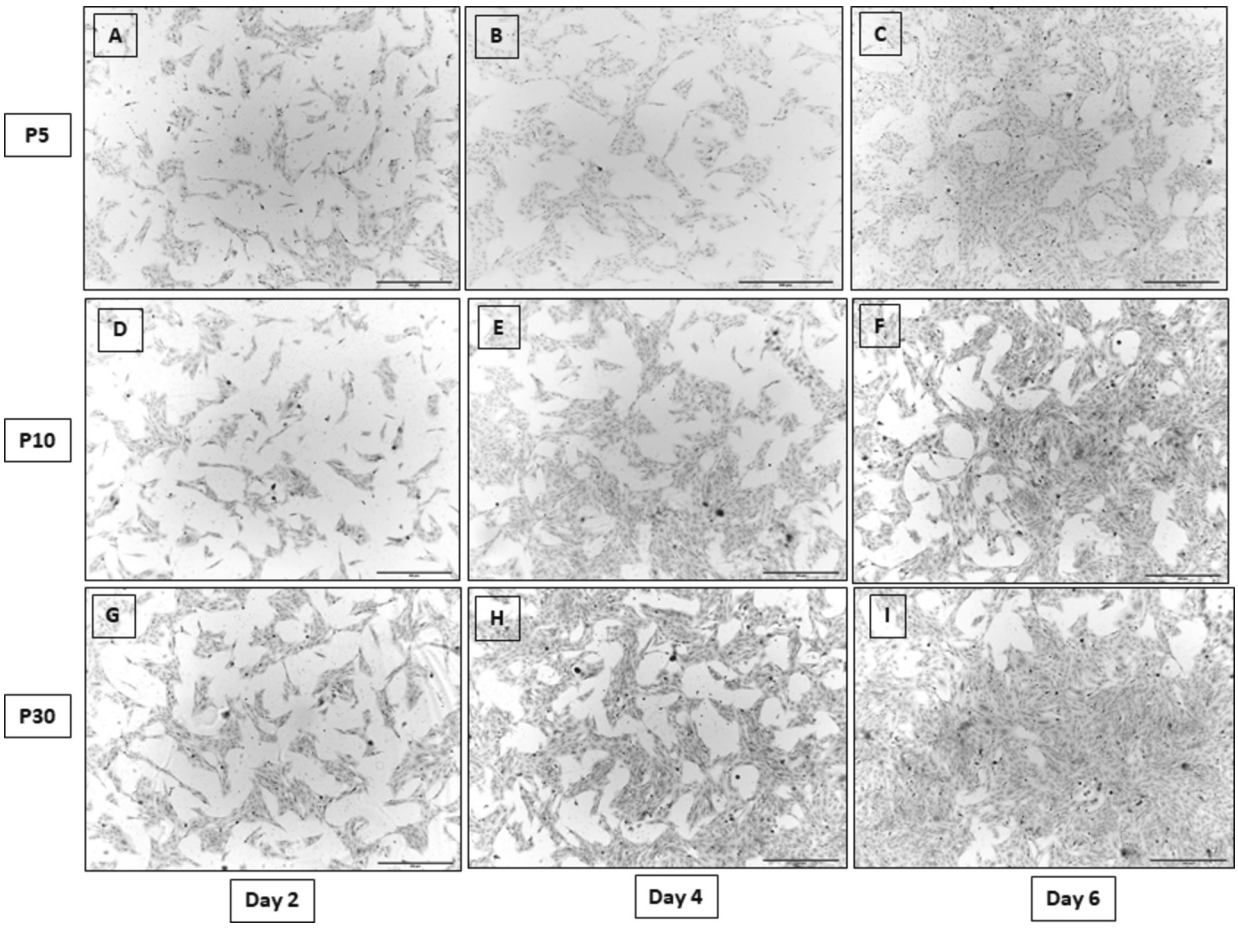

Fig. 1. Optical phase contrast images showing the morphologic development of the myogenic cells after immortalization of the cell line at diverse passages. Cells were seeded at 5 × 105 cells/ml in standard serum-rich medium (10 % FBS). In A, B, and C, images of cells at the passage P5 after 2, 4 and 6 days in culture respectively. In D, E, and F, images of cells at the passage P10 after 2, 4 and 6 days in culture respectively. In G, H, and I, images of cells at the passage P30 after 2, 4 and 6 days in culture respectively. As displayed in all the images, cells often align in parallel forming tight clusters, reflecting their muscle precursor nature. During the passages (P5, P10, P30 and more), cells retained their myogenic characteristics, such as the ability to proliferate rapidly while maintaining a mononuclear phenotype. Scale bar 500 μm.

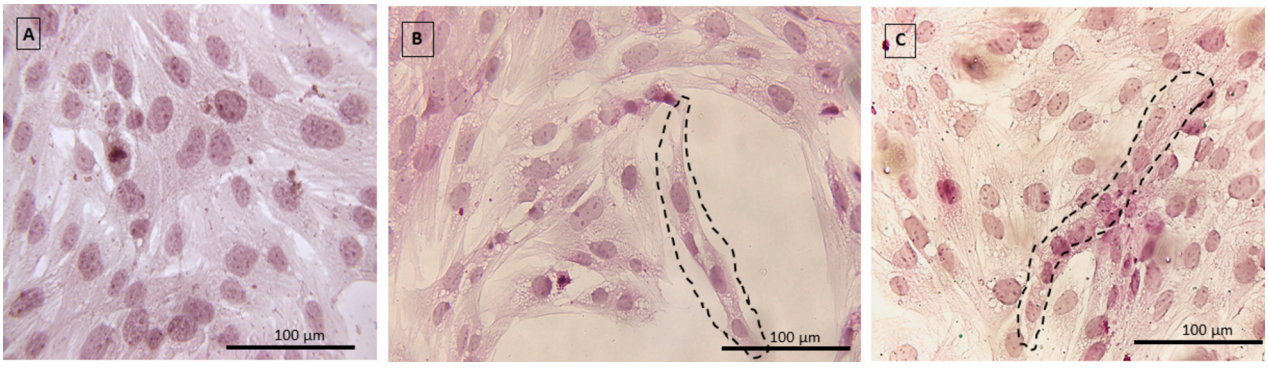

Fig. 2. Hematoxylin and eosin staining of Cuvier's' beaked whale myogenic cells in culture. In **A**, T0 stage, myogenic cells after 2 days in culture with 10 % of FBS; cells showed spindle shaped body, appeared mononucleated with the presence of central rounded nucleus; In **B**, T2 stage, myogenic cells after 2 days in culture with 1 % of FBS; black dotted line surrounds a multinucleate cell with elongated tubular shape. It is possible to note as cells start to aggregate leaving empty spaces on the well. In **C**, T4 stage, myogenic cells after 4 days in culture; with 1 % of FBS; black dotted line surrounds a multinucleate cell with elongated tubular shape. The cell fusion process appeared more evident and the cells became visible in groups with nuclei oriented along common direction.

mechanisms driving evolution (Lam et al., 2020). The establishment of our myogenic models obtained from the marine mammal *Ziphius cavirostris*, provide new system to study the molecular bases of the extreme physiological adaptations, as the ability to tolerate extreme hypoxia (low oxygen levels) and endure prolonged periods of food deprivation (Torres-Velarde et al., 2021, Allen et al., 2024). From this perspective, our *Ziphius cavirostris* cells model provides a new font of biological material to study the molecular basis of the extreme physiological adaptations evolved by Cuvier's beaked whale.

It must consider that in the marine environment, the POPs presence

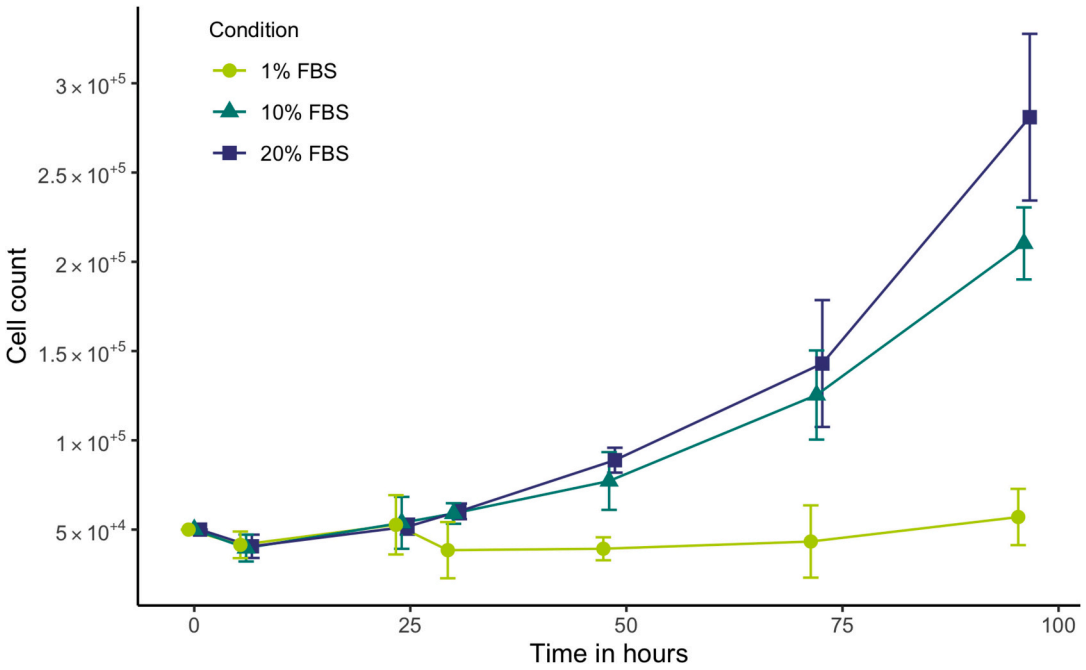

Fig. 3. Proliferation kinetics growth curves for Cuvier's beaked whale muscle-derived cells maintained in the culture media with FBS at the concentration of 1 %, 10 % and 20 % respectively, for 100 h of culture. Cells were counted after 6, 24, 30, 48, 72 and 96 h of culture. Data are presented as mean ± S.E.M ($N = 3$) of the number of cells at each time point; plotted in linear scale.

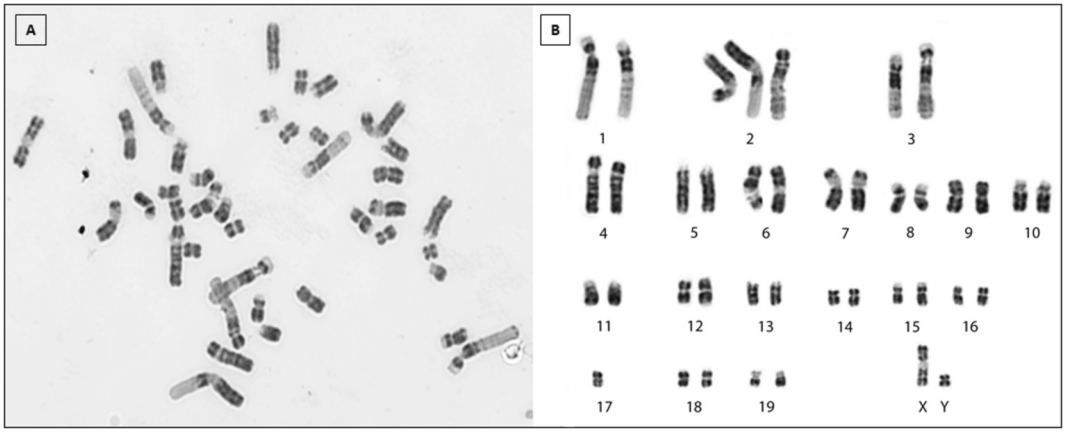

Fig. 4. GBG-banded metaphase (A) and relative karyotype (B) of the *Ziphius cavirostris* cells (2n = 42, XY).

represents a threat for cetacean species. For these reasons, dolphins and whales are considered sea sentinels of the status of marine environment and are protected under a wide legislative framework (CITES, 1973).

In this context, in Cuvier's beaked whale, only few post-mortem investigations have been carried out to determine the concentration of heavy metals (Hg, Se, Cd, Pb and Cr) and of methylmercury, demonstrating that high levels of total mercury and selenium were present in the liver, while a maximum concentration of cadmium was found in the kidneys (Martoja and Viale, 1977; Storelli et al., 1999). Recently, an ecotoxicological POPs assessment in Mediterranean animals was performed on skin biopsies revealing that the pattern of concentration for the polychlorinated biphenyl (PCBs) and for the polybrominated diphenyl ethers (PBDEs), were linked to age and sex, with adult males showing significantly higher levels than juvenile and 80 % of the individuals had PCB levels above the toxicity threshold (Baini et al., 2020). No studs have been performed in Cuvier's beaked whale *in vitro* model.

Despite the findings that these toxicants accumulate in the tissues of this species, the knowledge on their effects in the cellular physiology of marine mammals is still very poor, due to the impossibility of *in vivo* studies. The cell line we established, represents a new opportunity to understand normal physiology of cells or to investigate toxic mechanisms due to waters pollutants in the Cuvier's beaked whale myogenic cells.

2. Methods and materials

2.1. Cell isolation and cell line establishment

Primary cell cultures derived from fresh skeletal muscle tissue samples collected during the *post-mortem* examination of an adult male Cuvier's Beaked whale.

The animal died few hours after coast guard found it stranded in a beach along the Tuscany coastline (Livorno, Italy). The animal was and adult male, 5.3-m-long, 3800 kg weight. Two hours and half passed from the animal death to sample collection during the post-mortem

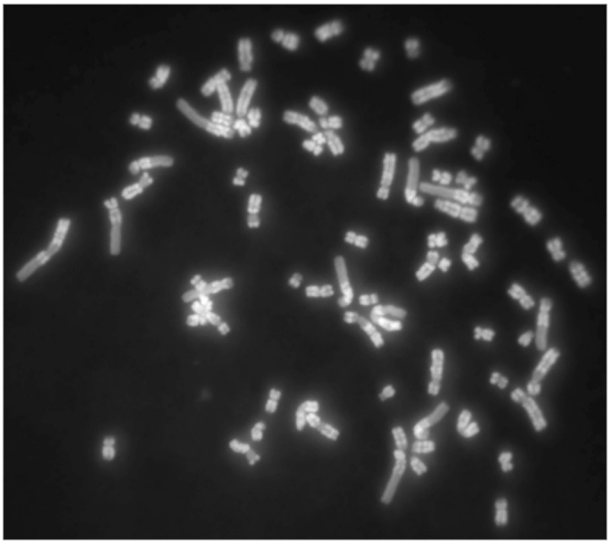

Fig. 5. Tetraploid *Ziphius cavirostris* QBH-banded metaphase (2n = 84).

examination in a clean and protected location.

The samples of muscle tissue were dissected under sterile conditions from a portion of the skeletal muscle (*Longissimus dorsi*) and placed in sterile phosphate buffered saline (PBS). Samples were minced into small fragments and washed in the PBS solution.

Fragments of muscle tissue were suspended in an ice-cold cell freezing medium composed of Dulbecco's modified Eagle's medium (DMEM) with 1 % HEPES (pH 7.4) plus 10 % FCS, and 10 %.

dimethylsulfoxide (DMSO). Cryotubes were slowly cooled to −80 °C. To perform primary cell cultures, frozen tissues were rapidly thawed in a water bath at 37 °C and minced into small fragments.

To dissociate cells, we used the papain dissociation system kit (Worthington Biochemical Corporation, Lakewood, NJ, USA) following the manufacturer instruction. Fragments of muscle tissue were introduced in 15 ml falcons containing the mixture with the papain and dissociated at 37C° under gently mechanical agitation for 2 h. Cells solution were than centrifuged at 2000 rpm. Cells were then suspended in a medium composed of Dulbecco's modified Eagle's medium (DMEM) consisting of a 1:1 mixture of DMEM and Ham's F-12 (Biowest®) supplemented with penicillin (30 mg l − 1), streptomycin (50 mg l − 1) (Pan Biotech™) and 10 % fetal bovine serum (FBS Good, Pan Biotech™). Cells were plated on glass coverslips, previously coated with poly-L-lysine (Sigma-Aldrich, Milan, Italy), at a density of 5×10^5 cells/ml of medium. Cells were maintained in an incubator under standard conditions at 37 °C with 5 % CO2 and humidified atmosphere. Cell culture medium was replaced with fresh media 24 h (h) after and every 2–3 days until cells reached 80 % confluence.

To obtain the immortalized cell line, primary cell cultures were transfected with pSV3neo plasmid (**ATCC** LGC Promochem, Teddington, UK) using GenJet™ *In Vitro* DNA Transfection Reagent (Ver. II, SignaGen® Laboratories) following the manufacturer instructions. Afterward, the selection medium was added and resistant cells were selected with the antibiotic G418 (400 μg/ml; Gibco, Life Techologies BRL).

To validate the cetacean species (*Ziphius cavirostris*) and confirm cells immortalization, short tandem repeat (STR) genetic profile analysis and polymerase chain reaction (PCR) analysis have been performed by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany.

2.2. Cell Cryopreservation and thawing

Immortalized myogenic cells were cryopreserved at −80 °C in a cryopreservation medium consisting of a mixture of 90 % (*v*/v) FBS and 10 % (v/v) dimethyl sulfoxide (DMSO). For recovery of the cells, cryovials were rapidly thawed in the water bath at 37 °C and cells were suspended in cell culture medium and seeded in culture flasks.

2.3. Proliferation kinetics growth curves

In the Cuvier's beaked whale myogenic cell line at T0 stage we performed the growth rate assay.

To determine the population doubling time (PDT), cells were seeded in 24 well plates at a density of 0.5 × 106 cells/well. Seeded cells were maintained in cell culture media with FBS concentrations of 1, 10 and 20 % respectively and counted after 6, 24, 30, 48, 72 and 96 h of culture respectively (3 replicates per condition each time). This procedure was repeated 3 time. Cell viability was determined using the Trypan Blue exclusion assay. The mean cell counts at each time point were then used to plot the growth curves, and the population doubling time (PDT) in h was calculated from the obtained growth curve as:

$$PDT = \frac{\Delta t}{log_2\left(\frac{\Delta N}{N_0} + 1\right)}$$

N_0 is the number of seeded cells at starting time (time-0); ΔN is the increase in number of cells during the period of time Δt (time-48 h).

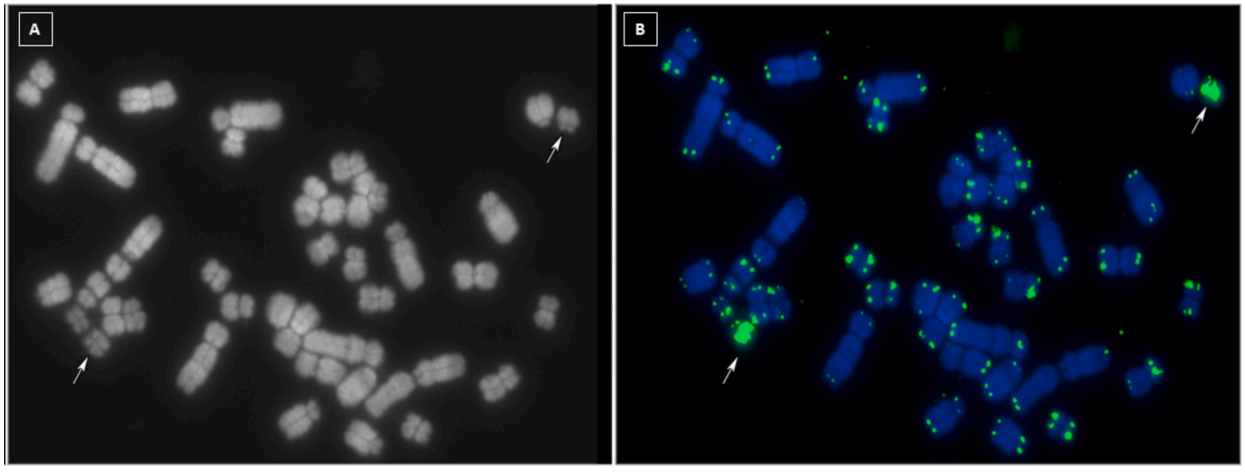

Fig. 6. In **A**. Sequential QBH-(A) and in **B.** FISH-mapping with PNA telomeric probes showing positive signals in all telomeric regions of all chromosomes. Note the strong signals in a small chromosome pair (arrows).

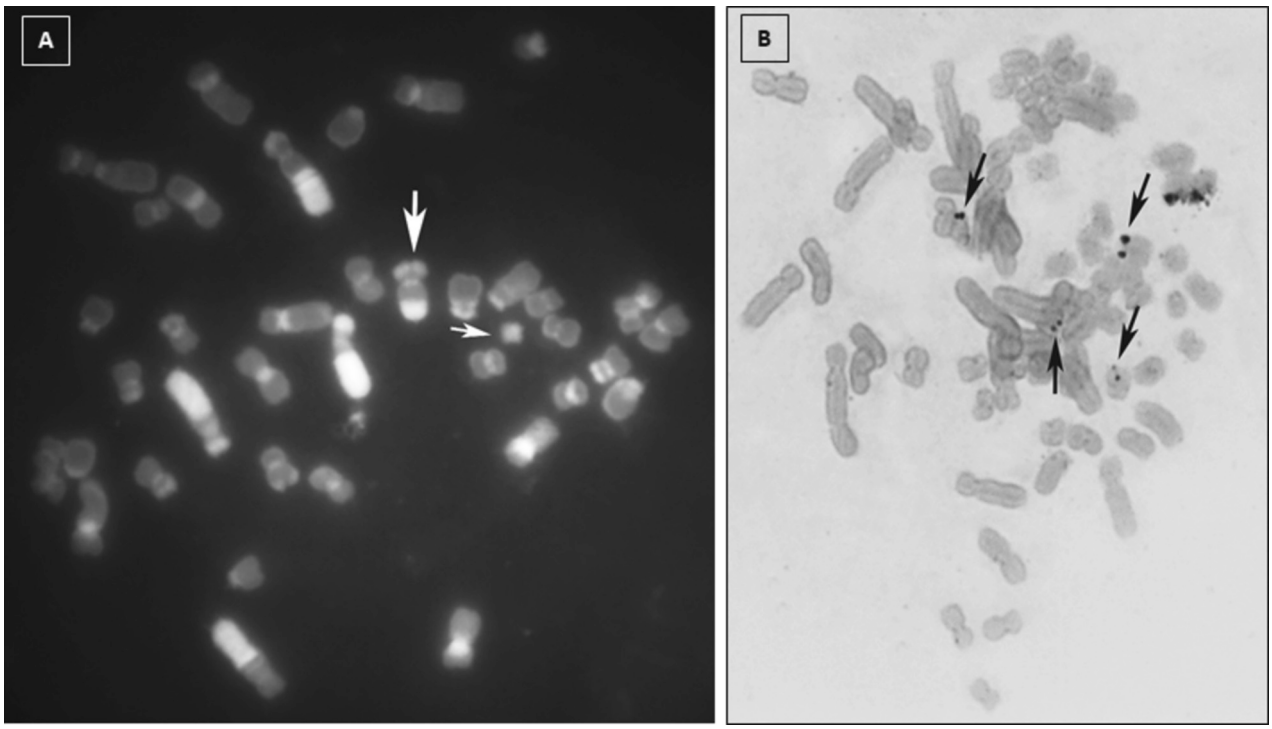

Fig. 7. In **A.** CBA-banding patterns in a metaphase plate of a male *Ziphius cavirostris* (2n = 42, XY). Note the large HC-blocks in several chromosomes, including the X (large arrow). The small Y-chromosome (small arrow) appears entirely heterochromatic but with low fluorescent C-banding. In **B.** A metaphase of *Ziphius cavirostris* treated for Ag-NORs technique. Clear NORs were located in two small chromosomes (arrows).

2.4. Karyotype analysis

In the myogenic cell line at T0 stage we performed the karyotype analysis.

Confluent fibroblasts were transferred from T25 flasks to T75 flasks by changing the medium and adding 5-BrdU (15 μg/ml). The following morning, the medium was removed from all flasks, the cells were washed twice with physiological solution, and fresh medium containing Thymidine (10 μg/ml) was added. After six hours, all cells were harvested following one hour of colcemid (0.1 μg/ml) treatment. This treatment facilitates the incorporation of BrdU exclusively in early-replicating regions (G-C-rich – euchromatin). No trypsin treatment was performed to remove the cells. Instead, a gentle yet effective tap was administered to each flask, removing only cells in mitosis. The medium and cells were then transferred into 15 ml tubes and centrifuged at 1000 rpm for 8 min. The supernatant was discarded, and a hypotonic solution (0.5 % KCl) was added. The cells were centrifuged again, and a fixative solution (Methanol/Acetic Acid 1:3) was added. After briefly vortexing the tubes, a second round of centrifugation and cell fixation was performed. Subsequently, two drops of the cell pellet were spread on clean, humid slides. The slides were treated with three different banding techniques: GBG-banding, QBH-banding, CBA-banding, and the Ag-NOR technique, as well as with the FISH-mapping technique (Iannuzzi and Di Berardino, 2008). The slides were examined using the cytogenetic workstations I (Leica systems) equipped with fluorescence microscopes, CCD cameras, and PCs with appropriate software (Leica).

2.5. Transmission Electron Microscopy (TEM) observation

In the myogenic cell line at T0 and T4 stage was performed the ultrastructural analysis. Immortalized cells at the T0 and T4 stage were seeded in 6-wells plates. At confluence, cells were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C. Samples were post-fixed with a mixture containing 1 % osmium tetroxide and 1 % potassium ferrocyanide in a 0.1 M sodium cacodylate buffer for 1 h at 4 °C. After three washes with water, samples were dehydrated by immersion in increasing concentrations of ethanol and embedded in epoxy resin (Sigma-Aldrich). Ultrathin sections (60–70 nm) were obtained with an Ultrotome V (LKB) ultramicrotome, counterstained with uranyl acetate and lead citrate. Samples were observed with a Tecnai G 2 (FEI) TEM operating at 100 kV and images were acquired with a Veleta digital camera (Olympus Soft Imaging System).

2.6. Cell differentiation, morphological analysis and cell fusion index

Immortalized cells were analyzed after 2 days in culture with 10 % of FBS (T0 stage). Moreover, to induce differentiation, after 24 h in culture (80 % cell confluence) cells were placed in the differentiation medium containing 1 % of FBS and analyzed after 2 days in culture (T2 stage) and 4 days in culture (T4 stage) respectively.

To estimate the cell fusion index, hematoxylin & eosin staining was performed for 10 min in the cells. Cells were considered fused if they contained two nuclei within one cytoplasmic continuity. By light microscope, the fusion index was determined in T2 and T4 myogenic cells as number of fused nuclei divided by the total number of nuclei in multiplied for 100 (100× objective) in 10 randomly fields (Lawson and Purslow, 2000).

2.7. Immunocytochemical analysis

Immunocytochemical analysis of cells was performed at the T0, T2 and T4 stage. After fixation with 4 % paraformaldehyde, cells were permeabilized with 0.1 % Triton X-100, treated with 5 % BSA in PBS 1× for 30 min to block endogenous nonspecific sites and then incubated overnight with the following primary monoclonal mouse antibodies: anti- vimentin (GeneTex Catt# GTX100619, dilution 1:200), anti-cytokeratin (Genetex, Clone C-11, Cat# GTX27753, dilution 1:100); anti-endothelial NO synthase (e-NOS; Abcam, dilution 1:250); anti-β-actin (Sigma-Aldrich, Clone AC-74, Cat# A2228, dilution 1:500); anti-desmin (Dako, Clone D33, Cat# M0760, dilution 1:100); anti-desmin

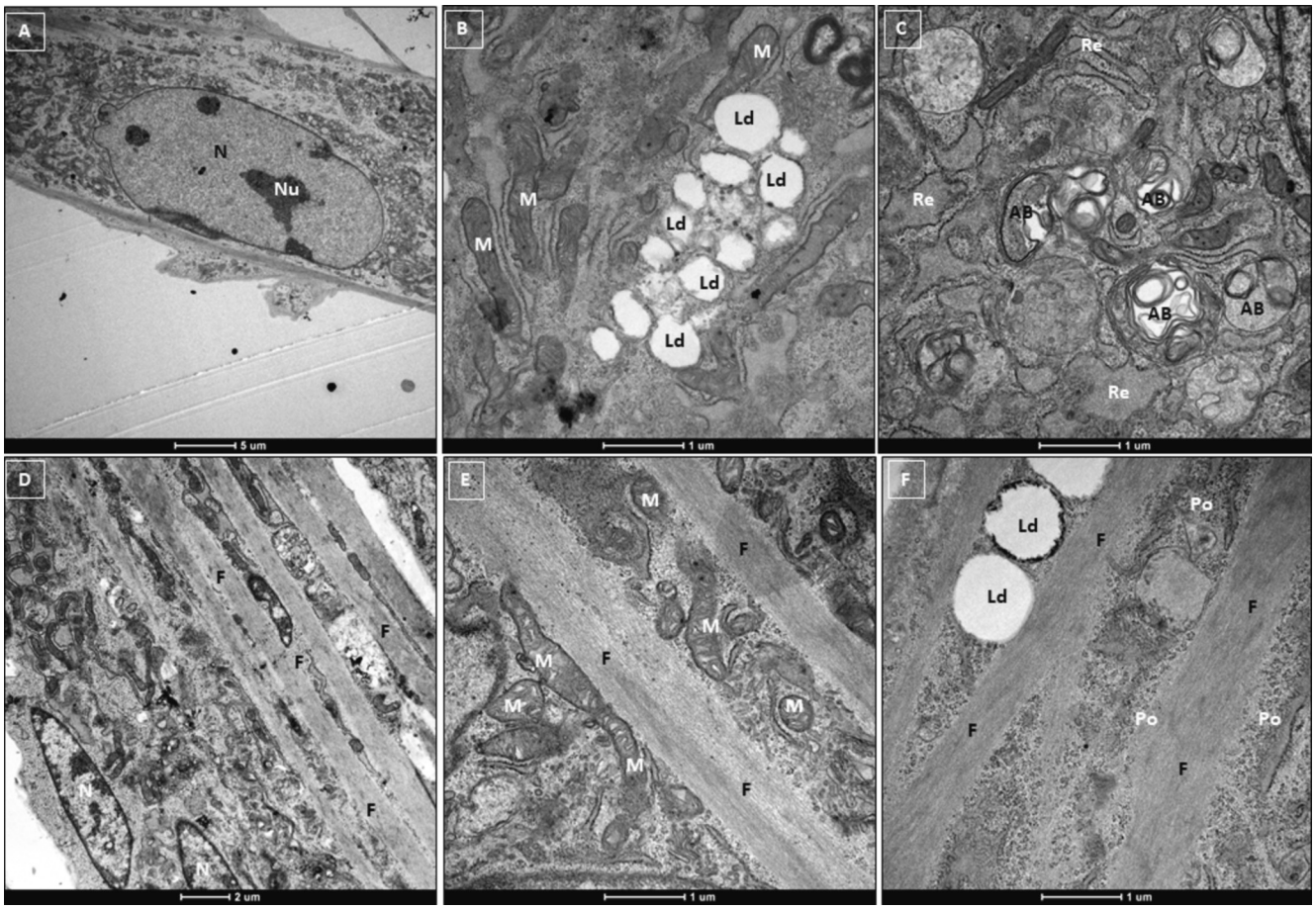

Fig. 8. Details of the ultra-structure of the Cuvier's beaked whale T0 and T4 cells. In **A**, image of a T0 myogenic cell section showing the nucleus (N) containing the nucleolus (Nu) surrounded by abundant cytoplasm (scale bar 5 μm). In **B**, detail of the cytoplasm containing a few grouped lipid droplets (Ld) and numerous mitochondria (M), (scale bar 1 μm). In **C**, detail of cytoplasm occupied by numerous autophagic bodies (AB) and cisternae of rough endoplasmic reticulum (Re), (scale bar 1 μm). In **D**, details of T4 myogenic cells showing the cell nuclei (N) surrounded by abundant cytoplasm, note the peripheral cytoplasm characterized by numerous filaments (F), (scale bar 2 μm). In **E**, detail of filaments (F) and interspersed numerous mitochondria (M), (scale bar 1 μm). In F, detail of cytoplasm enriched of filaments and grouped lipid droplets (Ld) and polyribosomes (Po), (scale bar 1 μm).

(Sigma, Cat# D 8281, dilution 1:100); anti-myogenin (Millipore, Cat#MAB3876, dilution 1:100) and anti- myosin (Sigma-Aldrich, Clone NOQ7.5.4D, Cat# M8421, dilution 1:100).

Cells were then incubated for 2 h with the secondary antibodies Alexa Fluor 488 (Invitrogen, goat anti-mouse IgG (H + L), Cat# A32733, dilution 1:500) and Alexa Fluor Plus 647 (Invitrogen, goat anti-rabbit IgG (H + L), Cat# A32733, dilution 1:500). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich). Blank controls were performed by substituting primary antibodies with bovine serum albumin in phosphate-buffered saline. Finally, the labeled cells were analyzed using the confocal microscope (Leica TCS SP5) and the high content screening microscope (Operetta, PerkinElmer®). Negative controls were performed by substituting primary antibodies with bovine serum albumin in PBS. Substitution of the primary antibodies with bovine serum albumin completely abolished immunostaining.

3. Results

3.1. Culture characteristic, differentiation and fusion index of Cuvier's beaked whale myogenic cells

Immortalization of *Ziphius cavirostris* cell line was confirmed by standard polymerase chain reaction (PCR) analysis performed by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Germany.

In culture, the immortalized myogenic cells formed a monolayer and demonstrated a higher proliferation rate. Morphologically, the myogenic cells resembled the non-immortalized cells under standard culture conditions. They exhibited a tendency to grow in tight clusters, often displaying clustering behavior (Fig. 1).

These myogenic T0 cells were mononuclear These cells showed round or elongated nucleus surrounded by abundant cytoplasm (Fig. 2A). At the T2 stage, cells started to aggregate (T2, fusion index 8.2 +/− 5 %). At the T4 stage, cells fusion increased (T4, fusion index 28 +/− 10 %). The appearance of cells in both T2 and T4 stage displayed fusiform elongated shape, cells started to align with one another and fuse itself, showing by tubular shape and the presence of polynucleated myotubes, probably resulting from the fusion of the myoblasts (Fig. 2B and C).

The Cuvier's Beaked whale muscle-derived cell line belongs to the patent Sea Sentinels System (S.S.S.), (patent n° 102,020,000,003,248; https://www.knowledge-share.eu/en/patent/sea-sentinel-system-for-environmental-studies/).*Ziphius cavirostris* cell line.

3.2. Growth kinetics results

Growth properties of Cuvier's beaked whale cell lines were stable and no growth reduction was observed to date (P30) under standard conditions for mammalian cell cultures (37 °C and 5 % CO_2). The growth curves of Cuvier's beaked whale cells generated in our study showed an

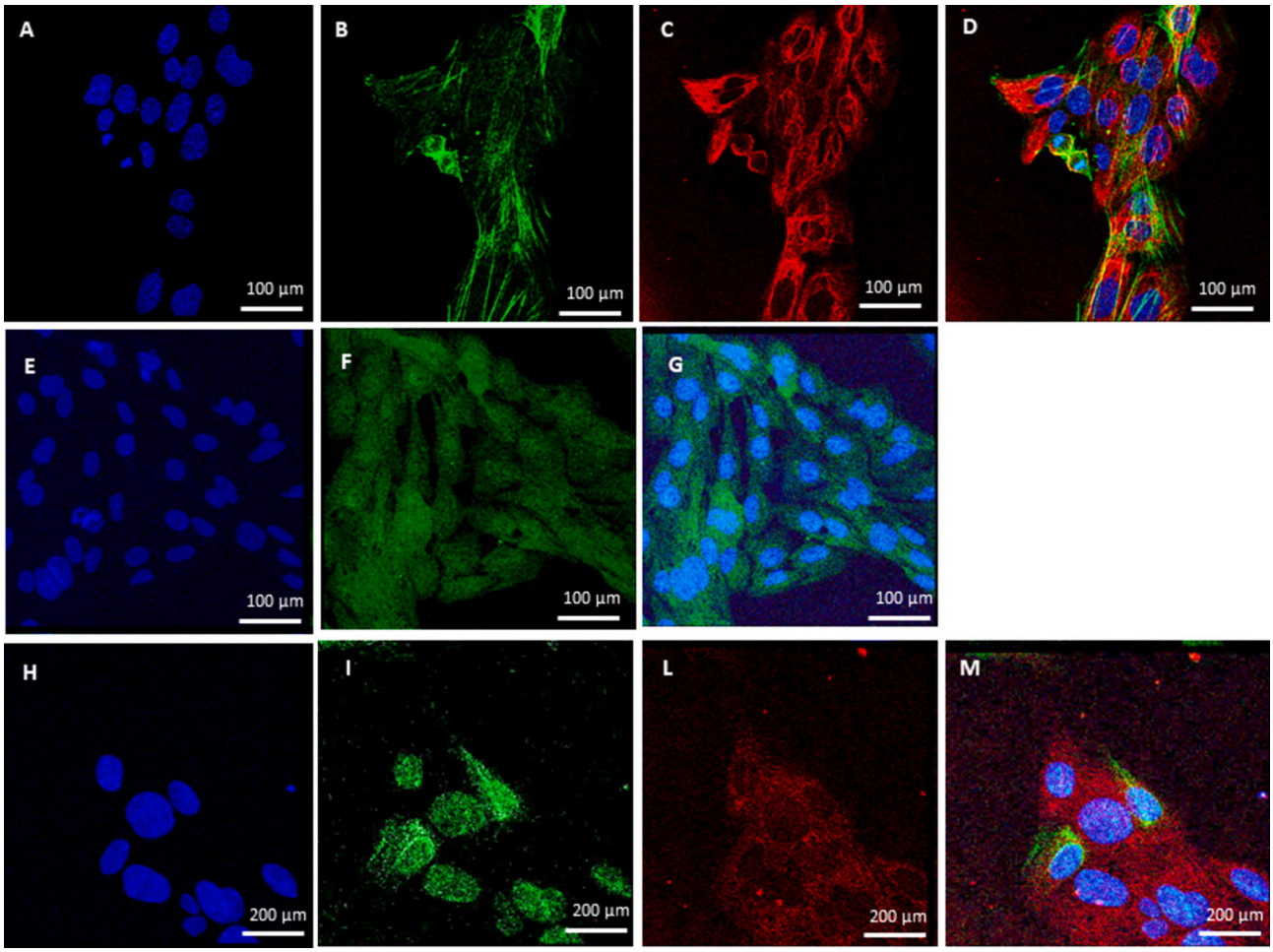

Fig. 9. Confocal images of immunocytochemical detection at the T0 stage in Cuvier's beaked myogenic cells. In **A** Hoechst-staining cell nucleus, in **B** actin-ir thin filaments in the cytoplasm of cells, in **C** vimentin-ir intermediated filaments in the cytoplasm of cells, in **D** superimposed image of A, B and C image. In **E** Hoechst-staining cell nuclei, in **F** myosin-ir thick filaments in the cytoplasm of cells, in **G** superimposed image of E and F images. In **H** Hoechst-staining nuclei, in **I** desmin-ir intermediated filaments in the cytoplasm of cells, in **L** vimentin-ir intermediated filaments in the cytoplasm of cells, in **M** superimposed image of H, I and L images.

initial phase (until 24 h) in which cells grew equally, independently from the FBS concentrations used (1 %, 10 % and 20 %). Then, 10 % and 20 % FBS promoted cell grow exponentially until 96 h while cell cultures maintained with FBS 1 % presented no growth, reaching a plateau-phase. Cells continued to grow exponentially even after reaching confluence of ~80 %, generating multiple cell layers when over-confluent. Cell culture doubling time was calculated to be ~54 h (Fig. 3).

3.3. Karyotype analyses

T0 cell metaphases treated for GBG-banding were used for karyotype analysis which revealed 2n = 42, XY (Fig. 4). About 5 % of cells examined showed the presence of tetraploid cells (2n = 84) (Fig. 5).

A sequential QBH-banding GBA-banding and FISH-mapping with PNA-telomeric probes revealed clear signal in all telomeric regions of all chromosomes. Only one small chromosome pair showed strong signals covering almost all chromosomes (Fig. 6).

The CBA-banding revealed large blocks of constitutive heterochromatin in various chromosomes, including the X-chromosome (Fig. 5). The Ag-NOR staining with silver nitrate revealed two small chromosome pairs with active NORs (Fig. 7).

3.4. Cells ultrastructure analysis

The ultrastructural analysis revealed differences between undifferentiated myogenic cells and differentiated T4 cells. Undifferentiated T0 cells exhibited spindle shape and ellipsoid single nucleus surrounded by abundant cytoplasm (Fig. 8A). The cytoplasm was enriched of grouped lipid droplets, mitochondria, endoplasmic reticulum and lamellar autophagic bodies (Fig. 8B and C). The differentiated T4 myogenic cells showed fused multinucleated cells containing many filaments in the peripheral cytoplasm and spindle nuclei (Fig. 8D). In the cytosol, numerous mitochondria, endoplasmic reticulum cisternae and polyribosomes were interposed between the filaments (Figs. 8 E and 8F).

3.5. Immunocytochemical characterization of the Cuvier's beaked whale cells

The immunocytochemical results revealed that in our *in vitro* model, we generated a heterogeneous population of Cuvier's beaked whale myogenic cells. In details, all myogenic cells at the T0, T2 and T4 stage, were vimentin-immunoreactive (−ir), β-actin-ir and myosin-ir. Representative images are showed in the (Figs. 9, 10 and 11). The immunocytochemical results pointed to the existence of a sub-population of cells (5 %) that reacted with the antibody against desmin (Fig. 9H, I, L; 10H, I, L and 11H, I, L). In detail, at the T0 stage all the cells were ellipsoidal mononuclear undifferentiated myoblasts and the 5 % of them showed intermediate filaments desmin-ir in the cytoplasm (Fig. 12A).

During the process of differentiation at the **T2 and T4** stage, part of myoblasts began to approach and fuse with each other; the myotube

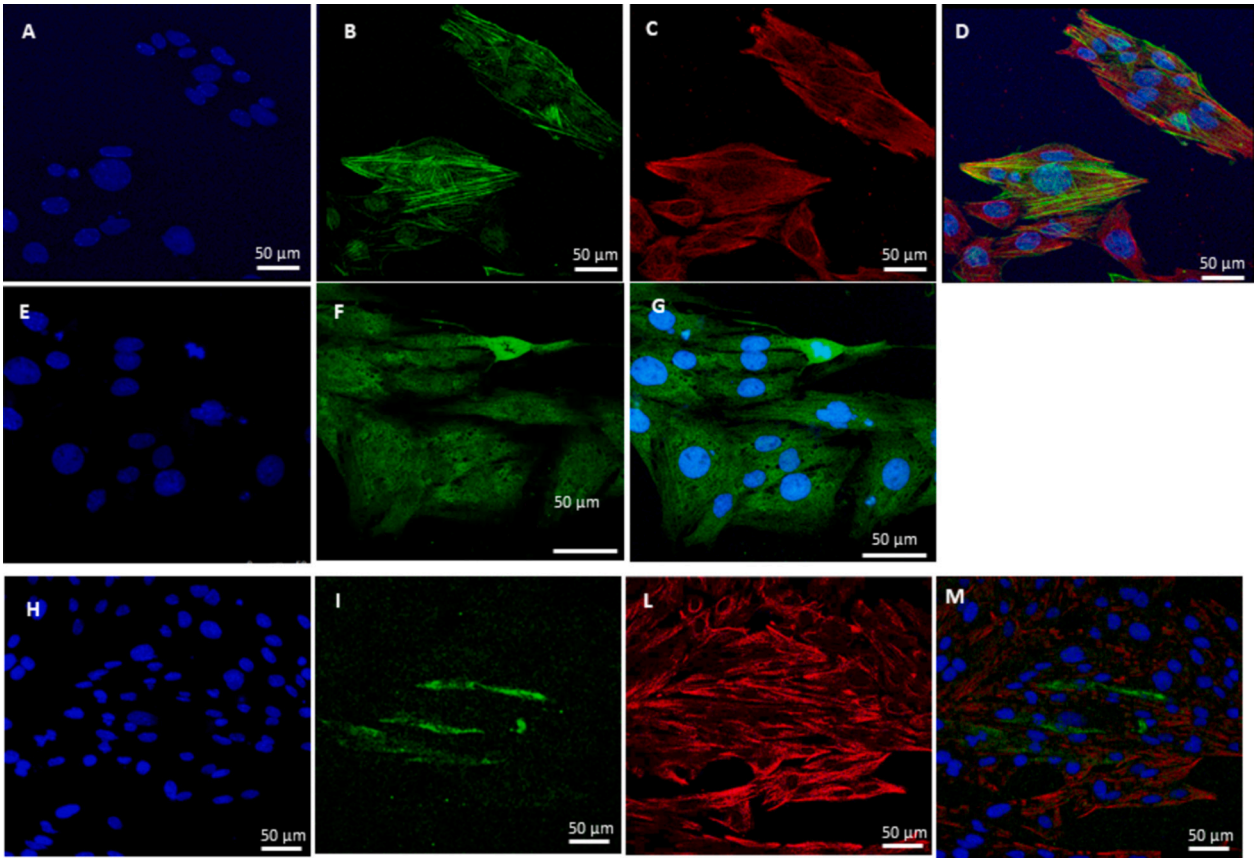

Fig. 10. Confocal images of immunocytochemical detection at the T2 differentiation stage in Cuvier's beaked whale myogenic cells. In **A** Hoechst-staining cell nucleus, in **B** actin-ir thin filaments in the cytoplasm of cells, in **C** vimentin-ir intermediated filaments in the cytoplasm of cells, in **D** superimposed image of A, B and C images. In **E** Hoechst-staining cell nuclei, in **F** myosin-ir thick filaments in the cytoplasm of cells, in **G** superimposed image of D and E images. In **H** Hoechst-staining cell nuclei, in **I** desmin-ir intermediated filaments in the cytoplasm of cells, in **L** vimentin-ir intermediated filaments in the cytoplasm of cells, in **M** superimposed image of H, I and L images.

shape was characterized by elongated cell body or rounded syncytial shape, probably resulting from the fusion of the myoblasts. (Fig. 12 C, D, E and F).

The Cuvier's Beaked whale cells did not show immunoreactivity to cytokeratin and e-Nos in all stages analyzed.

4. Discussion

In this study we successfully established a Cuvier's Beaked whale myogenic cell line obtained from frozen skeletal muscle tissue, sampled on a freshly dead adult animal.

Most of the marine mammal *in vitro* models involves the use of cell lines obtained from fresh samples of cetaceans. Only few studies have been established from frozen tissue samples. Here we report a procedure to obtain a myogenic cell line starting from cryopreserved muscle tissue.

The cell characterization in both undifferentiated and differentiated cells, revealed the presence of a heterogeneous population of myogenic cells; most of them were vimentin and myosin positive but desmin-negative, while a small population (5 %) was vimentin, myosin and desmin-positive.

Most of the knowledge on the process of skeletal muscle differentiation in mammals have been conducted on *in vitro* models, especially thanks to the availability of the mouse myoblast cell line model C2C12 (Burattini et al., 2004).

Our results showed that in the *Ziphius cavirostris* myogenic cells, serum modulation is able to induce cell differentiation. In details, 10 % of serum concentration in the medium promotes cell proliferation while reducing the serum percentage to 1 %, cell proliferation is reduced and cells started to differentiate and fuse.

Modulation of serum-concentration in the culture medium, provides a completely new environment to the myogenic cells, affecting the metabolic behavior, the proliferation and differentiation process (Jang et al., 2022). In this contest, it was demonstrated as the mouse C2C12 myoblasts are able to differentiate into myotubes and lose their ability to proliferate when the serum-rich medium is replaced with a serum-reduced medium (Ferri et al., 2009).

Also, in the human myoblasts in culture in was demonstrated as cell proliferation, the migration, the fusion and cell differentiation depending on serum concentration (Saini et al., 2018).

Despite at present, it has not been established as serum, add to the media or in serum-free media conditions, influence myoblast responses to different stimuli, it has been demonstrated as diverse biochemical responses (myoblast proliferation and myotube protein synthesis) in human myoblasts reflect the variable composition of mitogenic and anabolic factors in the serum (Saini et al., 2018).

The myogenic factors (such as Myf5, MyoD, myogenin) are the responsible of the skeletal muscle differentiation. They possess a specific set function that may regulate a distinct subset of muscle-specific genes at the onset of fusion (Dedieu et al., 2002).

The growth factors present in serum also influence fusion, such as insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), and other cytokines in serum play roles in regulating myoblast proliferation, differentiation, and fusion (Ahmad et al., 2023).

When serum is reduced, myoblasts are more likely to exit the cell cycle and enter a differentiation pathway (Flamini et al., 2018). In serum-free media or media with very low serum, myoblasts will

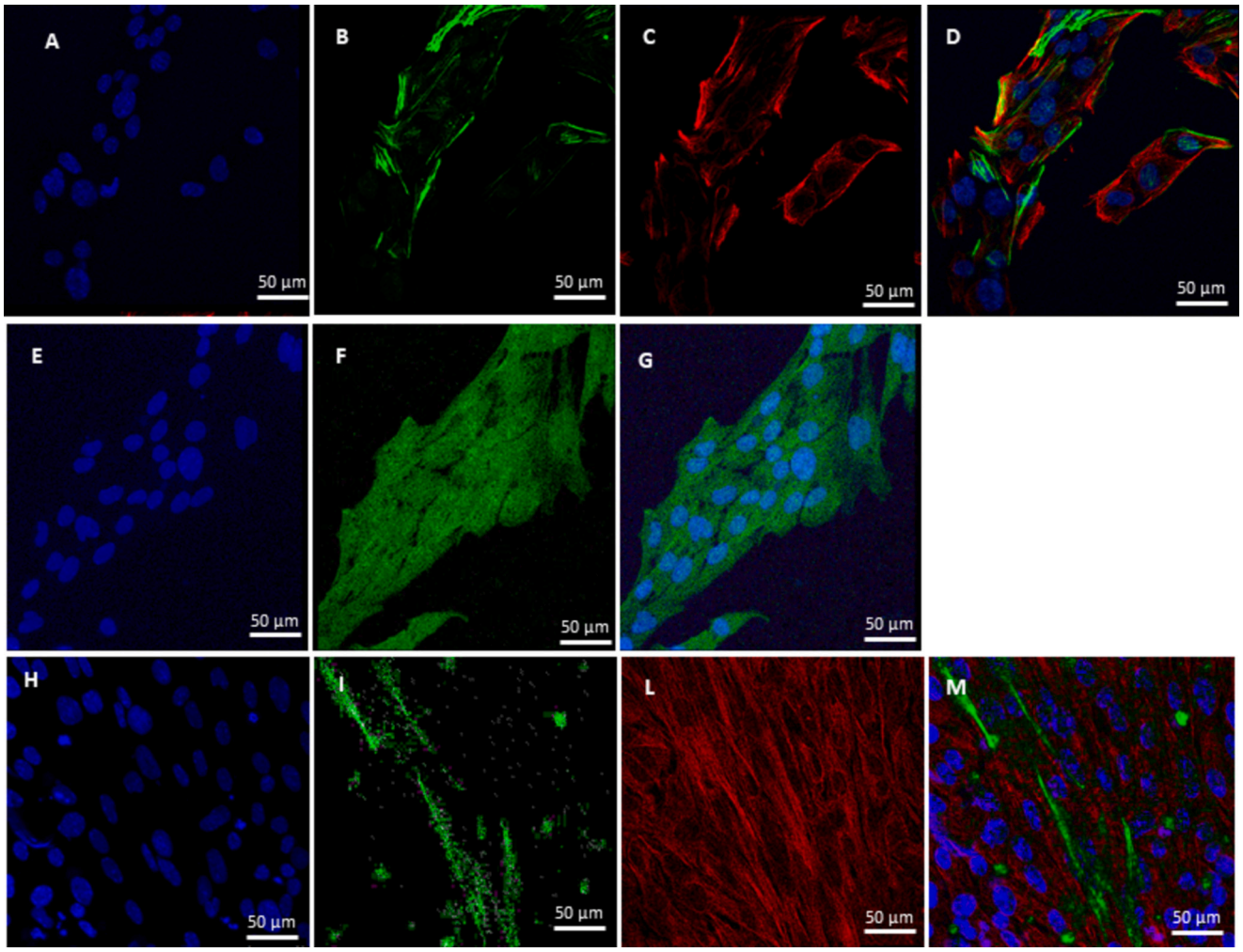

Fig. 11. Confocal images of immunocytochemical detection at the T4 differentiation stage in Cuvier's beaked whale myogenic cells. In **A** Hoechst-staining cell nucleus, in **B** actin-ir thin filaments in the cytoplasm of cells, in **C** vimentin-ir intermediated filaments in the cytoplasm of cells, in **D** superimposed image of A, B and C images. In **E** Hoechst-staining cell nuclei, in **F** myosin-ir thick filaments in the cytoplasm of cells, in **G** superimposed image of A and B images. In **H** Hoechst-staining cell nuclei, **I** desmin-ir intermediated filaments in the cytoplasm of cells, in **L** vimentin-ir intermediated filaments in the cytoplasm of cells, in **M** superimposed image of H, I and L images.

typically undergo a more mature differentiation process, which includes cell fusion into myotubes (Lawson and Purslow, 2000). Probably, this condition mimics the *in vivo* muscle development environment more closely, where growth factors and nutrients are more tightly regulated. In Cuvier's beaked whale, the T0 phase involved a process of high proliferation of myogenic cells with production of postmitotic mononuclear myoblasts. The T2 and T4 differentiation phase involved a decrease in proliferative cells number and the beginning of alignment of myoblasts and cell fusion with each other.

Despite studies have shown evolutionary conservation in the mechanisms of muscle cells differentiation among species as ovine, cattle, rodent, avian and amphibian (Hathaway et al., 1991; Allen et al., 1991); the presence of a heterogeneous population of myogenic cells and difference in desmin expression was determined also in these *in vitro* models. In a study performed in rat and bovine muscle satellite cells Allen and colleagues found that in bovine satellite cell cultures only 13 % of the cells express desmin-positive. Indeed, in rat muscle, many desmin-positive cells were present, and only a few cells stained positive for skeletal muscle myosin (Allen et al., 1991). Moreover, it should be noted that the role of desmin during myogenic cell differentiation is difficult to establish, since muscle cells can develop also without desmin (Li et al., 1997),

4.1. The karyotype analysis of *Ziphius cavirostris* cell line

In Cuvier's Beaked whale cell line the karyotype analysis shown the presence of 42 chromosomes (2n = 42, Fig. 2). The karyotype 2n = 42 was described in *Ziphius cavirostris* specie also by Benirschke and Kumamoto (Benirschke and Kumamoto, 1978). Interesting, about 5 % of cells examined showed the presence of tetraploidy (2n = 84, Fig. 3). In cetacean tetraploidy was observed for blue whales (*Balaenoptera musculus*) (Arnason et al., 1985). Diploid cells may become tetraploid through a variety of mechanisms, including endoreduplication, cell fusion, and cytokinesis failure 19 (Torres et al., 2010). Of course, scaling changes in chromosomes can affect cell physiology, but polyploidy could also play a role in the adaptive potential to likely environmental responses, promoting adaptability and diversity in proliferating cell lineages (Yant and Bomblies, 2015). However, given their rarity (5 %) in the population of the cells, it is supposed that these tetraploid cells become more unstable and thus prone to cell death. PNA-telomeric probes revealed clear signals at the telomeres of all chromosomes, exception of one small chromosome pairs which show very strong signals in about all chromosomes (Fig. 4) probably for the presence of repeated telomeric elements in this chromosome pair. NORs were found in two small chromosome pairs (Fig. 5), as found in other similar species (Bielek et al., 1997).

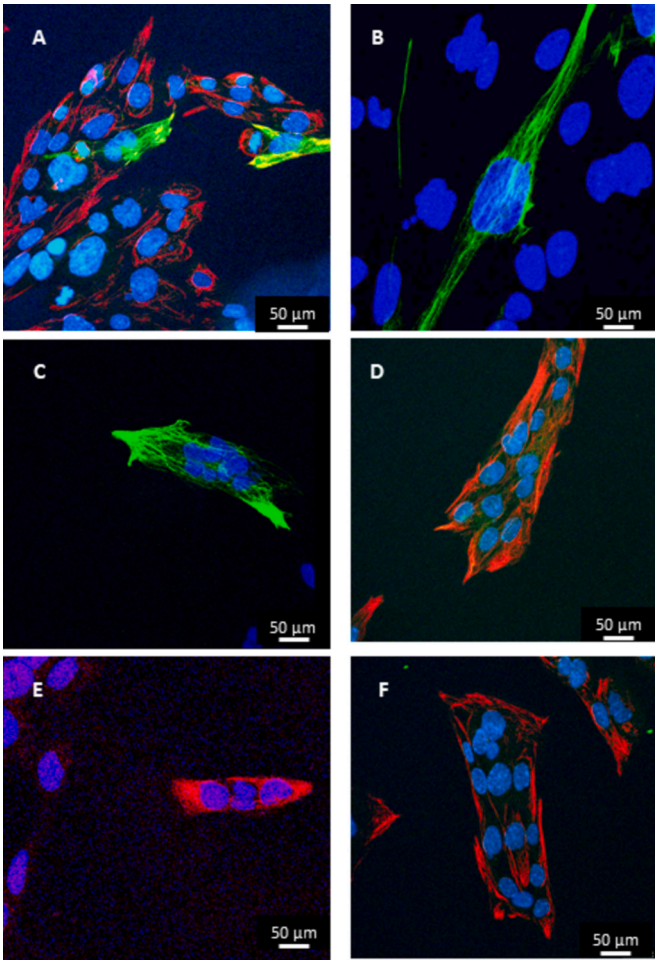

Fig. 12. Confocal superimposed images of Cuvier's beaked whale myogenic cells. In **A,** T0 stage myogenic cells vimentin positive (in red), desmin positive (in green) and Hoechst-staining nuclei (in blue). In **B,** detail of T0 undifferentiated mononucleated myoblasts, desmin positive (in green) and Hoechst-staining nuclei (in blue). In **C**, detail of T2 stage in witch myoblasts are fused in a syncytial structure, desmin positive (in green), Hoechst-staining nuclei (in blue). In **D**, detail of T2 myoblasts, vimentin positive (in red) and desmin negative began to approach with each other; Hoechst-staining nuclei (in blue). In **E**, detail of T4 stage myotube, probably originated from the fusion of myoblast; desmin positive (in red), Hoechst-staining nuclei (in blue). In **F**, detail of T4 stage cells in witch myoblasts vimentin positive (in red) and desmin negative began to fuse with each other in a syncytial structure); Hoechst-staining nuclei (in blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Marine mammals *in vitro* models as tool in cell biology, genetics and toxicology research.

In the last years, many studies report the use of marine mammals *in vitro* models as tool in cell biology, genetics and toxicology research to detect and quantify effects of water pollutants on toxicological endpoints (Johann et al., 2020). Moreover, it must be considered that biological responses are very integrative and accumulative in nature, so marine mammal cell lines represent a tool more sensitive than chemical analysis in detecting adverse conditions in the water environment.

Due to the impossibility to investigate in *in vivo* marine mammals the toxic effects of pollutants, this new *in vitro* model offers the opportunity to conduct experiments in Cuvier's beaked whale alive cells, establish new datasets under different culturing and compare results with those already published in other cetacean species (Marsili et al., 2012; Jin et al., 2013; Otero-Sabio et al., 2022).

Both Cetaceans as the *Ziphius cavirostris* and Pinniped as the elephant seals are marine mammals that undergo extreme and prolonged periods of fasting, diving, and long-term physical exertion, which may require specific metabolic shifts in muscle cells in response to glucocorticoids (such as cortisol), hormones involved in the stress response and metabolic regulation (Torres-Velarde et al., 2021). Moreover, these marine mammals are known to have highly specialized muscle physiology to support long periods of submersion during dives, high endurance swimming, and long fasting periods while foraging or migrating (Allen et al., 2024). While both cetaceans and pinnipeds face similar challenges related to long-duration fasting, diving, and physical exertion, their physiological traits may differ due to their unique adaptations. In this context, studying the physiology of the myogenic cell line obtained from the *Ziphius cavirostris*, the deep diver capable of reaching depths of more than 3000 m and hold their breath longer than any other cetacean, could contribute to reveal specialized mechanisms for long-duration dives endurance and the efficient utilization of fat as an energy source (Shearer et al., 2019).

5. Conclusion

Here we focus on Cuvier's beaked whale because, among the cetacean species, its biology and physiology are still poorly described, due to the fact that *Ziphius cavirostris* are inhabitants of the deep sea.

We present for the first time, an *in vitro* model based on a muscle-derived cell line from a Cuvier's Beaked whale male. The myogenic cells were able to grow, differentiate and evidence showed that they are capable to follow the myogenic program and/or fuse.

Since *Ziphius cavirostris* is a protected species and there are rare opportunities for sampling tissues suitable for cell culture from fresh animals, this *in vitro* cell line represents a new precious opportunity to investigate physiological and pathological responses in living cells.

Moreover, our study highlights how skeletal muscle tissue fragments can be cryopreserved and stored as a material for future experiments.

The studying the physiology of this *Ziphius cavirostris* myogenic cells could reveal specialized adaptations to endurance related to deep diving, long-duration fasting and physical exertion.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors declare that data and material can be freely given upon request. All data and images generated during this study are included in this published article and the authors consent for publication of the images in the figures and data in the table.

Funding information

The financial support of this study was approved by the "Proof of Concept (PoC) grant" (Bando Rep. n. 79/2021- Prot. n. 1097) from the University of Padova and from the Italian Ministry of Economic Development.

CRediT authorship contribution statement

Antonella Peruffo: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Irene Bassan:** Validation, Investigation. **Alice Gonella:** Investigation. **Lisa Maccatrozzo:** Validation, Investigation. **Cristina Otero-Sabio:** Methodology, Investigation. **Leopoldo Iannuzzi:** Writing – review & editing, Methodology. **Angela Perucatti:** Methodology, Investigation. **Ramona Pistucci:** Investigation. **Marta Giacomello:** Writing – review & editing, Methodology, Investigation. **Cinzia Centelleghe:** Writing – review & editing, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no conflict of interests. All authors have no relevant financial or nonfinancial interests to disclose.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

Data availability

All data and images generated during this study are included in this published article. Moreover, Cuvier's Beaked whale muscle-derived cell line belongs to the patent Sea Sentinels System (S.S.S.), (patent n° 102,020,000,003,248; and data can be seen in the link: https://www.knowledge-share.eu/en/patent/sea-sentinel-system-for-environmental-studies/).

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