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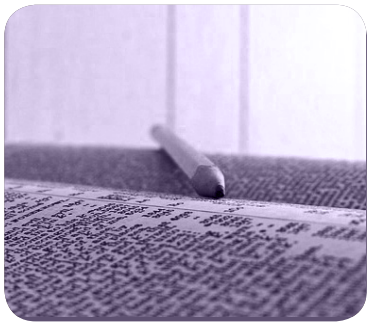

‘Muscle atrophy associated with aging: iPSCs cell-based therapy approaches’

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UAM EDICIONES

‘Muscle atrophy associated with aging: iPSCs cell-based therapy approaches’

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ABSTRACT

Sarcopenia is an important health problem characterized by muscle mass loss that occurs during aging. Due to the secondary effects and potential risks associated with the therapies used at this moment to treat this disease, new strategies are being developed to achieve an appropriate treatment. In the last few years, investigation is focused on the generation of inducible pluripotent stem cells (iPSCs) as a source of immune-tolerated cells for autologous cellular therapies.

In this work, CmC57-FiPS4F1 and CmC57-FiPS4F5 iPSC lines were generated from 'centenarian' mice fibroblasts by transduction of the Yamanaka factors OCT3/4, C-MYC, SOX2 and KLF4 using as delivery vector the non-integrative Sendai virus. We assessed the pluripotency of the generated lines with an analysis of alkaline phosphatase staining and by an immunofluorescence assay using antibodies against the pluripotency transcription factors NANOG, OCT4 and the surface marker for mouse undifferentiated stem cells, SSEA1.

Once these iPSC lines are fully characterized and appropriate control lines are also generated, they could be useful as a cellular model to study sarcopenia and other age-related diseases. Reprogramming senescent cells into iPSCs and its differentiation into muscle, the target tissue of the disease, could be a promising tool for its potential application in regenerative medicine.

1. INTRODUCTION

Muscle atrophy in aging (sarcopenia) is an important health problem characterized by muscle mass loss with consequent loss of muscular potency that affects up to 13% people aged 60-70 years and 50% of those aged 80+ years, leading to functional impairment and increased vulnerability to death (Lapasset et al., 2011; López-Otín et al., 2013). Sarcopenia is linked to the frailty syndrome which is related to disability, dependence hospitalization and mortality (Pareja-Galeano et al., 2016). In order to address this situation several hormonal and non-hormonal therapies have been proposed for its treatment, but they are accompanied by several secondary effects and potential risks (Sanchis-Gomar et al., 2011; Sanchis-Gomar et al., 2014).

The main biological hallmarks of aging are cellular senescence and a diminished tissue regenerative capacity (López-Otín et al., 2013). Accordingly, there are increasing research efforts to combat age-related diseases. In this sense, regenerative medicine has recently emerged as a potential novel therapy for numerous incurable degenerative diseases and can be applied for muscle atrophy and others age-related pathologies, being the ultimate goal to carry out stem cell therapies. Reprogramming has the capacity to reverse several molecular and cellular characteristics associated with aging, what suggests that these characteristics are reversible and rejuvenation can actually occur at the cellular level (Lunn et al., 2012; Mclaren et al., 2001; Pareja-Galeano et al., 2015).

Embryonic stem (ES) cells, derived from the inner cell mass of the early embryo at the blastocyst stage, seem to be promising donor sources for cell transplantation therapies. This is due to their capacity to self-renew indefinitely while maintaining pluripotency. This means that they are able to form the three germ layers – the ectoderm, endoderm and mesoderm –, specializing at some point to give rise to the tissues of the adult body. Despite their therapeutic potential, the use of ES cells raises some ethical concerns, being also important the problem of immune rejection when these cells are transplanted into patients (Müller et al., 2009; Pareja-Galeano et al., 2015; Sommer et al., 2013; Yamanaka et al., 2010).

One way to avoid these issues is by reprogramming patient-specific somatic cells into induced pluripotent stem cells (iPSCs). This can be achieved by the ectopic expression of some genes responsible for the pluripotency, thus acquiring similar molecular and functional characteristics as ES cells. Like ES cells, iPSCs also have the ability to differentiate into whatever cell type, ending up being a source of cells that could be used to replace those lost because of damage or disease (Müller et al., 2009; Pareja-Galeano et al., 2015; Sommer et al., 2013; Yamanaka et al., 2010). iPSCs were generated for the first time in 2006 by Shinya Yamanaka's group in Japan by retroviral transduction of a defined set of four transcription factors (OCT3/4, SOX2, KLF4 and C-MYC), the Yamanaka factors, into murine adult fibroblasts (Takahashi et al.,

2006). Later on, iPSCs were also generated from human somatic cells using the same retroviral system (Takahashi et al., 2007). For this important discovery, Yamanaka got the Nobel Prize of Medicine and Physiology in 2012 shared with John Gurdon.

The major limitation of using retroviral vectors is that foreign genes integrate into the host genome during reprogramming, thus existing a risk of tumor formation that affects iPSC-derived cells safety and limits the potential clinical application (Fusaki et al., 2009; Lieu et al., 2013; Nishimura et al., 2011; O'Malley et al., 2009). To overcome this problem, other delivery methods such as adenoviral vectors that do not integrate into the genome have been used but the reprogramming efficiency is much lower because of a difficulty to maintain sufficiently high levels of the reprogramming factors (Stadtfeld et al., 2008). Nowadays, more effective techniques based on the use of murine parainfluenza virus type 1, known as Sendai virus (SeV), have been developed. Sendai virus belongs to *Paramyxoviridae* family, replicating in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells. It is remarkable that SeV is a safe non-integrative vector able to infect various animal cells but not being pathogenic in humans and very efficient for introduction of foreign genes due to a transient but strong gene expression (Fusaki et al., 2009; Nishimura et al., 2011).

In this scenario, the generation of patient-specific pluripotent cell lines could be very useful for a wide variety of applications (figure 1) including: 1) Disease modelling, 2) High throughput drug screening that could result in pharmacological treatments and 3) Approaches with potential application in tissue replacement therapies, also existing the possibility of using recent advanced tools of genomic edition like the CRISPR/Cas9 system that allow the correction of the specific genetic alteration in the iPSCs (Müller et al., 2009; Pareja-Galeano et al., 2015; Soria-Valles et al., 2016).

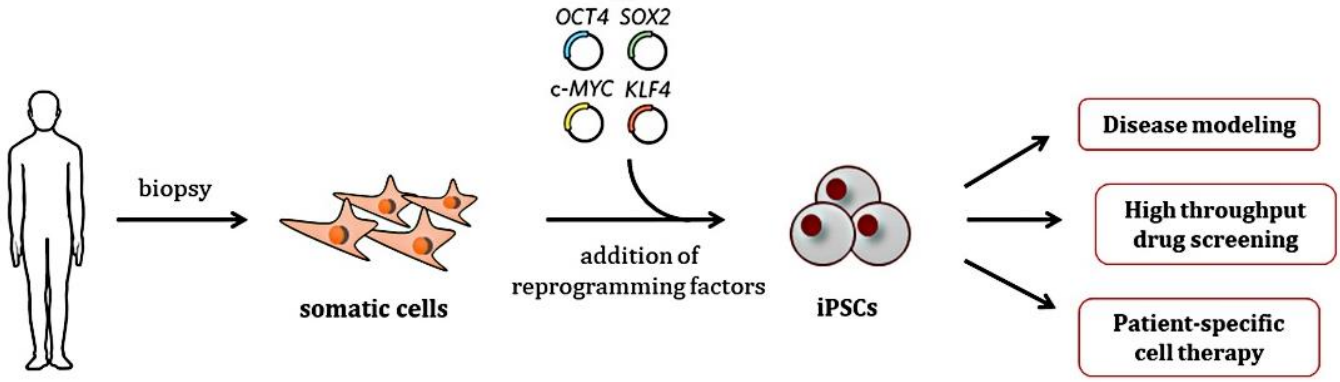

Figure 1. Applications of iPSCs. iPSCs are generated from somatic cells with Yamanaka factors and can be used to study disease mechanisms, for screening the efficacy of therapeutic drugs or for cell therapy to regenerate defective tissues.

In this work, the main aim has been to reprogram fibroblasts obtained from mice whose age is equivalent to the age of a centenarian ('centenarian' mice). As delivery vector of the Yamanaka factors, Sendai virus were used in order to generate iPSCs, which could be useful as a model to better understand age-related diseases and to assess the potential of cell transplantation for correcting sarcopenia in mice with muscle atrophy.

2. MATERIALS AND METHODS

2.1. Fibroblasts culture

'Centenarian' mouse tail-tip fibroblasts (strain: C57BL/6J) were kindly provided by Dr. Martínez, from the CIEMAT (Madrid).

Fibroblasts were cultured with DMEM high glucose containing L-glutamine and sodium pyruvate (Biowest) supplemented with 10% fetal bovine serum (FBS) hyclone (GE Healthcare Lifesciences) and penicillin/streptomycin (P/S) 1x (Gibco®, Life Technologies). Fibroblasts were expanded at 37ºC and 5% CO_2.

2.2. *Feeder* cells irradiation

Human commercial fibroblasts obtained in ATCC (CCD 1112Sk) were used as *feeder* cells. These fibroblasts were unfrozen and plated in a T75 flask with IMDM high glucose containing L-glutamine and HEPES (ATCC®) supplemented with 10% FBS ATCC® and P/S 1x (Gibco®, Life Technologies).

Fibroblasts were expanded until getting 6 T175 with fibroblasts (90% of confluence). Subsequently, they were mitotically inactivated by irradiation at 85 Gys for 30 minutes. Then, cells were counted using a Neubauer camera and different aliquots containing $1.4x10^6$, $2x10^6$ and $4x10^6$ cells were prepared. These aliquots were frozen in IMDM containing 5% dymethilsulfoxide (DMSO) Hybri-max® (Sigma-Aldrich®) at -80ºC at a speed of 1ºC/min in a *Mr Frosty* (Nalgene). Then fibroblasts were stored in liquid nitrogen tanks at -180ºC.

For preparing feeder plates, dishes treated with gelatin 0.1% were incubated at 37ºC and 5% CO_2 for 1 hour. Feeder aliquots were rapidly unfrozen. Right after, IMDM medium was added and cells were spin at 220g for 5 minutes. Finally, cells were resuspended in the desired volume of medium and plated.

2.3. Reprogramming of mouse fibroblasts into iPSCs

One day before reprogramming 'centenarian' fibroblasts into iPSCs, cells were seeded at different densities in a range from $5x10^4$ to $2x10^5$ cells per well in 6-well plates with DMEM high glucose medium. Cells were incubated overnight at 37ºC and 5% CO_2.

24h after plating, those wells with fibroblasts presenting an appropriate confluency were chosen for reprogramming. Fibroblasts were reprogrammed using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Life Technologies) following the instructions of the manufacturer. For this purpose, cells were counted in order to calculate the virus volume needed to reach the appropriate multiplicity of infection (MOI) (table 1), for which next formula was used:

$$\text{Virus volume (µl)} = [\text{MOI x number of cells}] / [\text{virus title x } 10^{-3} \text{ (µl/ml)}]$$

The virus title is specified in the kit used and it depends on the lot number (KOS title: 0,85 x 10^8 (CIU/ml); c-Myc title: 0,85 x 10^8 (CIU/ml); Klf4 title: 0,82 x 10^8 (CIU/ml)).

Number of cells in the transduction (day 0)	KOS (MOI=5) Volume (µl)	c-Myc (MOI=5) Volume (µl)	Klf4 (MOI=5) Volume (µl)
133.125	7,83	7,83	8,1
216.250	12,72	12,72	13,18

Table 1. Virus volume needed of each vector (KOS, c-Myc and Klf4) to reach an appropriate MOI for reprogramming 'centenarian' fibroblasts.

Then, cells were incubated for 3 days, being medium changed every day. At day 4, medium was replaced to LIF medium containing Neurobasal™ (Gibco®) and DMEM F-12 GlutaMAX™ (Gibco®) supplemented with B27® Supplement 1x (Gibco®), N-2 Supplement 1x (Gibco®), β-mercaptoethanol 55 µM (Gibco®), L-glutamine 1x (Gibco®) and penicillin/streptomycin 1x (Gibco®), PD0325901 1 µM (Sigma), CHIR99021 3 µM (StemCell Technologies) and LIF 1U/ml (Millipore). Cells were incubated at 37ºC and 5% CO_2 being LIF medium changed every day.

2.4. iPSCs culture on feeder layer

Day 7 after reprogramming, cells were splitted by trypsinization with 0,25% trypsin-EDTA solution (Life Technologies) in PBS for 3 min at room temperature. Then, cells were cultured on a 100 mm dish with about 1.5 million feeder cells, being the medium changed to LIF medium one hour before the passage in order to conditionate it. LIF medium was changed every day.

When iPSC colonies with ES-like morphology and growth behavior appeared in the 100 mm dish (around day 20 after reprogramming), they were individually picked using a Stripper micropipette (Origio) onto 35 mm dishes for expansion. This way, different colonies were expanded and frozen. In this work, only 'centenarian' 1 and 5 iPSCs lines were chosen to characterize its pluripotency.

For freezing the generated iPSC lines, first they were washed with PBS 1X and then incubated with 1mg/ml collagenase in Knock-out DMEM F12 (Gibco®) at 37ºC for 7 minutes. After incubation, the colonies were visualized under a microscope. When the edges of the colonies appeared slightly rounded up, collagenase was removed and iPSCs were washed again with PBS and were collected into a Falcon tube with an appropriate volume of medium. After that, they were centrifuged at 200g for 4 min and medium was aspirated off. Freezing medium A containing 50% knock-out serum and 50% LIF medium was added and colonies slightly resuspended on it. Then, freezing medium B containing 80% LIF medium and 20% dymethylsulfoxyde (DMSO) was added drop by drop and mixing. Colonies in freezing medium were slowly frozen in cryovials at -80ºC and then were stored in liquid nitrogen containers.

2.5. Mycoplasma test

Mycoplasma detection was performed by PCR analysis in 'centenarian' 1 and 5 iPSC lines in order to discard mycoplasma contamination.

1 ml of the cell culture supernatant was taken (3 days culture at ~80-90% confluence) and was heated at 95ºC for 5 min and spin at 12500g for 5 seconds. Supernatant was taken for the analysis. The primers used in the PCR are specified in Table 2, with an annealing temperature of 55ºC. The electrophoresis of the product was carried out in 1% agarose.

Oligonucleotide MGSO	*5'-TGCACCATCTGTCACTCTGTTAACCTC-3'*
Oligonucleotide GPO-3	*5'-GGGAGCAAACAGGATTAGATACCCT-3'*

Table 2. Sequences of the oligonucleotides used in the PCR analysis for mycoplasma detection.

2.6. Phosphatase alkaline analysis

Three or four iPSC colonies were seeded onto 35 mm feeder layer plates. When colonies had the appropriate size, phosphatase alkaline activity was determined using the phosphatase alkaline substrate solution kit (Sigma-Aldrich®, AB0300). Briefly, cells were washed twice with PBS 1X and incubated for 3-4 min with 1 ml fixer solution (4% formaldehyde in PBS 1X). Then, cells were incubated for 20 min in darkness with 1 ml substrate solution from the kit. Images of the colonies were taken before and after the analysis using Imaging Software NIS-Elements F.

2.7. Immunofluorescence analysis

The analysis is performed using antibodies against the pluripotency markers NANOG, SSEA1 and OCT4.

Before starting the immunofluorescence, around $3{,}3x10^5$ feeder cells were plated on 35 mm culture plates (Ibidi) and 3-4 colonies were picked on them, being incubated until presenting appropriate size. Then, samples were washed 3 times with 2 ml PBS 1X for 5 min and fixed with 1 ml 4% paraformaldehyde for 30 min. After that, samples were washed 3 times with 2 ml TBS+ (0,1% Triton (Sigma-Aldrich®) in Tris-Buffered Saline, TBS 1X) for 15 min and were incubated with 1 ml TBS++ (3% *donkey serum* (Sigma-Aldrich®) and 0,3% Triton (Sigma-Aldrich®) in TBS 1X) for 2 hours.

Samples were incubated with a solution of the primary antibody in TBS++ overnight at 4ºC in dark (antibodies are indicated in the table 3). Then, samples were washed 3 times with 2 ml TBS+ for 10 min and were blocked with 1 ml TBS++ for 1 hour. Afterwards, samples were incubated for 2 hours in dark at room temperature with the secondary antibody (antibodies are indicated in the table 3). From here on, samples were maintained in dark. Samples were washed twice with 2 ml TBS+ for 10 min and once with 2 ml TBS 1X for 15 min. After that, samples were incubated with 1 ml DAPI 1:1000 in TBS 1X (ThermoFisher Scientific) for 5 min and washed twice with 2 ml TBS 1X for 15 min. Following immunostaining, samples were mounted with Prolong Diamond (ThermoFisher Scientific) and a coverslip.

	Antibody	**Dilution**	**Company**
Primary antibodies	*Mouse anti-SSEA1*	1:100	*Abcam*
	Rabbit anti-NANOG	1:150	*Abcam*
	Mouse anti-OCT4	1:100	*Santa Cruz Biotechnology*
Secondary antibodies	*Goat anti-mouse IgG (H+L), Alexa Fluor® 488*	1:500	*ThermoFisher Scientific*
	Goat anti-rabbit IgG (H+L), Alexa Fluor® 546	1:500	*ThermoFisher Scientific*

Table 3. Primary and secondary antibodies for immunofluorescence analysis of the pluripotency markers *NANOG*, *SSEA1* and *OCT4*.

Samples were maintained overnight in the dark with the lid opened in dark and then were kept at 4ºC until were seen in the confocal microscopy service (IIB, Madrid). Acquired images were visualized with the program *Zen lite* (ZEISS) program and scale bars were added with *Image J* program.

3. RESULTS

3.1. iPSCs generation from 'centenarian' mice fibroblasts

In this work, two iPSC lines were generated by reprogramming 'centenarian' mice fibroblasts: 'Centenarian' 1 (CmC57-FiPS4F1) and 'centenarian' 5 (CmC57-FiPS4F5). Colonies 1 and 5 were selected based on its ES-like morphology and growth behavior. After the selection, colonies were picked under a steromicroscope using a stripper micropipette. Subsequently, colonies were expanded and a molecular and functional characterization of the pluripotency of these iPSC lines were carried out following the protocols described in the 'Materials and methods' section. The selected colonies are shown in figure 2.

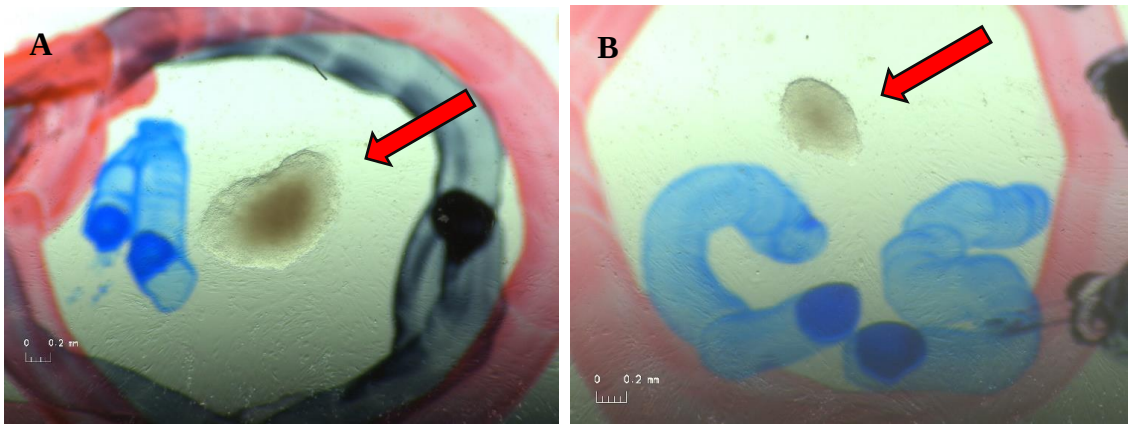

Figure 2. Colonies selected after the reprogramming procedure to be picked and expanded. iPSCs colonies were grown on top of human feeders with LIF medium (marked with red arrows). **A.** iPSC colony 'centenarian' 1 and **B.** iPSC colony 'centenarian' 5.

3.2. Molecular and functional characterization of the generated lines

3.2.1. Phosphatase alkaline analysis

A high phosphatase alkaline activity is a traditional marker of ES and iPS cells, since it has been shown to be up-regulated in pluripotent stem cells. Because of that, in order to confirm the pluripotency of the generated iPSC lines, phosphatase alkaline activity was analyzed as a first test of pluripotency. In figures 3A and 3C are shown the iPSC lines, CmC57-FiPS4F1 and CmC57-FiPS4F5. As it has been shown in these figures, iPSCs have an ES-like morphology forming compact colonies that have well-defined edges, presenting these cells a high nucleus-to-cytoplasm ratio. These colonies are both positive for phosphatase alkaline staining (figures 3B and 3D, respectively).

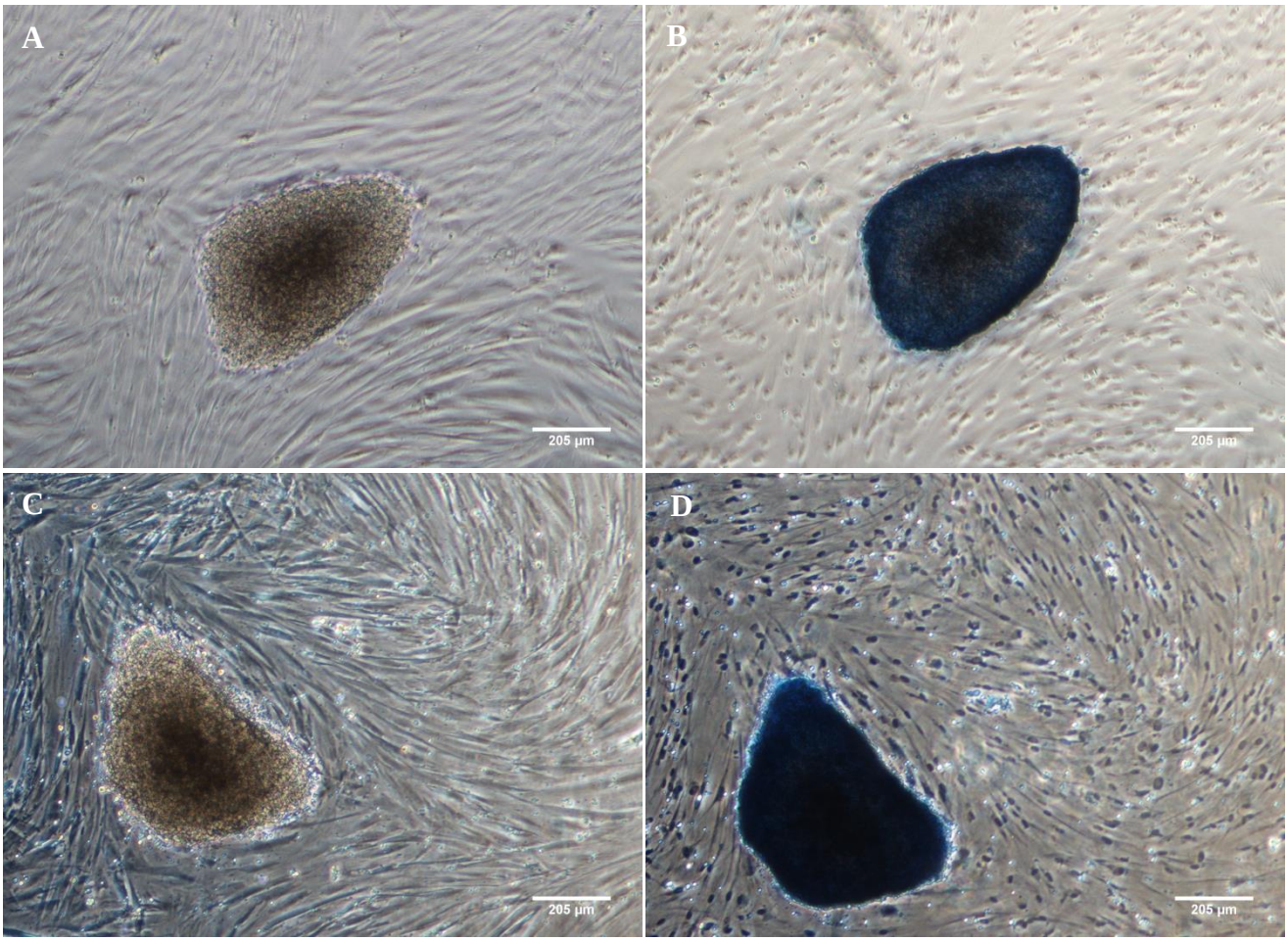

Figure 3. Positive phosphatase alkaline staining of the generated iPSCs colonies. **A.** iPSC CmC57-FiPS4F1 colony with a typical ES-like morphology. **B.** iPSC CmC57-FiPS4F1 colony positive for phosphatase alkaline activity. **C.** iPSC line CmC57-FiPS4F5 colony with a typical ES-like morphology. **D.** iPSC line CmC57-FiPS4F5 colony positive for phosphatase alkaline activity. Scale bar: 205 µm.

3.2.2. Immunofluorescence analysis

As phosphatase alkaline activity is not exclusive to ES and iPS cells, it is necessary to use this analysis in conjunction with other pluripotency tests. To confirm that CmC57-FiPS4F1 and CmC57-FiPS4F5 lines are pluripotent, an immunofluorescence analysis was also performed with antibodies against the transcription factors NANOG, SSEA1 and OCT4. These typical ES cells markers turn out to be fundamental to maintain the undifferentiated state, and they are detected on the generated iPSC lines CmC57-FiPS4F1 and CmC57-FiPS4F5 (as shown in figures 4 and 5, respectively).

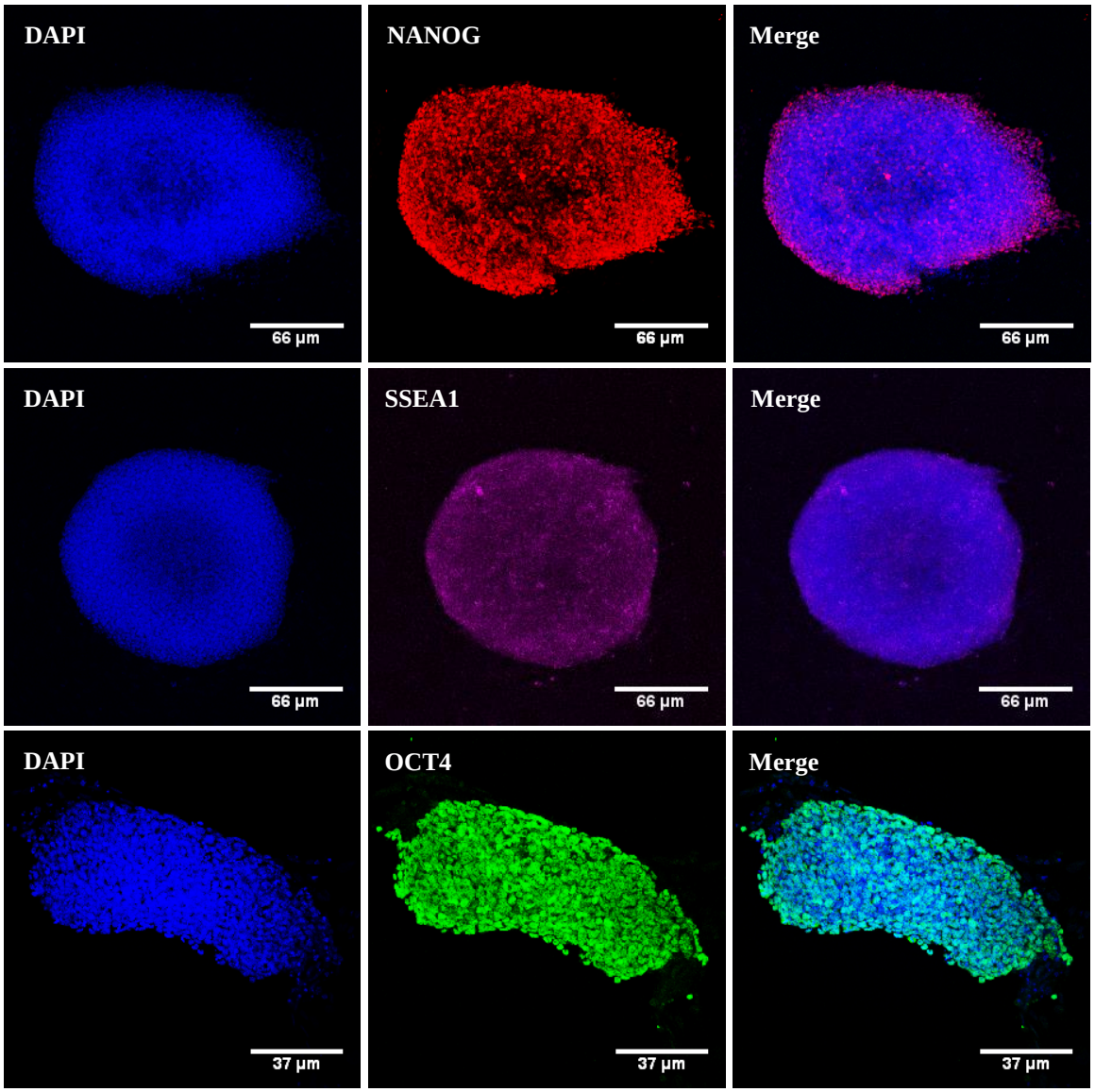

Figure 4. Expression of the typical pluripotent ES cell markers NANOG, SSEA1 and OCT4 detected by an immunofluorescence analysis of CmC57-FiPS4F1 line. DAPI is a nuclear marker. Scale bars: for NANOG and SSEA1 (66 µm) and for OCT4 (37 µm).

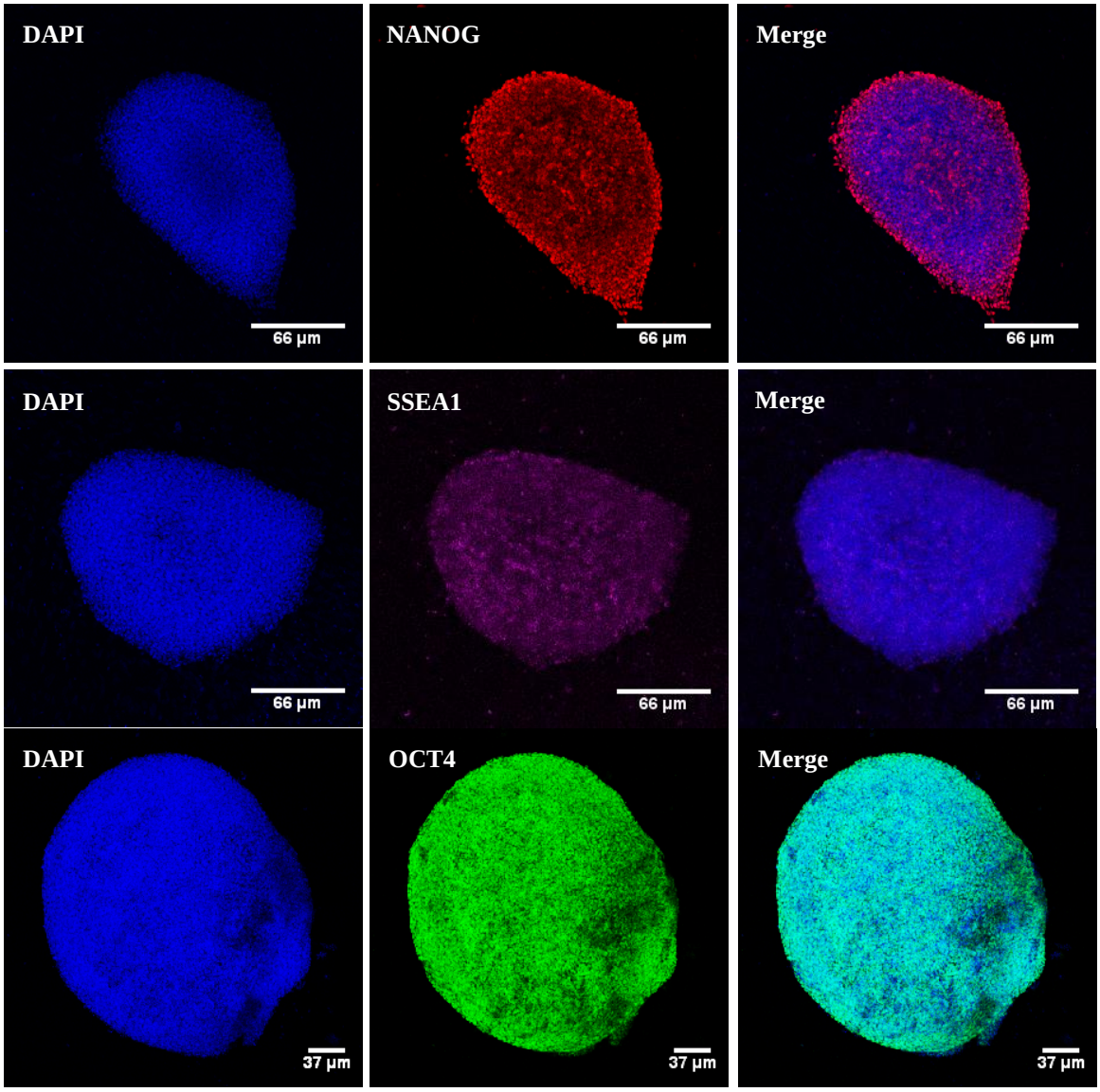

Figure 5. Immunofluorescence analysis of the iPSC line CmC57-FiPS4F5. Figure shows the expression of the typical pluripotent ES cell markers NANOG, SSEA1 and OCT4.. DAPI is a nuclear marker. Scale bars for NANOG and SSEA1 (66 µm) and 37 µm for OCT4.

3.3. Other analysis to confirm quality of the generated lines: Mycoplasma test

The maintenance of contamination-free iPSC lines is essential, being mycoplasma among the biggest contaminant concerns. Mycoplasma can alter cell metabolism and cause chromosomal aberrations. A PCR analysis was performed in order to detect mycoplasma in CmC57-FiPS4F1 and CmC57-FiPS4F5 lines. As shown in figure 6, generated iPSC lines are both negative for mycoplasma contamination.

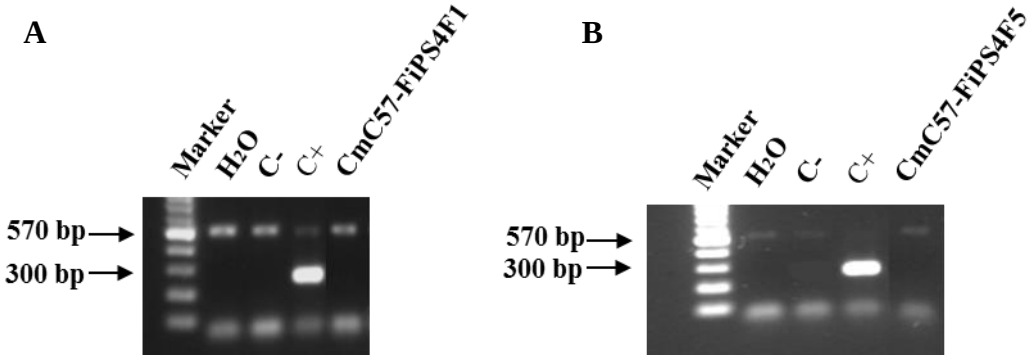

Figure 6. PCR analysis for testing mycoplasma in the generated iPSC lines. 300 bp band represents that the sample is positive for mycoplasma contamination. 570 bp band is an internal control to discard the inhibition of the Taq polymerase. C-, negative control; C+, positive control. **A.** PCR analysis for CmC57-FiPS4F1 line. **B.** PCR analysis for CmC57-FiPS4F5 line.

4. DISCUSSION

Nowadays, there are increasing research efforts to combat sarcopenia, an important health problem characterized by muscle mass loss that occurs during aging (Pareja-Galeano et al., 2016). Derivation of autologous iPSCs through direct reprogramming of easily accessible somatic cells has opened new horizons in the field of regenerative medicine. The reduction of the chances of immune rejection in transplantation, among other advantages, makes iPSC technology a promising tool for therapeutic applications (Sommer et al., 2010).

In this work, CmC57-FiPS4F1 and CmC57-FiPS4F5 lines were generated using Sendai virus as non-integrative method for delivering the Yamanaka factors, in order to reprogram 'centenarian' mice fibroblasts. Generated iPSC colonies present a characteristic ES-like morphology and growth behavior. Additionally, they have been confirmed to be pluripotent cells since they stain positive for alkaline phosphatase analysis and express the typical ES cell markers NANOG, SSEA1 and OCT4.

Other analysis are needed to fully characterize these lines (Galera et al., 2016). Among them, a quantitative real-time RT-PCR (qPCR) for quantifying the expression of endogenous pluripotency genes. It is also essential to confirm the clearance of the vectors and the exogenous reprogramming factor genes. In order to functionally demonstrate the pluripotency of the generated lines, an embryoid body based *in vitro* differentiation assay of the iPSCs into the three germ layers (endoderm, ectoderm and mesoderm) must be performed (Aasen et al., 2008). Other analysis need to be done in order to confirm the generation of *bona fide* iPSCs: 1) A karyotype analysis to show that the generated iPSC lines have maintained genetic stability, as it is already

known that prolonged culture of pluripotent cell lines can result in genetic abnormalities (Liang et al., 2013); 2) a DNA fingerprinting analysis using highly polymorphic regions to confirm that the generated lines come from the original fibroblasts (Galera et al., 2016).

In order to compare the different functional analysis that will be carried out with the 'centenarian' iPSC lines, control iPSC lines need to be generated from 'young' mice fibroblasts. These fibroblasts have been already reprogrammed, but they did not show an ES-like morphology and growth behavior (as shown in figure 7). For that reason, a new reprogramming experiment using a different fibroblast density must be performed.

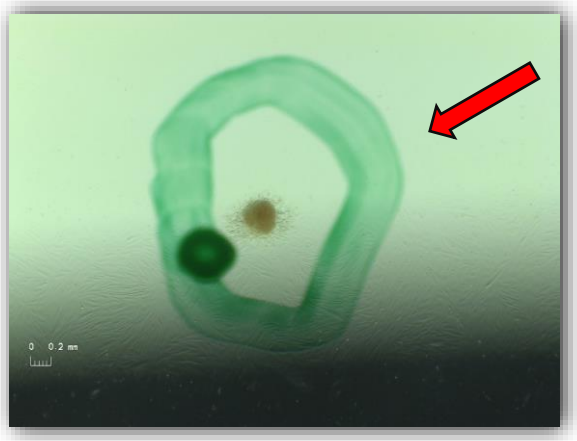

Figure 7. Partially reprogrammed colony after reprogramming of 'young' mice fibroblasts.

Once 'centenarian' iPSC lines are fully characterized, they would be useful as a cellular model for studying molecular mechanisms underlying age-related diseases, such as sarcopenia, and for pharmaceutical high throughput approaches that improve this condition.

These iPSCs could also be differentiated into muscle, the target tissue of the disease, having a potential application in regenerative medicine for correcting sarcopenia in mice with muscle atrophy. In 2011, Lapasset et al. showed that it was possible to reprogram senescent cells into iPSCs achieving the reversibility of major aspects of cellular phenotype associated with aging when differentiating them. This suggests that rejuvenation can actually occur at the cellular level when reprogramming old cells. In this context, the ultimate goal could be obtaining 'rejuvenated' myogenic precursors by differentiating the generated iPSCs from 'centenarian' cells to carry out autologous transplantation approaches and combat sarcopenia (Pareja-Galeano et al., 2016).

5. CONCLUSIONS

- Two iPSC lines, CmC57-FiPS4F1 and CmC57-FiPS4F5, have been generated by reprogramming 'centenarian' mice fibroblasts.
- iPSC lines must be generated from 'young' mice fibroblasts to be used as control in the different functional analyses that will be carried out.
- Analyses displayed up to now show that the generated iPSCs are pluripotent. However, other characterization analyses and quality tests must be carried out in order to confirm that they are *bona fide* iPSCs.

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