Title: Centenarians overexpress pluripotency-related genes

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ABSTRACT

Human mesenchymal cells can become pluripotent by the addition of Yamanaka factors *OCT3/4, SOX2, c-MYC, KLF4*. We have recently reported that centenarians overexpress *BCL-xL*, which has been shown to improve pluripotency, thus we aimed to determine the expression of pluripotency-related genes in centenarians. We recruited 22 young, 32 octogenarian and 47 centenarian individuals and determined the mRNA expression of Yamanaka factors and other stemness-related cell surface marker genes (*VIM*, *BMP4*, *NCAM*, *BMPR2*) in peripheral blood mononuclear cells by reverse transcription polymerase chain reaction. We found that centenarians overexpress *OCT3/4*, *SOX2*, *c-MYC*, *VIM*, *BMP4*, *NCAM* and *BMPR2*, when compared to octogenarians (p<0.05). We further tested the functional role of *BCL-xL* in centenarians' ability to express pluripotency-related genes: lymphocytes from octogenarians transduced with *BCL-xL* overexpressed *SOX2*, *c-MYC*, and *KLF4.* We conclude that centenarians overexpress Yamanaka Factors and other stemness-related cell surface marker genes, which may contribute to their successful aging.

Key words: Healthy aging, longevity, pluripotency, stemness.

List of abbreviations:

IPSCs: induced pluripotent stem cells

DPSCs: dental pulp stem cells.

PBMCs: peripheral blood mononuclear cells.

Yamanaka factors: *OCT4, SOX2, KLF4* and *c-MYC.*

Other pluripotency-related genes: *VIM*, *BMP4*, *NCAM*, *BMPR2*.

RT-PCR: reverse transcription polymerase chain reaction.

INTRODUCTION

Aging is characterized by a progressive loss in the ability to maintain homeostasis, leading to impaired function and increased vulnerability to death. One of the hallmarks of aging is stem cell exhaustion, this is, stem cells are no longer able to replenish tissues and to sustain tissue function due a progressive decline in their regenerative potential (1). In this regard, the stem cell theory of aging postulates that aging results from the inability of stem cells to self-renew and to act as an internal repair system (2–3). Further, it has even been shown that stem cell manipulation can extend lifespan (4).

In recent years, stem cell research has become one of the most intriguing fields, mainly since Yamanaka and coworkers demonstrated in 2006 that the introduction of four specific transcription factors, known as "Yamanaka Factors" (*OCT3/4*, *SOX2*, *KLF4* and *c-MYC*) could convert adult cells into induced pluripotent stem cells (iPSCs)(5). These cells,

as occurs with normal pluripotent stem cells, have the capacity to self-renewal and to give rise to every cell type of the adult body, but not extra-embryonic tissues. Just one year later, the same group reported the generation of iPSCs from human fibroblasts (6) and then Serrano and coworkers succeeded in reprogramming cells *in vivo* (7). Furthermore, the introduction of these transcription factors in progeria mice could partially revert age-related signs and extend their lifespan (8). In accordance with this, we recently showed that senescence is inversely related to pluripotency in dental pulp stem cells (DPSC), since Yamanaka factors' expression was reduced after long-term *in vitro* culture (9).

Centenarians are endowed with an outstanding ability to maintain homeostasis. They show such a remarkable compression of age-associated morbidity that their health span approximates their lifespan (10), thus they are considered as a model of healthy aging. Both environmental and genetic factors contribute to the complex phenotype of exceptional longevity that characterizes centenarians (11). However, existing evidence suggests that the genetic contribution to a healthy life span in centenarians may be greater than in the general population (12–13).

We have previously reported that centenarians exhibit a characteristic miRNA and mRNA profile, which differs from that of normal ageing, thus providing molecular basis for their remarkable maintenance of homeostasis (14). We further demonstrated that *BCL-xL*, which is an anti-apoptotic factor, is overexpressed in centenarians when compared to septuagenarians, playing a critical role in the mRNA profile of centenarians

(15). Interestingly, it has been suggested that *BCL-xL* is able to enhance the efficiency of Yamanaka factors-mediated iPSC generation in adult human peripheral blood mononuclear cells (PBMCs) (16). In fact, the inclusion of *BCL-xL* in the three factor combinations tested in PBMCs increased iPSCs generation efficiency by 10-fold, suggesting a role of *BCL-xL* in iPSCs long-term self-renewal and proliferation (16). This led us to hypothesize that PBMCs from centenarians overexpressing *BCL-xL* might display higher pluripotency-related gene expression when compared to young and octogenarians. We report that successful ageing (centenarians) is associated with higher levels of pluripotency-related genes, when compared to normal ageing (octogenarians) with BCL-xL playing a relevant role in this process.

METHODS

Participants

We recruited 22 young, 32 octogenarian and 42 centenarian individuals from Alzira (Spanish Centenarian Study) and Sardinia (Sardinian Cohort) as previously described (15). The Spanish Centenarian Study Group (RETICEF) began in 2007 as a population-based study of centenarians living within the 11th Health Department of the Valencian Community (Spain), which is composed of 240.000 inhabitants. Potential subjects were selected from the population data system. We found 31 centenarians of whom 20 fulfilled the inclusion criteria. Then, we randomly recruited 20 octogenarians of whom 11 satisfied the inclusion criteria and 20 young people of whom 14 met the inclusion criteria. The inclusion criteria included: to be born before 1908 for centenarians,

between 1928 and 1938 for octogenarians and between 1968 and 1988 for young individuals, to live in the 11th Health Department for at least the last 6 years and to sign an informed consent. The exclusion criterion was to be terminally ill for any reason. All experimental procedures were performed in accordance with the ethical standards and according to the Declaration of Helsinki and according to the national and international guidelines. The study was approved by the Committee for Ethics in Clinical Research of the Hospital de la Ribera, Alzira. All patients or their relatives were fully informed of the aims and scope of the research and signed an informed consent. The Sardinian cohort is described in (17). The age of the "young" individuals was 38 ± 11 years, that of octogenarians was 81 ± 4 and that of centenarians was 100 ± 2. The gender distribution (men/women) was 10/12 for young individuals, 17/15 for octogenarians and 18/29 for centenarians.

Sampling

Blood samples were drawn from the antecubital vein in VACUTAINER® CPT™ tubes (BD, Franklin Lakes, NJ) containing sodium heparin as anticoagulant, in order to obtain PBMCs. Within 0.5 hours of collection, blood was processed at the collection site according to the manufacturer's instructions by centrifugation at 3000 × g at room temperature for 15 minutes. After centrifugation, the CPTs were gently inverted several times to separate plasma, PBMCs, and erythrocytes. We collected the white ring containing PBMCs. PBMCs were then washed twice in PBS and frozen at –80°C for

subsequent RNA isolation. Laboratory personnel were all blinded to the sample's identity.

Analysis in lymphocytes from octogenarians

For PBMCs culture, they were isolated using lymphoprep following the manufacturer´s instructions (Axis shield, Oslo, Norway) from octogenarian individuals. After collecting and washing PBMCs, $2x10^6$ cells were infected with a lentivirus containing *BCL-xL* (pCDH-puro-Bcl-XL, Addgene, plasmid #46972) or the empty vector as previously described (18). Cells were maintained in RPMI high glucose media supplemented with 10% FBS, 2 mM L-glutamine and penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO_2 atmosphere, in the presence or absence of the mitogen PHA 1% (Sigma, St Louis, MO). Cell pellets were harvested for RNA analysis after 9 days in culture.

RNA extraction and cDNA synthesis

Total RNA was isolated using a mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's directions. RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA preparations was assessed by the 260/280 ratio.

cDNA was synthesized from 0.25µg total RNA using a Multi Scribe reverse transcriptase (RT) system kit of Applied Biosystems (High-Capacity cDNA Reverse Transcription Kits). The reaction was incubated as recommended by the manufacturer, for 10 min at 25°C,

followed by 120 min at 37°C, then for 5 min at 85°C, and finally cooled to 4°C to collect the cDNA and then stored at -20°C prior to the real-time PCR assay.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) analysis

The RT-qPCR was performed using the detection system 7900HT Fast Real-Time PCR System (Applied Biosystems) with Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas). Target and control were run in separate wells, using the following procedure: 10 min at 95°C and then 40 cycles of: denaturation 95°C for 15 s and annealing and extension at 62°C for 1 min per cycle. All experiments were repeated at least three times for each sample.

Specific primers employed, sense and antisense for each gene respectively, were: *OCT3/4*, 3'-GATCCTCGGACCTGGCTAAG-5' and 5'-GACTCCTGCTTCACCCTCAG-3'; *SOX2*, 3'-AAAACAGCCCGGACCGCGTC-5' and 5'-CTCGTCGATGAACGGCCGCT-3'; *KLF4*, 3'-CCCACATGAAGCGACTTCCC-5' and 5'-CAGGTCCAGGAGATCGTTGAA-3'; *c-MYC*, 3'-CGCCCTCCTACGTTGCGGTC-5' and 5'-CGTCGTCCGGGTCGCAGATG-3'; *VIM*, 3'-GACAATGCGTCTCTGGCACGTCTT-5' and 5'-TCCTCCGCCTCCTGCAGGTTCTT-3'; *BMP4,* 3'-GATCTTTACCGGCTTCAGTCTGGG-5' and 5'-ACCTCGTTCTCAGGGATGCTGC-3'; *NCAM,* 3'-GGA GCA CTC AAG TGT GAC GA-5' and 5'-CAT CCA GAC AGA CAG CCT CA-3'; *BMPR2,* 3'-CTTTACTGAGAATTTTCCACCTCCTG-5' and 5'-GCCAAAGCAATGATTAT TGTCTCATC–3'.

Results were normalized according to *RPLPO* (3'GGCGACCTGGAAGTCCAACT- 5' and 5'CCATCAGCACCACAGCCTTC) (housekeeping control) quantification in the same sample

reaction. We chose RPLPO as the housekeeping control for these samples, and not GAPDH, because the former varied among the studied groups and the first one remained consistent across the groups. The threshold cycle (CT) was determined and then the relative gene expression was calculated as follows: Relative amount = $2^{-\Delta (\Delta CT)}$, where ΔCT =CT target – CT housekeeping (control) and Δ(ΔCT) = ΔCT studied– ΔCT baseline. Gene level in young group was chosen as the baseline.

Statistical analysis

Statistical analysis was performed using PASW v.21 (SPSS Inc., Chicago, IL, USA). All dependent variables complied with the assumption of normality (Shapiro–Wilks normality test). Standard statistical methods were used to obtain the mean and the standard deviation of the mean. The statistical analysis was performed using the one way analysis of variance (ANOVA) test to check any possible statistically significant difference between groups, followed by the adequate post hoc tests. The *p*- value was set at level <0.05.

RESULTS

Centenarians overexpress Yamanaka factors when compared to octogenarians.

We analyzed the mRNA expression levels of *OCT3/4*, *SOX2*, *KLF4* and *c-MYC* in young, octogenarians and centenarians from Alzira and Sardinia cohorts. As shown in Figures 1a, 1b and 1c, centenarians display higher *OCT3/4*, *SOX2* and *c-MYC* mRNA levels than octogenarians (p<0.05). We also observed that *OCT3/4* and *c-MYC* mRNA levels were

lower in octogenarians, when compared to young people (p<0.05). We did not find any statistical significant difference in *KFL4* mRNA expression between groups (Figure 1d).

Centenarians overexpress stemness-related cell surface marker genes when compared to octogenarians.

Next, we aimed to determine if centenarians overexpress stemness-related cell surface marker genes, when compared to young and octogenarians. Based on our previous functional transcriptomic analysis using Genechip Human Gene1.0 ST array, we observed that centenarians overexpress several stemness surface markers when compared to octogenarians (whole data-set not shown). We selected those that were reported in the literature, such as *VIM* (p=0.00025), *BMP4* (p=0.00124), *NCAM* (p=0.02619) and *BMPR2* (p=0.01008) and aimed to validate them by RT-PCR. As shown in Figure 2 (panels a, c and d), centenarians maintain a high expression of *VIM, NCAM* and *BMPR2* (i.e. similar to young persons), whereas octogenarians do not (p<0.05). The same profile can be observed in the case of *BMP4* (p<0.01) (Figure 2d). The general picture that emerges from these results is than centenarians have a higher pluripotency and stemness-related cell surface marker gene expression than octogenarians and that they display a similar pattern to that of young people.

Bcl-xL enhances pluripotency-related gene expression in lymphocytes from octogenarian individuals in primary culture.

In order to confirm the role of BCL-xL in centenarians' ability to delay stem cell exhaustion, we transduced lymphocytes from octogenarians with *BCL-xL*. As shown in

Figure 3a, this resulted in a seven-fold increased *BCL-xL* mRNA expression, when compared to control lymphocytes from octogenarians. Figure 3b shows that *BCL-xL* overexpression in lymphocytes from octogenarians significantly increased their *SOX2*, *c-MYC* and *KLF4* mRNA levels, when compared to control lymphocytes (p<0.05). Unexpectedly, *OCT 3/4* mRNA expression was not increased, but decreased after BCL-xL overexpression. This is in keeping with a previous study (15), where we reported that mitochondrial membrane potential (ΔΨm) as assessed by JC-1 cytometry was significantly higher in PBMCs obtained from centenarians versus septuagenarians as well as young people, and Cytochrome c levels were decreased in centenarians. These results indicated that centenarians exhibit a low intrinsic apoptosis. We also reported that overexpresion of Bcl-xL in lymphocytes from septuagenarians resulted in a decreased expression of cell cycle inhibitors levels (i.e. p16Ink4a, p14Arf, and p21Cip) and an increased proliferation rate, suggesting an improved renewal capacity.

DISCUSSION

There is increasing evidence that the aging process involves adverse effects on stem cells. Accordingly, it has been suggested that aging-induced deterioration of stem cell functions may play a critical role in the process of aging itself and the pathophysiology of many aging-associated disorders. In the current study, we show that PBMCs from centenarians overexpress Yamanaka Factors, (i.e. *OCT3/4*, *SOX2* and *c-MYC*), as well as some other genes that are considered as stem cells surface markers, such as *VIM*,

NCAM, *BMP4* and *BMPR2*, when compared to octogenarians. This may be one of the reasons why centenarians maintain such an efficient cell homeostasis.

We used PBMCs because they are the most easily accessible and thus can be ethically available from healthy centenarians in sufficient amounts to obtain workable quantities of RNA. Indeed, they express 80% of the genes encoded by the human genome and are therefore considered as a useful cell model for genomics and proteomics studies. Since PBMCs can respond to environmental changes in the body, they have been described as "sentinel" biomarkers ideal as surrogates for organism-wide genetic regulation (19). Furthermore, PBMCs contain many distinct multipotent progenitor cell populations and have the potential to differentiate into almost all cells of the three embryonic layers (20).

Transcription factors regulating pluripotency have been extensively studied, and of these, *OCT3/4* and *SOX2* have been found to play pivotal roles in the molecular mechanism to maintain pluripotency. *OCT3/4* and *SOX2* can synergistically activate OCT-SOX enhancers, by which many pluripotent stem cell-specific genes are regulated (21). Our results on Yamanaka factors indicated a 2- fold increase in *OCT3/4* and *SOX2* mRNA expression in centenarians, when compared to octogenarians. Interestingly, the pattern observed in centenarians was similar to that found in young people. We found a similar result regarding to *c-MYC*, a potent gene with roles in transcriptional regulation of metabolism, differentiation, cell lifespan, cell cycle and cell size control. All these functions are relevant to stem cell renewal and maintenance (22). Indeed, we found

that *c-MYC* mRNA expression was higher in centenarians, when compared to octogenarians. Regarding *KLF4,* it was recently observed that it is associated with longevity in *C. Elegans* (23). However, we did not find an increased expression of this gene in centenarians.

We report here that some genes considered as stemness cell surface-markers, such as *VIM*, *BMP4*, *BMPR2* and *NCAM* are higher in centenarians, when compared to octogenarians. Indeed, *VIM* is considered as a skin-derived precursor stem cell marker, *BMP4* and *BMPR2* are considered osteoblasts stem cell markers. *NCAM* is also a well-known neurological marker in neural stem cells formed in the central nervous system and is involved in their migration and differentiation.

In previous studies, we reported that the expression patterns of miRNAs and mRNAs in centenarians are very different from octogenarians and similar to young people (14–15). We also found that *BCL-xL*, that inhibits the intrinsic mitochondrial pathway to apoptosis, thus promoting cell survival, was elevated in centenarians. This is probably involved in centenarians' ability to protect cells from apoptosis and immunosenescence (15). In this study, we observe that lymphocytes from octogenarians transduced with *BCL-xL in vitro* increase *SOX2*, *c-MYC* and *KLF4* mRNA expression, when compared to control lymphocytes. Thus, in this regard, cells from octogenarians, when they overexpress BCL-xL, behave in a similar way to those from centenarians, as we previously suggested (15). In line with our results, it has been demonstrated that the inclusion of *BCL-xL* together with several combinations of Yamanaka Factors

(*OCT3/4+SOX2*, *OCT3/4+SOX2+KLF4* and *OCT3/4+SOX2+KLF4+c-MYC*) in PBMCs is able to increase iPSCs generation efficiency up to 10 fold, thus suggesting a role of *BCL-xL* in iPSCs long-term self-renewal and proliferation (16).

Aging tissues exhibit a progressive decline in homeostatic and regenerative abilities, which has been attributed to degenerative changes in tissue-specific stem cells, stem cell niches and systemic cues that regulate stem cell activity. As stem cells age, their renewal capacity and their ability to differentiate into the various cell types is diminished (24). Together, our results on *BCL-xL* and Yamanaka Factors may indicate that centenarians (healthy aging) maintain their stemness, this is, the ability to self-renew and replenish their damaged tissues better than octogenarians (normal aging). In this regard, we recently published that senescent cells downregulate Yamanaka Factors and that environmental factors, such as oxygen tension, plays a critical role in *in vitro* senescence (9). Thus, our results showing that centenarians upregulate pluripotency-related genes may indicate that they are better protected against cellular senescence than octogenarians.

To conclude, the main finding reported here is that centenarians overexpress pluripotency and stemness related genes, and that BCL-xL seems to regulate their expression. This provides new insight into the molecular machinery that endows centenarians with the ability to maintain such an optimal health status that their health span approximates their lifespan.

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CONFLICT OF INTERESTS:

The authors declare no Potential Conflicts of Interest.

AUTHORS CONTRIBUTION

MI and C M-B performed the experimental work in human samples, A B-E and A M performed the experimental work and in the *in vitro* studies, P S and J-A A collected the samples, and CB and JV directed the work.

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FIGURE LEGENDS:

Figure 1. Yamanaka Factors' expression in PBMCs from centenarians. a) OCT3/4 mRNA expression in PBMCs from young (n=11), octogenarians (n=27) and centenarians (n=34). **b)** SOX2 mRNA expression in PBMCs from young (n=11), octogenarians (n=26) and centenarians (n=36). **c)** c-MYC mRNA expression in PBMCs from young (n=11), octogenarians (n=28) and centenarians (n=36). **d)** KLF4 mRNA expression in PBMCs from young (n=11), octogenarians (n=28) and centenarians (n=35). Data are expressed as means ±SE. *= $p<0.05$ vs. octogenarians; #= $p<0.05$ vs. young.

Figure 2 Stemness-related cell surface marker gene expression in PBMCs from centenarians. a) VIM mRNA expression in PBMCs from young (n=22), octogenarians (n=29) and centenarians (n=48). **b)** NCAM mRNA expression in PBMCs from young (n=21), octogenarians (n=31) and centenarians (n=49). **c)** BMP4 mRNA expression in PBMCs from young (n=22), octogenarians (n=32) and centenarians (n=47). **d)** BMPR2 mRNA expression in PBMCs from young (n=22), octogenarians (n=29) and centenarians (n=42). Data are expressed as means ±SE. *= $p<0.05$ vs. octogenarians; **= $p<0.01$vs. octogenarians; #= $p<0.05$ vs. young; ##= $p<0.01$vs. young.

Figure 3. Yamanaka Factors' expression in lymphocytes from octogenarian individuals transduced with BCL-xL. a) BCL-xL mRNA expression in lymphocytes from octogenarian individuals transduced with BCL-xL, compared to control lymphocytes. **b)** OCT 3/4, SOX2, c-MYC and KLF4 mRNA expression in lymphocytes from octogenarian individuals

transduced with BCL-xL, compared to control lymphocytes. Data are expressed as means ±SE. **= $p<0.01$ vs. control; *= $p<0.05$ vs. control.

Figure 1

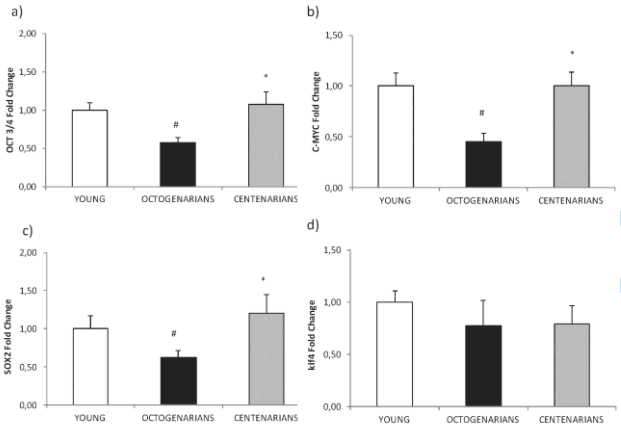

Figure 2

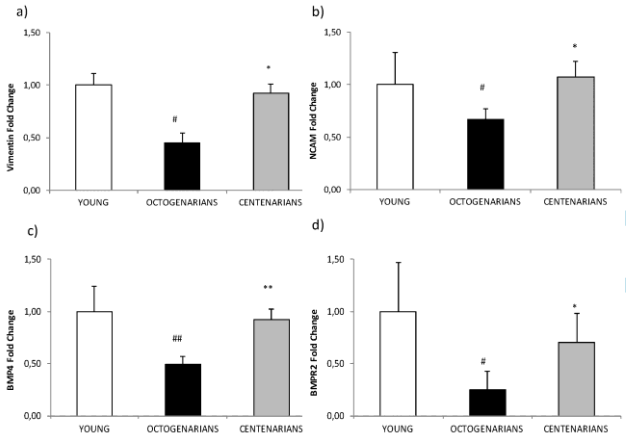

Figure 3