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SOX2 expression diminishes with ageing in several tissues in mice and humans

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ABSTRACT

SOX2 (Sex-determining region Y box 2) is a transcription factor expressed in several foetal and adult tissues and its deregulated activity has been linked to chronic diseases associated with ageing. Nevertheless, the level of SOX2 expression in aged individuals at the tissue level has not previously been examined. In this work, we show that *SOX2* expression decreases significantly in the brain with ageing, in both humans and rodents. The administration of resveratrol for 6 months in mice partly attenuated this reduction. We also identified an age-related decline in SOX2 mRNA and protein expression in several other organs, namely, the lung, heart, kidney, spleen and liver. Moreover, peripheral blood mononuclear cells (PBMCs) from elderly expressed lower levels of *SOX2* than those from young individuals. Mechanistically, *SOX2* expression inversely correlates with $p16^{Ink4a}$ levels. Together, these data show a widespread decrease in SOX2 with age, suggesting that the decline in SOX2 expression might be used as a biomarker of ageing.

1. Introduction

Ageing is a multifactorial process that affects most cells, organs and tissues. It is characterized by a decline in the ability of tissues to maintain homeostasis and the impaired regeneration and/or repair of lost or damaged cells, which leads finally to mortality. In addition, ageing contributes to the development and progression of the pathology of several chronic diseases. It is well-known that ageing promotes complex molecular changes affecting the structure and activity of organs and tissues throughout the body. Recently, however, research has started to unravel the key mechanisms that underlie such changes, and consequently, that serve as hallmarks of ageing and drivers of disease (Lopez-Otin et al., 2013; Hodes et al., 2016; Kennedy et al., 2014). Stem cell exhaustion has been described as a hallmark of ageing, driver of disease, and constitutes a pillar of geroscience (Lopez-Otin et al., 2013; Hodes et al., 2016; Kennedy et al., 2014).

The existence of biomarkers suitable for measuring or predicting the degree of biological ageing at either population or individual levels would have utility as surrogate endpoints. Although currently no definition of a biomarker of ageing or criteria for its selection are universally recognized, the American Federation for Aging Research proposed three criteria for a biomarker of ageing: (i) it must predict a person's rate of ageing; in other words, it should measure biological rather than chronological age and predict the future onset of age-related diseases; (ii) it must work in humans and in laboratory animals; and (iii) it must be able to be tested without harming the individuals.

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Over recent decades, ongoing efforts to identify reliable and readily-measured biomarkers of ageing have identified several candidates (Comfort, 1969; Baker and Sprott, 1988; Johnson, 2006; Lara et al., 2015; Burkle et al., 2015). Among them, the increase in the expression of *p16^{Ink4a}* cell cycle inhibitor is one of the most robust and best-established biomarkers of ageing known to date (Kim and Sharpless, 2006). Its expression is very low or undetectable during development and in young healthy individuals, but increases progressively in most tissues with chronological age in all mammalian species tested (Kim and Sharpless, 2006; Krishnamurthy et al., 2004; Zindy et al., 1997). Expression of p16^{INK4a} also increases in human peripheral blood cells with age (Liu et al., 2009a). Human genome-wide association studies identified genetic variants in regions near p16^{INK4a} that confer an increased risk of several age-related diseases including atherosclerotic vascular diseases, type 2 diabetes and glaucoma (Jeck et al., 2012; Liu et al., 2009b). It has also been shown that p16^{INK4a} expression regulates stem cell activity in several niches such as in the hematopoietic system, the pancreas and the central nervous system (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). Apart from the knowledge of p16^{INK4a} as a stem cell marker and biomarker of ageing, little is known about the expression and function of genes involved in stem cell activity on ageing (Oh et al., 2014).

Sex-determining region Y box 2 (SOX2) is a transcription factor that maintains the pluripotency of early embryonic cells and plays critical roles during the formation of several cell types and tissues in the embryo (Avilion et al., 2003; Takahashi and Yamanaka, 2006). Moreover, SOX2 sustains adult stem cells (Arnold et al., 2011), acting intrinsically to confer stem cell properties, but also more broadly by regulating the expression of critical niche factors, as shown, for example, in the central nervous system (Favaro et al., 2009). Moreover, it is expressed in several adult epithelial tissues including the testes, lung, lens, glandular stomach, oesophagus, forestomach, tongue, anus, and cervix, as well as glands associated with the oral cavity, trachea and cervix (Arnold et al., 2011). In humans, *SOX2* inactivating mutations cause syndromic microphthalmia-3 and anophthalmia-oesophageal-genital (AEG) syndrome, genetic diseases characterized in particular by severe eye defects and abnormalities in the hypothalamic-pituitary-gonadal axis (Fantes et al., 2003; Kelberman et al., 2006; Williamson et al., 2006). Clinical features observed in such patients also include developmental delay, poor growth, cognitive deficit, brain malformations, seizures, mesial temporal motor disorder, sensorineural hearing loss, anterior pituitary hypoplasia, oesophageal malformations, horseshoe kidney, and male genital abnormalities (Williamson et al., 2006; Osborne et al., 2011), reflecting its roles in multiple and different organs. Moreover, SOX2 is overexpressed in the majority of solid cancers including those of the lung, oesophageal, brain, skin, and breast, sarcomas and colorectal human cancers where it acts to control tumour progression and cancer cell activity (Bass et al., 2009; Sarkar and Hochedlinger, 2013; de la Rocha et al., 2014). SOX2 overexpression is associated with genetic amplification in several cancers including squamous cell carcinomas of the lung, oesophagus, and glioblastoma (Bass et al., 2009; Alonso et al., 2011). Nevertheless, little is known about its expression and activity in relation to mammalian ageing. It has been shown that its levels qualitatively decreased in different areas of the brain in middle age mice (Brazel et al., 2005). In this study, we investigated the expression of SOX2 in different tissues in both rodents and humans with ageing. We also tested the levels of SOX2 in human-derived PBMCs. Finally, we assessed whether there is any correlation with p16^{INK4a} levels.

2. Methods

2.1. Human and animal studies

The Ethic Committee in Clinical Research of the Basque Country approved the study. Written informed consent was obtained from all patients prior to sample collection. PBMCs were obtained from community-dwelling independent individuals of two different cohorts, one from Basque Country (range of age; young from 30 to 45 (n = 4) and elderly from 80 to 87 (n = 14)) and the other from an area near Valencia (range of age; young from 25 to 35 (n = 11) and elderly from 74 to 87 (n = 27)), both in Spain and described previously (Alberro et al., 2016; Borras et al., 2016). The BIOMICs group provided the human brain samples from cortex and hippocampus of same individuals (range of age; young from 22 to 32 (n = 10) and elderly from 83 to 96 (n = 9)).

The Biodonostia and University Pablo Olavide Animal Care Committees approved the animal experimentation in accordance with the Spanish and European relevant guidelines. *C57Bl6* mice were housed under specific pathogen-free conditions in barrier facilities of the Biodonostia Health Research Institute and University Pablo Olavide. For the resveratrol experiment, a group of eight 18-month-old male *C57Bl6* mice were used. They were divided into two groups of four each: Control non treated and fed with liquid containing resveratrol for 6 months, following the international rules for animal research (Tung et al., 2015). Old mice were euthanized upon signs of morbidity.

2.2. RNA analysis

Total RNA was extracted with Trizol (Life Technologies). Reverse transcription was performed using random priming and SuperScript Reverse Transcriptase (Life Technologies), according to the manufacturer's guidelines. Quantitative real-time PCR was performed using Absolute SYBR Green Mix (Thermo Scientific) in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Variations in input RNA were corrected for by subtracting the number of PCR cycles obtained for *ß-actin* and *GAPDH* in murine and human samples respectively.

2.3. Immunohistochemistry

Murine tissues were dissected, fixed in buffered 4% formaldehyde solution (Sigma) and embedded in paraffin. Subsequently, 4-μm-thick slices were cut, deparaffinized, rehydrated through a graded series of alcohols and heated in citrate buffer for 10 min for antigen retrieval. Sections were stained with anti-Sox2 antibody (rabbit polyclonal to Sox2; 1:200 dilution, ab97959, Abcam). The sections then were washed and incubated with MACH 3 Rabbit Probe and MACH 3 Rabbit HRP-Polymer (M3R531, Biocare Medical). Immunostaining was developed with a 3,3′-diaminobenzidine (DAB) solution.

2.4. Statistics

Data are presented as mean values ± SEM with the number of experiments (n) in parenthesis. Unless otherwise indicated, statistical significance (*p*-values) was calculated using the Studenťs *t*-test.

3. Results

3.1. SOX2 expression decreases in rodent and human brain with ageing

In order to quantitatively evaluate the expression of SOX2 with age in different tissues, we first examined its levels in mouse brain tissue. For this, we homogenized brain samples from animals of different ages, and found that over 24-month-old aged mice expressed significantly lower levels of *Sox2* than young ones (Fig. 1A). *Sox2* expression was also decreased specifically in the neurogenic niche of the hippocampus in aged mice (0.29 fold) (Fig. 1B). As expected, *p16Ink4a* was elevated (3 and 18 fold) in the same aged group compared to young mice (Fig. 1A,B).

Next, we tested the expression of *SOX2* in a set of hippocampus and cortex sections obtained from human brains. The expression of *SOX2* in

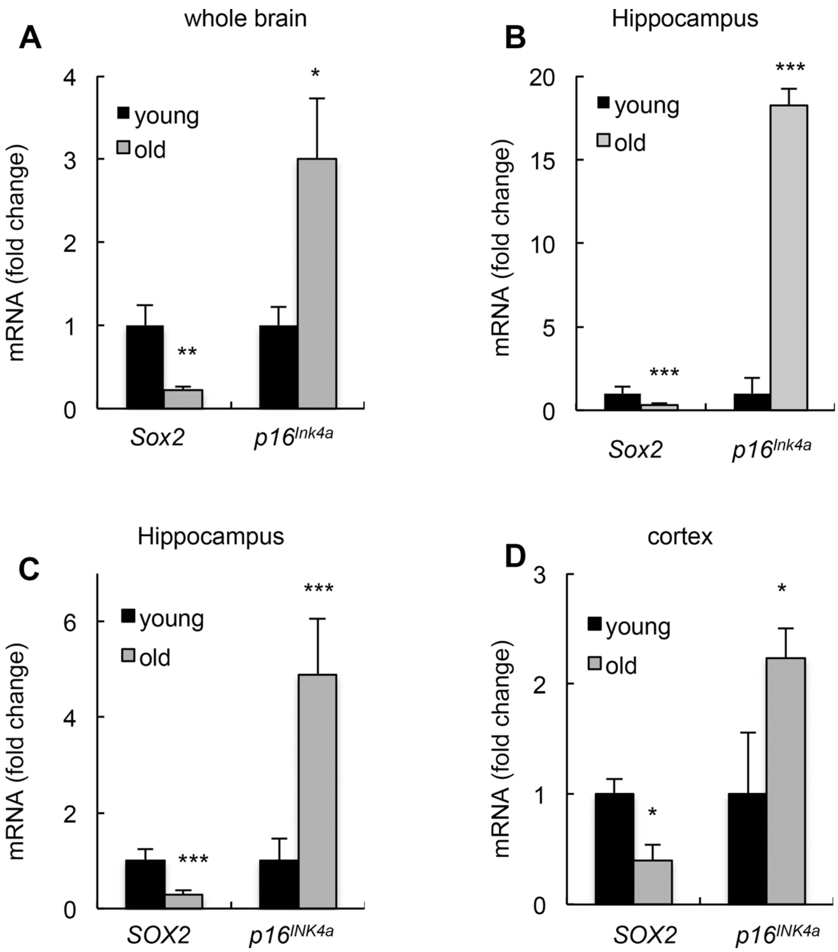

Fig. 1. *SOX2* expression decreases in the brain with ageing. **(A)** *Sox2* (p = .011) and *p16Ink4a* (p = .02) mRNA levels in the whole brain from mice (n = 4) of different ages (8 and 24 months). **(B)** *Sox2* and *p16Ink4a* mRNA levels in the hippocampus from 2 (young) and 24 (old) months-old mice ($n \geq 6$). **(C,D)** Quantification of *SOX2* and *p16INK4a* mRNA levels in the hippocampus ($p < 0.001$) and cortex (p = .05) area of brains from young (20–30) and aged (over 80 years-old) human individuals. Data are mean ± SEM, and the number of individuals per group is $n \geq 4$. Asterisks (*, **, and ***) indicate statistical significance ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively).

both areas of individuals aged between 80 and 95 years was significantly lower than that in 20–35–year-olds (Fig. 1C,D). Similarly to mice, the levels were 70% lower in aged than young human hippocampus brain samples. Further, in the human tissues, the expression of *p16INK4a* was higher in samples from elderly than young individuals (Fig. 1C,D).

Resveratrol delays ageing in several species (Gambini et al., 2015) and modulates the expression of SOX2 to facilitate the maintenance of self-renewal and multipotency (Yoon et al., 2014). Thus, we examined SOX2 levels in the whole brain of aged mice fed with resveratrol for 6 months, and compared them with non-treated controls. Interestingly, we noted 3-fold higher *Sox2* mRNA expression in the resveratrol-treated than the non-treated mice (Fig. 2A). Moreover, analysis including the expression in young mice showed that resveratrol treatment significantly attenuated the decline in *Sox2* levels observed in aged mice, although expression did not remain as high as in young mice (Fig. 2B). In parallel, we observed a significant increase on $p16^{Ink4a}$ levels in non-treated aged mice, an accumulation which was slightly ameliorated in the group fed with resveratrol (Fig. 2C). These results show that a decrease in *Sox2* levels correlates with brain tissue ageing in rodents and humans, whilst the anti-ageing treatment resveratrol, maintains *Sox2* expression.

3.2. SOX2 expression decreases in rodent tissues with ageing

In order to further explore the expression of Sox2 with ageing, we measured its levels in cells isolated from different tissues from young (2 months-old) and aged (over 24 months-old) mice. Intriguingly, *Sox2* levels were markedly diminished (< 0.5-fold) in all tissues studied including from the lung, kidney, spleen and heart (Fig. 3A). In contrast, the expression of $p16^{Ink4a}$ was significantly elevated in all tissues, with fold changes of 4–10 (Fig. 3B). These results confirm the inverse

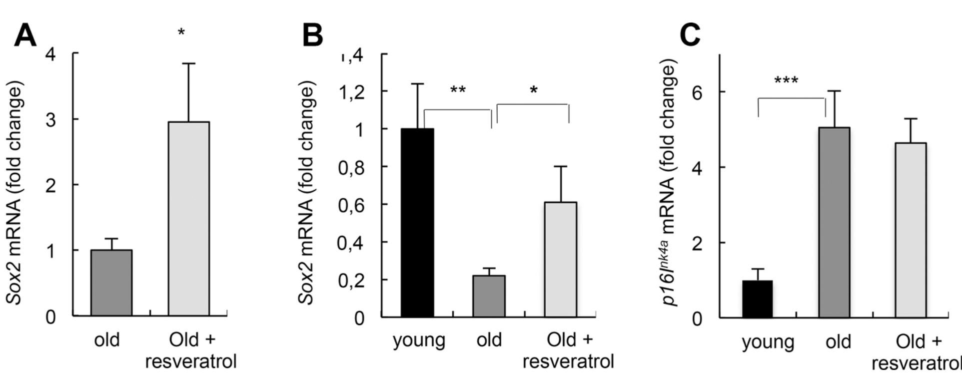

Fig. 2. Resveratrol ameliorates ageing-related *SOX2* decrease in the brain. **(A)** *Sox2* expression in the whole brain from old mice that had and had not been treated with resveratrol (p = .038) for 6 months (n = 4). **(B)** Relative quantification of *Sox2* levels in the aforementioned old animals compared to young animals (p = .01). **(C)** Relative quantification of $p16^{Ink4a}$ levels in the aforementioned animals. Asterisks (*, **, and ***) indicate statistical significance ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively).

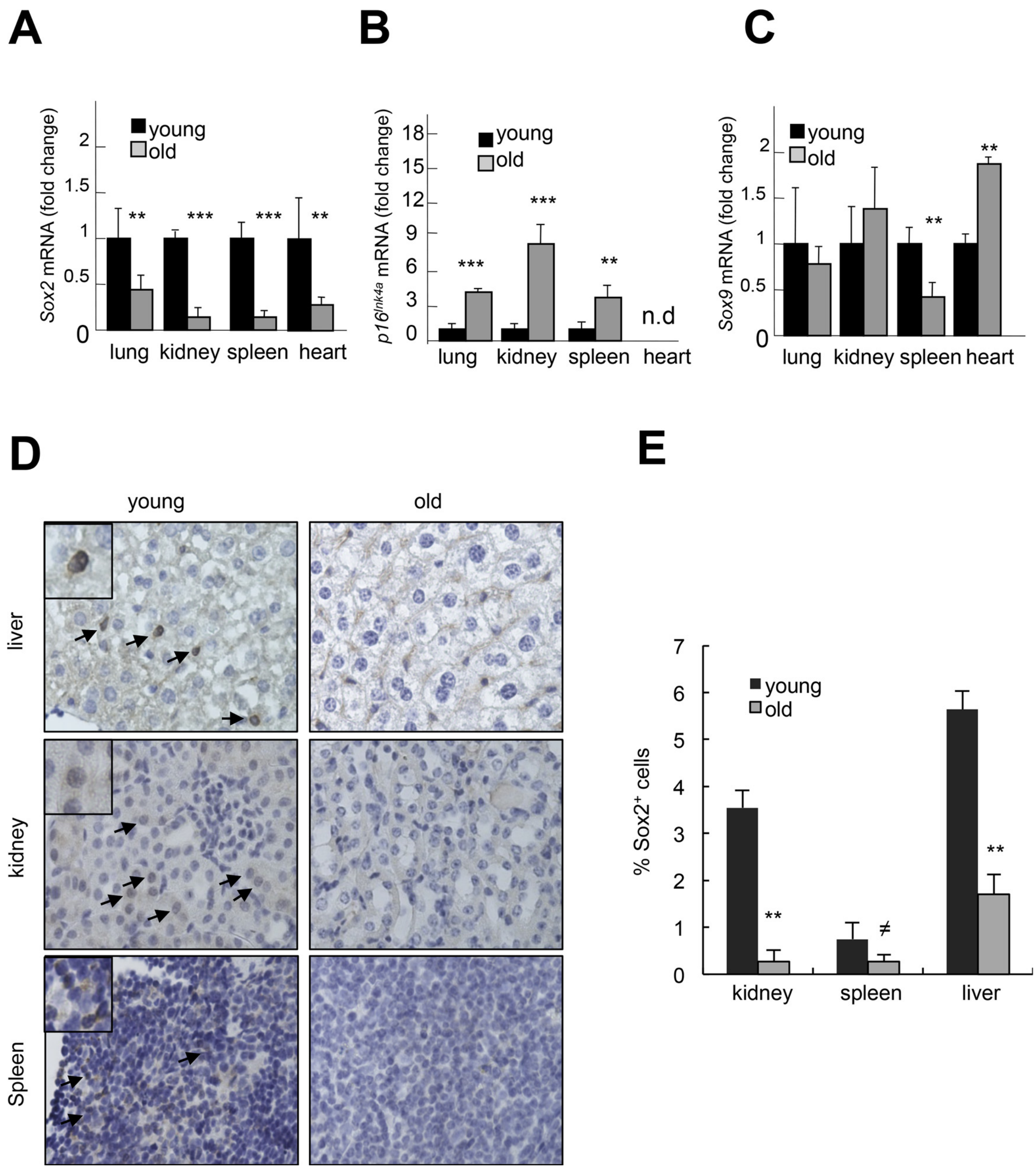

Fig. 3. SOX2 mRNA and protein levels decrease in mouse tissues with ageing.
(A) Quantification of *Sox2*, **(B)** *p16Ink4a* and **(C)** *Sox9* mRNA in *ex vivo* cells from the lung, kidney, spleen and heart of young (2 months) and old (≥24 months) mice. The number of mice per group is n ≥ 4. **(D)** Representative images of SOX2 immunohistochemistry at x40 magnification in sections from the kidney, spleen and liver of 2- and 24-month-old mice (n = 4). **(E)** Quantification of SOX2-positive cells in tissues from the aforementioned young and old mice (n = 4) (p = .001, 0.1 and 0.001, respectively).

correlation between *Sox2* and $p16^{Ink4a}$ expression with ageing at tissue level.

SOX2 belongs to SOX family, and might share biochemical properties and have overlapping expression patterns with other members of the family. In particular, SOX2 expression correlates with SOX9 in homeostasis and in some pathological contexts in the brain (Scott et al., 2010; Hoffmann et al., 2014; Garros-Regulez et al., 2016). Therefore, we measured the expression of *Sox9* in the above-indicated tissues. In this case, we did not detect a regular decline in its levels with ageing (Fig. 3C).

Next, we performed additional analysis measuring SOX2 protein expression by immunohistochemistry in kidney, spleen and liver. We noted that the number of SOX2-positive cells differed across the tissue types but, in all cases, there were fewer in tissues from over 24-month-old mice (Fig. 3D). Specifically, the percentage of SOX2-positive cells in the kidney, spleen and liver ranged respectively from 3.5, 0.75 and 5.65 in young mice to 0.27, 0.26 and 1.71 in aged mice (Fig. 3E). These results show that there is a robust correlation between Sox2 decline in mRNA and protein expression and organismal ageing across different tissues.

3.3. SOX2 expression decreases in PBMCs with ageing

Finally, we extended the *SOX2* characterization in human samples, by checking its levels in blood cells. We detected 0.75-fold lower levels of *SOX2* in PBMCs in a cohort of Basque octogenarians than in young individuals (Fig. 4A). In these PBMCs, the expression of *p16INK4a* was higher in samples from elderly (Fig. 4A). Given that the Basque

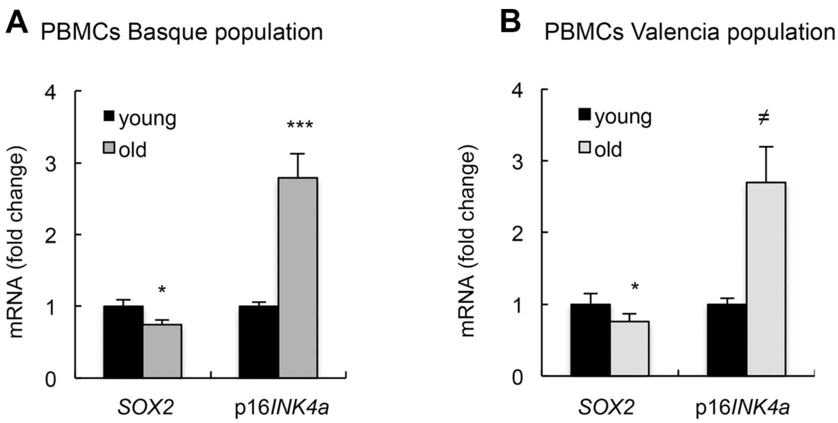

Fig. 4. *SOX2* levels diminish in human PBMCs with ageing. **(A)** qRT-PCR analysis of *SOX2* ($p = .02$) and $p16^{INK4a}$ ($p = .0008$) in PBMCs from young ($n = 4$) and aged (old, $n = 14$) individuals from the Basque country. **(B)** Measurement of *SOX2* ($p = 0.05$) and $p16^{INK4a}$ ($p = .06$) levels in PBMCs from an independent cohort of individuals from Valencia ($n \geq 7$). Asterisks (≠, *, and **) indicate statistical significance ($p \leq 0.1$, $p \leq 0.05$, and $p \leq 0.01$, respectively).

population is genetically distinct (Behar et al., 2012), we wondered whether the decrease in SOX2 might be specific to this region. Therefore, we additionally measured *SOX2* levels in PBMCs from a cohort of aged individuals from Valencia, a region located on the Spanish Mediterranean coast. These individuals also showed diminished *SOX2* expression (Fig. 4B), which was correlated with $p16^{INK4a}$ elevation (Fig. 4B). Moreover, the magnitude of decrease was similar in both populations (25–30%), further confirming our original finding. Together, these results demonstrate that *Sox2* levels decrease in human PBMCs with age.

4. Discussion

The identification of biomarkers for age-related changes in mammalians is a topic that has been intensively studied for several decades (Baker and Sprott, 1988), providing findings that allowed the American Federation for Aging Research to propose the following three criteria for a biomarker of ageing; (i) it must predict a person's rate of ageing; (ii) it must work in human and laboratory animals; and (iii) it must be testable without harming the individuals. Our results show that SOX2 expression in a wide range of organs including the brain, lung, liver, spleen and kidney is lower in aged than young individuals. Our data also reveal that SOX2 levels diminish similarly in human and mice samples and inversely correlate with those of *p16INK4a*, currently considered one of the most robust biomarkers of ageing. Therefore, we can conclude that Sox2 decline is a marker of chronological ageing. Moreover, this decrease is attenuated after long-term treatment with resveratrol, a well-established agent that extends lifespan and delays ageing in several animal models (Gambini et al., 2015), and hence, Sox2 might also be considered a biomarker of physiological ageing. In line with this idea, Sox2 levels have been observed to be elevated in different areas of the brain in a mouse model with elongated lifespan and delayed ageing (Matheu et al., 2007; Carrasco-Garcia et al., 2015). Finally, *SOX2* levels also reduce with ageing in PBMCs of human individuals, meaning that the level of SOX2 expression could be detected through a non-invasive blood test. With this, we have demonstrated that the decline in SOX2 expression fulfils all three criteria of the American Federation for Aging Research, and therefore propose SOX2 as a novel biomarker of ageing.

We found that *SOX2* expression is lower in the brains of 75-year-old individuals and over-2 year-old mice. In agreement with these data, SOX2 levels are reduced in middle-aged (1-year-old) mice, across different areas of the brain (Brazel et al., 2005). These areas include neurogenic areas such as the subventricular zone and hippocampus, where stem cells reside, and others such as the olfactory bulbs, third ventricle, spinal cord and cerebellum (Brazel et al., 2005). Since there is a decrease in the population of neural stem cells with age (Lopez-Otin et al., 2013; Capilla-Gonzalez et al., 2015), it is tempting to speculate that SOX2 reduction marks the decline in neural stem cell numbers with ageing. Future work should test this hypothesis, but there is already evidence supporting the idea: SOX2 is a marker of the nervous system from early development stages (Avilion et al., 2003), it is expressed in embryonic and adult neural stem cells (Brazel et al., 2005; Graham et al., 2003; Pevny and Nicolis, 2010), and high levels of SOX2 can be used to distinguish quiescent neural stem cells from neuroblasts and other differentiating/differentiated cells (Andersen et al., 2014). The widespread expression of SOX2 in multiple adult stem cell compartments (Arnold et al., 2011), might allow to extend the idea of the correlation between the decrease in SOX2 and the decline in stem cell and progenitor numbers to different organs. For example, we observed a significant decrease in *Sox2* in the lung, where it is required for normal development and maintenance in both the embryo and adult, and SOX2 expression is restricted in the epithelium of the trachea and airways, areas where stem cells are found (Que et al., 2009). Nevertheless, it should be emphasized that SOX2 is not only expressed in stem cells and progenitors, and *SOX2* is also important for the differentiation and maintenance of diverse cell types such as neurons and oligodendrocytes in the brain (Hoffmann et al., 2014; Cavallaro et al., 2008; Ferri et al., 2004), and in neutrophils (Xia et al., 2015), indicating that the decline in SOX2 with ageing might not be restricted to stem cell/progenitor populations. For example, SOX2 expression in neutrophils and its function as a sensor to initiate innate immunity might explain the decline we observed in spleen and human PBMC levels of *SOX2* with age.

Intriguingly, *Sox2* levels were detected and found to be downregulated in the kidney, spleen and heart from aged mice, tissues in which previously no GFP signal was detected in a *knock-in* Sox2-GFP mouse model, where GFP was integrated in one allele under the control of the endogenous locus-regulatory regions of *Sox2* gene (Arnold et al., 2011). This is likely to be a consequence of the small number of SOX2-positive cells and this may also indicate that SOX2 levels are lower in these that in other tissues. The decrease in the kidney and spleen is particularly remarkable. Kidney injuries are the most common age-associated pathology (Matheu et al., 2007), and the decrease in SOX2 expression might indicate a predisposition to organ failure. In line with this idea, ectopic *Sox2* is able to reprogramme and dedifferentiate murine and human kidney cells *in vivo* (Abad et al., 2013; Montserrat et al., 2012). Future work will need to elucidate whether the lower expression of SOX2 with age might be responsible for pathologies in some situations. In support of this idea, *Sox2* deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain (Ferri et al., 2004); Sox2 activity functions in a dose-dependent manner (Cavallaro et al., 2008; Taranova et al., 2006; Que et al., 2007). Regarding human pathologies, SOX2 levels are decreased in samples from patients with Alzheimer's disease (Crews et al., 2010; Sarlak and Vincent, 2016); and loss-of-function point mutations and deletions of SOX2 have been linked to severe abnormalities in multiple different human tissues, where SOX2 is expressed during embryonic development and at adult stages (Arnold et al., 2011; Kelberman et al., 2006; Williamson et al., 2006; Osborne et al., 2011).

Mechanistically, we have identified that there is a significant inverse correlation between SOX2 and p16^{INK4a} in human brain and PBMCs and in mice-isolated brain, lung, kidney and spleen samples. Moreover, we measured the expression of *Sox9*, another important member of SOX family, which expression is associated to SOX2 in different contexts and cell types in the brain (Scott et al., 2010; Hoffmann et al., 2014; Garros-Regulez et al., 2016). Interestingly, we did not observe a similar pattern in *Sox2* and *Sox9* expression or detect an inverse correlation between *Sox9* levels and ageing in the tissues studied. These data suggest that the widespread decrease of SOX2 with age might be unique among SOX members.

In summary, our results demonstrate that SOX2 levels decrease with ageing in different tissues in both rodents and humans. We also show an inverse correlation with p16^{Ink4a} levels. Taken together, our data show that the decline in SOX2 expression is a biological marker of ageing,

Author contributions

EC-G, LM-C, IG, CB and MR performed experimental work and analysed data; AI, GL, MMP and IV, provided reagents and biological samples, JV and AM directed experimental work, and AM designed the research study and wrote the paper.

Conflict of interest

The authors have declared that no conflict of interest exists.

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