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Markers of senescence are often associated with neuronal differentiation in the developing sensory systems

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Summary. It has been shown that senescent cells accumulate in transient structures of the embryo that normally degenerate during tissue development. A collection of biomarkers is generally accepted to define senescence in embryonic tissues. The histochemical detection of β-galactosidase activity at pH 6.0 (β-gal-pH6) is the most widely used assay for cellular senescence. Immunohistochemical detection of common mediators of senescence which block cell cycle progression, including p16, p21, p63, p15 or p27, has also been used to characterize senescent cells in the embryo. However, the reliability of this techniques has been discussed in recent publications because non-senescent cells are also labelled during development. Indeed, increased levels of senescent markers promote differentiation over apoptosis in developing neurons, suggesting that machinery used for the establishment of cellular senescence is also involved in neuronal maturation. Notably, it has recently been argued that a comparable state of cellular senescence might be adopted by terminally differentiated neurons. The developing sensory systems provide excellent models for studying if canonical markers of senescence are associated with terminal neuronal differentiation.

Key words: β-Galactosidase, Cell differentiation, Developmental cell senescence, Histochemistry, Olfactory epithelium, Sensory system, Vertebrate embryo

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Introduction

Senescent cells undergo permanent inhibition of cell replication, change their morphology to more flat and large cells, alter gene expression patterns, change chromatin organization, and express and secrete cytokines, growth factors and pro-apoptotic factors (Hernández-Segura et al., 2018; Gorgoulis et al., 2019; Schafer et al., 2020; Wagner and Wagner, 2022; Weng et al., 2022). Cellular senescence is mainly associated with cellular and organismal aging and aging-related diseases (Rhinn et al., 2019; Borgonetti and Galeotti, 2022; Kudlova et al., 2022; Liu, 2022; Nacarino-Palma et al., 2022; Ring et al., 2022; Wagner and Wagner, 2022).

More recently, it has been demonstrated that senescence occurs from early stages of embryo development to adulthood, and plays roles in normal development, maintains tissue homeostasis, regulates healing and regeneration, and limits tumor progression (Muñoz-Espín and Serrano, 2014; Ritschka et al., 2017; McHugh and Gil, 2018; Chia et al., 2021). Developmental senescence is a conserved feature of embryonic development across vertebrates (Czarkwiani and Yun, 2018; Ring et al., 2022). It appears in discrete developmental windows during embryogenesis in evolutionary distant organisms such as fish (Villiard et al., 2017; Da Silva-Álvarez et al., 2020), amphibians (Davaapil et al., 2017; Villiard et al., 2017), birds (Nacher et al., 2006; Lorda-Díez et al., 2015a, 2019; Sánchez-Fernández et al., 2019, 2020, 2021; de Mera-Rodríguez et al., 2021), and mammals (Muñoz-Espín et al., 2013; Storer et al., 2013; Wanner et al., 2021). Markers of cellular senescence, such as p21, p63, and p73, are detected in the developing limb (Lorda-Díez et al., 2015a; Sánchez-Fernández et al., 2020; de Mera-Rodríguez et al., 2021), pronephros and mesonephros (Nacher et al., 2006; Davaapil et al., 2017; Da Silva-Álvarez et al., 2018), heart (Lorda-Díez et al., 2019) and

central nervous system (Domínguez-Bautista et al., 2021). In most of these structures, senescence is primarily involved in structural degeneration, and senescent labelled cells coincide with areas affected by intense cell death (see below). Many authors have demonstrated that cellular senescence is followed by apoptosis in different systems (Muñoz-Espín et al., 2013; Storer et al., 2013; Lorda-Díez et al., 2015b).

Cellular senescence markers

Cellular senescence has been proposed to have a role in both ageing and tumor suppression, therefore, identifying senescent cells *in vivo* and *in vitro* has important diagnostic and therapeutic potential (Basisty et al., 2020). A certain amount of consensus has been established on how to identify senescent cells (Gorgoulis et al., 2019). Senescent cells lack markers of cell proliferation such as Ki67 or PCNA (Rhinn et al., 2019). They also activate cell cycle inhibitors p16 (INK4a) and $p14^{ARF}$/$p19^{ARF}$ which function inducing the expression of Rb protein and p53 transcription factor respectively in senescent cells (Lowe and Sherr, 2003; Wagner and Wagner, 2022). However, senescent cells in the developing embryo show enhanced expression of p21, but not that of p53, p16, or p19 (Muñoz-Espín et al., 2013; Storer et al., 2013). Other genes such as p63 and p73 also block cell cycle progression in senescent cells in the developing embryo (Lorda-Díez et al., 2015a; Sánchez-Fernández et al., 2020).

Nevertheless, one of the best-characterized and simplified methods to identify senescence *in vitro* and *in vivo* is the β-galactosidase histochemical staining at pH 6.0 (β-gal-pH6, also called "senescence-associated β-galactosidase, SA-β-GAL) (Dimri et al., 1995). Endogenous β-gal activity catalyses the hydrolysis of β-galactosides into monosaccharides. When acid β-gal is histochemically traced at pH 4.0, it appears strictly confined to lysosomes (Groh and von Mayersbach, 1981). In the case of senescent cells, this enhanced enzymatic activity is also related to increased lysosomal content and altered cytosolic pH (Kurz et al., 2000; Severino et al., 2000; Lee et al., 2006; Hernández-Segura et al., 2018; de Mera-Rodríguez et al., 2019).

β-gal-pH6 labelling is also detected in transient developmental structures in whole mount embryos (Muñoz-Espín et al., 2013; Storer et al., 2013; Lorda-Díez et al., 2015a,b; 2019; de Mera-Rodríguez et al., 2021) and is closely related with progression of the apoptotic process in some models. It suggests that, in some developing tissues, senescence is followed by apoptosis in cells with unrepairable damage. Nonetheless, some researchers detect intense β-gal-pH6 activity in a wide range of healthy post-mitotic cells. For instance, intense histochemical signal is found in the visceral endoderm of the early mouse embryo (Huang and Rivera-Pérez, 2014), in macrophages and osteoclasts in mature (Bursuker et al., 1982; Kopp et al., 2007; Hall et al., 2017) and developing tissues (de Mera-Rodríguez et al., 2021). Other authors have shown that the expression of well-known senescence markers, such as p16, p19, and p21 is highly dynamic in many organs of embryos, early postnatal, mature, and old mice (Zindy et al., 1997; Safwan-Zaiter et al., 2022). These data might implicate these senescence markers in developmental and physiological processes in addition to their well-known function in the build-up of cellular senescence.

In the case of the nervous system, it has been shown that the number of neurons labelled with senescent markers increases during aging in long-term *in vitro* neuronal cultures (Bhanu et al., 2010; Jurk et al., 2012). However, populations of healthy postmitotic neurons located in different regions of the brain of young, adult, and old animals are also strongly positive for β-gal-pH6 histochemistry (Jurk et al., 2012; Piechota et al., 2016; de Mera-Rodríguez et al., 2021). Other authors have also described intense β-gal-pH6 activity in populations of neurons located in the roof plate of the neural tube and in motoneurons of vertebrate embryos (Muñoz-Espín et al., 2013; Storer et al., 2013; Domínguez-Bautista et al., 2021). Additionally, an up-regulation of the p16, p19, and p21 mRNAs and proteins is detected in different sub-populations of neuronal cells in the brain of embryos, adults, and old mice (Safwan-Zaiter et al., 2022), and populations of healthy β-gal-pH6-labeled neurons are also positive for p16 and p21 (Raffaele et al., 2020). In other cases, strong histochemical signal of β-gal-pH6 coincides in early embryonic neural tissues with areas of intense mitotic activity (de Mera-Rodríguez et al., 2019). Again, high levels of β-gal-pH6 and other senescence markers are found in neuronal cells in absence of disease or advanced aging.

All these data suggest that some structures of the sensory systems constitute excellent models for studying the chronotopographical distribution of β-gal-pH6 and other senescence markers to clarify their potential role in development and their possible involvement in developmental senescence. Moreover, apoptosis has long been known to be essential for the formation and preservation of tissue patterning in the retina (Knabe et al., 2000; Mayordomo et al., 2003; Candal et al., 2005; Rodríguez-Gallardo et al., 2005; Chavarría et al., 2007, 2013; Bejarano-Escobar et al., 2011, 2013; Álvarez-Hernán et al., 2021a), in the developing and mature olfactory epithelium (Carr and Farbman, 1992; Deckner et al., 1997; Robinson et al., 2002; Kondo et al., 2010), and in the developing inner ear (Nishizaki et al., 1998; Frago et al., 2003; León et al., 2004). Therefore, the analysis of senescence markers in developing retinal, olfactory, and inner ear neuronal cells could provide substantial evidence on the relationship between cellular senescence and cell death. Here, we outline evidence that increased levels of the senescent markers promote differentiation over apoptosis in developing neurons of sensory systems.

Cellular senescence markers in the developing visual system

In the case of the visual system, β-gal-pH6 histochemistry has been used to identify senescence in cultured primary monkey retinal pigment epithelial (RPE) cells in cultures (Chen et al., 2021). Intense β-gal-pH6 signal is detected in the developing avian (Fig. 1A) (de Mera-Rodríguez et al., 2019, 2021) and mammalian RPE (Hjelmeland, 1999; Mishima et al., 1999; Lamoke et al., 2015) under physiological and pathological conditions (Kozlowski, 2012). β-gal-pH6 activity is also observed in endothelial and smooth muscle cells of aged retinal blood vessels (López-Luppo et al., 2017). Some subpopulations of neural retinal cells located in the amacrine cell layer (Oubaha et al., 2016; López-Luppo et al., 2017) and in the ganglion cell layer (GCL) (Oubaha et al., 2016) appear strongly stained in the retina of mammals. β-gal-pH6 activity increases in the GCL after ischemic injury (Li et al., 2017).

The layered embryonic avian retina shows a similar staining pattern of β-gal-pH6 histochemistry to that described in the mammalian mature retina (de Mera-Rodríguez et al., 2019, 2021). Subpopulations of neurons located in the GCL, amacrine cell layer, and horizontal cell layer appear strongly stained (Fig. 1A). During development of the visual system, different areas are affected by intense apoptosis, such as the anterior wall of the developing lens during detachment of the lens vesicle from the cephalic ectoderm (Bejarano-Escobar et al., 2011, 2013). β-gal-pH6 senescent cells coincide temporo-spatially with this degenerating area (Lorda-Díez et al., 2015b; de Mera-Rodríguez et al., 2021), but, is this senescent marker linked to apoptosis in the developing retina? At E10, cell death is intense in the chicken retina and is detected in the GCL and in the inner nuclear layer (INL) (Cook et al., 1998) and TUNEL-positive nuclei were clearly concentrated in the GCL, in the amacrine cell layer, and in the bipolar cell layer (Fig. 1B). However, β-gal-pH6 staining is detected homogenously in the GCL, amacrine cell layer, and horizontal cell layer (Fig. 1B). There is therefore no correlation between the distribution of apoptotic cells and β-gal-pH6 activity in the developing avian retina (de Mera-Rodríguez et al., 2019, 2021).

In the non-laminated retina, β-gal-pH6-positive cells are mainly located in the vitreal surface of the neuroblastic layer that also express p21 (Fig. 1C,D), a senescence marker in embryonic tissues (Georgakilas et al., 2017). At these stages, these β-gal-pH6-positive cells located in the presumptive GCL can be identified with markers of differentiated ganglion cells in the avian retina, such as anti- neuron-specific class III β-tubulin antibody (TUJ1) (Fig. 1E,F) (Álvarez-Hernán et al., 2020, 2021b). In the early developing avian retina, β-gal-pH6 staining is absent from the vitreal surface, but is detected in the scleral surface and in the presumptive RPE, correlating with immunohistochemistry against cathepsin D (Fig. 1G,H), a reliable marker of lysosomes (Bejarano-Escobar et al., 2011; de Mera-Rodríguez et al., 2019). A similar staining pattern has been described at early postnatal ages in the mouse (Oubaha et al., 2016).

Taken together, these data suggest that some populations of healthy differentiated neurons in the developing retina show elevated levels of β-gal-pH6 or p21, typical markers of cell senescence. Thus, β-gal-pH6 activity corresponds to lysosomal β-gal, and β-gal-pH6 does not correlate with the distribution of apoptotic cells.

Cellular senescence markers in the developing olfactory epithelium

Like other sensory systems, the olfactory epithelium of aged vertebrates often shows degenerative changes, including in humans (Attems et al., 2015; Kondo et al., 2020). In 32/35-month-old rats, proliferative activity and the number of mature and immature olfactory neurons decrease significantly in aged rodents (Loo et al., 1996; Ueha et al., 2018). In aging-senescence-accelerated mice, an up-regulation of the β-gal gene has been detected, indicating a higher level of cellular senescence in the olfactory mucosa (Getchell et al., 2004). Surprisingly, when vertebrate embryos are stained for β-gal-pH6 activity, a strong labelling area of senescent cells is detected in the olfactory epithelium (Villiard et al., 2017). These authors demonstrated that β-gal-pH6-stained cells are not proliferative. The detailed staining pattern of β-gal-pH6 in the mouse developing olfactory epithelium has been described recently (de Mera-Rodríguez et al., 2022). β-gal-pH6 staining is located in a thin band at perinatal ages (Fig. 2A,B). This band progressively increases in width during the first postnatal weeks (Fig. 2C-F) (de Mera-Rodríguez et al., 2022). TUNEL-positive cells are also found in the same region where β-gal-pH6-positive cells are located, but apoptotic cells are very sparse, suggesting that there is no correlation between senescence markers and cell death in the developing olfactory epithelium. Are these β-gal-pH6-positive cells proliferative? The analysis of proliferative activity by using anti-PCNA (Fig. 3A) and anti-pHisH3 (Fig. 3B,C) antibodies clearly shows that proliferative nuclei and mitotic cells are located basally to the band of β-gal-pH6 staining, in the globose basal cell layer (Fig. 3A-C). CyK5-immunoreactive horizontal basal cells are located basally to the β-gal-pH6 positive band (Fig. 3D), while CyK8-immunopositive supporting cell bodies are always found apically (Fig. 3E). The β-gal-pH6 staining pattern correlates with the pattern of neuronal maturation described in the mouse olfactory epithelium (Graziadei et al., 1980). Indeed, the cell somata of the OMP-positive neurons in the P4 mouse is located in a row that coincides topographically with the β-gal-pH6-positive band (Fig. 3F). The cell somata of mature DBA-positive neurons is also located inside the β-gal-pH6 signal in the P60 mouse (Fig. 3G). However,

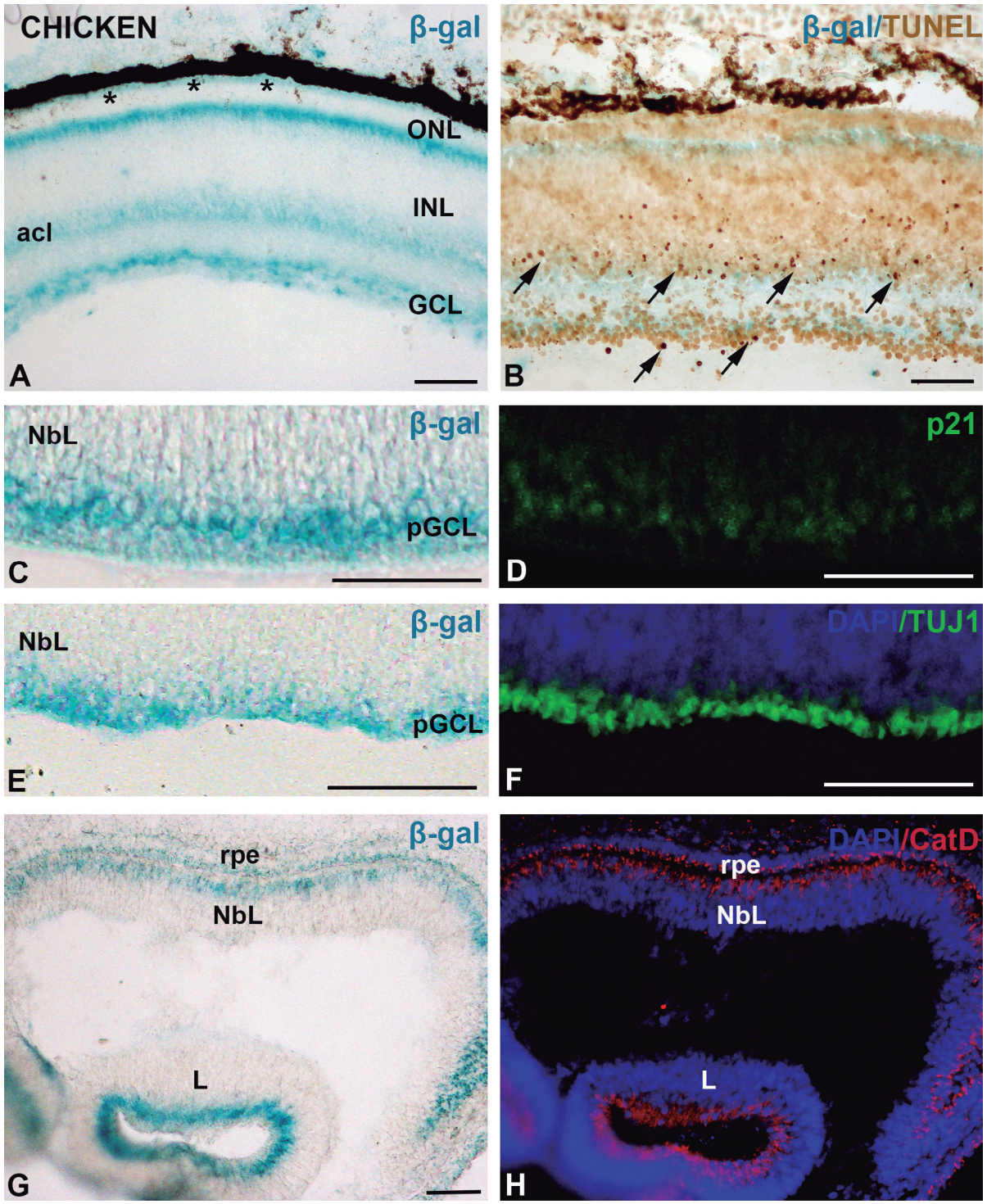

Fig. 1. Senescence markers, cell death, and cell differentiation in chicken (*G. gallus*) retina. Cryosections were stained for SA-ß-GAL histochemistry **(A)**, or doubly stained with SA-ß-GAL histochemistry and TUNEL histochemistry **(B)**, antibodies against p21 **(C, D)**, TUJ1 **(E, F)** and CatD **(G, H)**. Some sections were counterstained with DAPI **(E-H)**. SA-ß-GAL staining is detected in the ganglion, amacrine, and horizontal cell layers in the E14 embryonic laminated retina **(A, B)**. SA-ß-GAL staining is also detected in the RPE (asterisks in **A**). TUNEL-positive nuclei were mainly detected in sparse nuclei in the GCL and in the inner half of the INL (arrows). SA-ß-GAL staining in E6 embryos was mainly concentrated in the vitreal surface of the neuroblastic layer, in the region where the first postmitotic ganglion cells are found **(C, E)**. These differentiating ganglion cells are detected with anti-p21 **(D)** and TUJ1 **(F)** antibodies. In the undifferentiated retina, SA-ß-GAL staining was mainly concentrated in the scleral surface of the retina, in the RPE, and in the inner surface of the lens vesicle, in contact with the lens cavity **(G)**. The staining pattern is highly coincident with CatD immunoreactivity. Abbreviations: acl, amacrine cell layer; GCL, ganglion cell layer; INL, inner nuclear layer; L, lens; NbL, neuroblastic layer; ONL, outer nuclear layer; pGCL, presumptive ganglion cell layer; RPE, retinal pigment epithelium. Scale bars: 100 μm

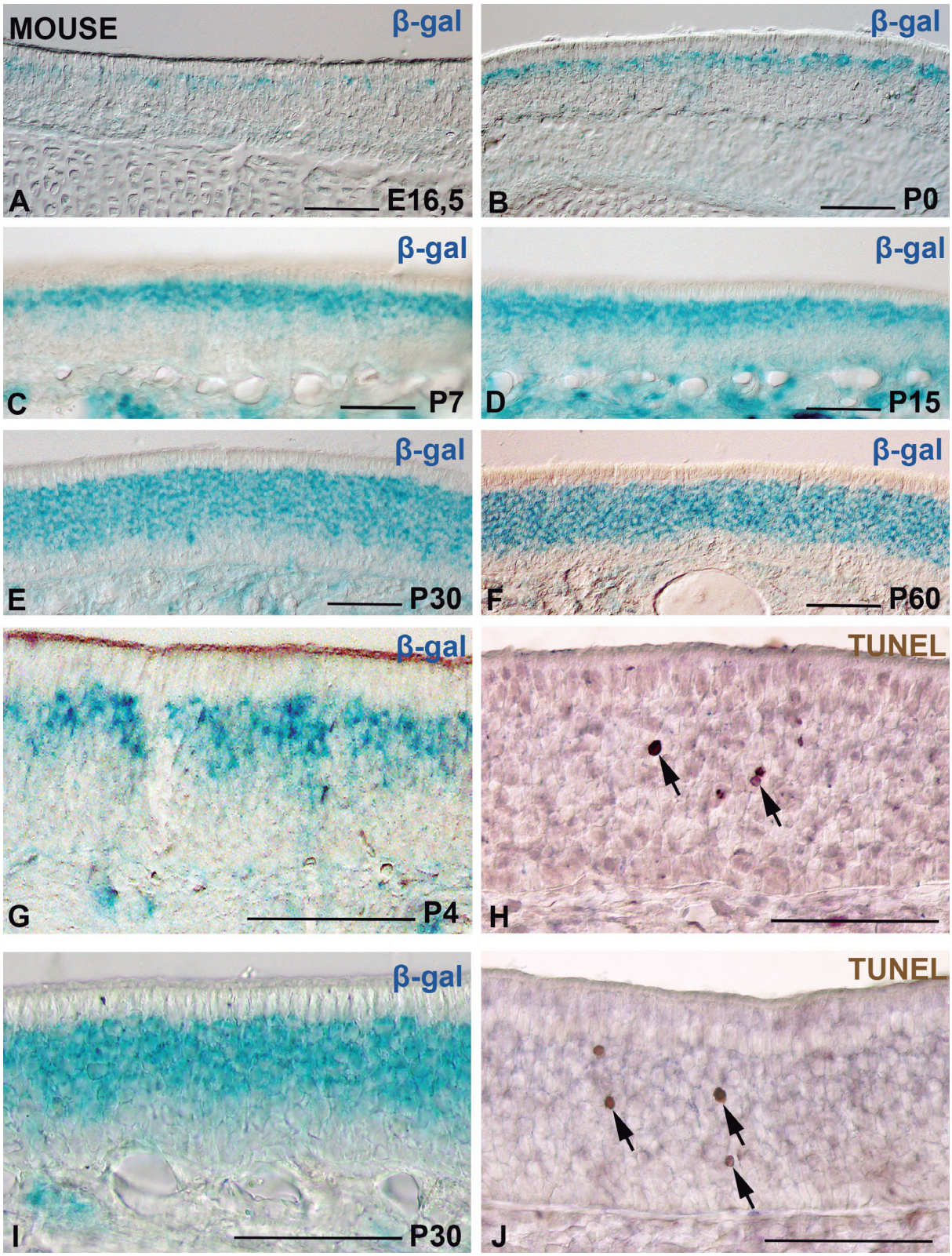

Fig. 2. The presence of SA-ß-GAL histochemical staining in the developing mouse olfactory epithelium of E16,5 **(A)**, P0 **(B)**, P7 **(C)**, P15 **(D)**, P30 **(E, I)**, P60 **(F)**, P4 **(G)**, and TUNEL histochemistry at P4 **(H)** and P30 **(J)**. SA-ß-GAL staining was restricted to the intermediate zone of the olfactory epithelium **(A-F, G, I)**. The width of the SA-ß-GAL staining band increases progressively with age **(A-F)**. Sparse TUNEL-positive nuclei were detected in the middle of olfactory epithelium (arrows in **H, J**). Scale bars: 100 μm

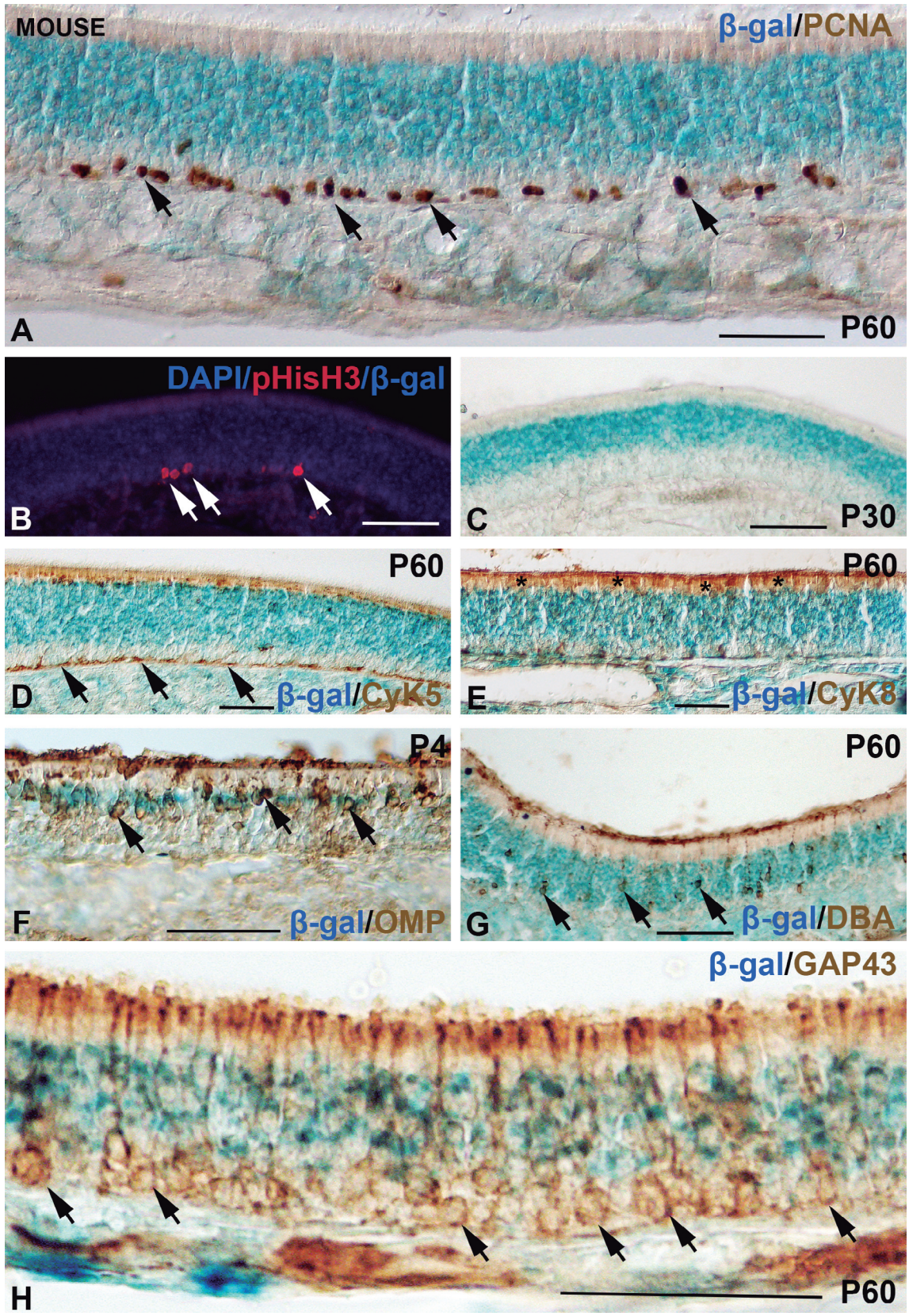

Fig. 3. Relationship between SA-ß-GAL histochemical staining and different markers of cell differentiation in the olfactory epithelium of postnatal mice. Cryosections were doubly stained for SA-ß-GAL histochemistry **(A, C-H)**, antibodies against PCNA **(A)**, pHisH3 **(B)**, CyK5 **(D)**, CyK8 **(E)**, OMP **(F)**, and GAP43 **(H)**, and lectin DBA **(G)**. DAPI staining was used in section **B, C**. PCNA immunoreactive nuclei (arrows in **A**) and pHisH3 immunoreactive figures (arrows in **B**) are located basally to the SA-ß-GAL staining. CyK5-immunoreative horizontal basal cells (arrows in **D**) are located basally to the SA-ß-GAL histochemical staining. CytK8 immunoreactive supporting cell bodies were located apically to the SA-ß-GAL labeling (asterisks in **E**). The cell somata of OMP-immunoreactive (arrows in **F**) and DBA-positive (arrows in **G**) olfactory neurons are located inside the band of SA-ß-GAL staining. The cell somata of GAP43-immunoreactive neurons are mainly located in the basal surface of the olfactory epithelium, in a region negative for SA-ß-GAL staining (arrows in **H**). Scale bars: 100 *μ*m.

most of the cell somata of the GAP-43-immunoreactive immature neurons are located basally to the band of histochemical staining (Fig. 3H) (de Mera-Rodríguez et al., 2022).

Overall, these data suggest that increased levels of β-gal-pH6 activity are linked with differentiation in developing olfactory neurons. Furthermore, while the histochemical staining is intense in mature olfactory neurons, differentiating neurons are devoid of β-gal-pH6 activity.

Cellular senescence markers in the developing inner ear

From early stages of development, morphogenesis of the inner ear is accompanied by the presence of areas of cells suffering apoptosis (León et al., 2004). Senescence is also linked to apoptosis, autophagy, and differentiation in the inner ear development (Varela-Nieto et al., 2019). In this sense, intense staining of β-gal-pH6 is found at early stages of otic development around the otic pore (Gibaja et al., 2019), an area of known intense apoptosis (Sanz et al., 1999). By these stages, β-gal-pH6 staining is intense in the otic epithelium (Gibaja et al., 2019). Later in development, β-gal-pH6 activity is particularly prominent in the endolymphatic sac of the inner ear of birds (Gibaja et al., 2019) and mammals (Muñoz-Espín et al., 2013), coinciding also with abundant non-dividing-p21-positive cells. The number of β-gal-pH6-labeled cells increases when the otocyst is exposed to TGFβ2 (Gibaja et al., 2019). Surprisingly, TGFβ2 induces neuronal differentiation during the acoustic-vestibular-ganglion formation, by promoting cell cycle arrest and neuritogenesis of neuroblasts, altering neither cell senescence nor cell death (Magariños et al., 2020).

Therefore, the coordination of cellular senescence with other developmental processes such as cell death or neuronal differentiation has started to be understood in the developing inner ear, but there are still many open aspects that deserve further studies.

Conclusions

The previous view we had linking senescence and cell death during organ development has now been challenged by the discovery that terminally differentiated neurons in the central nervous system might also adopt a senescent-like state as assessed by different markers. In the case of developing neurons in the sensory systems, senescence markers are expressed in subpopulations of postmitotic neurons even at early stages of cell differentiation, suggesting that these molecules may also play an essential role in terminal differentiation of neurons, that is accompanied by irreversible exist from the cell cycle and expression of neuronal phenotypes. In addition, cell senescence is not linked to ontogenetic cell death during development of the sensory neurons.

Contributions. J.F.-M. designed research; J.S-F, G.M.-P., and Y.G. analyzed data; G.Á.-H., J.A.d.M.-R. performed research; J.R.-L. and J.F.-M. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the University of Extremadura (protocol code 264/2019, 29 June 2020).

Informed Consent Statement. Not applicable.

Data Availability Statement. Some or all data used during the study are available from the corresponding author by request.

Conflicts of Interest. The authors declare no conflict of interest.

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