Complementary and distinct roles of autophagy, apoptosis and senescence during early inner ear development

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Abstract

The development of the inner ear complex cytoarchitecture and functional geometry requires the exquisite coordination of a variety of cellular processes in a temporal manner. At early stages of inner ear development several rounds of cell proliferation in the otocyst promote the growth of the structure. The apoptotic program is initiated in exceeding cells to adjust cell type numbers. Apoptotic cells are cleared by phagocytic cells that recognize the phosphatidylserine residues exposed in the cell membrane thanks to the energy supplied by autophagy. Specific molecular programs determine hair and supporting cell fate, these populations are responsible for the functions of the adult sensory organ: detection of sound, position and acceleration. The neurons that transmit auditory and balance information to the brain are also born at the otocyst by neurogenesis facilitated by autophagy.

Cellular senescence participates in tissue repair, cancer and aging, situations in which cells enter a permanent cell cycle arrest and acquire a highly secretory phenotype that modulates their microenvironment. More recently, senescence has also been proposed to take place during vertebrate development in a limited number of transitory structures and organs; among the later, the endolymphatic duct in the inner ear.

Here, we review these cellular processes during the early development of the inner ear, focusing on how the most recently described cellular senescence participates and cooperates with proliferation, apoptosis and autophagy to achieve otic morphogenesis and differentiation.

Keywords: Endolymphatic duct; IGF-1; AKT; Morphogenesis; Otic vesicle; SASP

1 A brief introduction to inner ear development

Auditory and vestibular organs form the adult vertebrate inner ear, both contain highly differentiated and specialized sensory epithelia that receive sound and position stimuli, respectively, and convey information to the brain (Magariños et al., 2012). Sensory patches of the inner ear contain sensory cells of two types, outer and inner hair cells, several types of supporting cells and neurons (Magariños et al., 2012; Burns et al., 2015). These cells do not regenerate in adult mammals, despite it has been described that there are small populations of resident stem cells in the adult inner ear (Oshima et al., 2007). Both sensory organs have a common developmental origin in the sensory otic placode, a thickening of the ectoderm that invaginates towards the neural tube forming the otic cup, which later closes up and forms the otic vesicle, also named otocyst (Adam et al., 1998; Magariños et al., 2012).

The otic vesicle is an embryonic transitory organ that contains the information required to generate most cells of the adult inner ear (Magariños et al., 2014). The otic vesicle can be explanted and the whole organ cultured *in*

Fig. 1 Cellular processes during early development of the chicken inner ear. **A)** Schematic view of the *ex vivo* culture of otic vesicles. The cellular processes responsible for initial acoustic-vestibular ganglion development (AVG) and endolymphatic duct (Ed) morphogenesis can be followed in HH18 cultured otic vesicle after 20 h in serum-free culture medium (OS). **B) Apoptosis (a-b).** Blocking apoptosis in *ex vivo* otocyst cultures with BOC disrupts dorsal morphogenesis and AVG development (asterisks). **Autophagy (c-d).** Apoptotic cells visualized by TUNEL (green) accumulate when autophagy is inhibited (3-MA) due to reduced expression of the “eat-me” signal detected by An-V (red). **Senescence (e-f).** The elimination of senescent cells abolishes endolymphatic duct morphogenesis (asterisk). **Proliferation (g-i).** (g) EdU incorporation (red) shows proliferating cells in the otocyst and AVG (co-labelled with the neuronal marker G4, green). Magnification in g' shows no proliferation in the endolymphatic duct region at this stage while the rest of the otic vesicle shows EdU incorporation (g''). (h-i) Cell proliferation visualized by BrdU incorporation (red) is highly reduced by the RAF-MEK-ERK inhibitor Sor. **Cell differentiation (j).** Early neurogenesis is shown as an example of otic cell differentiation at HH18. Immature neural population is labelled by the neuroblast marker Islet-1 (green) and post-mitotic neurons show the neuronal marker G4 (magenta). **C)** Cartoon showing apoptosis, autophagy, senescence and cell differentiation participation in the remodeling events of a HH18 otic vesicle. Senescent (blue) and apoptotic (green) cells facilitate dorsal morphogenesis that includes the protrusion of the endolymphatic duct. Apoptosis is also required to eliminate exceeding neuroblasts during neurogenesis and cell differentiation (yellow) generates mature neurons. Activation of autophagy (red) assists cell clearance of apoptotic cells and allows neuronal differentiation. Scale bar: a-i, 150 μ M. Abbreviations: A, anterior; An-V, Annexin-V; AVG, acoustic-vestibular ganglion; BOC, Boc-D-FMK (pan-caspase inhibitor); D, dorsal; Ed, endolymphatic duct; HH, Hamburger and Hamilton classification; M, medial; Sor, Sorafenib (RAF-MEK-ERK inhibitor). 3-MA, 3-methyladenine. Adapted from [Magariños et al. \(2010\)](#) and [Aburto et al. \(2012b\)](#).c. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Finally, otic neurons are specified from ventromedial otic vesicle neurosensory progenitors ([Fig. 1 B, j and C](#)). Epithelial neuroblasts delaminate from the otocysts and differentiate giving rise to the bipolar neurons of the embryonic auditory and vestibular ganglion (AVG), cochleovestibular ganglion in mammals ([Sanchez-Calderon et al., 2007](#)). Later in development, the AVG will generate distinct auditory and vestibular ganglia that connect sensory hair cells with the central brainstem ([Coate and Kelley, 2013](#)). Likewise, understanding the molecular basis of neurogenesis, neuronal specification and connections has been the focus of much attention in development ([Magariños et al., 2010](#); [Davies, 2011](#); [Aburto et al., 2012b](#)) and regenerative medicine studies ([Chen et al., 2012](#)).

2 Apoptosis and autophagy contribute to morphogenesis and cellular specification

2.1 Apoptosis during early inner ear development

Apoptosis is a programmed cell death suicide mechanism crucial during the development of organisms ([Degterev and Yuan, 2008](#); [Green, 2011](#)). This regulated cell death program runs through a series of concrete stages that can be identified both by the morphology of the cell and by the collection of molecular markers that have been exhaustively described over the last decades ([Galluzzi et al., 2015](#)).

The earliest detectable event is the exposure to the extracellular milieu of phosphatidylserine residues, followed by cell shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation that form part of the morphological alterations characteristic of the cellular process of self-degradation, which ends with the phagocytosis of the apoptotic cells and looks for minimal impact in the surrounding cells. Central elements of the self-degradation process are the caspases family of specific Asp residues cysteine proteases. Caspases are subdivided into initiator caspases (–2, –8, –9, –10, –11, and –12) and their downstream substrates the effector caspases (–3, –6, and –7), which are activated by cleavage and in turn execute apoptosis by cleaving cellular proteins ([Pradelli et al., 2010](#)). Caspases activation is tightly regulated and requires additional inputs, as the release of cytochrome c, among other pro-apoptotic molecules, from damaged mitochondria, which together with caspase-9 and APAF1 will form the apoptosome and mark the irreversibility of the cell death process ([Riedl and Salvesen, 2007](#); [Acehan et al., 2002](#)). Anti-apoptotic ligands, including growth factors and neurokines, activate AKT, which in turn phosphorylates and inactivates the pro-apoptotic protein BAD, a member of the BCL-2 family. Thus preventing and blocking the subsequent apoptotic steps including the expression of BIM and FASL and the activation of FOXO transcription factors. AKT also activates the I κ B kinase/NF- κ B pathway thus promoting the transcription of pro-survival genes ([Chen et al., 2001](#); [Song et al., 2005](#)).

During the development of the inner ear, apoptosis has been reported to play an essential role in the shaping of the otocyst and in the control of AVG neurogenesis ([Fekete et al., 1997](#); [León et al., 2004](#)). Apoptosis shows a defined pattern during development that associates with specific developmental events in the otic epithelium, as the closure of the otic cup, the formation of the endolymphatic duct and, later in development, in the formation of the vestibular semicircular channels ([Fig. 1 B, a-b and C](#); [Fekete et al., 1997](#); [Frago et al., 1998](#); [Chang et al., 2004](#)). In the otic vesicle stage, apoptosis is associated with the thinning of the endolymphatic duct and with the control of the populations that are proliferating ([Frago et al., 1998](#)). Blockade of caspase activation disturbs both processes ([Fig. 1 B, a-b](#)). Apoptosis is also required to control the populations of epithelial and neural neuroblasts, as well as for the adjustment of the auditory and vestibular neurons to the hair sensory cells that they innervate ([León et al., 2004](#)), as an example of the neurotrophic theory ([León et al., 1999](#); [Sanz et al., 1999a,b](#)). Thus, neurotrophic factors are secreted from the primordium of the sensory otic epithelia to guide neurons during the process of innervation. Embryonic neurons are primed to die but express neurotrophic factors receptors that, upon activation, initiate intracellular signaling to block apoptosis. Only those otic neurons receiving neurotrophic factor signaling from the otic vesicle will epithelia survive ([Frago et al., 2003](#); [Aburto et al., 2012b](#)).

Intracellular pathways leading to apoptosis have been studied in the embryonic otic vesicle ([León et al., 1999](#); [Sanz et al., 1999a,b](#)). Extracellular factors leading to cell survival converge in the activation of the PI3K/AKT pathway, among these factors insulin-like growth factor 1 (IGF-1) and neurotrophins have a central role ([Frago et al., 2003](#); [Aburto et al., 2012b](#)). Neurotrophins have a dual function depending on the neurotrophin receptor involved, thus when p75

nerve growth factor (NGF) is activated in the absence of the tyrosine kinase high affinity receptors TRK it leads to cell death by apoptosis ([Bandtlow and Dechant, 2004](#)). Signaling pathways described so far include the balance between ceramide pro- and anti-apoptotic metabolites ([Frago et al., 1998](#)) and the activation of the RAF kinase and the MAPK cascade also lead to cell survival but, in addition, promote cell division and otocyst growth ([Fig. 1 g-h](#); [Sanz et al., 1999a,b](#); [Magariños et al., 2010](#)). Apoptotic signaling leads to the activation of the c-JUN-N-terminal kinase (JNK) that is a central element of the transcriptional machinery of programmed cell death during development ([León et al., 1998](#); [Sanz et al., 1999a](#); [Lahlou et al., 2018](#)).

In the adult inner ear, apoptosis is a crucial mechanism to maintain neuronal and sensory cell populations, being this a field of renewed research interest to identify drug-targets and develop novel therapies to prevent cell death and hearing loss ([Morrill and He, 2017](#); [Wang and Puel, 2018](#)).

2.2 Autophagy during inner ear development

Autophagy emerged as an evolutionary conserved mechanism of cell death, which was later shown to play other central roles in mammalian development, metabolic homeostasis, human disease and ageing ([Booth et al., 2014](#); [Magariños et al., 2017](#); [Doherty and Baehrecke, 2018](#)). Autophagy is activated by stress conditions, which include growth factor deprivation, and depending on the cellular context it is classified as macroautophagy, microautophagy and chaperone-mediated autophagy, only reported in mammals ([Hansen et al., 2018](#); [Kaushik and Cuervo, 2018](#)). Morphological and molecular characteristics of autophagy have been extensively described ([Ariosa and Klionsky, 2016](#); [Klionsky et al., 2016](#)). Macroautophagy (hereafter referred as autophagy) signaling is the best studied pathway. Downstream AKT, mechanistic target of rapamycin (mTOR) is the key sensor of nutrients and regulates cell homeostasis by modulating lysosomal biogenesis and autophagy. During autophagy the intracellular components to be degraded are encased into a phagophore that evolves to form the autophagosome, a transport vesicle, which will fuse with the lysosomes to complete the degradation. This complex process involves more than thirty autophagy related genes (ATG), whose precise roles and regulation are still under study. Thus, autophagy is used by cells to degrade cytoplasmic macromolecules and dysfunctional organelles to recycle intracellular components and to generate energy, being an essential process during the development of the nervous system ([Aburto et al., 2012a](#); [Magariños et al., 2017](#)).

During early inner ear development autophagy plays a central role that has been recently reviewed ([Magariños et al., 2017](#), [Fig. 1 C](#)). Autophagy helps apoptosis providing energy to carry out the ATP-consuming programmed death cell and facilitates epithelial neuroblasts delamination from the otic vesicle ([Fig. 1 B, j](#); [Aburto et al., 2012a,c](#)). Autophagy has also been reported to be essential for the development of the mouse vestibular system ([Mariño et al., 2010](#)) although much research effort should be placed into studying this process during mammalian inner ear development. Later in development and lifelong autophagy plays a role in inner ear homeostasis ([de Iriarte Rodríguez et al., 2015](#); [Magariños et al., 2017](#)) and response to injury ([Taylor et al., 2008](#); [Yuan et al., 2015](#)) in the mouse.

3 Cell senescence in inner ear development

3.1 Overview of cell senescence

Cell senescence is a stable form of cell cycle arrest, with a relevant role in cell balance in different pathological and physiological situations ([Fig. 2 A, a and b](#); [Pérez-Mancera et al., 2014](#); [Salama et al., 2014](#); [He and Sharpless, 2017](#)). Through inhibition of proliferation followed by clearance mediated by immune cells, senescence facilitates the elimination of cells that either suffer an unrepairable level of damage or are no longer required for normal tissue function. A series of distinctive morphological and molecular markers distinguishes senescence from other non-proliferative states ([Fig. 2 B](#)). These include increased lysosomal β -galactosidase activity (known as senescence-associated β -galactosidase or SA β G), a characteristic morphology with extended and vacuolated cytoplasm, changes in nuclear and chromatin structure and a characteristic secretory phenotype (senescence-associated secretory phenotype or SASP, see below) ([Salama et al., 2014](#)). Retinoblastoma and p53 tumor suppressor pathways generally play an essential role in senescence regulation, most frequently involving p16INK4A and ARF, products of *CDKN2A* locus ([Salama et al., 2014](#)). Of note, none of these markers are universal or completely specific for senescence, making advisable the use of a combination of several markers to identify senescent cells.

A) Types of senescence

B) Characteristics of senescent cells

C) Elimination of developmental cell senescence

Fig. 2 Classification and characteristics of senescent cells. A) Schematic representation of senescence cellular phenotypes. (a) Senescence is associated to pathological situations such as cancer (oncogene-induced senescence) where it can be beneficial (tumor suppression) or detrimental (tumor promotion), as well as to aging (replicative senescence). (b) Developmental senescence is present at specific time windows in vertebrate embryos acting through p15, p21, TGF β and PI3K/FOXO. Senescence functions include cell clearance and population balance contributing to the morphogenesis of embryonic structures. Developmental senescence has been reported in most vertebrates. **B) Characteristics of senescent cells.** A variety of cellular insults trigger the senescent program that results in a stable cell cycle arrest. Senescent cells show upregulation of cell cycle inhibitors (p16, p53 and p21), increased lysosomal content with high levels of senescence-associated β -galactosidase activity (SA β G) and a strong secretory phenotype known as the senescence-associated secretory phenotype (SASP), which includes pro-inflammatory cytokines, metalloproteinases and growth factors to influence neighboring cells. **C) Clearance of developmental senescent cells.** Cells to be removed during development could be eliminated either by apoptosis or by senescence, either way cells will be finally phagocytosed. Senescent cells may eventually enter the apoptotic program.

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Senescence can be activated by a variety of cellular alterations that can be regarded as stress or damage signals (Fig. 2B). Many of these are commonly associated with the tumorigenic process, such as DNA damage or oncogene activation. Accordingly, senescence acts as an effective tumor suppressive mechanism blocking the uncontrolled proliferation of cells with potentially oncogenic alterations (Collado and Serrano, 2010). Cellular senescence is also involved in a variety of physiological and pathological situations, beyond its well-established role in tumor suppression (Muñoz-Espín and Serrano, 2014; Childs et al., 2017; He and Sharpless, 2017). Senescence has been implicated in a growing list of pathologies, many of them aging-related, including atherosclerosis, osteoarthritis, fibrosis or diabetes. The relevance of senescence in disease is supported by studies of senescence markers in human samples and, most significantly, from recent studies on the impact of selective elimination of senescent cells in vivo (Baker et al., 2011; Demaria et al., 2014). Strikingly, the role of senescence in disease is highly context dependent, with examples of both protective and pathogenic roles for senescence (Muñoz-Espín and Serrano, 2014; Childs et al., 2017; He and Sharpless, 2017). Notably, in addition of these links to disease, senescence also has a role in the control of cell balance and tissue homeostasis in the context of normal physiology and embryonic development, as will be discussed in detail below (Fig. 2A and b; Muñoz-Espín et al., 2013; Storer et al., 2013). Pathologies associated with senescence could be explained by

the dysregulation of the normal senescence program, for example by defective activation of senescence, or aberrant accumulation of senescent cells and impaired regeneration as in aging (Muñoz-Espín et al., 2013; He and Sharpless, 2017). The growing evidence of the implication of senescence in disease has fuelled the interest in exploiting manipulation of senescence for therapeutical purposes. A number of compounds have been recently identified with the ability to induce senescence (senogenics) or selectively kill senescent cells (senolytics). These promising findings have set the ground for therapeutical interventions based on senescence (He and Sharpless, 2017; Kirkland and Tchkonja, 2017; Soto-Gamez and Demaria, 2017).

Senescent cells produce and release a complex combination of secreted factors, known as the SASP (Fig. 2 B; Pérez-Mancera et al., 2014; Malaquin et al., 2016; Ito et al., 2017), which typically includes inflammatory cytokines, IL-6 and IL-8, matrix metalloproteases, and components of several key signalling pathways, such as TGFβ or WNT (Acosta et al., 2008; Coppé et al., 2008; Malaquin et al., 2016). The SASP is under a complex regulatory circuit that includes NFκB, C/EBPβ, mTOR and p38MAPK (Acosta et al., 2013, 2008; Kuilman et al., 2008; Malaquin et al., 2016). The SASP is central to the communication of senescent cells with their environment and participates in opposing outcomes of senescence. It promotes the senescent response through the cell-autonomous reinforcement of senescence, the paracrine spreading of senescence, and the recruitment of immune cells for the clearance of senescent cells (Xue et al., 2007; Krizhanovsky et al., 2008; Kuilman et al., 2008; Acosta et al., 2008, 2013). Paradoxically, in addition to these beneficial actions, the SASP can also elicit detrimental effects, such as proliferation of tumor cells, epithelial-mesenchymal transition and invasiveness or an immunosuppressive environment that allows tumor growth (Coppé et al., 2008; Ruhland et al., 2016). As discussed in detail below, the SASP is also a key player in developmental senescence and in the link of senescence to cell plasticity. Recent investigations also indicate that exosomes released by senescent cells play an important role in cellular communication in senescence, although the relative importance of SASP soluble factors or exosome-associated cargo in this context is still unclear (Takasugi, 2018).

3.2 Developmental senescence

As described above, senescence had been traditionally associated to cellular damage and pathological situations. In contrast to this view, the existence of “physiological” senescence during vertebrate development was described (Fig. 2A and b; Muñoz-Espín et al., 2013; Storer et al., 2013). Using SAβG as a senescence marker, these studies identified senescent cells in several discrete anatomical structures in mouse embryos in mid to late stages of development, from E9.5 to E18.5 (Muñoz-Espín et al., 2013; Storer et al., 2013). Specific SAβG staining was detected in the otic vesicle, neural roof plate, limb buds, and the mesonephros, among other locations. Although these phenotypes have been characterized in detail in mouse embryos, developmental senescence has also been identified in human, chick, quail, axolotl, xenopus and zebra fish embryos (Nacher et al., 2006; Muñoz-Espín et al., 2013; Storer et al., 2013; Lorda-Diez et al., 2015; Davaapil et al., 2017; Villiard et al., 2017), indicating that it represents an evolutionary conserved mechanism. Interestingly, physiological senescence has also been observed in the placenta, where it has been associated to trophoblast cell fusion (Chuprin et al., 2013), as well as in embryonically-derived membranes, where its dysregulation could be related to pregnancy and parturition defects (Cha and Aronoff, 2017). From an evolutionary point of view, it appears that developmental senescence may represent a primitive form of senescence, directed to elicit cell-cycle arrest, cell clearance (Fig. 2 C) and possibly instructive signals necessary for morphogenesis. Accordingly, adult, stress-induced senescence would have appeared as an adaptation of this physiological process in order to respond to stress or damage situations sustained during adult life (Banito and Lowe, 2013).

In the developing inner ear, senescence is restricted to the endolymphatic duct in a time window that spans from the onset of its formation in the dorsal tip of the epithelium of the otic vesicle around E9.5, up to E14.5 when the morphogenesis of this structure is complete (Muñoz-Espín et al., 2013). The main function of the endolymphatic duct and sac is the control of the homeostasis of the endolymph, the fluid in contact with sensory cells in the cochlea and vestibule. The ion composition, volume and pressure of the endolymph are critical for efficient transduction of sound and balance stimuli into nerve signals. The endolymphatic system seems to mediate the resorption of endolymph that balances its generation from the perilymph. Additional functions of the endolymphatic sac may include the macrophage-mediated immune surveillance of the inner ear and the clearance of cellular debris from inner ear ducts (Couloigner et al., 2004). Of note, several human diseases of the endolymphatic duct and sac are known. These include Meniere's disease, characterized by vertigo and tinnitus and linked to changes in the endolymph content and pressure (Nakashima et al., 2016), endolymphatic sac epithelial tumors, frequently associated to the Von-Hippel-Lindau syndrome (Wick et al., 2015), and the Enlarged Vestibular Aqueduct, linked to dilated endolymphatic sac and duct epithelia (Griffith and Wangemann, 2011). Given the well-established involvement of senescence in many diseases and its physiological occurrence in endolymphatic sac morphogenesis, it would be interesting to investigate the potential role of senescence in endolymphatic sac pathologies.

3.3 Regulation of developmental senescence

Notably, developmental senescence seems to depend on a specific regulatory network that differs from canonical adult senescence. In the endolymphatic sac, senescence is accompanied by increased expression of the cell-cycle inhibitors and senescence regulators p21CIP1 and p15INK4b. In contrast, p16INK4a and p19ARF, which are critical mediators of adult mouse senescence, are not involved in developmental senescence (Muñoz-Espín et al., 2013). Senescent cells in the endolymphatic sac display other hallmarks of senescence, like reduced proliferation or heterochromatin foci, but they show no evidence of DNA damage, a common marker of many forms of adult senescence (Muñoz-Espín et al., 2013; Pérez-Mancera et al., 2014). Genetic analysis revealed that p21CIP1 (hereafter p21) seems to be the major mediator of senescence during development, as shown by reduced senescence markers in p21-null embryos. In contrast, genetic manipulation of senescence mediators like p16INK4a/p19ARF, p53 or p27 had no significant impact in this phenotype (Muñoz-Espín et al., 2013; Storer et al., 2013). p21 is a well-known transcriptional target of the tumor suppressor p53 and plays an important role in p53-mediated cell cycle arrest in adult senescence and other p53-mediated responses (Pérez-Mancera et al., 2014). However, since p53 is apparently not involved in

developmental senescence, the role of p21 in this setting seems to be p53-independent. Notably, *p21* expression in mouse embryos faithfully correlates with known locations of developmental senescence (Vasey et al., 2011). In addition to p21, other senescence effectors may also be important. One attractive candidate is p15INK4b, which is also prevalent marker of inner ear developmental senescence. Genetic analyses of the impact of p15INK4b loss in developmental senescence would be interesting to determine its contribution to this process.

p21 expression and developmental senescence in the inner ear are under the coordinated control of the TGF β and FOXO/PI3K pathways (Muñoz-Espín et al., 2013). FOXO1 and P-SMAD2, the latter indicative of active TGF β signalling, are elevated in senescent cells and the genetic or pharmacological inhibition of either pathway results in reduced levels of senescent markers (Muñoz-Espín et al., 2013). Interestingly, gene expression analysis in the senescent mesonephros also suggested the possible implication in programmed senescence of additional signalling pathways relevant in development, such as SHH or WNT, but their involvement in the otic vesicle or other senescent structures remains to be determined. Of note, some of the pathways linked to senescence, like TGF/BMP, SHH or WNT, are known for their central role in specifying dorsal-ventral polarity on the otic vesicle (Bok et al., 2007; Magariños et al., 2012). Since senescence occurs in the endolymphatic sac in the dorsal-most extreme of the otic vesicle and elimination of senescent cells at the otic vesicle stage abolish endolymphatic duct morphogenesis (Fig. 1 B, e-f and C; Gibaja et al., 2019; submitted) it is tempting to speculate that the regulation of senescence might somehow be connected to the general program of dorso/ventral patterning of the otocyst.

A similar regulatory mechanism to that of the endolymphatic sac appears to be in action in additional locations of developmental senescence. p21 (and to some extent p15INK4b) is also overexpressed in senescent cells in the mesonephros, the apical ectodermal ridge (AER) of the limb bud, and the neural roof plate. Accordingly, senescence in these structures is impaired in p21-null embryos (Muñoz-Espín et al., 2013; Storer et al., 2013). However, in addition to this common layer of regulation, specificities may also exist in each individual senescent structure. For instance, the TGF β or PI3K pathways have only been investigated so far in the endolymphatic sac and the mesonephros, but its possible role in other senescent sites is not known. Instead, senescence in the AER seems to be mediated by ERK signalling emanating from the surrounding mesenchyme. Thus, although the existing evidence suggests a common regulatory theme, further studies are required to fully characterize the regulatory machinery of developmental senescence and identify common as well as structure-specific mechanisms.

3.4 Functional significance of developmental senescence

What is the physiological function of senescence during development in the inner ear and other locations? In many cases, developmental senescence occurs in transient structures, which undergo active remodelling coinciding with the presence of senescence. In these cases, senescence has been associated to cell clearance leading to patterning during development. This is clearly the case for the AER (Storer et al., 2013) and the mesonephros (Muñoz-Espín et al., 2013). Both structures normally undergo massive remodelling, which is impaired in senescence-defective *p21*-null embryos. The case of the endolymphatic sac seems more complex as senescence markers are detected at early stages of the formation of the sac (since E9.5), and this structure is retained in the adult inner ear, without presence of overt remodelling processes. Interestingly, senescent-defective *p21*-null embryos, display abnormal folding of the endolymphatic sac at later developmental stages (E18.5). To explain this phenotype, it has been suggested that senescence could control the balance between specific cell populations required for proper morphogenesis of the sac. Senescence would block the proliferation of the major population of epithelial cells characterized by the absence of pendrin, a membrane anion transporter key for endolymph absorption, in favour of the minority of pendrin-positive cells (Muñoz-Espín et al., 2013). In addition to this role at later stages, senescence plays a role in the formation of the endolymphatic sac and its modulation modifies otic vesicle morphology (Fig. 1 B, e and C; Gibaja et al., 2019; submitted). These findings raise the possibility that senescence may play distinct roles at different stages morphogenesis. Interestingly, similar defects both at early and late stages of endolymphatic sac have been described in embryos defective in ephrin signalling, a pathway linked to senescence (Zhu et al., 2015). Indeed, genetic inactivation of *Efnb2* in the inner ear results in endolymphatic sac delayed morphogenesis and malformation that includes abnormal distribution of pendrin-positive cells (Raft et al., 2014).

Somewhat unexpectedly, impaired developmental senescence seems to have a limited impact in the inner ear in adult mice. The defects in the endolymphatic sac found in *p21*-null mice appear to be compensated at later stages by a process involving macrophage infiltration and apoptosis, explaining that adult *p21*-null mice show no obvious ear anatomical defects or impaired hearing (Muñoz-Espín et al., 2013; Chang et al., 2016). Similarly, increased apoptosis is observed in the AER in *p21*-null mice, presumably to compensate for the lack of senescence, providing an explanation for the lack of overt limb phenotypes in these mice. These observations are reminiscent of the relatively normal embryo phenotypes observed in mice defective in the apoptotic machinery (Lindsten et al., 2000), and most likely reflect the existence of robust compensation mechanisms directed to preserve correct embryo development, even in the presence of severe alterations in developmentally relevant processes. Moreover, redundancy with other potential regulators of developmental senescence, such as p15 or p27 is also possible. Interestingly, the impact of defective senescence depends on the organ. The consequences of defective senescence in *p21*-null mice were most evident in the mesonephros, where the female Wolffian duct fails to degenerate completely, leading to vagina abnormalities (Muñoz-Espín et al., 2013).

3.5 Role of the SASP in developmental senescence

Considering the importance of the SASP in many aspects of adult senescence, it is important to understand its contribution to developmental senescence. The signature of genes upregulated in senescent cells in the endolymphatic sac, AER or the mesonephros, include several genes associated with the SASP, such as C/EBP, a key transcriptional regulator of the SASP, the BMP receptor 1 of the TGF pathway or IGFBP5. Notably, inflammatory

cytokines, such as IL-6, which are major SASP components in many forms of senescence, were not up-regulated in this context (Muñoz-Espín et al., 2013; Storer et al., 2013). As mentioned, one major function of the SASP in adult senescence is to recruit immune cells for the clearance of senescent cells (Xue et al., 2007; Krizhanovsky et al., 2008). Macrophage-mediated cell clearance has so far been described in the senescent mesonephros and the limb bud, but not in the inner ear. Thus, recruitment of immune cells, possibly mediated by the SASP, appears to be relevant for patterning at least in some senescent locations. In addition to cell clearance, the SASP can also relay paracrine transmission of the senescent phenotype to surrounding cells, influencing tissue microenvironment (Acosta et al., 2013). Whether this type of SASP-mediated signaling contributes to developmental senescence is unclear. In support of an active role of senescence in instructive signalling, is the fact that impaired AER epithelium senescence in *p21*-null mice results in reduced FGF expression and decreased proliferation in the adjacent mesenchyme. Conversely, ERK-mediated paracrine signals from the surrounding non-senescent mesenchyme can influence senescence in the AER epithelium (Storer et al., 2013). Interestingly, this pathway is also involved in instructive signalling from the mesenchyme necessary for limb morphogenesis. Similarly, some of the SASP-related signalling pathways linked to senescence in the otic vesicle, such as SHH or WNT also underpin instructive signals originating from mesoderm or neural plate critical for shaping of the developing inner ear (Bok et al., 2007). Thus, it is feasible that common molecular pathways can orchestrate the coordinated regulation of senescence and complementary biological responses in order to ensure correct morphogenesis.

4 Senescence links to apoptosis, autophagy and differentiation in inner ear development

4.1 Apoptosis

Apoptosis and senescence seem to have mutually exclusive outcomes in many settings (Childs et al., 2014). Indeed, many types of senescent cells are protected from apoptosis by the elevated expression of antiapoptotic factors, including members of the BCL2 family (Zhu et al., 2015; Chang et al., 2016; Yosef et al., 2016). In fact, this vulnerability has been exploited for the development of senolytic drugs for selective elimination of senescent cells (Kirkland and Tchkonja, 2017). Moreover, in addition to its function in proliferation, p21 can also have a protective role from apoptosis during development and in other settings, at least in part through regulation of the SASP (Roninson, 2002; Vasey et al., 2011). The crosstalk between senescence and apoptosis during development appears to be complex, and the relative contribution of apoptosis to the cell fate of developmental senescent cells is still poorly understood. In the AER, clearance of senescent cells apparently follows a multi-step process, where senescent cells initially disappear from the limb bud epithelium and can be found predominantly in the surrounding mesenchyme where they then become apoptotic and are cleared by macrophages (Storer et al., 2013). However, the precise timing and functional connection between apoptosis and macrophage clearance in this context remains to be defined. Clearing of senescent cells in the mesonephros similarly involves macrophage-mediated clearance, but in this case the process is apparently unrelated to apoptosis (Muñoz-Espín et al., 2013). To date, it is unclear whether senescence in the otic vesicle may be linked to programmed cell clearance either through macrophage phagocytosis or apoptosis. Interestingly, apoptosis can be detected in the endolymphatic duct epithelium at early stages of morphogenesis in a time window overlapping with senescence, and apoptosis dysregulation is linked to defective endolymphatic sac formation (Cecconi et al., 2004; Raft et al., 2014). Further studies should determine whether senescence and apoptosis might be related processes also in the otic vesicle. Interestingly, apoptosis can also act as a compensatory mechanism when senescence is impaired, as shown in the endolymphatic sac and the AER of *p21*-deficient mice (Storer et al., 2013). In apparent contrast to the notion that developmental senescent cells disappear over time, either by active clearance or as a consequence of cellular rebalancing (Fig. 2 C), a recent study suggests that senescent cells may, at least in part, be retained at later developmental stages. Using lineage tracing in mice to follow the fate of cells that are p21-positive, and presumably senescent, at early developmental stages (E10.5 to E11.5), the study finds that a fraction of these cells can be detected at later stages of development where they can even resume proliferation (Li et al., 2018). Further studies should clarify the fate of senescent cells during development.

4.2 Autophagy

Several studies have investigated the link between autophagy and senescence. In the context of stress-related senescence, it is thought that general autophagy prevents senescence, by counteracting cellular damage or stress that would trigger this response (Gewirtz, 2013; Kwon et al., 2017). For instance, in muscle satellite cells, autophagy prevents senescence in young muscle by opposing proteotoxicity. In aging, autophagy is impaired and, as a consequence, cellular damage accumulates, leading to senescence (García-Prat et al., 2016). On the other hand, autophagy seems to be necessary to maintain the viability of senescent cells through different mechanisms, mainly related to the SASP (Pérez-Mancera et al., 2014). Autophagy could counteract the elevated cellular stress characteristic of senescence, which is largely due to intense SASP production leading to ER stress and unfolded protein response (Dörr et al., 2013). Also, autophagy may support the intensive synthesis of SASP factors, by providing building blocks for protein synthesis through recycling of cytoplasmic components (Young et al., 2009; Narita et al., 2011). Finally, the increased mitochondrial respiration typical of senescent cells might be dependent, at least in part, on autophagy (Dörr et al., 2013; Pérez-Mancera et al., 2014). In addition to general autophagy, substrate-selective autophagy has also a relevant, but context-dependent, role in senescence with examples of both reduced and augmented autophagy-mediated degradation of senescence mediators (Dou et al., 2015; Kang et al., 2015). Based on the strong evidence of the link of autophagy to stress senescence, it is feasible that it may also play a role in developmental senescence. Interestingly, chemical and genetic manipulation of autophagy indicates that this process is essential for vertebrate inner ear development, where it has been implicated in diverse processes, including clearance of apoptotic cells, migration of epithelial neuroblasts and formation of otoconia in the vestibular system, possibly linked to the elimination of ROS (Magariños et al., 2017). The existence of crosstalk between autophagy and senescence during inner ear development has not been described experimentally yet, however, it could be speculated that some of the forms of autophagy could participate in the acquisition and maintenance of the developmental senescent phenotype. Further studies are necessary to investigate the potential link between autophagy and senescence in this context.

4.3 Differentiation

A series of recent investigations has uncovered the connection of senescence with cellular plasticity in different contexts. On one hand, senescence can oppose pluripotency and promote differentiation. Indeed, senescence acts as a cell-autonomous roadblock to reprogramming with Yamanaka factors (Banito et al., 2009), and increased senescence is linked to diminished self-renewal capacity in aged adult stem cells (Sousa-Victor et al., 2014). In contrast, in vivo studies using mouse models or limb regeneration in amphibians have shown that senescence can promote cell plasticity in adjacent cells in a process mediated by the SASP (Demaria et al., 2014; Yun et al., 2015; Mosteiro et al., 2016; Chiche et al., 2017; Ritschka et al., 2017). This effect has primarily been described in the context of tissue or cell damage, where this pro-plasticity action of senescence could contribute to tissue repair. Whether a similar link between senescence and plasticity occurs during development is not known. The balance between stem/progenitor and differentiated phenotypes is at the core of the morphogenetic events that take place during development. It is thus possible that senescence may not only mediate cell clearance and remodelling but also participate in differentiation and cell fate decisions during development. It has also been suggested that senescence may be related to changes in regenerative capacity during development and in adult life (Yun et al., 2015). Clearly, further studies would be informative in identifying the potential crosstalk between senescence and cell plasticity during development.

5 Conclusions and perspectives

Developmental proliferation generates an excessive number of otic cells that are later eliminated by a number of mechanisms of refinement exquisitely regulated. Senescence jointly with apoptosis is part of cell clearing mechanisms during development, which for example sculpt the endolymphatic duct. The precise connection between apoptosis and senescence in ear development is still unclear. It is feasible that apoptosis may play an active part in the elimination senescent cells, as in other sites of developmental senescence. On the other hand, autophagy supplies energy for migrating otic neuroblasts and also to carry out the clearing of apoptotic cells. To study the autophagy status of programmed cell senescent cells is particularly interesting considering their highly secretory nature.

Although the existing evidence is still limited, the emerging picture described here supports a role for programmed cell senescence in inner ear development.

The coordination of senescence with other developmental processes has started to be understood but there are still many open aspects that deserve further studies. The mechanisms and extracellular factors that trigger senescence in the otic epithelia are not well defined. The impact of the secretome of senescent cells in their microenvironment, and therefore, on surrounding developmental cells, is also unknown.

Finally, cellular senescence is being increasingly involved in the ethiology of various pathologies. It is an attractive hypothesis to suggest that its dysfunction could be the underlying cause of inner ear human developmental disorders.

Conflicts of interest

The authors declare that they have no conflict of interest.

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References

- Aburto María R., Hurlé J.M., Varela-Nieto I. and Magariños M., Autophagy during vertebrate development, *Cells* **1**, 2012a, 428–448 <https://doi.org/10.3390/cells1030428>.
- Aburto M.R., Magariños M., Leon Y., Varela-Nieto I. and Sanchez-Calderon H., AKT signaling mediates IGF-I survival actions on otic neural progenitors, *PLoS One* **7**, 2012b, e30790–e30790 <https://doi.org/10.1371/journal.pone.0030790>.
- Aburto M.R., Sánchez-Calderón H., Hurlé J.M., Varela-Nieto I. and Magariños M., Early otic development depends on autophagy for apoptotic cell clearance and neural differentiation, *Cell Death Dis.* **3**, 2012c, e394–e394 <https://doi.org/10.1038/cddis.2012.132>.
- Acehan D., Jiang X., Morgan D.G., Heuser J.E., Wang X. and Akey C.W., Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation, *Mol. Cell* **9**, 2002, 423–432.
- Acosta J.C., Banito A., Wuestefeld T., Georgilis A., Janich P., Morton J.P., Athineos D., Kang T.-W., Lasitschka F., Andrulis M., Pascual G., Morris K.J., Khan S., Jin H., Dharmalingam G., Snijders A.P., Carroll T., Capper D., Pritchard C., Inman G.J., Longerich T., Sansom O.J., Benitah S.A., Zender L. and Gil J., A complex secretory program orchestrated by the inflammasome controls paracrine senescence, *Nat. Cell Biol.* **15**, 2013, 978–990

<https://doi.org/10.1038/ncb2784>.

- Acosta J.C., O'Loughlen A., Banito A., Guijarro M.V., Augert A., Raguz S., Fumagalli M., Da Costa M., Brown C., Popov N., Takatsu Y., Melamed J., d'Adda di Fagagna F., Bernard D., Hernando E. and Gil J., Chemokine signaling via the CXCR2 receptor reinforces senescence, *Cell* **133**, 2008, 1006-1018 <https://doi.org/10.1016/j.cell.2008.03.038>.
- Adam J., Myat A., Le Roux I., Eddison M., Henrique D., Ish-Horowicz D. and Lewis J., Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sense-organ development, *Development* **125**, 1998, 4645-4654.
- Ariosa A.R. and Klionsky D.J., Autophagy core machinery: overcoming spatial barriers in neurons, *J. Mol. Med.* **94**, 2016, 1217-1227 <https://doi.org/10.1007/s00109-016-1461-9>.
- Baker D.J., Wijshake T., Tchkonia T., LeBrasseur N.K., Childs B.G., van de Sluis B., Kirkland J.L. and van Deursen J.M., Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders, *Nature* **479**, 2011, 232-236 <https://doi.org/10.1038/nature10600>.
- Bandtlow C. and Dechant G., From cell death to neuronal regeneration, effects of the p75 neurotrophin receptor depend on interactions with partner subunits, *Sci. STKE* 2004, pe24, 2004 <https://doi.org/10.1126/stke.2352004pe24>.
- Banito A. and Lowe S.W., A new development in senescence, *Cell* **155**, 2013, 977-978 <https://doi.org/10.1016/j.cell.2013.10.050>.
- Banito A., Rashid S.T., Acosta J.C., Li S., Pereira C.F., Geti I., Pinho S., Silva J.C., Azuara V., Walsh M., Vallier L. and Gil J., Senescence impairs successful reprogramming to pluripotent stem cells, *Genes Dev.* **23**, 2009, 2134-2139 <https://doi.org/10.1101/gad.1811609>.
- Bok J., Dolson D.K., Hill P., Rüther U., Epstein D.J. and Wu D.K., Opposing gradients of Gli repressor and activators mediate Shh signaling along the dorsoventral axis of the inner ear, *Development* **134**, 2007, 1713-1722 <https://doi.org/10.1242/dev.000760>.
- Booth L.A., Tavallai S., Hamed H.A., Cruickshanks N. and Dent P., The role of cell signalling in the crosstalk between autophagy and apoptosis, *Cell. Signal.* **26**, 2014, 549-555 <https://doi.org/10.1016/j.cellsig.2013.11.028>.
- Burns J.C., Kelly M.C., Hoa M., Morell R.J. and Kelley M.W., Single-cell RNA-Seq resolves cellular complexity in sensory organs from the neonatal inner ear, *Nat. Commun.* **6**, 2015, 8557 <https://doi.org/10.1038/ncomms9557>.
- Cecconi F., Roth K.A., Dolgov O., Munarriz E., Anokhin K., Gruss P. and Salminen M., Apaf1-dependent programmed cell death is required for inner ear morphogenesis and growth, *Development* **131**, 2004, 2125-2135 <https://doi.org/10.1242/dev.01082>.
- Cha J.M. and Aronoff D.M., A role for cellular senescence in birth timing, *Cell Cycle* **16**, 2017, 2023-2031 <https://doi.org/10.1080/15384101.2017.1371888>.
- Chang J., Wang Y., Shao L., Laberge R.-M., Demaria M., Campisi J., Janakiraman K., Sharpless N.E., Ding S., Feng W., Luo Y., Wang X., Aykin-Burns N., Krager K., Ponnappan U., Hauer-Jensen M., Meng A. and Zhou D., Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice, *Nat. Med.* **22**, 2016, 78-83 <https://doi.org/10.1038/nm.4010>.
- Chang W., Brigande J.V., Fekete D.M. and Wu D.K., The development of semicircular canals in the inner ear: role of FGFs in sensory cristae, *Development* **131**, 2004, 4201-4211 <https://doi.org/10.1242/dev.01292>.
- Chen W., Jongkamonwiwat N., Abbas L., Eshtan S.J., Johnson S.L., Kuhn S., Milo M., Thurlow J.K., Andrews P.W., Marcotti W., Moore H.D. and Rivolta M.N., Restoration of auditory evoked responses by human ES-cell-derived otic progenitors, *Nature* **490**, 2012, 278-282 <https://doi.org/10.1038/nature11415>.
- Chen W.S., Xu P.Z., Gottlob K., Chen M.L., Sokol K., Shiyanova T., Roninson I., Weng W., Suzuki R., Tobe K., Kadowaki T. and Hay N., Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene, *Genes Dev.* **15**, 2001, 2203-2208 <https://doi.org/10.1101/gad.913901>.
- Chiche A., Le Roux I., von Joest M., Sakai H., Aguin S.B., Cazin C., Salam R., Fiette L., Alegria O., Flamant P., Tajbakhsh S. and Li H., Injury-induced senescence enables in vivo reprogramming in skeletal muscle, *Cell Stem Cell* **20**, 2017, 407-414, e4 <https://doi.org/10.1016/j.stem.2016.11.020>.
- Childs B.G., Baker D.J., Kirkland J.L., Campisi J. and van Deursen J.M., Senescence and apoptosis: dueling or complementary cell fates?, *EMBO Rep.* **15**, 2014, 1139-1153 <https://doi.org/10.15252/embr.201439245>.
- Childs B.G., Gluscevic M., Baker D.J., Laberge R.-M., Marquess D., Dananberg J. and van Deursen J.M., Senescent cells: an emerging target for diseases of ageing, *Nat. Rev. Drug Discov.* **16**, 2017, 718-735

<https://doi.org/10.1038/nrd.2017.116>.

Chuprin A., Gal H., Biron-Shental T., Biran A., Amiel A., Rozenblatt S. and Krizhanovsky V., Cell fusion induced by ERVWE1 or measles virus causes cellular senescence, *Genes Dev.* **27**, 2013, 2356-2366

<https://doi.org/10.1101/gad.227512.113>.

Coate T.M. and Kelley M.W., Making connections in the inner ear: recent insights into the development of spiral ganglion neurons and their connectivity with sensory hair cells, *Semin. Cell Dev. Biol.* **24**, 2013, 460-469

<https://doi.org/10.1016/j.semcdb.2013.04.003>.

Collado M. and Serrano M., Senescence in tumours: evidence from mice and humans, *Nat. Rev. Canc.* **10**, 2010, 51-57 <https://doi.org/10.1038/nrc2772>.

Coppé J.-P., Patil C.K., Rodier F., Sun Y., Muñoz D.P., Goldstein J., Nelson P.S., Desprez P.-Y. and Campisi J., Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor, *PLoS Biol.* **6**, 2008, 2853-2868 <https://doi.org/10.1371/journal.pbio.0060301>.

Couloigner V., Teixeira M., Sterkers O., Rask-Andersen H. and Ferrary E., [The endolymphatic sac: its roles in the inner ear], *Med. Sci.* **20**, 2004, 304-310 <https://doi.org/10.1051/medsci/2004203304>.

Davaapil H., Brockes J.P. and Yun M.H., Conserved and novel functions of programmed cellular senescence during vertebrate development, *Development* **144**, 2017, 106-114 <https://doi.org/10.1242/dev.138222>.

Davies D., Cell-extracellular matrix versus cell-cell interactions during the development of the cochlear-vestibular ganglion, *J. Neurosci. Res.* **89**, 2011, 1375-1387 <https://doi.org/10.1002/jnr.22664>.

de Iriarte Rodríguez R., Pulido S., Rodríguez-de la Rosa L., Magariños M. and Varela-Nieto I., Age-regulated function of autophagy in the mouse inner ear, *Hear. Res.* **330**, 2015, 39-50

<https://doi.org/10.1016/j.heares.2015.07.020>.

Degterev A. and Yuan J., Expansion and evolution of cell death programmes, *Nat. Rev. Mol. Cell Biol.* **9**, 2008, 378-390 <https://doi.org/10.1038/nrm2393>.

Demaria M., Ohtani N., Youssef S.A., Rodier F., Toussaint W., Mitchell J.R., Laberge R.-M., Vijg J., Van Steeg H., Dollé M.E.T., Hoeijmakers J.H.J., de Bruin A., Hara E. and Campisi J., An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA, *Dev. Cell* **31**, 2014, 722-733 <https://doi.org/10.1016/j.devcel.2014.11.012>.

Doherty J. and Baehrecke E.H., Life, death and autophagy, *Nat. Cell Biol.* 2018 <https://doi.org/10.1038/s41556-018-0201-5>.

Dörr J.R., Yu Y., Milanovic M., Beuster G., Zasada C., Däbritz J.H.M., Lisec J., Lenze D., Gerhardt A., Schleicher K., Kratzat S., Purfürst B., Walenta S., Mueller-Klieser W., Gräler M., Hummel M., Keller U., Buck A.K., Dörken B., Willmitzer L., Reimann M., Kempa S., Lee S. and Schmitt C.A., Synthetic lethal metabolic targeting of cellular senescence in cancer therapy, *Nature* **501**, 2013, 421-425 <https://doi.org/10.1038/nature12437>.

Dou Z., Xu C., Donahue G., Shimi T., Pan J.-A., Zhu J., Ivanov A., Capell B.C., Drake A.M., Shah P.P., Catanzaro J.M., Ricketts M.D., Lamark T., Adam S.A., Marmorstein R., Zong W.-X., Johansen T., Goldman R.D., Adams P.D. and Berger S.L., Autophagy mediates degradation of nuclear lamina, *Nature* **527**, 2015, 105-109 <https://doi.org/10.1038/nature15548>.

Elliott K.L., Fritsch B. and Duncan J.S., Evolutionary and developmental biology provide insights into the regeneration of organ of corti hair cells, *Front. Cell. Neurosci.* **12**, 2018, 252

<https://doi.org/10.3389/fncel.2018.00252>.

Fekete D.M., Homburger S.A., Waring M.T., Riedl A.E. and Garcia L.F., Involvement of programmed cell death in morphogenesis of the vertebrate inner ear, *Development* **124**, 1997, 2451-2461.

Frago L.M., Cañón S., de la Rosa E.J., León Y. and Varela-Nieto I., Programmed cell death in the developing inner ear is balanced by nerve growth factor and insulin-like growth factor I, *J. Cell Sci.* **116**, 2003, 475-486.

Frago L.M., León Y., de la Rosa E.J., Gómez-Muñoz A. and Varela-Nieto I., Nerve growth factor and ceramides modulate cell death in the early developing inner ear, *J. Cell Sci.* **111** (Pt 5), 1998, 549-556.

Galluzzi L., Bravo-San Pedro J.M., Vitale I., Aaronson S.A., Abrams J.M., Adam D., et al., Essential versus accessory aspects of cell death: recommendations of the NCCD 2015, *Cell Death Differ.* **22**, 2015, 58-73

<https://doi.org/10.1038/cdd.2014.137>.

García-Prat L., Martínez-Vicente M., Perdiguero E., Ortet L., Rodríguez-Ubreva J., Rebollo E., Ruiz-Bonilla V., Gutarra S., Ballestar E., Serrano A.L., Sandri M. and Muñoz-Cánoves P., Autophagy maintains stemness by preventing senescence, *Nature* **529**, 2016, 37-42 <https://doi.org/10.1038/nature16187>.

- Gewirtz D.A., Autophagy and senescence: a partnership in search of definition, *Autophagy* **9**, 2013, 808-812 <https://doi.org/10.4161/auto.23922>.
- Green D., Means to an End: Apoptosis and Other Cell Death Mechanisms Cold Spring Harbor, 2011, Cold Spring Harbor Laboratory Press; NY, ISBN 978-0-87969-888-1.
- Griffith A.J. and Wangemann P., Hearing loss associated with enlargement of the vestibular aqueduct: mechanistic insights from clinical phenotypes, genotypes, and mouse models, *Hear. Res.* **281**, 2011, 11-17 <https://doi.org/10.1016/j.heares.2011.05.009>.
- Groves A.K., Zhang K.D. and Fekete D.M., The genetics of hair cell development and regeneration, *Annu. Rev. Neurosci.* **36**, 2013, 361-381 <https://doi.org/10.1146/annurev-neuro-062012-170309>.
- Hansen M., Rubinstein D.C. and Walker D.W., Autophagy as a promoter of longevity: insights from model organisms, *Nat. Rev. Mol. Cell Biol.* **19**, 2018, 579-593 <https://doi.org/10.1038/s41580-018-0033-y>.
- Hawkins R.D., Bashirdes S., Powder K.E., Sajan S.A., Bhonagiri V., Alvarado D.M., Speck J., Warchol M.E. and Lovett M., Large scale gene expression profiles of regenerating inner ear sensory epithelia, *PLoS One* **2**, 2007, e525 <https://doi.org/10.1371/journal.pone.0000525>.
- He S. and Sharpless N.E., Senescence in health and disease, *Cell* **169**, 2017, 1000-1011 <https://doi.org/10.1016/j.cell.2017.05.015>.
- Ito Y., Hoare M. and Narita M., Spatial and temporal control of senescence, *Trends Cell Biol.* **27**, 2017, 820-832 <https://doi.org/10.1016/j.tcb.2017.07.004>.
- Kang C., Xu Q., Martin T.D., Li M.Z., Demaria M., Aron L., Lu T., Yankner B.A., Campisi J. and Elledge S.J., The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4, *Science* **349**, 2015, 561-562 <https://doi.org/10.1126/science.aaa5612>.
- Kaushik S. and Cuervo A.M., The coming of age of chaperone-mediated autophagy, *Nat. Rev. Mol. Cell Biol.* **19**, 2018, 365-381 <https://doi.org/10.1038/s41580-018-0001-6>.
- Kirkland J.L. and Tchkonja T., Cellular senescence: a translational perspective, *EBioMedicine* **21**, 2017, 21-28 <https://doi.org/10.1016/j.ebiom.2017.04.013>.
- Klionsky D.J., Abdelmohsen K., Abe A., Abedin M.J., Abeliovich H., et al., Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition), *Autophagy* **12**, 2016, 1-222 <https://doi.org/10.1080/15548627.2015.1100356>.
- Krizhanovsky V., Yon M., Dickins R.A., Hearn S., Simon J., Miething C., Yee H., Zender L. and Lowe S.W., Senescence of activated stellate cells limits liver fibrosis, *Cell* **134**, 2008, 657-667 <https://doi.org/10.1016/j.cell.2008.06.049>.
- Kuilman T., Michaloglou C., Vredeveld L.C.W., Douma S., van Doorn R., Desmet C.J., Aarden L.A., Mooi W.J. and Peeper D.S., Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network, *Cell* **133**, 2008, 1019-1031 <https://doi.org/10.1016/j.cell.2008.03.039>.
- Kwon Y., Kim J.W., Jeoung J.A., Kim M.-S. and Kang C., Autophagy is pro-senescence when seen in close-up, but anti-senescence in long-shot, *Mol. Cell.* **40**, 2017, 607-612 <https://doi.org/10.14348/molcells.2017.0151>.
- Lahlou H., Lopez-Juarez A., Fontbonne A., Nivet E. and Zine A., Modeling human early otic sensory cell development with induced pluripotent stem cells, *PLoS One* **13**, 2018, e0198954 <https://doi.org/10.1371/journal.pone.0198954>.
- León Y., Sánchez-Galiano S. and Gorospe I., Programmed cell death in the development of the vertebrate inner ear, *Apoptosis* **9**, 2004, 255-264.
- León Y., Sanz C., Frago L.M., Camarero G., Cañón S., Varela-Nieto I. and Giráldez F., Involvement of insulin-like growth factor-I in inner ear organogenesis and regeneration, *Horm. Metab. Res.* **31**, 1999, 126-132.
- León Y., Sanz C., Giráldez F. and Varela-Nieto I., Induction of cell growth by insulin and insulin-like growth factor-I is associated with Jun expression in the otic vesicle, *J. Comp. Neurol.* **398**, 1998, 323-332.
- León Y., Vazquez E., Sanz C., Vega J.A., Mato J.M., Giraldez F., Represa J. and Varela-Nieto I., Insulin-like growth factor-I regulates cell proliferation in the developing inner ear, activating glycosyl-phosphatidylinositol hydrolysis and Fos expression, *Endocrinology* **136**, 1995, 3494-3503 <https://doi.org/10.1210/endo.136.8.7628386>.
- Li Yi, Zhao H., Huang X., Tang J., Zhang S., Li Yan, Liu X., He L., Ju Z., Lui K.O. and Zhou B., Embryonic senescent cells re-enter cell cycle and contribute to tissues after birth, *Cell Res.* **28**, 2018, 775-778 <https://doi.org/10.1038/s41422-018-0050-6>.

- Lindsten T, Ross A.J., King A., Zong W.X., Rathmell J.C., Shiels H.A., Ulrich E., Waymire K.G., Mahar P, Frauwirth K., Chen Y., Wei M., Eng V.M., Adelman D.M., Simon M.C., Ma A., Golden J.A., Evan G., Korsmeyer S.J., MacGregor G.R. and Thompson C.B., The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues, *Mol. Cell* **6**, 2000, 1389-1399.
- Lorda-Diez C.I., Garcia-Riart B., Montero J.A., Rodriguez-León J., Garcia-Porrero J.A. and Hurle J.M., Apoptosis during embryonic tissue remodeling is accompanied by cell senescence, *Aging (Albany NY)* **7**, 2015, 974-985 <https://doi.org/10.18632/aging.100844>.
- Magariños M., Aburto M.R., Sánchez-Calderón H., Muñoz-Agudo C., Rapp U.R. and Varela-Nieto I., RAF kinase activity regulates neuroepithelial cell proliferation and neuronal progenitor cell differentiation during early inner ear development, *PLoS One* **5**, 2010, e14435-e14435 <https://doi.org/10.1371/journal.pone.0014435>.
- Magariños M., Contreras J., Aburto M.R. and Varela-Nieto I., Early development of the vertebrate inner ear, *Anat. Rec.* **295**, 2012, 1775-1790, Hoboken, N.J.: 2007.
- Magariños M., Contreras J. and Varela-Nieto I., Early development of the vertebrate inner ear, In: Romand R. and Varela-Nieto I., (Eds.), *Development of Auditory and Vestibular Systems*, fourth ed., 2014, 1-30, 2014.
- Magariños M., Pulido S., Aburto M.R., de Iriarte Rodríguez R. and Varela-Nieto I., Autophagy in the vertebrate inner ear, *Front. Cell Dev. Biol.* **5**, 2017, 56 <https://doi.org/10.3389/fcell.2017.00056>.
- Malaquin N., Martinez A. and Rodier F., Keeping the senescence secretome under control: molecular reins on the senescence-associated secretory phenotype, *Exp. Gerontol.* **82**, 2016, 39-49 <https://doi.org/10.1016/j.exger.2016.05.010>.
- Mariño G., Fernández A.F., Cabrera S., Lundberg Y.W., Cabanillas R., Rodríguez F., Salvador-Montoliu N., Vega J.A., Germanà A., Fueyo A., Freije J.M.P. and López-Otín C., Autophagy is essential for mouse sense of balance, *J. Clin. Invest.* **120**, 2010, 2331-2344 <https://doi.org/10.1172/JCI42601>.
- Morrill S. and He D.Z.Z., Apoptosis in inner ear sensory hair cells, *J. Otolaryngol.* **12**, 2017, 151-164 <https://doi.org/10.1016/j.joto.2017.08.001>.
- Mosteiro L., Pantoja C., Alcazar N., Marión R.M., Chondronasiou D., Rovira M., Fernandez-Marcos P.J., Muñoz-Martin M., Blanco-Aparicio C., Pastor J., Gómez-López G., De Martino A., Blasco M.A., Abad M. and Serrano M., Tissue damage and senescence provide critical signals for cellular reprogramming in vivo, *Science* **354**, 2016 <https://doi.org/10.1126/science.aaf4445>.
- Muñoz-Espín D., Cañamero M., Maraver A., Gómez-López G., Contreras J., Murillo-Cuesta S., Rodríguez-Baeza A., Varela-Nieto I., Ruberte J., Collado M. and Serrano M., Programmed cell senescence during mammalian embryonic development, *Cell* **155**, 2013, 1104-1118 <https://doi.org/10.1016/j.cell.2013.10.019>.
- Muñoz-Espín D. and Serrano M., Cellular senescence: from physiology to pathology, *Nat. Rev. Mol. Cell Biol.* **15**, 2014, 482-496 <https://doi.org/10.1038/nrm3823>.
- Nacher V., Carretero A., Navarro M., Armengol C., Llombart C., Rodríguez A., Herrero-Fresneda I., Ayuso E. and Ruberte J., The quail mesonephros: a new model for renal senescence?, *J. Vasc. Res.* **43**, 2006, 581-586 <https://doi.org/10.1159/000096076>.
- Nakashima T., Pyykkö I., Arroll M.A., Casselbrant M.L., Foster C.A., Manzoor N.F., Megerian C.A., Naganawa S. and Young Y.-H., Meniere's disease, *Nat. Rev. Dis. Primers* **2**, 2016, 16028 <https://doi.org/10.1038/nrdp.2016.28>.
- Narita Masako, Young A.R.J., Arakawa S., Samarajiwa S.A., Nakashima T., Yoshida S., Hong S., Berry L.S., Reichelt S., Ferreira M., Tavaré S., Inoki K., Shimizu S. and Narita Masashi, Spatial coupling of mTOR and autophagy augments secretory phenotypes, *Science* **332**, 2011, 966-970 <https://doi.org/10.1126/science.1205407>.
- Oshima K., Grimm C.M., Corrales C.E., Senn P., Martinez Monedero R., Géléoc G.S.G., Edge A., Holt J.R. and Heller S., Differential distribution of stem cells in the auditory and vestibular organs of the inner ear, *J. Assoc. Res. Otolaryngol.* **8**, 2007, 18-31 <https://doi.org/10.1007/s10162-006-0058-3>.
- Pérez-Mancera P.A., Young A.R.J. and Narita M., Inside and out: the activities of senescence in cancer, *Nat. Rev. Canc.* **14**, 2014, 547-558 <https://doi.org/10.1038/nrc3773>.
- Pradelli L.A., Bénétteau M. and Ricci J.-E., Mitochondrial control of caspase-dependent and -independent cell death, *Cell. Mol. Life Sci.* **67**, 2010, 1589-1597 <https://doi.org/10.1007/s00018-010-0285-y>.
- Raft S., Andrade L.R., Shao D., Akiyama H., Henkemeyer M. and Wu D.K., Ephrin-B2 governs morphogenesis of endolymphatic sac and duct epithelia in the mouse inner ear, *Dev. Biol.* **390**, 2014, 51-67 <https://doi.org/10.1016/j.ydbio.2014.02.019>.
- Riedl S.J. and Salvesen G.S., The apoptosome: signalling platform of cell death, *Nat. Rev. Mol. Cell Biol.* **8**, 2007, 405-413 <https://doi.org/10.1038/nrm2153>.

- Ritschka B., Storer M., Mas A., Heinzmann F., Ortells M.C., Morton J.P., Sansom O.J., Zender L. and Keyes W.M., The senescence-associated secretory phenotype induces cellular plasticity and tissue regeneration, *Genes Dev.* **31** 2017, 172-183 <https://doi.org/10.1101/gad.290635.116>.
- Roninson I.B., Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts, *Cancer Lett.* **179**, 2002, 1-14.
- Ruhland M.K., Loza A.J., Capietto A.-H., Luo X., Knolhoff B.L., Flanagan K.C., Belt B.A., Alspach E., Leahy K., Luo J., Schaffer A., Edwards J.R., Longmore G., Faccio R., DeNardo D.G. and Stewart S.A., Stromal senescence establishes an immunosuppressive microenvironment that drives tumorigenesis, *Nat. Commun.* **7**, 2016, 11762 <https://doi.org/10.1038/ncomms11762>.
- Salama R., Sadaie M., Hoare M. and Narita M., Cellular senescence and its effector programs, *Genes Dev.* **28**, 2014, 99-114 <https://doi.org/10.1101/gad.235184.113>.
- Sanchez-Calderon H., Milo M., Leon Y. and Varela-Nieto I., A network of growth and transcription factors controls neuronal differentiation and survival in the developing ear, *Int. J. Dev. Biol.* **51**, 2007, 557-570 <https://doi.org/10.1387/ijdb.072373hs>.
- Sanz C., León Y., Cañón S., Alvarez L., Giraldez F. and Varela-Nieto I., Pattern of expression of the jun family of transcription factors during the early development of the inner ear: implications in apoptosis, *J. Cell Sci.* **112** (Pt 22), 1999a, 3967-3974.
- Sanz C., León Y., Troppmair J., Rapp U.R. and Varela-Nieto I., Strict regulation of c-Raf kinase levels is required for early organogenesis of the vertebrate inner ear, *Oncogene* **18**, 1999b, 429-437 <https://doi.org/10.1038/sj.onc.1202312>.
- Song G., Ouyang G. and Bao S., The activation of Akt/PKB signaling pathway and cell survival, *J. Cell Mol. Med.* **9**, 2005, 59-71.
- Soto-Gamez A. and Demaria M., Therapeutic interventions for aging: the case of cellular senescence, *Drug Discov. Today* **22**, 2017, 786-795 <https://doi.org/10.1016/j.drudis.2017.01.004>.
- Sousa-Victor P., Gutarra S., García-Prat L., Rodríguez-Ubrea J., Ortet L., Ruiz-Bonilla V., Jardí M., Ballestar E., González S., Serrano A.L., Perdiguero E. and Muñoz-Cánoves P., Geriatric muscle stem cells switch reversible quiescence into senescence, *Nature* **506**, 2014, 316-321 <https://doi.org/10.1038/nature13013>.
- Stojanova Z.P., Kwan T. and Segil N., Epigenetic regulation of Atoh1 guides hair cell development in the mammalian cochlea, *Development* **143**, 2016, 1632 <https://doi.org/10.1242/dev.137976>.
- Storer M., Mas A., Robert-Moreno A., Pecoraro M., Ortells M.C., Di Giacomo V., Yosef R., Pilpel N., Krizhanovsky V., Sharpe J. and Keyes W.M., Senescence is a developmental mechanism that contributes to embryonic growth and patterning, *Cell* **155**, 2013, 1119-1130 <https://doi.org/10.1016/j.cell.2013.10.041>.
- Takasugi M., Emerging roles of extracellular vesicles in cellular senescence and aging, *Aging Cell* **17**, 2018 <https://doi.org/10.1111/accel.12734>.
- Taylor R.R., Nevill G. and Forge A., Rapid hair cell loss: a mouse model for cochlear lesions, *J. Assoc. Res. Otolaryngol.* **9**, 2008, 44-64 <https://doi.org/10.1007/s10162-007-0105-8>.
- Torres M. and Giraldez F., The development of the vertebrate inner ear, *Mech. Dev.* **71**, 1998, 5-21.
- Vasey D.B., Wolf C.R., Brown K. and Whitelaw C.B.A., Spatial p21 expression profile in the mid-term mouse embryo, *Transgenic Res.* **20**, 2011, 23-28 <https://doi.org/10.1007/s11248-010-9385-6>.
- Villiard É., Denis J.-F., Hashemi F.S., Igelmann S., Ferbeyre G. and Roy S., Senescence gives insights into the morphogenetic evolution of amniotes, *Biol. Open* **6**, 2017, 891-896 <https://doi.org/10.1242/bio.025809>.
- Wang J. and Puel J.-L., Toward cochlear therapies, *Physiol. Rev.* **98**, 2018, 2477-2522 <https://doi.org/10.1152/physrev.00053.2017>.
- Wick C.C., Manzoor N.F., Semaan M.T. and Megerian C.A., Endolymphatic sac tumors, *Otolaryngol. Clin. North Am.* **48**, 2015, 317-330 <https://doi.org/10.1016/j.otc.2014.12.006>.
- Xue W., Zender L., Miething C., Dickins R.A., Hernando E., Krizhanovsky V., Cordon-Cardo C. and Lowe S.W., Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas, *Nature* **445**, 2007, 656-660 <https://doi.org/10.1038/nature05529>.
- Yosef R., Pilpel N., Tokarsky-Amiel R., Biran A., Ovadya Y., Cohen S., Vadai E., Dassa L., Shahar E., Condiotti R., Ben-Porath I. and Krizhanovsky V., Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL, *Nat. Commun.* **7**, 2016, 11190 <https://doi.org/10.1038/ncomms11190>.

Young A.R.J., Narita Masako, Ferreira M., Kirschner K., Sadaie M., Darot J.F.J., Tavaré S., Arakawa S., Shimizu S., Watt F.M. and Narita Masashi, Autophagy mediates the mitotic senescence transition, *Genes Dev.* **23**, 2009, 798-803 <https://doi.org/10.1101/gad.519709>.

Yuan H., Wang X., Hill K., Chen J., Lemasters J., Yang S.-M. and Sha S.-H., Autophagy attenuates noise-induced hearing loss by reducing oxidative stress, *Antioxidants Redox Signal.* **22**, 2015, 1308-1324 <https://doi.org/10.1089/ars.2014.6004>.

Yun M.H., Davaapil H. and Brookes J.P., Recurrent turnover of senescent cells during regeneration of a complex structure, *Elife* **4**, 2015 <https://doi.org/10.7554/eLife.05505>.

Zhu Y., Tchkonja T., Pirtskhalava T., Gower A.C., Ding H., Giorgadze N., Palmer A.K., Ikeno Y., Hubbard G.B., Lenburg M., O'Hara S.P., LaRusso N.F., Miller J.D., Roos C.M., Verzosa G.C., LeBrasseur N.K., Wren J.D., Farr J.N., Khosla S., Stout M.B., McGowan S.J., Fuhrmann-Stroissnigg H., Gurkar A.U., Zhao J., Colangelo D., Dorronsoro A., Ling Y.Y., Barghouthy A.S., Navarro D.C., Sano T., Robbins P.D., Niedernhofer L.J. and Kirkland J.L., The Achilles' heel of senescent cells: from transcriptome to senolytic drugs, *Aging Cell* **14**, 2015, 644-658 <https://doi.org/10.1111/accel.12344>.

Highlights

- ~~The development of the inner ear requires the coordination in time and space of different strictly regulated cellular processes.~~The development of the inner ear requires the coordination in time and space of strictly regulated cellular processes.
- ~~Cellular senescence emerges as one of these processes that is complementary to those of apoptosis and autophagy to sculpt the developing inner ear.~~Cellular senescence emerges as a complementary process to apoptosis and autophagy to sculpt the developing inner ear.
- ~~Understanding the molecular mechanisms that underlie otic cell specification and survival should help the development of regenerative medicine strategies.~~Understanding the mechanisms of otic cell specification and survival should help the development of regenerative strategies.

Queries and Answers

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Answer: The development of the inner ear requires the coordination in time and space of strictly regulated cellular processes.

Cellular senescence emerges as a complementary process to apoptosis and autophagy to sculpt the developing inner ear.

Understanding the mechanisms of otic cell specification and survival should help the development of regenerative strategies.

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