Research Highlight in Developmental Biology

Senescence— Before Birth

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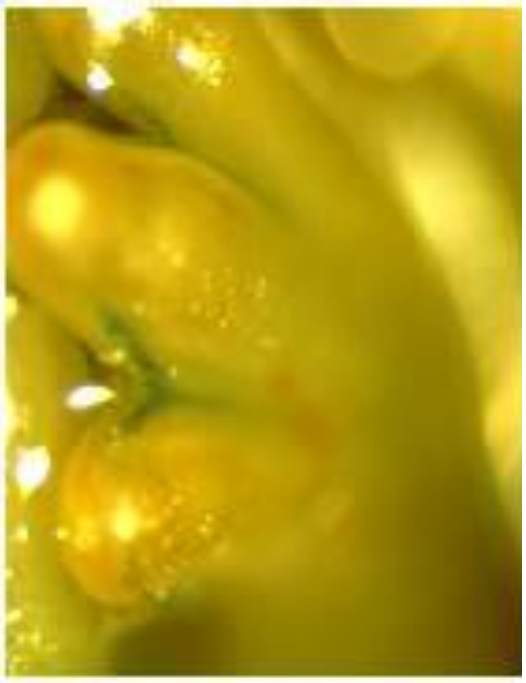
Cellular senescence— defined as a process of stable cell-cycle arrest—was first identified as a biological state that marked the end of a cell's replicative life (Hayflick, 1965). In the investigations that followed, senescence was established as a component of disease states {e.g. DNA damage} (Kuilman et al., 2010), and was correlated with aging-associated disorders (Keyes et al., 2005; Baker et al., 2011). It was also shown that cells attain oncogene-induced senescence {OIS} when triggered by intense oncogene signaling {e.g. *ras*} (Serrano et al., 1997). Senescent cells also participate in extensive signaling to the immune system for their own clearance due to their distinct senescence-associated secretory phenotype (Kuilman et al., 2010). Overall, senescence was widely considered a component of cellular response to disease states.

Cellular senescence, inescapably, in its pathological context and features, parallels yet another critical cellular process: apoptosis. Apoptosis, however, was understood to include both disease states and normal stages of development. Meanwhile, the role of cellular senescence in organismal development was unclear. Two reports, published in the journal Cell (Munoz-Espin et al., 2013; Storer et al., 2013), provide compelling evidence to suggest that cellular senescence may be at play during embryogenesis as well, ensuring normal development of the organism.

In the study by Muñoz-Espín et al. senescence was verified as a programmed developmental phenomenon in both mouse and human. Focusing their efforts to characterize the



Interdigital webs of the mouse embryo positively identified for senescence by senescence-associated β-galactosidase activity (blue). *Image courtesy of M. Serrano and W.M. Keyes*

relevance of senescence in the mesonephros {transiently operating embryonic kidney} and the endolymphatic sac of the developing inner ear, they propose that developmentally programmed senescence preceded the

damage-induced senescence during evolution. Muñoz-Espín et al. began their investigations by noting the occurrence of senescent tissue in various structures such as the neural tube, apical ectodermal ridge, interdigital webs {See Image}, vibrissae, and mesonephros during mouse development. In the mesonephric tubules and the endolymphatic sacs, structures that were investigated in detail, the onset of senescence was accompanied by a drop in cell proliferation, thus confirming a typical feature of the senescence status. Epithelial cells from both structures also tested positive for heterochromatic markers of senescence such as HP1γ and H3K9me3 while the stromal cells tested negative. The mesonephros and the endolymphatic sac also expressed cellular senescence mediators such as p21, p27, and p15. These results identified the occurrence of developmentally programmed senescence in selective cell clusters of the embryo.

Testing for senescence in p21 and p53 null embryos revealed the requirement of developmentally programmed senescence on p21, independently of p53, in the wild type {WT} mesonephric tubules and the endolymphatic sac. The authors also excluded a role for DNA-damage signaling kinases in developmental senescence. Using the standard markers for senescence, Muñoz-Espín et al. noted the occurrence of developmental senescence in human mesonephros and endolymphatic sac as well. Gene set enrichment analysis of microarray data from WT and p21 null mouse embryos along with results from earlier investigations revealed a possible role for TGF-β pathway in p21-mediated developmentally programmed senescence (Acosta et al., 2013). Earlier results pointed to the possibility that TGF-β induced transcription of p21 gene occurred through SMAD complexes (Datto et al., 1995). After verification of active nuclear localization of phosphorylated SMAD2 in the mesonephros and endolymphatic sac during developmental senescence, Muñoz-Espín et al. treated pregnant mice with a TGF-β pathway inhibitor. Treatment with the TGF-β pathway inhibitor significantly reduced the expression of senescence markers and the senescence mediator p21 thus causally linking developmentally programmed senescence to TGF-β/SMAD pathway. Based on previously reported findings that SMAD proteins and FOXO proteins form complexes that bind and activate the p21 promoter (Seoane et al., 2004), Muñoz-Espín et al. used additional genetic and chemical approaches to reveal the dependence of developmentally programmed senescence also on the PI3K/FOXO pathway. These experiments indicated that an intact TGF-β pathway and PI3K/FOXO pathway, acting in parallel, induce developmentally programmed senescence through p21 transcription.

Muñoz-Espín et al. also observed that cells undergoing developmentally programmed senescence were cleared by macrophages in the absence of widespread apoptosis. The functional significance of developmentally programmed senescence was highlighted by histological analyses showing that genetic ablation of developmental senescence in p21 null embryos compromised the normal timing of regression in the mesonephros. Meanwhile, compensatory apoptotic programs ensured developmental progression. In the endolymphatic sac, however, developmental senescence was shown to be critical for the regulation of balance between different cell populations.

Although regression is the developmental fate of the mesonephros, the portion of tissue that

becomes the wolffian duct {a structure that differentiates into the epididymis and vas deferens in males} persists. In females, all of wolffian duct with the exception of the caudal portion that contributes to morphogenesis of the vagina is subject to degeneration during normal development. Muñoz-Espín et al. noted significant expression of senescence markers in the female wolffian duct but not in males. Upon investigation of the vaginal morphology of p21 null females, the authors noted a significantly higher percentage of occurrences of septate vagina compared to WT females. The p21 null females also had a lower number of pups compared to WT females indicating compromised fertility. In summary, the work by Muñoz-Espín et al. demonstrates senescence as a developmentally programmed phenomenon with functional significance during mammalian development.

The second study, by Storer et al. (Storer et al., 2013), complements the findings of Muñoz-Espín et al. by investigating the role of developmentally programmed senescence in limb patterning, a paradigm that is known to involve apoptosis during embryogenesis (Montero and Hurle, 2010). Storer et al. began by identifying distinct structures that tested positive for senescence associated markers during the development of both mouse and chick embryos. In particular, they noticed that senescence in the developing limb enveloped the apical ectodermal ridge {AER}, a critical signaling center involved in proximo-distal limb pattering (Fernandez-Teran and Ros, 2008). They verified that the AER tested positive for p21, a mediator of senescence, also discussed above in the work by Muñoz-Espín et al. Then, they performed gene-expression profiling on AER, and overlapped the data set with previously published data sets from IMR90 human fibroblasts, induced into senescence by oncogene activation, to identify commonly upregulated genes between developmentally programmed and OIS states. Remarkably, they found that both p21 (*CDKN1A*) and p15 (*CDKN2B*) were common to both the AER and senescent human fibroblasts. Gene ontology analysis revealed that changes in many developmental pathways mark the induction of OIS.

After identifying p21 as a common link between developmentally programmed senescence and OIS, Storer et al. examined mice with p21 deficiency. They noticed that p21 deficiency resulted in an increase of cell death in the AER associated with decreased proliferation of the mesenchymal cells directly below the AER. Two key proliferation signals – FGF8 and FGF4 – that are passed on from the AER to the underlying mesenchyme were decreased in p21 deficiency compared to WT, along with changes in expression of several genes involved in limb patterning. These results showed the requirement for p21 for normal senescence and limb patterning. When p21 was knocked down by small interfering RNA in IMR90 human fibroblasts – under OIS – there were changes in the expression of developmental mediators compared to the controls. These results suggested that p21 mediated senescence involving developmental signals are partially conserved in OIS.

Storer et al. also observed that senescent cells of the AER closely followed the fate of the AER itself – a transient signaling center that functions during a precise developmental window and regresses at 14.5 days of mouse development. They also observed, using histological analyses, that the senescent cells in the limbs were subjected to apoptosis and

cleared by macrophages. Based on prior findings that the AER instructs the mesenchyme via growth factor signaling which in turn activates phospho-ERK (pERK) in the mesenchyme for reciprocal paracrine signling, the authors treated pregnant mice with a MEK inhibitor to offset ERK phosphorylation *in vivo*. The pERK inhibition was applied to overlap with the time window of developmental senescence in the AER. pERK inhibition resulted in a reduction of the number of senescent cells in the AER. Culturing embryonic limbs in MEK inhibitor also resulted in the loss of senescent cells within the AER with the ultimate effect of abnormal limb patterning. These final set of experiments suggested a critical role for developmentally programmed senescence in patterning the mammalian limb.

In summary, the experiments by Storer et al. provide evidence for developmentally programmed senescence during mouse and chick embryogenesis. They also provide evidence for the role of common molecular signaling cascades between developmentally programmed and oncogene-induced senescence.

Together, the work by Muñoz-Espín et al. and Storer et al. suggest a critical role for cellular senescence during normal development in amniotes. They also suggest an evolutionarily ancient developmental program for senescence that was adapted and reactivated during tumor suppression and other disease states such as liver fibrosis that also involve cellular senescence. These experiments also provide a conceptual framework for development in which, along with proliferation, differentiation, migration, and cell death, senescence also plays a fundamental role in normal embryogenesis.

Future studies may shed light on the specific role of developmentally programmed senescence in various other organs. They might also define the role of other important developmental pathways such as the Hedgehog and WNT signaling pathways during senescence. The identification of molecules mediating immune-mediated clearance of senescent cells during development also remains for future exploration.

Acknowledgements

I would like to thank Dr. Deborah Andrew for supporting my work. I also thank the anonymous reviewers for their thoughtful suggestions for improving this article.

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