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The Senescence Marker p16 in Development, Adulthood, and Aging

Hasan Safwan Zaiter

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THÈSE DE DOCTORAT

Le marqueur de sénescence p16Ink4a dans le développement, l'âge adulte et le vieillissement

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Le marqueur de sénescence p16Ink4a dans le développement, l'âge adulte et le vieillissement

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Le marqueur de sénescence p16Ink4a dans le développement, l'âge adulte et le vieillissement

Resumé

Le vieillissement est une caractéristique biologique qui se caractérise par une dégénérescence progressive de la fonction des cellules, des tissus, des organes ou d'un organisme intact en raison de l'accumulation de facteurs environnementaux et de stress avec le temps. Plusieurs facteurs ont été attribués au vieillissement tels que le stress oxydatif, le raccourcissement des télomères, les dommages à l'ADN et surtout, le dépôt de cellules sénescents. Ce sont des cellules irréversiblement mitotiquement inactives, mais métaboliquement actives. La raison sous-jacente à leur sénescence réside dans les bras extrinsèques et intrinsèques. Le bras extrinsèque est principalement caractérisé par l'expression et le profil sécrétoire connu sous le nom de phénotype sécrétoire associé à la sénescence (SASP). Le bras intrinsèque résulte de l'impact de plusieurs gènes censés réguler le cycle cellulaire, tels que les gènes suppresseurs de tumeurs p16Ink4a (p16), p19ARF (p19) et p21. P16 est un suppresseur de tumeur et un régulateur du cycle cellulaire qui a été lié au vieillissement et à la sénescence. Des recherches approfondies ont révélé que l'expression de p16 est significativement augmentée dans les cellules sénescents, ainsi que lors du vieillissement naturel ou de pathologies liées à l'âge. De ce fait, p16 est considéré comme un biomarqueur spécifique pour détecter les cellules sénescents et le vieillissement. Alors qu'un rôle potentiel de p19 et p21 a été démontré dans le développement embryonnaire, p16 a été moins bien documenté. Pour étudier le rôle potentiel de p16 dans le développement, nous avons mené une étude d'expression développementale de p16, à côté des suppresseurs de tumeurs p19ARF et p21, et examiné leur expression d'ARN dans le cerveau, le cœur, le foie et les reins de souris au stade embryonnaire, postnatal, adulte et les vieux âges. L'immunohistochimie a été utilisée pour examiner l'expression de P16 au niveau de la protéine. Nous avons constaté que l'expression de p16 était très dynamique dans tous les organes au cours des stades embryonnaire et postnatal, et qu'elle était significativement plus élevée chez les souris âgées par rapport à p19 et p21. De plus, nous avons constaté que l'ARNm et la protéine p16 étaient plus répandus dans les cellules endothéliales hépatiques (CE) que les cellules parenchymateuses chez les souris âgées. Ces

découvertes indiquent un rôle possible de p16 dans le développement embryonnaire, ainsi qu'un rôle sélectif potentiel de p16 dans les CE hépatiques.

Par conséquent, nous avons cherché à mieux comprendre le rôle de p16 dans les processus biologiques des CE hépatiques. Par conséquent, nous avons utilisé de petites constructions en épingle à cheveux (shRNA) et un vecteur ADNc-GFP p16 transduit via un lentivirus, pour inhiber et surexprimer p16 in vitro, afin d'évaluer la perte et le gain de fonction dans deux types de foie ECs, ECs vasculaires CD31+ et cellules endothéliales sinusoidales CD146+ (LSEC). Les cellules ont été isolées du foie par un test de tri magnétique des cellules activées (MACS) à l'aide d'anticorps monoclonaux attachés à des billes magnétiques contre les marqueurs de surface CD31 et CD146. Une séquence non codante et des cellules transduites par un vecteur GFP vide ont été utilisées comme contrôle pour les cellules transduites par shRNA et p16-GFP, respectivement.

Mots clés: Vieillesse, sénescence cellulaire, développement p16Ink4a, foie, cellules endothéliales.

The Senescence Marker P16Ink4a in Development, Adulthood, and Aging

Abstract

Aging is a biological feature that is characterized by gradual degeneration of function of cells, tissues, organs, or an intact organism due to accumulation of environmental factors and stresses with time. Several factors have been attributed to aging such as oxidative stress, telomere shortening, DNA damage and most importantly, the deposit of senescent cells (SnCs). These are irreversibly mitotically inactive, yet metabolically active cells. The reason underlying their senescence lies within the extrinsic and the intrinsic arms. The extrinsic arm is mainly characterized by the expression and the secretory profile known as the senescence associated secretory phenotype (SASP). The intrinsic arm results from the impact of several genes meant to regulate the cell cycle, such as the tumor suppressor genes p16Ink4a (p16), p19ARF (p19) and p21. P16 is a tumor suppressor and cell cycle regulator that has been linked to aging and senescence. Extensive research has revealed that p16 expression is significantly increased in SnCs, as well as during natural aging or age-related pathologies. Based on this fact, p16 is considered as a specific biomarker for identifying SnCs and aging. Whilst a potential role of p19 and p21 has been demonstrated in embryonic development, yet p16 has been less well documented. To investigate p16's potential role in development, we conducted a developmental expression study of p16, beside the tumor suppressors p19 and p21, and examined their RNA expression in the brain, heart, liver, and kidney of mice at embryonic, postnatal, adult, and old ages. Moreover, immunohistochemistry (IHC) was used to examine p16 expression at the protein level. We found that p16 expression was highly dynamic in all organs during embryonic and postnatal stages, and it was significantly more upregulated in old mice compared to p19 and p21. Furthermore, we found that p16 mRNA and protein were more prevalent in liver endothelial cells (ECs) than parenchymal cells in old mice. These findings point to a possible role for p16 in embryonic development, as well as a potential selective role for p16 in liver ECs.

Therefore, we aimed at better understanding the role of p16 in biological processes of liver ECs. Hence, we used small hairpin (shRNA) constructs and a p16 cDNA-GFP vector transduced via a

lentivirus, to knock-down and over-express p16 in-vitro, in order to assess the loss and gain of function in two types of liver ECs, CD31+ vascular ECs and CD146+ liver sinusoidal endothelial cells (LSECs). Cells were isolated from the liver through magnetic activated cells sorting (MACS) assay using a magnetic bead-attached monoclonal antibodies against CD31 and CD146 surface markers. Non-coding sequence and an empty-GFP vector transduced cells were used as a control for shRNA and p16-GFP transduced cells, respectively.

Keywords: Aging, cellular senescence, development p16Ink4a, liver, endothelial cells.

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LIST OF ABBREVIATIONS

| | |
|---|--|
| AECII - Alveolar type II epithelial cells | FGF - Fibroblast Growth Factor |
| AD - Alzheimer's disease | GCV – Ganciclovir |
| AKI - Acute kidney injury | GP - Glycoprotein |
| ATN - Acute tubular necrosis | GDNF - Glial cell-derived neurotrophic factor |
| AGEs - Advanced Glycation End-products | HDAi - Histone Deacetylase inhibitor |
| AT - Antithrombin | HFD - High-Fat Diet |
| ATIII - Antithrombin III | HNF - Hepatocyte nuclear factors |
| BMP - Bone morphogenetic protein | HP1α - Heterochromatin Protein 1α |
| BM-MSCs - Bone marrow mesenchymal stem cells | HSC - Hematopoietic Stem Cells |
| BMM - Bone Marrow-Derived Macrophages | ICM - Inner Cell Mass |
| CCN1 - Cysteine-rich angiogenic inducer 61 | ICSA - International Cell Senescence Association |
| CDK/i - Cyclin Dependent Kinase/inhibitor | IGF - Insulin-like Growth Factor |
| CDK4 - Cyclin-dependent kinase 4 | IM - Intermediate Mesoderm |
| COPD - Chronic obstructive pulmonary disease | IPF - Idiopathic Pulmonary Fibrosis |
| CSCs - Cardiac stem cells | ICAM-1 - Intercellular Adhesion Molecule-1 |
| CS - Cigarette smoking | KCs - Kupffer Cells |
| CCL2 - Monocyte Chemoattractant Protein-1 | LSEC - Liver sinusoidal endothelial cells |
| CD31 - Cluster of Differentiation 31 | LDL - Low-density lipoprotein |
| CD146 - Cluster of Differentiation 146 | LPS - Lipopolysaccharide |
| CLEC-1 - C-type Lectin Domain Family 1 | miRNAs - MicroRNAs |
| cGMP - Cyclic Guanosine Monophosphate | MM - Metanephric Mesenchyme |
| ECM - Extracellular Matrix | mtDNA - Mitochondrial DNA |
| ECs - Endothelial Cells | MAPK - Mitogen-Activated Protein Kinase |
| eNOS - Endothelial Nitric Oxide Synthase | MCP-1 - Monocyte Chemoattractant Protein-1 |
| DDR - DNA Damage Response | MMPs - Matrix Metalloproteinases |
| DEJ - Dermal epidermal joint | NFTs - Neurofibrillary tangles |
| DIS - Damage Induced Senescence | ND - Nephric Duct |
| DSB - Double Strand DNA Break | NPCs - Neural Progenitor Cells |
| ECM - Extracellular Matrix | NSCLC - Non-small cell lung carcinoma |
| EMT - Epithelial-Mesenchymal Transition | NAFLD - Nonalcoholic Fatty Liver Disease |

NF- κ B - Nuclear Factor Kappa B
NK - Natural Killer
NO - Nitric Oxide
OIS - Oncogene Induced Senescence
p14/p19 - p14ARF/p19ARF
p16 - p16Ink4a
p21 - Cyclin-dependent kinase inhibitor 1
p53 - Tumor protein p53
pRB - Retinoblastoma protein
POT1 - Protection of telomeres 1
RAP1 - TRF2 interacting protein
PAMPs - Pathogen-Associated Molecular Patterns
PECAM-1 - Platelet/Endothelial Cell Adhesion Molecule 1 (also known as CD31)
PGI2 - Prostacyclin
ROS - Reactive oxygen species
SA β -gal - Senescence-Associated β -Galactosidase
SAHF - Senescence-Associated Heterochromatin Foci
SASPs - Senescence Associated Secretory Phenotypes
SCC - Squamous cell carcinoma
SCs - Stem cells
SnCs - Senescent Cells
SOD - Superoxide Dismutase
scRNA-seq - Single-cell RNA sequencing
TIN2 - TRF1-interacting nuclear factor 2
TRF - Telomeric Repeat Binding Factor
TF - Tissue Factor
TFPI - Tissue Factor Pathway Inhibitor
TM – Thrombomodulin
tPA - Tissue-type Plasmin Activator
TLRs - Toll-Like Receptors
TNF- α - Tumor Necrosis Factor Alpha
UBE2S - Ubiquitin-conjugating enzyme E2S

UV - Ultraviolet
VSMC - Vascular Smooth Muscle Cell
VAP-1 - Vascular Adhesion Protein-1
VCAM-1 - Vascular Cell Adhesion Molecule-1
vWF - von Willebrand Factor
VEGF - Vascular Endothelial Growth Factor
VEGFR2 - Vascular Endothelial Growth Factor Receptor 2
ECs – Endothelial Cells
PECAM-1 - platelet/endothelial cell adhesion molecule-1

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Thank You for Being Part of my Journey

“

From the depths of perseverance and the heights of accomplishment, I stand proudly as a newly accomplished Doctor. This challenging journey, adorned with obstacles and marred by moments of self-doubt, has now reached its magnificent culmination. Like a stormy sea, my path was strewn with countless challenges, each one testing the very limits of my endurance. Yet, amidst the turbulent tides, I discovered an unwavering resolve that refused to be extinguished. In those moments when the light seemed faint, I unearthed a wellspring of resilience termed “The Goal” that propelled me forward. By setting goals, maintaining focus, and steadily progressing towards them, I could navigate the winds of uncertainty. The lows may have cast shadows upon my aspirations, but they also awakened me for the purposes of my life. Today, as I bask in the radiance of achievement, I find comfort in the knowledge that the obstacles I faced were but steppingstones on the path to glory. They ignited sparks of joy and nurtured a flame of unyielding passion within me. It is through this confluence of struggle and success that I emerge, not only as a scholar, but as an indomitable human spirit. My heart swells with gratitude for every setback, for they fostered resilience, and for every success, for they kindled the fires of happiness. I now embark upon a new chapter, carrying the invaluable lessons of my PhD, forever grateful for the arduous yet rewarding expedition that shaped the person I have become.”

”



CHAPTER I





INTRODUCTION

PART I: The Tumor Suppressor p16Ink4a in Senescence and Aging

I. Aging

1. Definition

Aging can be defined as the progressive, time-dependent decline in an organism's functional capacity that leads to increased susceptibility to disease and death. This decline in function occurs at all levels of organization, from the molecular and cellular to the organismal and systemic levels [1–3]. The aging process is complex and multifactorial and is influenced by both genetic and environmental factors. The exact mechanisms that contribute to aging are not completely understood, but researchers have identified several theories. The most well-known is the programmed theory of aging, which suggests that aging is a natural, genetically determined process that occurs as a result of an organism's natural lifespan. According to this theory, each species has a set lifespan that is predetermined by its genetic makeup. Another theory is the damage or error theory of aging, which proposes that aging occurs as a result of accumulated damage or errors in the cells and tissues of the body over time. This damage can be caused by various factors, including environmental stressors such as exposure to toxins, radiation, and pollutants, as well as normal cellular processes such as metabolism and replication [2,4–6].

There are two main types of aging. The chronological aging, that is the natural process of aging which occurs with the passage of time. It is simply measured by counting the number of years a person has lived. The biological aging is characterized by gradual degeneration of function of cells, tissues, organs, or an intact organism due to accumulation of environmental factors and stresses with time [2,4]. This age-related functional degeneration affects each organism that pass through developmental phases up to aging, as it is experienced by single cellular and multicellular organisms. In mammals, aging is associated with a variety of pathologies, and has been classified as the leading predictive factor of many chronic diseases that account for the majority of morbidity and mortality worldwide [7]. These diseases include neurodegenerative (Alzheimer's and Parkinson), cardiovascular, pulmonary, renal, and bone disorders, cancers, and many others [8–13]. What makes aging a common risk factor is the fact that it arises from molecular mechanism and pathologic pathways that are cornerstones for the development of all these diseases and which are now highly considered as hallmarks for aging identification [2]. This includes oxidative stress, overproduction of inflammatory cytokines, oncogenes activation, genomic instability, telomeres shortening, epigenetic alteration, mitochondrial dysfunction and consequently accumulation SnCs [14–19].

II. Cellular Senescence

1. Definition

Cellular senescence is defined as an irreversible proliferative arrest caused by two major intrinsic and extrinsic stimuli [20]. It was first demonstrated in the early 1960s by Leonard Hayflick and Paul Moorhead, when they found that the normal fetal fibroblast ceased growth after several passages. This phenomenon was termed “replicative senescence” or the Hayflick’s limit [21,22].

Although SnCs undergo a permanent cell cycle arrest, several morphological, molecular, biochemical and epigenetic hallmarks have been identified to characterize senescent cells [23]. Morphologically, SnCs exhibit increased size compared to normal cells of the same type, have a flattened shape, and occasionally have multiple nuclei. However, at the molecular and biochemical level, these cells revealed a distinct expression profile [24–27]. The increased activity of the lysosomal SA β -gal is a common characteristic feature of SnCs [24,28]. In addition, upregulated expression of the cell cycle regulators p16 and p21 have been frequently associated with senescence detection as well and they are part of the intrinsic triggers of cellular senescence. Besides that, the senescence associated secretory phenotypes (SASPs), which are a mix of pro-inflammatory cytokines, proteases, and growth factors, are upregulated and secreted by SnCs and are part of the extrinsic arm of cellular senescence [29]. These features collectively contribute to the senescent phenotype and influence neighboring cells and the tissue microenvironment. As no single reliable marker for the detection of senescence *in vivo* exists, the recent consensus from the International Cell Senescence Association (ICSA) requests at least a combination of more than two markers to identify a cell as senescent [30].

A plethora of factors or events have been attributed to trigger the onset of cellular senescence. These triggers can be intrinsic such as telomere shortening, DNA damage, oncogenes activation, epigenetic alterations, and tumor suppressors activation. Nevertheless, other triggers of cellular senescence are extrinsic, such as chronic inflammation, SASPs, oxidative stress and mitogenic signals [25,27,31–38]. It's worth noting that these causes can often interact and reinforce each other, contributing to the complex process of cellular senescence.

Cellular senescence was first considered a protective mechanism against damaged cells or potential development of cancer [39]. However, depending on its specific triggers and context, cellular senescence can exhibit diverse functions that give rise to diverse types of senescence. Therefore,

the functions of cellular senescence are intricately linked to the underlying causes that initiate and drive its establishment [40–43].

2. Characteristics and Hallmarks

Cellular senescence is defined by a set of distinct characteristics that contribute to the aging process and the development of age-related diseases. These characteristics have been thoroughly researched and commonly found in cellular senescence. However, there is not yet a gold standard biomarker or set of markers that can verify whether a cell is senescent or not. Similarly, not all SnCs feature the same markers identified for cellular senescence [3,44].

One of the significant hallmarks of senescence is the development of a complex secretory program called the SASP, which was first described by the Campisi group [45]. The SASP is characterized by the release of a variety of growth factors, chemokines, cytokines, extracellular matrix (ECM) components, and proteases. SnCs modulate their surrounding environment via the SASP, resulting in a variety of pathophysiological effects [46]. Cellular senescence has been linked to many biological processes, including aging, tumor progression or suppression, development, wound healing, and even regeneration. The SASP is critical in mitigating these effects [44,47].

Besides that, SnCs exhibit a permanent growth arrest, which is a hallmark feature of senescence. This arrest prevents the cells from further dividing and proliferating. The arrest is primarily mediated by the activation of the p53/p21 and p16/pRb tumor suppressor pathways, which regulate the cell cycle. These pathways induce cell cycle inhibitors, such as p21 and p16, leading to cell cycle arrest in the G1 phase [20,48]. P16, a cyclin-dependent kinase inhibitor, is overexpressed and plays an important role in inducing and maintaining cell cycle arrest in SnCs. It achieves this by inhibiting cyclin-dependent kinase 4/6 (CDK4/6) and preventing subsequent retinoblastoma (pRb) protein phosphorylation (discussed in detail below). The induction of cellular senescence and the senescence-associated secretory phenotype (SASP) are linked to the upregulation of p16. This tumor suppressor protein is now widely used as a marker in culture and tissues to identify SnCs [48,49]. While p16 expression is typically low or undetectable in normal cells and tissues, it becomes readily detectable in cells that have been driven to senesce by various stimuli [20,50,51]. Furthermore, p16 expression increases with age in a variety of vertebrate tissues [49].

Although proliferative arrest is a necessary feature of SnCs, it still insufficient. Therefore, SnCs acquire a distinctive morphology compared to normal cells, which is another useful feature for

SnCs identification. These modifications include enlarged and flattened cell shape, increased granularity, and cytoskeleton changes. The enlarged and flattened morphology is caused primarily by changes in the actin cytoskeleton and reorganization of the nuclear lamina. These morphological changes are linked to changes in cell adhesion and the expression of the SASP [28,52].

At the biochemical level, the SA- β gal is the most commonly used biomarker for identification of SnCs. SnCs have increased activity of the enzyme SA- β gal, which catalyzes a colorimetric reaction at acidic pH to give a blue staining in the presence of X-gal. Increased SA- β gal activity is a popular biomarker for detecting SnCs in culture and tissues. SA-gal accumulation is linked to lysosomal enlargement, increased number and activity [28,53]. However, not only SnCs might give positive SA- β -gal staining, some non-senescent macrophages showed a false positive staining, as well as some embryonic tissue in absence of p21 [44,54]. Likewise, SnCs lacking Glb1 gene, might show a false negative for SA- β gal staining [53].

Another characteristic of SnCs is that they often develop apoptosis resistance. Because of this resistance, SnCs can remain in tissues and contribute to aging and age-related diseases. SnCs are resistant to apoptosis due to the upregulation of anti-apoptotic factors such as Bcl-2 family members and the downregulation of pro-apoptotic factors such as Bax and Bak [55,56]. Moreover, SnCs usually demonstrate an altered metabolism compared to normal cells. These modifications include, altered nutrient sensing pathways, increased aerobic glycolysis, dysfunctional mitochondria, and increased ROS production [2,57,58]. Mitochondrial dysfunction is characterized by decreased mitochondrial mass, altered mitochondrial morphology, increased ROS production, and mtDNA damage, as well as impaired oxidative phosphorylation [57]. Nevertheless, the endoplasmic reticulum (ER) is another cellular compartment that is affected by the onset of senescence. ER stress, as an outcome of senescence, yields misfolded protein, imbalanced redox, and disturbed calcium homeostasis which chronically associate with senescence induction [59,60].

Altogether, these features shape the senescence phenotype. However, it is important to note that different senescence-inducing stimuli and cell types may exhibit varying combinations of these

features. Therefore, a careful analysis of multiple markers and features is crucial for accurate senescence identification (**Figure 1**).

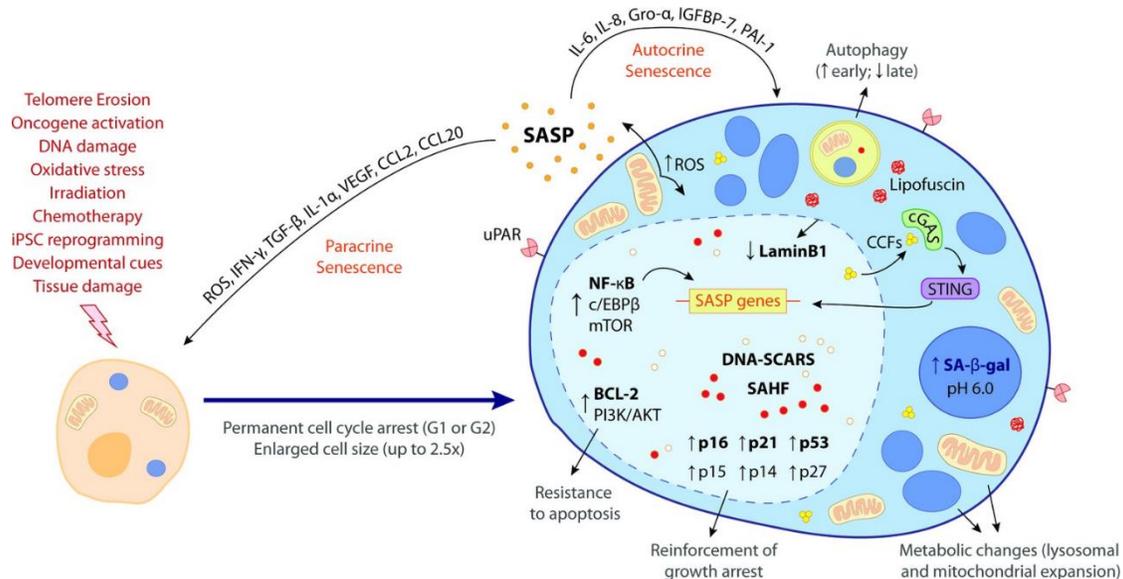


Figure 1. Hallmarks of Cellular Senescence. Senescent cells exhibit changes like enlarged size, altered metabolism, and increased production of reactive oxygen species. They also display a senescence-associated secretory phenotype (SASP) involving the release of various factors that influence neighboring cells. Chromatin modifications and the appearance of DNA-SCARS and SAHF contribute to the senescence process. Additionally, the cGAS-STING pathway and urokinase-type plasminogen activator receptor (uPAR) play roles in the inflammatory response associated with senescence. The manifestation of these features can vary based on the senescence trigger, cell type, and timing.

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3. Causes

The causes of cellular senescence can be broadly categorized into two arms: the intrinsic arm, which involves cellular processes and alterations inherent to the cell itself, and the extrinsic arm, which encompasses external factors that influence cellular senescence.

i. Intrinsic Arm

a. Telomeres Shortening

Telomeres, repetitive DNA sequences located at the ends of chromosomes, are protected by a complex called "Shelterin" which helps maintain their structural integrity [61]. The Shelterin complex is made up of six proteins: telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TRF2 interacting protein (RAP1), TRF1-interacting nuclear factor 2 (TIN2), protection of telomeres 1 (POT1), and adrenocortical dysplasia protein homolog (TPP1)

[62–65]. Moreover, telomeres have a G-rich leading strand and a C-rich lagging strand. The leading strand, has a single stranded 3' overhang sequence that is thought to facilitate the telomere-loop formation via binding to the double stranded region [66]. Telomeres shorten due to the "end-replication problem" in most somatic cells, in which the C-rich telomere on the lagging strand is incompletely replicated. RNA primers initiate DNA replication during lagging-strand synthesis, but when the last primer at the 3' end is removed, the newly synthesized strand becomes a few nucleotides shorter, resulting in telomere repeat loss [67,68]. The progressive loss of telomere repeats during cell division can cause shelterin components to be displaced and the telomere-loop structure to be destabilized. This exposes the telomere end, which the DNA repair machinery recognizes as a double-strand DNA break (DSB) [66,69,70]. Experimental evidence has shown that deletion of the shelterin component TRF2 or the 3' overhang binding protein Pot1a in cells activates the DNA damage response (DDR) proteins, such as p53 binding protein 1 (53BP1), the Mre11 complex, and phosphorylated forms of ataxia telangiectasia mutated (ATM), H2AX, and Rad17. Nevertheless, replicative senescence associated with critical telomeres shortening, also accumulate DDR proteins at telomere regions which in turns arrests cell cycle via activation of the p53 pathway [69,71]. Therefore, altogether these evidences further support the link between telomere shortening, DDR activation, and cellular senescence [72–74].

b. Oncogene Activation

Oncogenes are mutant version of normal genes that when activated are capable to trigger cellular transformation and cancer development. Point mutations, gene amplification, or changes in gene expression regulation can all activate oncogenes. Oncogene activation promotes uncontrolled cell proliferation and inhibits normal cellular functions, which contributes to tumorigenesis [75]. However, as a counteracting mechanism, the oncogene induced abnormal proliferative activity can cause genetic stress, DSBs, altered signaling pathway and induce irreversible growth arrest, a type of senescence termed oncogene-induced senescence (OIS) [76–78]. A first example of oncogene-induced senescence was demonstrated in human lung fibroblast primary culture as a result of the *Ras* oncogene mutation [34]. Subsequently, other oncogenes have been unraveled in OIS such as *AKT*, *E2F1*, *cyclin E*, and *BRAF* [79]. The mechanism through which oncogenes induce senescence starts with the DNA damage that launches a DDR. DDR subsequently activates the tumor suppressor pathways such as p53/p21 and p16/pRB which maintain cellular cycle arrest and promotes formation of senescence-associated heterochromatin foci (SAHF). These pathways when

activated, inhibit the activity of cyclin-dependent kinases (CDKs) and prevent cell cycle progression, leading to cell cycle arrest [36,80,81,79,77,34,82,83].

c. DNA Damage

A significant and prevalent trigger for the establishment and maintenance of the senescence phenotype is persistent DNA damage. SnCs often exhibit ongoing DNA damage that can originate from multiple sources such as telomere attrition, ROS, genotoxic stress, and replication stress. The accumulation of DNA damage activates pathways known as the DDR, resulting in cell cycle arrest, the onset of senescence [20,47]. In addition to the replicative senescence and the OIS, which are induced as a consequence of telomere attrition and oncogene activation respectively, some DNA damaging agents are capable of inducing the senescence phenotype independently from telomeres shortening [84–88]. Oxidative stress induced upon hydrogen peroxide (H₂O₂) treatment was associated with acute DSBs, upregulation of p53 and p21 and subsequent cell growth arrest in stressed cells, that in turn results the induction of premature senescence [87,89]. Moreover, ectopic expression of mutant TRF2 has shown to launch a DDR with the DDR factors 53BP1, γ H2AX, Rad17, ATM, and Mre11 and induce premature senescence as a result of telomere uncapping and consequent disrupted telomeric function without affecting telomeres length [72,90] suggesting the importance of functional telomeres as another factor besides their length.

Thus, strong evidence presented above suggests that DNA damage is the common underlying cause of cellular senescence, including replicative senescence and premature senescence brought on by various stressors. The primary causes of endogenous oxidative DNA damage in cells are ROS. Therefore, it would be expected that any disruption of biological systems that raises intracellular ROS levels would hasten senescence. A study on a copper-zinc-containing superoxide dismutase (SOD1) demonstrated that, SOD1 inhibition causes premature senescence in human fibroblasts [91]. SOD1 is a key defense against ROS by detoxifying the superoxide anion. Furthermore, it was discovered that overexpression of the Protein kinase B (Akt), a crucial cell signaling molecule, inhibited the FOXO3a transcription factor and increased intracellular ROS, both of which later caused a cell growth arrest that resembled senescence in a p53/p21 dependent manner [92]. On the other hand, raising SOD levels slows telomere shortening and delays the senescence of primary fibroblasts [93].

The signals and pathways that trigger cellular senescence are beyond the limits of discussion, however, it is becoming increasingly clear that they indispensably require the p53/p21/Rb pathway

to promote senescence primarily brought on by telomere shortening and the p16-Rb pathway to bring about premature senescence [94–98].

d. Epigenetic Perturbation

Epigenomic modification highly contributes to induction of cellular senescence [37]. The transition between the two chromatin states, euchromatin and heterochromatin, decides the extent of gene expression and it is controlled by histone modifications such as methylation or acetylation [99]. For instance, in human fibroblasts, histone deacetylase inhibitor (HDAi) induces senescence by causing open euchromatin and mediating p21 and p16 expression which in turn promotes SAHF formation [36,38]. In mouse fibroblasts, HDAi-induced cellular senescence seems to be more p53 dependent. Although, the clear mechanism by which HDAi induces cellular senescence is intricate, yet it suggests the importance of pRB interference [38,100]. Other factors that affect chromatin organization and trigger p16 expression include poor c-MYC or p300 histone acetyltransferase activity [101]. Additionally, even in the absence of actual DNA damage, epigenomic perturbations can cause a DNA damage response (DDR). For instance, HDAi activate the DDR protein ATM, starting a DDR without actual DNA damage [100,102].

Epigenetically induced senescence predominantly involves the upregulation of p16 and mostly do not rely on DNA damage or stress induction. Conversely, DNA damage-induced senescence relies more on p21 via induced DDR [103]. These epigenetic modifications during senescence are diverse, varying across cell types and contexts. Moreover, replicative senescence is associated with alterations in DNA methylation, while oncogene-induced senescence does not exhibit significant DNA methylation changes [104,105]. Hence, understanding the role of epigenetic modifications in senescence is of utmost importance, as it sheds light on the cause and mechanisms of senescence and holds potential for therapeutic interventions, particularly in cancer treatment.

e. Tumor Suppressor Activation

The induction and maintenance of cellular senescence in response to various stimuli primarily involve the activation of the p53/p21 and p16/pRB tumor suppressive pathways [20,37,50]. These pathways, which are complex and interconnected, play a crucial role in establishing the senescence growth arrest and regulating gene expression changes. Both pathways can cross-regulate each other, and their activation is key to the senescence response [106–109]. Their chronic stimulation can guarantee the senescence induction, however, their disruption grants cellular avoidance of senescence [52,110,111].

- **The p53/p21/pRb and p16/pRb pathways+**

The tumor suppressor protein p53 is essential for regulating the senescence process. Its level is primarily regulated by MDM2-mediated degradation. p53 levels are kept low in normal, undamaged cells, and its stabilization and activation occur in response to various signals such as DNA damage or oncogene activation [112]. The p19Arf (p14Arf in human), is another tumor suppressor, upstream to p53, that arises from alternative reading frame of the CDK2NA gene that encodes the p16. P19 activates the p53 axis by trapping and inhibiting the action of the p53-degrading ubiquitin ligase MDM2 and therefore prevents p53 degradation [113].

One of the critical mediators of p53's functions in the context of senescence is its transcriptional target p21Cip1/Waf1. Early p21 induction is thought to be important for senescence initiation and a key driver in developmental senescence. However, studies have shown that p21 levels are not maintained over time, raising the possibility that its importance in senescence is limited to the early stages [86,114–116]. Otherwise, once activated by p53, the cyclin-dependent kinase inhibitor (CDKi) p21 inhibits the activity of cyclin-CDK complexes, specifically CDK2, preventing the phosphorylation of retinoblastoma family proteins (RB1/pRB, p107/RBL1, p130/RBL2). When these proteins are hypophosphorylated, they have a higher affinity for E2F transcription factors and form RB-E2F repressive complexes. This results in the transcriptional repression of genes required for cell cycle progression, which helps to establish and maintain growth arrest [117].

The DNA damage induction of senescence occurs in two stages. As is typical of p53's response to DNA damage, the initial response is rapid and transient, fading within 24-48 hours [106]. However, after the initial rapid phase, low-level activation of p53 and p21 persist in cases of damages severe enough to induce senescence [118–120]. However, prolonged DNA damage response signaling activates other pathways, including the stress-responsive p38MAPK and protein kinase C pathways, which increase the production of reactive oxygen species, and participate in senescence induction [121–123]. The mechanisms underlying the activation of these new signaling pathways are still unknown. Subsequently, these pathways contribute to the expression of p16, which, in conjunction with pRB, reinforces the irreversibility of the growth arrest [111].

In conclusion, the induction and maintenance of cellular senescence involves complex signaling pathways, particularly the p53/p21 and p16/pRB pathways. These pathways interact to control senescence growth arrest and other senescence-related features. Persistent DDR signaling, as well

as the subsequent activation of additional signaling pathways, are required for the initiation and reinforcement of the senescence response.

ii. Extrinsic Arm

a. Senescence-Associated Secretory Phenotype (SASP)

Senescence-associated secretory phenotype (SASP) expression is the defining feature of the extrinsic arm during cellular senescence induction. Despite being unable to proliferate, SnCs can produce a wide range of secreted molecules that interact with their surroundings [26]. While the SASP's exact composition remains intricate, it is thought to include growth factors, cytokines, chemokines, and proteins involved in extracellular matrix (ECM) remodeling [25,124,125]. Furthermore, the SASP composition can vary depending on the cell type and senescence-inducing stimuli, leading to a diverse array of secreted factors [26,57]

The SASP factors secretion is highly regulated and dynamic process. The DDR, the aftermath of a plethora of stimuli such as DNA damage, telomeres attrition, epigenetic perturbation, oncogene activation and mitogenic signal, is the primary activator of SASP to a divers extent [25,102,120,126]. On the contrary, senescence induced by p16 ectopic expression did not induce SASP [127]. Furthermore, the transcription factors NF- κ B, C/EBP β , p53, and GATA4 were found to be significant transcriptional regulators of SASPs [126,128,129]. Nevertheless, the p38 MAPK pathway also regulates SASP secretion in a DNA damage-independent manner [122]. During the onset of senescence, Notch1 has been identified as an orchestrator of SASP composition [130]. Furthermore, epigenetic regulation contribute to the control of SASP gene expression following senescence induction [131–134]. Although the precise differences in SASP composition in response to various stimuli or between different cell types are not yet fully understood, it is evident that the strength and mode of senescence induction are reflected in the SASP. For instance, senescence induced by oncogenes like Ras genes or DNA damage leads to a more pronounced SASP compared to other factors [26,120].

The main role attributed to the SASP included the induction and promotion of cell cycle arrest by cytokines like IL6 or IL8 acting via the CCR2 receptor [126,128]. The SASP components not only act on nearby cells but also have autocrine effects on SnCs themselves. These factors can reinforce the senescence phenotype by maintaining a state of growth arrest and reinforcing the senescence-associated cell cycle inhibitors, such as p53 and p16 [135]. This autocrine feedback loop

contributes to the stability and maintenance of senescence. Additionally, it was found that long-term exposure to the SASP can cause paracrine signaling, which causes senescence in nearby cells [25]. Several studies have demonstrated that, certain SASP proteins, including Csf1, Ccl2, and IL8 (Cxcl8), promote the recruitment of immune cells, such as macrophages and natural killer (NK) cells, which eliminate SnCs [136–138]. Additionally, recent studies have demonstrated the release of chromatin fragments from nuclei of SnCs as a new feature for regulation of the SASP [139]. These nuclear fragments activate the cGAS-STING pathway, which aids in the control of SASP by inducing the anti-viral defense response [140–142]. However, it has also been found that SnCs and the SASP have biological functions beyond simple tumor suppression or aging, including the ability to promote proliferation, angiogenesis, or epithelial-mesenchymal transition (EMT) in nearby or cancer cells [27,45,143].

In summary, the SASP represents the extrinsic arm of the senescence program, enabling SnCs to communicate with their surrounding environment. The SASP is a complex secretome consisting of growth factors, cytokines, chemokines, and ECM-related proteins. Its secretion is regulated by transcription factors, signaling pathways, and epigenetic regulators. The composition of the SASP varies depending on the mode and strength of senescence induction, with distinct profiles observed in response to different stimuli or cell types.

4. Cellular Senescence: Various Types and Functions

i. Damage-Induced Senescence

Damage-induced senescence (DIS) is a type of cellular senescence caused by various types of DNA damage, telomeric or non-telomeric, or cellular stresses [35,43,129,144,145]. It occurs when cells sustain significant and irreversible damage, such as DNA double-strand breaks, oxidative stress, oncogene activation, epigenome perturbation and many other triggers. However, the diversity of cellular senescence types, is highly dependent on the trigger underlying its initiation [52,146–148]. For instance, **replicative senescence** was the first context of DIS described. It refers to the phenomenon where cells reach a state of irreversible growth arrest after a certain number of cell divisions [21]. This process is primarily driven by DDR generated from telomere shortening, which occurs with each cell division (as described in the causes of senescence) [32,35,68]. The primary function of replicative senescence is to limit the proliferative capacity of cells and prevent the propagation of cells with damaged or unstable genomes. It contributes to tissue homeostasis

and plays a role in aging and age-related diseases. It also acts as a safeguard mechanism against uncontrolled cell growth and the accumulation of genetic aberrations that could lead to the development of cancer [20,43].

Stress-Induced Premature Senescence (SIPS), is another form of damage-induced senescence that is induced independently from telomere shortening. SIPS refers to the accelerated and irreversible growth arrest of cells in response to stressors such as oxidative stress, DNA damage, telomere dysfunction, oncogene activation, and exposure to certain chemotherapeutic agents [35,102,129,144–146,148,149]. It is similar to replicative senescence, but it occurs to cells prematurely before they reach their proliferative limits. The function of SIPS is to prevent the proliferation of stressed or damaged cells that could otherwise contribute to tissue dysfunction or transformation. It acts as a protective mechanism to maintain tissue integrity and prevent the propagation of potentially harmful cells. However, it is worth noting that, SnCs, with insufficient clearance by immune cells and prolonged accumulation in the tissue, demonstrate an antagonistic role and highly contribute to several pathologies exacerbation [20,43] (**Figure 2**).

ii. Senescence-Induced Senescence (Secondary Senescence)

Recent studies have described the secondary senescence, also known as senescence-induced senescence or paracrine senescence. Secondary senescence is a phenomenon where primary senescence, arisen from damage signals, can induce senescence in neighboring cells through paracrine and juxtacrine signaling [25,150–152]. Previously, it was believed that secondary senescence primarily occurred through the action of SASP factors secreted by primary SnCs, acting on neighboring cells in a paracrine manner [25,151,152]. However, recent findings have revealed a more intricate scenario involving additional mechanisms that are independent of the SASP. These mechanisms require direct cell-to-cell contact and are referred to as juxtacrine signaling, specifically involving the Notch-JAG1 pathway [130,150,153].

Research has revealed that the SASP has two distinct effects on target cell populations, leading to different senescence endpoints [154]. The primary endpoint, marked by Ras activation, involves the upregulation of pro-inflammatory cytokines. This promotes the recruitment of lymphocytes and enhances senescence surveillance. On the other hand, the secondary endpoint of oncogene-induced senescence requires Notch signaling in addition to SASP factors. In this case, the SASP response is blunted, showing immunosuppressive characteristics [130,150,153].

Primary senescence primarily relies on SASP factors to induce senescence, while secondary senescence driven by Notch signaling promotes a "lateral induction of senescence" through juxtacrine communication between cells. Interestingly, inhibiting Notch activity during senescence enhances the primary endpoint in vivo. This suggests that Notch activity plays a role in controlling the composition of the SASP during senescence [150] (**Figure 2**).

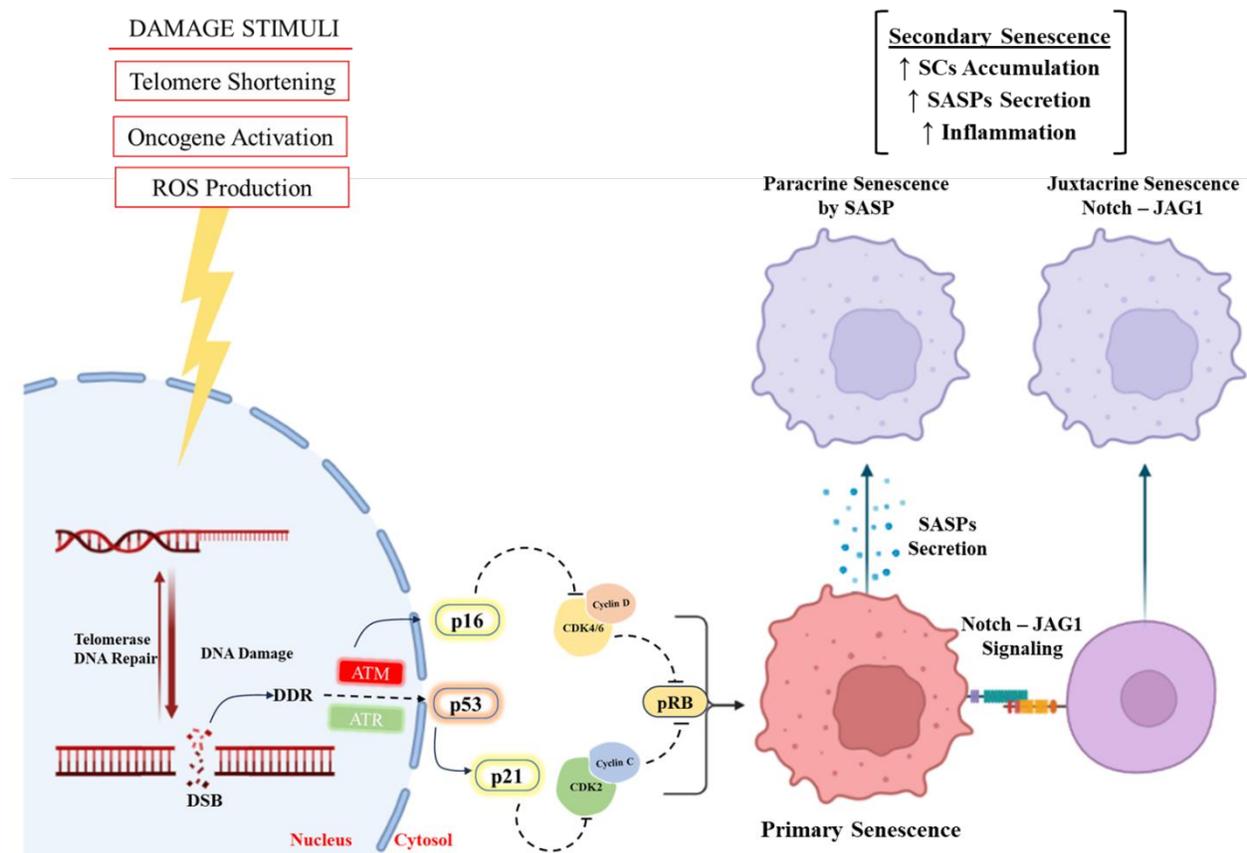


Figure 2. A Schematic illustration that shows the general mechanism of damage-induced senescence that is caused by telomeres shortening in replicative senescence or other DNA damaging stimuli such as oncogene activation or oxidative stress in premature senescence. On the other hand, the figure also represents how a damage-induced senescent cell (primary senescence) can trigger secondary senescence in the neighboring cells by two pathways. Either in paracrine manner through SASPs secretion or juxtacrine manner (lateral inductions) through direct contact signaling of Notch-JAG1.

iii. Developmental senescence

The discovery of SnCs during embryonic development has sparked interest and provided valuable insights into the physiological roles of senescence. These cells have been observed in the development of a variety of organisms, including mice, humans, naked mole rats, chicken, quail, *Xenopus*, axolotl, and zebrafish [155–161]. In mice, these cells were found at early embryonic stage in various tissues, including the hindbrain roof plate, mesonephros, developing limb, neural tube, pharyngeal arches, endolymphatic sac, tail tip, and gut endoderm [155,156]. Furthermore, in other organisms, such as *Xenopus*, axolotl, and zebrafish, SnCs were identified in tissues like the pronephros, olfactory epithelium, nerve fascicles, yolk sac, midbrain, and hindbrain [157–161].

In developing embryos, SnCs exhibited increased activity of the SA- β -gal and increased expression of p21, while other senescence markers such as p53, p16, and DNA damage response (DDR) markers were absent [155,156]. This suggests that developmental senescence has a distinct phenotype compared to senescence observed later in life, highlighting the importance of p21 in this context. Disruption of p21 or senolytic treatment leads to the loss of senescence and patterning abnormalities in various structures [155–157,159]. However, these patterning defects are transient and can be compensated for by other mechanisms, particularly apoptosis. This implies that SnCs are implicated in embryonic development, but they are not indispensable.

In conclusion, the current evidence suggest that developmental senescence is not a cellular damage consequence, but rather a highly organized and programmed process with distinct spatiotemporal patterns. Other cues, such as biophysical forces during morphogenesis, could also play a role in this process. SnCs appear to contribute to tissue remodeling during development by regulating cell population balance, fine-tuning cell fate specification, mediating morphogenetic signaling, and facilitating structural degeneration via macrophage-dependent elimination (**Figure 3**).

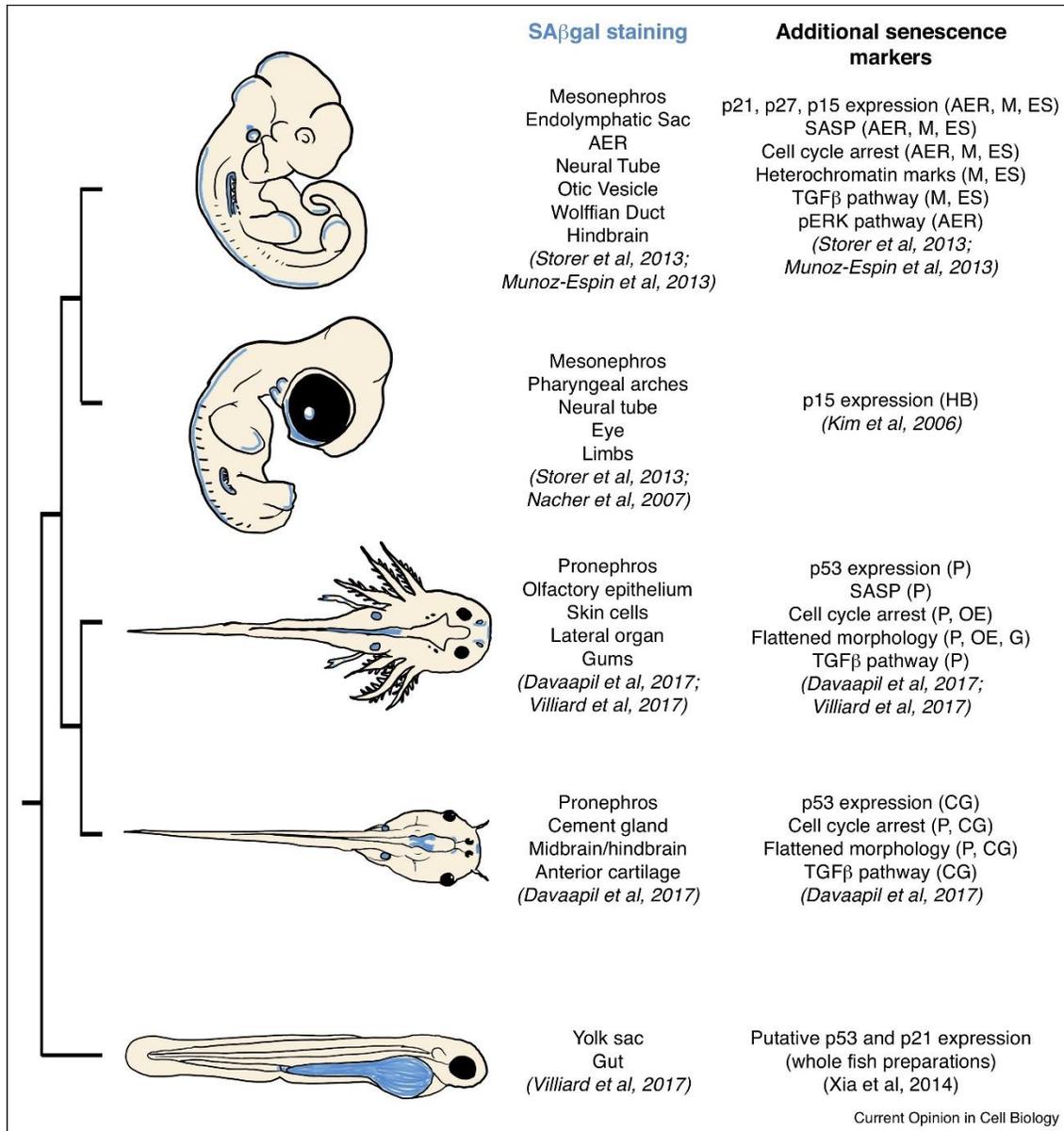


Figure 3. The experimental evidence of cellular senescence in developing embryos is demonstrated through SA-βgal staining localization in mouse, chick, axolotl, frog, and zebrafish embryos. The right side presents additional senescence hallmarks identified in each species, such as the expression of tumor-suppressor genes p53 or p21, lack of proliferation, or expression of components of the senescence-associated secretory phenotype (SASP). The embryonic structures where these hallmarks were found, including AER (apical ectodermal ridge), CG (cement gland), ES (endolymphatic sac), G (gums), HB (hindbrain), M (mesonephros), and P (pronephros), are indicated.

Czarkwiani A et al. Out with the old, in with the new: senescence in development. Current Opinion in Cell Biology. 2018

iv. Cellular senescence: Time-Gated Good or Bad

Depending on the circumstances, the impact of cellular senescence on organism health and disease can be both beneficial and detrimental. Factors such as the type of recipient cells, the specific tissue involved, the composition of the SASP, and the duration and quantity of senescent cell presence within the tissue all have an impact on the effects. However, the effect of SnCs appears to be primarily time gated. While transient expression of SnCs can be beneficial in terms of tissue balance, restoration, and functions such as tissue healing or cancer suppression. SnCs are typically removed by the immune system in such cases. If SnCs are not removed efficiently, they can accumulate and disrupt tissue homeostasis, resulting in detrimental outcomes such as tumor progression, damage, inflammation, and the development of various pathologies [3,23,29].

In this regard, the detrimental effects of senescence are partly attributed to the SASP's chronic secretion of pro-inflammatory molecules. The SASP can have an effect on neighboring cells, with the extent of the effect depending on the duration of exposure. Long-term SASP exposure can impair stem cell functions and promote paracrine senescence, amplifying the presence and signaling of SnCs [163]. This imbalance has the potential to impair organ and tissue function [23,29].

Furthermore, SnCs tend to accumulate with age, for reasons that are still not completely understood [23,164]. Despite different reasons, the accumulation of SnCs in aged organisms contributes significantly to the onset and progression of various age-related diseases [43]. In animal models, efforts to selectively eliminate SnCs with senolytic drugs or modulate the SASP with senomorphics have yielded promising results in terms of improving health and lifespan [164–167]. These strategies are being investigated in order to develop interventions that target the adverse effects of senescence and improve overall health. Understanding the molecular mechanisms at the basis of the detrimental effects of senescence is essential in advancing these approaches (**Figure 4** [168]).

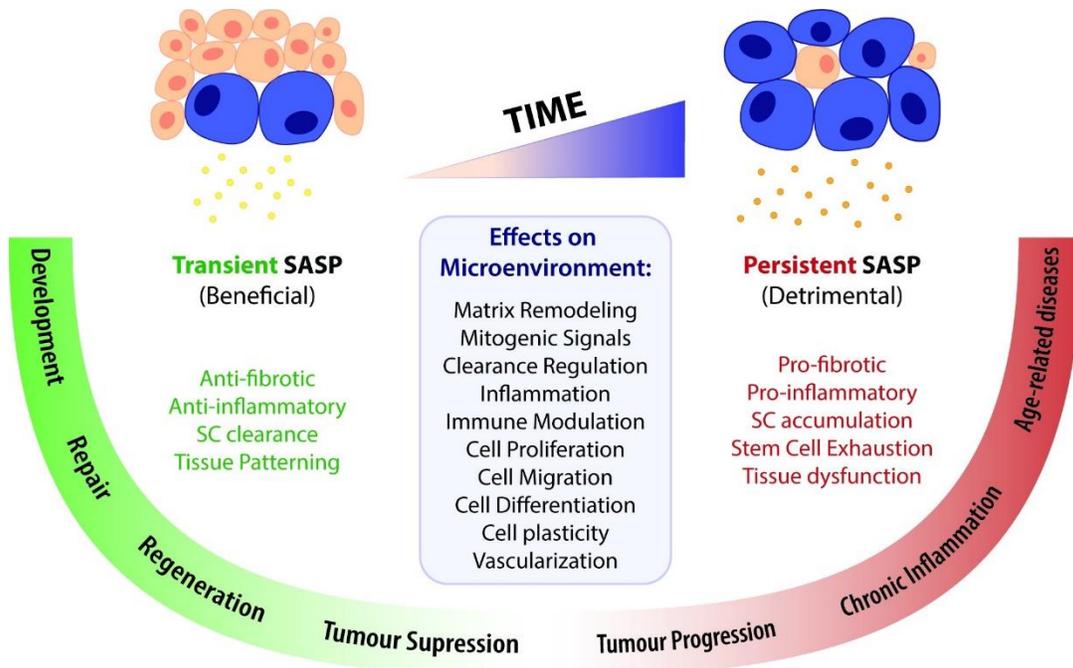


Figure 4. The SASP has a variety of effects on the microenvironment, including matrix remodeling, signaling for cell growth, regulating clearance, inflammation, and immune modulation. These effects can be beneficial or harmful depending on the duration of the senescence program and SASP response. Transient SASP responses have positive effects, such as tissue repair and immune clearance of SnCs. However, prolonged SASP responses lead to tissue dysfunction, chronic inflammation, and aging-related diseases. Long-lasting senescence also depletes stem cell pools, impairing tissue regeneration. The role of SASP in cancer is complex, as it can have both tumor-suppressive and tumor-promoting effects depending on the stage of cancer progression. Early on, the SASP suppresses tumor growth, but in later stages, it creates a pro-inflammatory environment that supports tumor development.

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III. The Tumor Suppressor and Cell Cycle Regulator: p16Ink4a

**(Information in this section is mostly taken from our review article: [169],
“p16Ink4a – More Than a Senescence Marker”)**

1. Definition and History

P16 is a tumor suppressor gene, which has been termed with several names such as the multiple tumor suppressor-1 (MTS-1), the inhibitor of cyclin-dependent kinase 4a (INK4A), or the cyclin-dependent kinase inhibitor 2a (CDKN2A). The human p16 gene is located on the short arm of chromosome (9p21.3). The p16 transcript is composed of three exons which encode 156 amino acids. The use of an alternative reading frame generates the human p14Arf protein (p19Arf in mice) [170–172].

The primary function attributed to p16 was that of a cell cycle regulator. It accomplishes this by inhibiting the activity of the cell cycle regulators CDK4 and CDK6. Shortly after, the discovery of p16 as a tumor suppressor protein was closely related to the study of familial melanoma, a type of skin cancer that is genetically inherited and has a risk to pass through generations of the family. In the early 1990s, researchers were looking into genetic changes linked to familial melanoma, and they were particularly interested in a region on chromosome 9p21. In melanoma and other types of cancer, this region was found to be frequently deleted or mutated [173–175]. Further research revealed that p16 was not only linked to familial melanoma but was also linked to a variety of other cancers. Loss of p16 function was discovered to be a common event in cancer development, either through genetic mutations or epigenetic silencing. The functional significance of p16 as a tumor suppressor and its role in cell cycle regulation has gotten a lot of attention in cancer research [176–178].

Nonetheless, since its discovery, p16 has grown a significant importance in aging and cellular senescence domain. It has formed the bedrock for demonstrating aging and cellular senescence processes and their correlation with other tumor suppressors and cell cycle regulators such as p21 and p19ARF [49,82,179]. Several studies have shown that p16 has a dramatically increased expression in a variety of tissues of old rodents, including the lungs, lymph nodes, adrenal gland, and uterus. Furthermore, removing p16 positive SnCs delayed the occurrence and progression of age-related pathologies in mice in vivo, as well as prolonging the lifespan of premature and natural aging mice [164,167]. All these combined made p16 a hallmark of aging and age-related diseases.

2. The p16Ink4a Gene and Protein Structure

The *CDKN2A* gene belongs to the *INK4* genes family. *CDKN2A* encodes for p16 and p14ARF (p19Arf in mice), while *CDKN2B* encodes for p15Ink4B, *CDKN2C* for p18Ink4C, and *CDKN2D* for p19Ink4D. They share biological properties in cell cycle regulation and tumor suppression [180,181]. The *CDKN2A* gene consists of exons, E1 β , E1 α , E2, E2 γ , and E3. Alternative splicing generates four different transcript variants including p16 (E1 α , E2, and E3), p19Arf (E1 β , E2, and E3), (the murine orthologue of the human p14ARF), in addition to p16 γ and p12. Thus, the difference between p16 and p19ARF transcript variants lies within the alternative splicing of E1 α versus E1 β , which then splice onto common exons E2 and E3 [182,183] (**Figure 5** [169]).

The p16 protein is made up of 156 amino acids and has a molecular weight of about 16 kDa. It is distinguished by the presence of several significant structural features. The p16 protein's structure is made up of four ankyrin repeats (ARs). Ankyrin repeats are conserved motifs made up of 31-34 amino acids arranged in a helix-turn-helix configuration. These repeats are linked together by loops of varying lengths that are perpendicular to the helical axes. The four ankyrin repeats are arranged in a helical structure that is involved in binding with target proteins.

When p16 interacts with cyclin-dependent kinase 6 (CDK6), it binds to the cavity of CDK6, exposing the catalytic cleft of CDK6. This binding causes an electrostatic interaction between two specific amino acids residues, D84, in p16 and R31 in CDK6, which potentially results in a reduction of CDK6's kinase activity. Mutations in p16 that are associated with cancer often occur at residue D84, disrupting this inhibitory effect and leading to uncontrolled cell proliferation [182,184] (**Figure 6**).

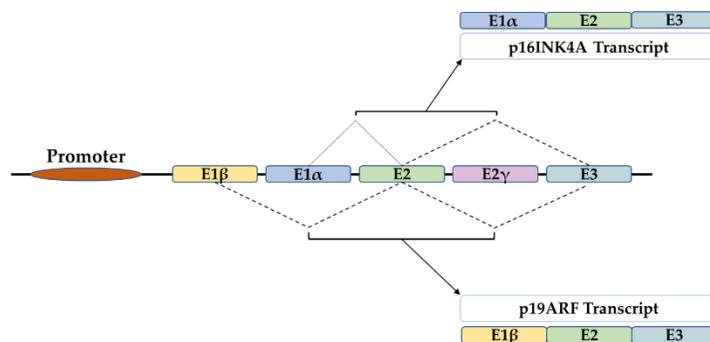
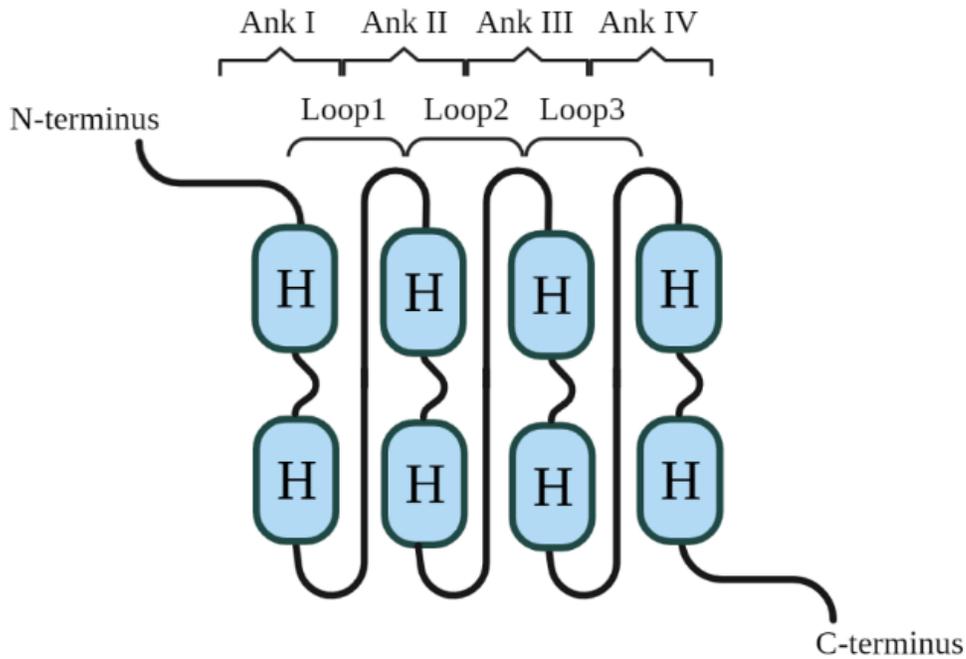


Figure 5. The structure of the INK4A locus gives rise to several transcripts through alternative splicing. P16Ink4a and p19ARF are two transcripts of 3 exons which differ only in the first exon that is E1 α for p16 and E1 β for p19ARF [169].

A.



B.

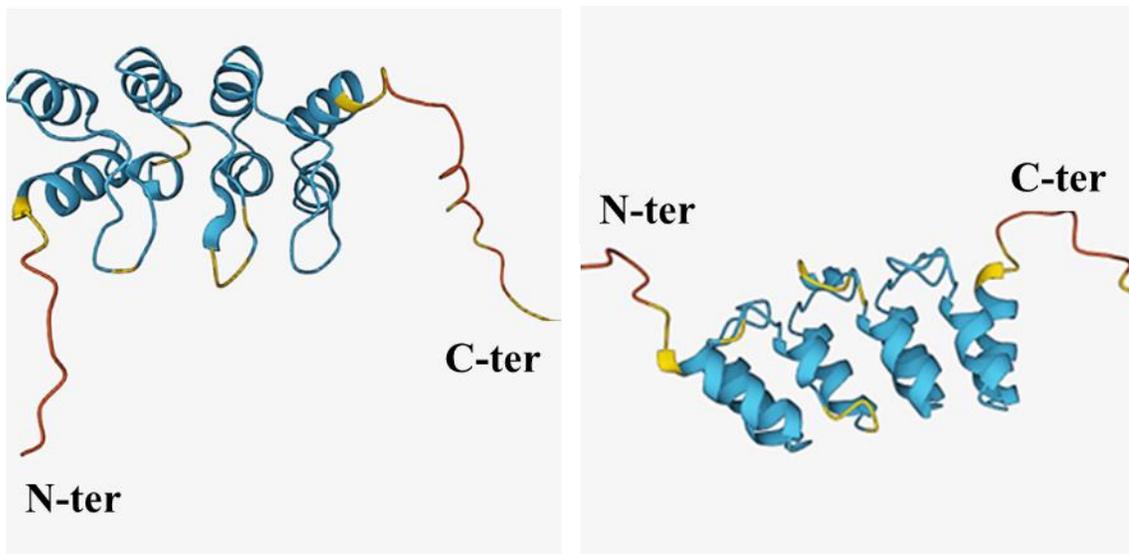


Figure 6. CDKN2A protein structure. (A) Schematic representation of the p16 protein. Each ankyrin repeat consists of a helix-turn-helix (H-T-H) structure. The four H-T-H motifs are connected by three loops. (B) 3D structural model of p16 protein from two different orientations. N-terminus and C-terminus indicated. Eight helices observed on the left and the four ankyrins observed on the right (in blue).

(A): Scheme illustrated on [Biorender.com](https://www.biorender.com/).

(B): Images obtained from UniProtKB webpage - P42771 entry (AlphaFold Protein Structure Database).

3. The Cell Cycle

i. Overview

The cell cycle is a meticulously orchestrated process that is divided into four stages: G1 (Gap 1), S (Synthesis), G2 (Gap 2), and M (Mitosis). Cells grow and perform their specialized functions during the G1 phase. The length of this phase is influenced by external cues and signaling pathways, which determine whether cells continue to replicate their DNA or exit the cell cycle. The G1 phase is a critical checkpoint in which the decision to divide or differentiate is made. Following G1, the S phase is characterized by DNA synthesis. The genome replicates, resulting in the formation of two identical copies of the genetic material. This phase is strictly controlled to ensure accurate DNA replication and the repair of any DNA damage. The G2 phase is a checkpoint phase in which cells prepare to divide. Additional organelle growth and synthesis take place, and the cell ensures that DNA replication is complete and error-free. Cells monitor DNA integrity and repair any damage that has occurred during this phase. Finally, the M phase includes mitosis, which is divided into several stages: prophase, prometaphase, metaphase, anaphase, and telophase. The replicated chromosomes are accurately segregated into two daughter cells during mitosis (**Figure 7**) [185–187].

ii. Regulation

The cell cycle is tightly controlled to ensure proper progression, accurate DNA replication, and cell division. Cyclins, cyclin-dependent kinases (CDKs), and their inhibitors are important regulatory components. Cyclins are proteins that accumulate and activate CDKs during specific stages of the cell cycle. Cyclin-CDK complexes regulate cell cycle phase transitions by phosphorylating target proteins. Specific transitions, such as G1 to S or G2 to M, are governed by different cyclin-CDK complexes. CDK activity is further regulated by a variety of mechanisms. CDKs must be phosphorylated at specific sites in order to be activated, and cyclin binding causes conformational changes that increase kinase activity. CDK inhibitors, such as p16, p19, and p21 proteins, can bind to cyclin-CDK complexes, inhibiting their activity and controlling cell proliferation. Additionally, checkpoints are also present throughout the cell cycle to ensure DNA integrity and proper progression. Checkpoints are regulated by tumor suppressor proteins such as p53, which can halt the cell cycle in response to DNA damage or abnormalities. These checkpoints

allow cells to repair DNA damage before moving on to the next phase, preventing errors from being passed down to daughter cells (**Figure 7**) [187,188].

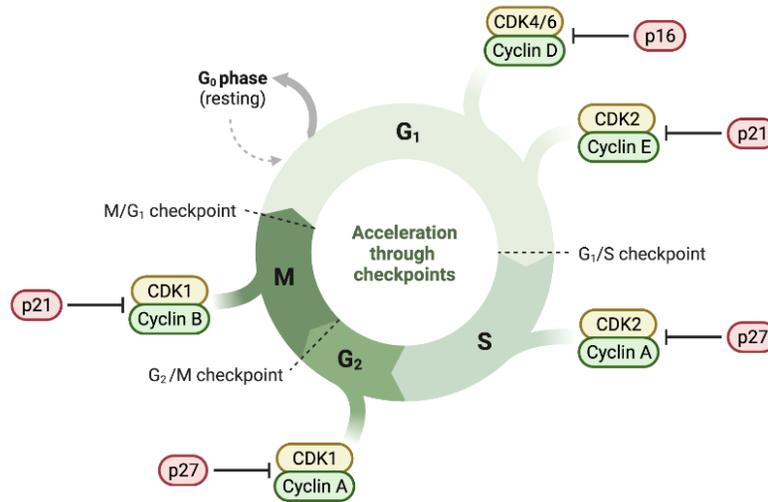


Figure 7. The cell cycle checkpoints and regulatory proteins. This figure illustrates the intricate regulation of the cell cycle and the pivotal role of cyclin-dependent kinases (CDKs) and tumor suppressors in orchestrating progression through its distinct phases. At the G₁/S transition, the CDK4/6-cyclin D and CDK2-Cyclin E complexes activity is selectively inhibited by p16 and p21, respectively, preventing the entry into the S phase. As cells traverse through the S phase and G₂ the tumor suppressor p27 becomes the key regulator which controls the activity of CDK2-cyclinA (S phase) and CDK1-cyclinA (G₂ phase) inhibiting the progression through G₂/M checkpoint. The final checkpoint marks the transition from M phase to G₁ phase. The CDK1-cyclin B complex is tightly regulated by p21 during the M phase, preventing premature entry into G₁. From Biorender.com

4. P16Ink4a Expression and Cell Cycle Regulation

The cell cycle is a complex loop of events consisting of doubling the genetic material of the mother cell in the S phase, which is afterward accurately segregated into two identical daughter cells in the M phase. Once out of the quiescent G₀ phase and prior to the S phase, the cell enters a critical preparatory gap phase termed G₁. There, the cell fate hinges to decide at this restriction point whether to progress through the cycle. Generally, the driving forces of progression through the cell cycle are the regulatory cyclin-dependent kinases (CDKs). CDKs are activated by binding to their corresponding cyclins. Specifically, at the G₁/S checkpoint, the progression through the cell cycle requires the cyclin D-CDK 4/6 assembly [176,188].

P16 is a specific inhibitor of the cyclin-dependent kinases CDK4 and CDK6 and it mainly prevents cell transition from G₁ to S phase and causes subsequent proliferation arrest by rendering

retinoblastoma protein (pRB) in a hypophosphorylated state. In G₀, pRB is unphosphorylated. At the beginning of G₁, cyclin D-Cdk4/6 monophosphorylates pRB, which is bound to E2F transcription factors. When the cell passes the restriction point, cyclin E-Cdk2 hyperphosphorylates pRB, which dissociates from the E2F factors, which in turn translocate to the nucleus and activate transcription of S phase genes [172,182,189,190]. However, in an alternative model proposed by Ahlander and Bosco, hypophosphorylated pRB binds E2F transcription factors on the chromatin, and recruits histone deacetylase (HDAC) and other chromatin-remodeling enzymes, which act all together to inhibit S-phase gene transcription and cell cycle progression. On the contrary, hyperphosphorylated pRB dissociates from E2F which in turn activates transcription and cell cycle progression [191]. P16 binding to CDK 4/6 can directly inhibit their activities in addition to non-p16 CDK inhibitors such as p27 for further hypophosphorylation of pRB [192] (**Figure 8**). This p16–pRB–E2F axis can also be enhanced through direct interaction of p16 with the gene-associated retinoid-IFN-induced mortality-19 (GRIM-19) [193].

Hereby, p16 expression seems to be credited to a feedback loop with pRB. In other words, pRB phosphorylation provokes E2F activation and induces p16 expression. Thus, p16 inhibits CDK 4/6 and increases hypophosphorylated pRB which tends to downregulate p16 [194]. P16 expression can be affected by epigenetic modification through promoter hypermethylation [195]. PRC1 and PRC2 complexes are involved in this response and provoke heterochromatin formation and p16 suppression [196,197]. The hypermethylation is mediated by the PRC2 core protein Ezh2. Ezh2 adds a trimethyl to H3K27 in an association with the polycomb protein Bmi-1, a member of the PRC1 complex. Bmi-1 dissociation from the suppression complex can restore p16 expression [198,199]. In addition, as a major cause of cellular senescence and aging, both exogenous- and endogenous-induced oxidative stress and reactive oxygen species (ROS) production resulted in upregulation of p16 expression in pathways that involve the extracellular signal-regulated kinases ERK1/2 and the stress-activated protein kinases p38 [200,201].

Furthermore, several studies have shown other mechanisms of p16 inhibition of the cell cycle independently from the pRB-E2F pathway [202,203]. One in which p16 interacts with the CDK7 subunit of the general transcription factor TFIIH to inhibit the phosphorylation of the carboxyl-terminal domain (CTD) of the large subunit of the RNA polymerase II, contributing to cell cycle arrest [202,204]. Besides that, it also inhibits the activities of the c-jun N-terminal kinase (JNK1 and JNK3) which in turn blocks AP-1 activity and inhibits cell transformation [203].

Moreover, the case of oncogene-induced senescence through elevated p16 expression was observed in primary cells with activated RAS or its downstream stream effectors Raf and MEK [34,205]. More precisely, the Ets family members, Ets1 and Ets2 which are transcription factors activated by the RAS-Raf-MEK cascade [206], bind and activate the p16 promoter with 5 to 10-fold potentiation [81]. Independently, RAS can also increase p16 expression, and induce cellular senescence by inducing the ectopic expression of the transcription factor HBP-1 [207] or activation of the H3K27 histone demethylase JMJD3 and down-regulation of Ezh2 [208].

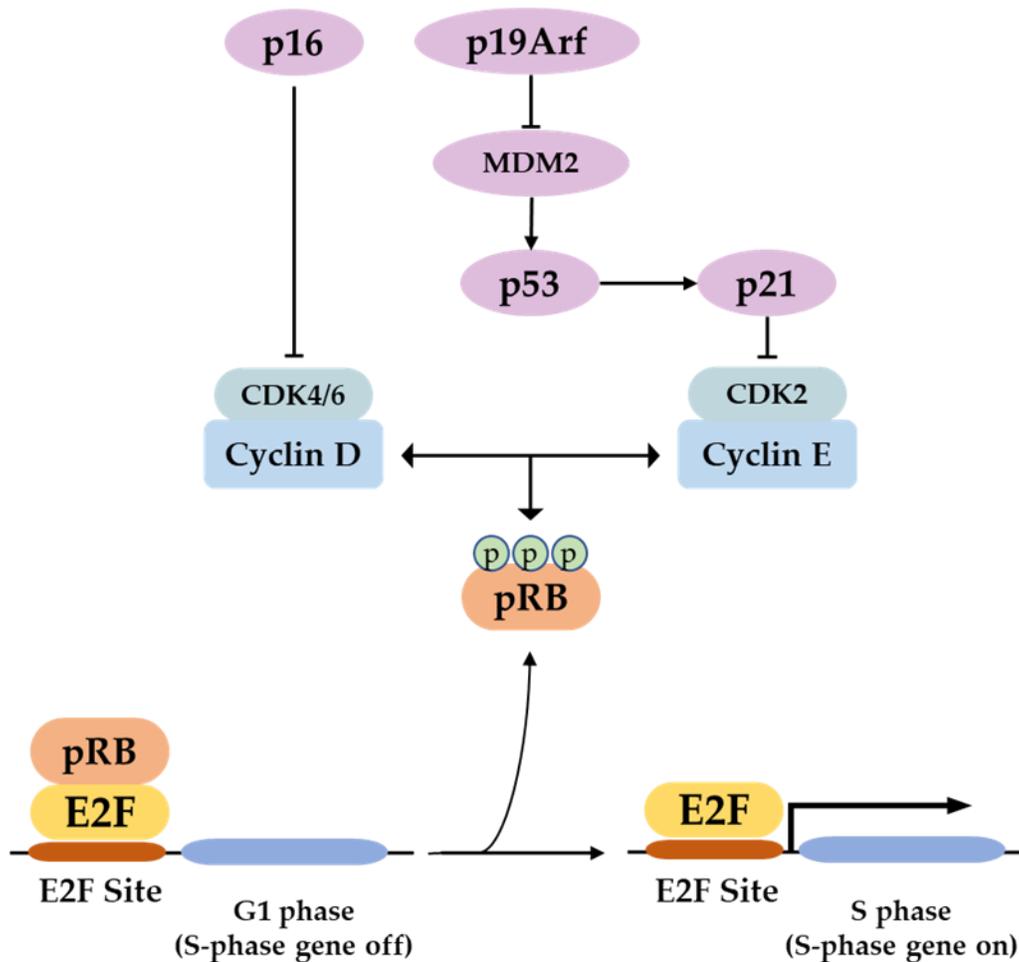
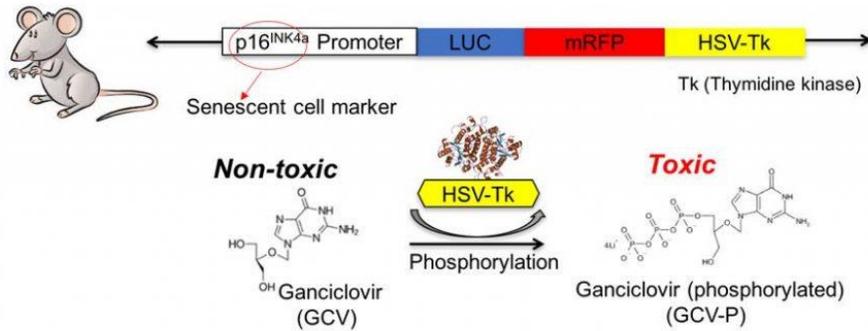


Figure 8. In the p16/pRB pathway, p16 inhibits Cdk4/6–cyclin D complex formation and induces subsequent pRB hypophosphorylation. Similarly, in the p53/p21 pathway, p19ARF traps MDM2 and prevents p53 degradation, which consequently activates p21. This works similarly as p16 but by inhibiting Cdk2–cyclin E complex, and, therefore, induces pRB phosphorylation. Dephosphorylated pRB binds E2F transcription factor at the E2F sites and blocks G1–S phase transition, blocking the cell cycle. However, in the absence of p16 and p21, hyperphosphorylated pRB detaches from E2F transcription factors, which consequently activates S-phase genes and induces progression of the cell cycle [169].

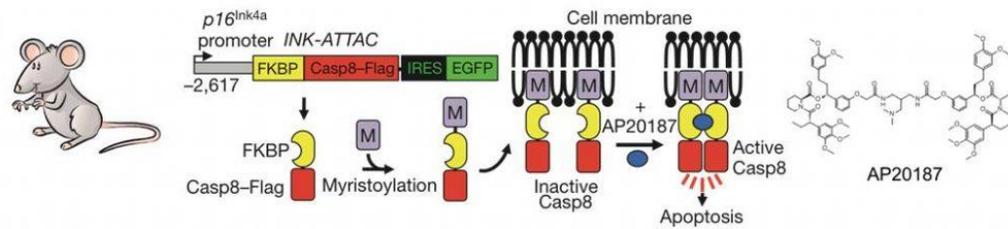
5. p16Ink4a Animal Models

Multiple studies investigating p16 functions used the p16-INK-ATTACK [164], p16-3MR [209], p16-Cre [210], and Super-INK4A/ARF mice. The p16-INK-ATTACK transgene consists of a 2.6 kb p16 promoter construct controlling expression of green fluorescent protein (GFP) and a FKBP–Casp8 “killing cassette”, which upon treatment with AP20187 induces apoptotic death of FKBP–Casp8 expressing cells [164]. GFP expression in these animals increased significantly between 12 and 18 months of age [167]. The p16-3MR transgene consists of a 50 kb p16 BAC clone containing the 3MR (trimodality reporter) fusion protein, with a synthetic Renilla luciferase (LUC), monomeric red fluorescent protein (mRFP), and truncated herpes simplex virus 1 thymidine kinase (HSV-TK). HSV-TK allows the killing of positive cells by ganciclovir. A significant increase in luciferase activity was detected by 18 months of age [209]. P16-Cre mice represent knock-in models in the endogenous p16 locus either as constitutively active Cre or Tamoxifen-inducible CreERT2 versions. These mice were crossed with mTmG reporter or diphtheria toxin deleter strains. Some GFP-positive cells were detectable by 2 months of age and the number increased significantly at 12 months [210] (**Figure 9**). The Super-Ink4a/Arf mouse strain carries an additional transgenic copy of the entire Ink4a/Arf locus [211]. Recently, p21-CreERT2 [212] and p21-INK-ATTACK [213] mice were established. Data from these animals suggest that p16 and p21 mark different populations of SnCs and have different functions. Additionally, the SASPs of p16 and p21 cells are functionally different [214]. Most of the studies showed that elimination of the small number of SnCs improves tissue health [164,167,213,215–223], but some studies also provided evidence for a physiological function of SnCs and the SASP [209,210]. As beneficial effects were observed in tissues where the p16-INK-ATTACK transgene is not expressed [167,218], p16 knockdown inhibits SASP factor expression [224], and very recently it has been shown that blood or plasma transfer from old to young mice induces senescence and aging features [225], it is likely that the SASP plays the most important role for the observed alterations.

A. p16-3MR Mouse



B. INK ATTAC Mouse



C. p16-Cre Mouse

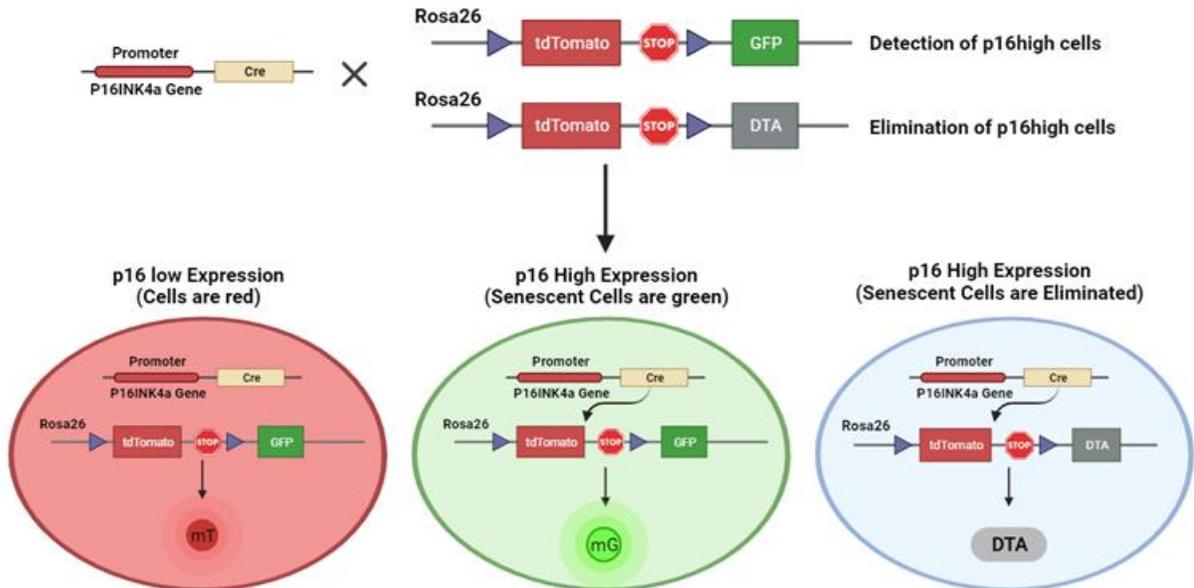


Figure 9. Schematic Representation of p16-3MR, INK ATTAC, p16-cre-mTmG/DTA Mouse Models. (A) Conditional expression of the p16^{INK4a} promoter-driven Trimodality Reporter (3MR) system, allowing visualization and ablation of p16^{INK4a} expressing cells. (B) Conditional caspase-based apoptosis induction under the p16^{INK4a} promoter, facilitating targeted apoptosis in p16^{INK4a}-expressing cells upon AP20187 treatment. (C) Utilizes the Cre-loxP system for p16^{high} cells tracing, with p16^{INK4a} promoter-driven Cre recombinase inducing a switch from red (mT) when p16 is low to Green (mG) fluorescence when p16 is high. In parallel, this model enables targeted ablation of high p16^{INK4a}-expressing cells through DTA-mediated toxicity. (A) Demaria et al. Dev Cell. 2014. (B) Baker et al. Nature. 2011, (C) adapted from Grosse et al. Cell Metabolism. 2020, schematized on Biorender.com

6. P16Ink4a in Senescence, Aging and Beyond

P16 has been demonstrated to play a crucial role in aging and cellular senescence processes. In addition, it has formed the basis for identifying these processes and their correlation with other tumor suppressors and cell cycle regulators such as p21 and p19ARF [49,82,179]. Several studies have shown that p16 has a dramatically increased expression in a variety of tissues of old rodents, including the lungs, lymph nodes, adrenal gland, and uterus. Furthermore, removing p16 positive SnCs delayed the occurrence and progression of age-related pathologies in mice *in vivo*, as well as prolonging the lifespan of premature and natural aging mice [164,167]. All these results combined made of p16 a hallmark of aging and age-related diseases. However, p16 along with other tumor suppressors characterize the intrinsic arm of cellular senescence by forming two pathways. The p16-pRB where p16 acts as an upstream regulator of pRB, and the p53-p21 where p21 is a downstream effector of p53 [50,108,172,190]. The major regulatory key of these pathways is the inhibition of the cyclin dependent kinases CDK4/6 and CDK2 which then control cell cycle and implicate them in physiological processes dependent on cellular proliferation such as cancer, tissue regeneration, aging, cellular senescence and development [44,108,226]. As a result, p16 upregulation or baseline expression is not solely associated with aging and senescence, but it may simply reflect cell cycle inhibition as p16 upregulation is not always associated with increased SASP expression [127]. In addition, p16 suppression restores proliferation and provokes senescence bypass associated with decreased SASP expression [224,227] and promotes tumorigenesis [228–231].

Therefore, while the primary role of p16 in various tissues and organs has been mainly restricted with its involvement in cellular senescence, aging, and age-related diseases, further research has revealed additional beneficial roles of p16 within these tissues despite its classical function as a cell cycle regulator. Hereby, demonstrated in several selected organs, the well-known function of p16 in senescence and aging discussed in parallel with its implication in several other biological processes apart from that.

i. In the Skin

The skin is the largest tissue in the human body. It serves as a physical barrier to both biological and nonbiological threats. Being exposed to the outside environment places the skin in direct contact with environmental hazards, making it extremely vulnerable. The skin is made up of two

layers: the outer epidermis, which is divided into four sublayers with keratinocytes predominating in the spinous, granular, and cornified sublayers, and pigment-producing melanocytes that confer photoprotection in the basal sublayer. The underlying dermis contains connective tissue with fibroblasts, collagen, and elastin as well as sebaceous and sweat glands and is connected to the epidermis by the dermal epidermal joint (DEJ) [232]. Skin aging is caused by both intrinsic (genetic, time, etc.) and extrinsic (pollution, UV exposure, sunlight, etc.) factors, and it has both biological and functional implications. Aged skin has thinner epidermis, dermis, and DEJ than younger skin, which is due to keratinocytes' decreased proliferation and renewal ability [24,233–235].

As major biomarkers of senescence, both the SA- β -gal and p16 determination has shown elevated expression upon in vitro exposure of fibroblasts and keratinocytes to UV light [236–238]. Furthermore, telomere shortening, DNA damage, and UV exposure increased the activity of the p16/pRB and P19ARF/p53/P21 cascades, resulting in an accumulation of SnCs and skin stem cell dysfunction and loss of regeneration capacity [235]. An in vivo study, on the other hand, claimed that UV light exposure has accelerated cellular senescence by increasing p21 expression [238].

In contrast to the previous results, the presence of p16 has been shown to play an important role in several biological processes that are beneficial to the skin. Starting with its tumor suppression function, p16 inactivation due to mutation or promoter methylation has been linked to a variety of cancers, including familial and sporadic melanoma [177,239–241]. These studies identified 55 out of 60 melanoma cell lines that were dependent on complete or partial p16 aberration, implicating this pathway in the development of melanomas. Furthermore, the level of p16 expression could be used as a melanoma predictive and prognostic biomarker. In other words, lower p16 levels were associated with higher Ki67 expression as a proliferation marker, and metastatic melanoma lesions were associated with even lower p16 levels and predicted poor patient survival [242]. Benign nevi had higher p16 levels than nonmetastatic melanoma, which had even higher p16 levels than metastatic melanoma [243]. Furthermore, in primary mouse fibroblasts (PMFs), human melanocytes, and a human melanoma cell line (A375), the loss of p16 correlated with increased mitochondrial mass, attenuated respiration, and altered morphology associated with augmented superoxide production and higher cellular motility. Forced p16 expression restored mitochondrial homeostasis, dynamics, and motility in a CDK4/pRB independent pathway [244]. Surprisingly, oxidative stress-induced p16 has attenuated ROS production in skin in vivo and in vitro. In

addition, elevated intracellular ROS and DNA damage were obtained in p16-deficient cells. This was restored in skin fibroblasts transduced with p16 using lentivirus [200]. These findings suggest a pRB-independent tumor suppression function of p16. As another mechanism, p16 has been found to transactivate the promoter of the tumor suppressor miRNAs, miRNA-141 and miRNA-146b-5p, in melanocyte through physical interaction with the transcription factor Sp1 and CDK4, via the p16 fourth ankyrin repeat. Mutation in this ankyrin repeat attenuated Sp1 binding and miRNA-141 and miRNA-146b-5p transactivation without affecting the expression level of Sp1 [245]. In addition, this p16–Sp1–CDK4 interaction and consequent miRNA-141 and miRNA-146b-5p transactivation has also been implicated in cellular response to UV-radiation-induced damage and apoptosis.

P16 has been shown to be an important factor in wound healing. ECs and fibroblasts were identified as p16-positive cells at the site of injury in the p16-3MR mouse model a few days after injury. These transiently appearing SnCs aimed to accelerate wound closure by inducing myofibroblast differentiation via platelet-derived growth factor AA secretion as part of the SASP [209]. Elimination of these cells delayed the wound healing process. The matricellular protein CCN1 has been identified as a key player in the induction of fibroblast senescence at the wound healing margins. By inducing DNA damage and p53 activity, CCN1 induces oxidative stress and provokes p16 upregulation, which leads to fibroblast senescence and antifibrotic gene activation [246]. Furthermore, coexpression and activation of the laminin 5/p16 response has been identified in migrating keratinocytes. The laminin 5/p16 response caused hypermotility and growth arrest in keratinocytes, leading to wound re-epithelialization [247]. This pathway has also been identified in critical stage neoplastic progression as a tumor suppressing pathway. This might suggest a protective effect of the induced p16 upregulation upon the exposure of skin to UV radiation [248]. Moreover, p16-orchestrated expression is required for stem cell self-renewal and differentiation. More precisely, p16 repression by epigenetic regulators is indispensable for SCs proliferation. On the contrary, its promoter epigenetic regulation and orchestrated expression level have been found crucial for keratinocyte differentiation beside many other differentiation genes [249–253]. Moreover, the balance between growth and differentiation requires a balanced expression of p16 and other cell cycle regulators [250–253]. For instance, Id-1, Id-2, and Id-3 are repressors of p16 and are upregulated in dividing keratinocytes, whereas they become downregulated in differentiated cells [254]. Activators of p16 transcription promoted keratinocyte differentiation via

acting on epidermal differentiation complex genes [255]. Therefore, unravelling the precise mechanism underlying p16 regulation of expression might provide a targeted approach which confers maintenance of epidermis regenerative capacity and avoids premature skin aging or cancer development (**Figure 10 and Table 1**).

ii. In the Bones

Two major types of cells are involved in maintaining skeletal homeostasis: osteoblasts, which are derived from osteoprogenitor cells and are in charge of bone growth, mineralization, and remodeling, and osteoclasts, which are descended from myeloid lineages and mediate bone resorption and breakdown [256]. Osteocytes are the most prevalent long-lived cell type in bone matrix and are in charge of maintenance of bone mass [257]. Skeletal aging is characterized by bone mass loss and is a significant risk factor for osteoporosis because it results from an increase in osteoclasts and a decrease in osteoblasts count [258–261]. Cellular senescence has been linked to bone aging and the development of aging-related osteo-pathologies [262]. More precisely, senescent osteocytes have been detected in aging bones with increased expression of p16.

In contrast to osteocytes, senescent osteoblasts are characterized by increased expression of p21 only [257,260,263–265]. Moreover, the selective elimination of p16-expressing cells using INK-ATTACK transgene resulted in increased bone mass in 20 months old mice [266]. Furthermore, using the p16-3MR transgene, which is based on the elimination of P16-expressing cells upon treatment with ganciclovir (GCV), has effectively abrogated age-related increases in osteoclastogenesis of the myeloid lineage but had no effect on bone formation. This might indicate that p16, rather than direct targeting of senescent osteocytes, contributes to osteoclastogenic potential without major impact on age-related bone loss [267].

However, other implications of p16 have been demonstrated in bone. P16 degradation by the ubiquitinated regulator UBE2S is an important step in the progression of prostate cancer bone metastasis [268]. Furthermore, patients with p16-positive oropharyngeal squamous cell carcinoma had a higher incidence of bone metastasis than p16-negative patients [269]. Lower expression of p16 in osteosarcoma patients was correlated with reduced response to primary chemotherapy [270], which, therefore, shows the importance of p16 as a prognostic and predictive biomarker and therapeutic target for cancer and metastasis.

Aside from p16 in cancer, although only p21-positive cells were able to prevent radiation-induced osteoporosis [213], p16 deletion inhibited oxidative stress, osteocyte senescence, and osteoclastic bone resorption, which led to osteogenesis and osteoblastic bone formation, indicating a promising mechanism to prevent estrogen deficiency-induced osteoporosis [271]. Furthermore, p16 deletion promoted migration, proliferation, and differentiation of bone marrow mesenchymal stem cells (BM-MSCs) and chondrocytes. It also stimulated osteoblastogenesis and vascularization, which improved bone fracture healing. Consequently, p16 modification might offer a novel strategy for treating fractured bones in elderly patients [272] (**Figure 10 and Table 1**).

iii. In the Lungs

Cellular senescence and aging have both been linked to increased lung damage and functional impairment [273]. Growing evidence suggested aging as another determinant of the chronic obstructive pulmonary disease (COPD) and showed higher prevalence of the disease in elderly [274–276]. Similarly, even though there are no certain causes of idiopathic pulmonary fibrosis (IPF), aging associated with cellular senescence and p16 overexpression has emerged as a main risk factor [277,278].

Cigarette smoking (CS) is a major risk factor attributed to COPD [279]. CS can alter cellular proliferation and induce apoptosis, reactive oxygen species production, and promote oxidative stress, cause DNA damage, and trigger cellular senescence [280,281]. Furthermore, mice exposed to chronic cigarette smoking at both young and old ages showed increased activation of the senescence marker beta-galactosidase as well as upregulation of p16 compared to their respective air-exposed controls. Older air-exposed mice had higher levels of beta-galactosidase and p16 than younger mice. Therefore, CS-induced senescence and natural-aging-associated senescence are both affected by the p16 pathway [282]. This was confirmed in human COPD patients who had higher p16 expression compared to normal smokers and nonsmokers [283]. Furthermore, after CS exposure, wild type mice had more senescent alveolar type II (AECII) epithelial cells than p16 knockout mice, which had normal pulmonary function. Moreover, p16 deletion has rescued the adverse effects induced by CS in the lungs via the insulin growth factor1 (IGF1)/Akt1 signaling pathway [283].

However, p16 expression is a differentiation key between cervical squamous cell carcinoma (SCC) with pulmonary metastasis and pulmonary SCC. Immunohistochemistry of both cervical SCC

without and with pulmonary metastasis has shown an intense staining of p16 in almost all cases studied. On the contrary, cases with pulmonary SCC demonstrated p16 expression in 7 out of 33 cases, 3 of which, showed weak p16 staining. This implies the usefulness of p16 as distinguishing marker between cervical SCC with lung metastasis and pulmonary SCC [284]. Furthermore, the fact that aberrant p16 methylation occurs at early stages of lung cancer renders p16 an early diagnostic biomarker for monitoring and prevention [285]. Moreover, p16 low expression and gene mutation were associated with early and late stage non-small cells lung carcinoma (NSCLC), respectively [286,287]. As a result, it has been identified as a predictable prognostic factor in NSCLC particularly at early stage.

On the other hand, p16 expression is not only linked with disease progression but also with lung protection. P16 loss was linked with poor survival after lung injury. In addition, p16 expression was found to be crucial for protection of lung epithelium against oncogenic stress and lung injury [288]. Moreover, injured p16-positive mesenchymal cells enhanced epithelial progenitor proliferation, whereas deletion of p16 attenuated normal epithelial repair in the lungs [289]. Furthermore, prevalent usefulness was demonstrated for p16 as a target for COPD therapy. Higher p16 expression was found in human COPD lungs compared to normal patients, and when CS induced impaired pulmonary function and augmented emphysema in WT mice, p16 knockout mice exhibited normal pulmonary function with reduced emphysema and increased alveolar progenitor proliferation [283] (**Figure 10 and Table 1**).

iv. In the Brain

Aging-induced p16 overexpression and cellular senescence have been linked to decreased subventricular zone progenitor proliferation and neurogenesis of the olfactory bulb, to diminished multipotent progenitor cell frequency and self-renewal potency [290]. Moreover, chronic accumulation of SnCs and the resulting inflammation in the brain has been linked to the development of Alzheimer's disease (AD) and other neurodegenerative diseases [291,292]. In two out of five AD models, Dorigatti *et al.* [293] found evidence of cellular senescence marked by a significant increase in p16, p21, and p53 expression, as well as increased SASPs expression and beta-galactosidase activity [293]. Another study found that tau-containing neurofibrillary tangles (NFTs), a hallmark of Alzheimer's disease, are age-dependent and strongly associated with senescence induction and upregulation of p16 and p21 [294,295]. Nonetheless, astrocytes play an

important role in neuronal homeostasis and functions, and as we age, they undergo senescence in response to multiple stresses, resulting in impaired brain function [296–299]. In a study to investigate the presence of senescent astrocytes in aging and Alzheimer's disease, tissue from the brains of elderly people and Alzheimer's patients was compared for p16 and SASPs expression with fetal tissue as a control. The findings revealed that aged brain tissue contains significantly more p16-positive astrocytes than fetal tissue. When compared to non-AD adults of the same age, AD brain tissue contains more p16 positive astrocytes [300].

As previously discussed for other tumors, unsurprisingly, p16 homozygous deletion was found in both primary glioblastoma and their derived xenografts [301]. In addition, p16-cdk4/cyclin D1-pRb pathway inactivation was found in the majority of glioblastomas [302]. P16 loss was linked to significantly poor outcome in all glioma patients, which indicates a predictive prognostic usefulness of p16 in brain tumors [303]. On the contrary, p16 null glioma cells demonstrated higher chemosensitivity to paclitaxel and topotecan compared to exogenous wild type p16 overexpression [304].

P16 overexpression has been shown to exert a protective function of neurons against CDK overexpression-induced apoptosis [305]. Moreover, increased expression of p16 and p21, induced by stress conditions, has protected female but not male astrocytes from transformation [306]. In another promising strategy, the selective elimination of p16-positive senescent astrocytes diminished cognitive impairment induced by whole brain irradiation [307]. Lastly, dihydromyricetin (DMY), through the downregulation of p16, p21, and p53, was able to inhibit oxidative stress and neuroinflammation and to attenuate brain aging and improve cognitive function in mice [308] (**Figure 10 and Table 1**).

v. In the Heart

Remarkable p16 expression and cellular senescence were found in cardiac chronological aging and heart failure [309,310]. For example, elevated p16 expression and beta-galactosidase activity were found in cardiomyocytes gathered from Langendorff heart perfusion with aging [218]. In addition to that, cardiac progenitor cells isolated from elderly (>70 years old) people expressed high levels of p16 and SASPs, besides shortened telomeres and increased SA- β -gal [219]. Furthermore, remarkable telomere shortening and senescent associated increased p16 expression were found in cardiomyocytes isolated from old rats compared to younger ones [311]. Older patients with heart

failure had higher p16 expression, which was associated with senescence and cell death, as well as shorter telomere length, when compared to healthy elderly people. This suggests that p16-induced senescence, telomere attrition, and cell death are features of heart failure in aging [310]. Furthermore, vascular smooth muscle cell (VSMC) senescence in atherosclerotic plaques was marked by increased p16, p21, and p53 expression in addition to increased beta-galactosidase activity [312].

The recovery of cardiac function and cardiac remodeling have been correlated with cardiac stem cells (CSCs) regeneration and differentiation ability [313,314]. Cellular senescence has an impact on CSCs and cardiac function, which might provide a concept of therapies by targeting SnCs for cardiac functional improvement and extended lifespan in elderly people [313]. With aging, a significant portion of human CSCs become senescent with elevated expression of p16, SA- β -gal, and SASPs, which contribute to CSCs senescence and impaired cardiac regeneration. However, INK-ATTAC or senolytic elimination of senescent CSCs reactivated resident CSCs and increased cardiomyocyte proliferation [313] reflecting the importance of p16-positive senescent CSCs as therapeutic approach for cardiac functional improvement. P16-positive cells that accumulate during adulthood have a negative impact on lifespan and promote age-dependent changes in the heart. The removal of p16-positive cells delayed age-related heart deterioration. Thus, the therapeutic removal of these cells may be an appealing approach to extend healthy lifespan [167]. On the contrary to the previous studies, the existence of p16 high cells detected in p16-CreERT2-tdTomato mouse model, was found indispensable for health span, and their elimination has induced cardiac fibrosis [210]. Furthermore, p16 overexpression has been detected in the infarction zone after myocardial infarction. The increased expression of p16 was associated with protected cardiac function and plays an important role for cardiac remodeling after myocardial infarction [315] (**Figure 10 and Table 1**).

vi. In the Kidneys

Several studies have linked p16 induction and subsequent cellular senescence to renal aging, diseases, and allograft rejection [316–318]. Age-dependent p16 upregulation in cortical tubular and interstitial cells was observed in humans. In addition, p16 and p27 expression were higher in the glomeruli, tubules, and interstitial cells of rejected grafts compared to normal kidneys [317]. Whether this reflects senescence as the underlying mechanism for chronic allograft rejection as

suggested or might correspond to reduced proliferation and repair or to an increased immune reaction remains to be determined. In line with this, in human kidney specimens ranging from 8 weeks to 88 years of age, p16 induction was negatively correlated with the proliferation marker Ki-67 [319], which is in agreement with the role of p16 as a cell cycle inhibitor. Levels of p16 in glomerular and interstitial cells were significantly higher in kidneys with glomerular disease than in normal aged kidneys and kidneys with tubular interstitial nephritis. P16 expression was higher in kidneys with proteinuria, with fibrosis, or interstitial inflammation [320]. Whether this increased P16 expression is cause or consequence of glomerular disease remains an open question. Similarly, increased p16 expression was observed in kidneys of hypertensive animals and patients and kidneys with type 2 diabetic nephropathy [321,322]. Blood pressure lowering reduced p16 expression [321], which argues against a close relation between p16 and irreversible senescence in this model. Increased p16 expression has been reported in acute kidney injury (AKI) and in acute tubular necrosis (ATN) [323]. P16 deletion ameliorated ATN and improved kidney function in animal models [323]. Similarly, p16 deletion in Bmi-1 deficient mice rescued kidney aging features including function and structure, ameliorated tubulointerstitial fibrosis and inhibited epithelial mesenchymal transition of renal interstitial fibroblasts [324] (**Figure 10 and Table 1**).

vii. In the Liver

Although the majority of liver functions appear to be preserved with age, evidence of aging and cellular senescence associated with liver functional decline, reduced regenerative capacity, and diseases are well-documented [325–327]. P16 expression was higher in elderly hepatectomy patients compared to younger ones, and the increased p16 expression was associated with decreased liver regeneration [328]. This is in agreement with the attenuated proliferative response of hepatocytes in old rat liver compared to young animals [329]. P16 upregulation was observed in liver tissue and liver sinusoidal endothelial cells (LSEC) in an aged rat model compared to young animals [330]. The p16 CreERT2 tdTomato mouse model also demonstrated that p16 high cells were detectable in the liver, and that they were enriched with aging. The majority of the P16-positive liver cells found were vascular endothelial, and their removal caused steatohepatitis and perivascular tissue fibrosis [210,331].

With respect to liver metabolism, the extra copy of p16 carried by the “Super-INK4A/ARF” mouse model prevented the development of glucose intolerance with aging. Instead, increased activation

of insulin receptors and high insulin sensitivity were obtained. This reveals a protective role of INK4A/ARF locus against age-induced insulin resistance [332], whereas increased insulin secretion, attenuated insulin sensitivity, and reduced hepatic insulin clearance were observed upon loss of function mutation of the *Cdkn2a* gene [333]. On the contrary, p16 deficiency improved fasting-activated glucose production in the liver, via the activation of PKA-CREB-PGC1 α [334]. Altogether, these studies show the importance of p16 in glucose homeostasis. However, p16 has not been only implicated in glucose but also in fat metabolism. P16 has been found to regulate fasting-induced fatty acid oxidation and lipid droplet accumulation in the liver in vivo and in vitro. In addition, p16 deficiency was correlated with increased expression of fatty acids catabolism genes in primary hepatocytes [335]. Furthermore, p16-positive senescent cell accumulation has been correlated with hepatic fat deposition and steatosis. Elimination of these cells in the INK-ATTAC mouse model or senolytics treatment (dasatinib plus quercetin) attenuated liver fibrosis [215]. However, the feedback loop between lipid accumulation and increased p16 expression remains intriguing. Senescence in hepatocytes triggered fat accumulation [215], while high fat diet provoked significantly elevated p16 expression [336]. A possible mechanism could be that due to high fat diet and with aging, increased p16 induces senescent cell accumulation in the liver which in turn impairs lipid metabolism and provokes liver fibrosis. Therefore, p16-positive SnCs might be a promising target for liver fibrosis therapy. On the other hand, elimination of p16-positive cells also provokes liver fibrosis [210]. In primary sclerosing cholangitis, p16 downregulation demonstrated a protective effect against biliary damage and fibrosis [337]. However, its upregulation was required for the regulation of reactive oxygen species in hepatic stellate cells and modulation of liver fibrosis [338].

Nonetheless, several studies have also described p16 functions in liver cancers. P16 hypermethylation and consequent p16 inactivation has a pivotal role in the development of hepatocellular carcinoma and liver cirrhosis [339]. Wong et al. reported aberrantly methylated p16 in the plasma of liver cancer patients, suggesting the usefulness of these circulating liver-cancer-methylated DNA for the monitoring of tumors [340]. Therefore, all this information combined suggests that p16 regulation and meticulously unravelling the molecular mechanisms regulating p16 expression in liver physiology and liver pathologies require further elucidation and could unveil novel therapeutic strategies for maintaining normal liver function and extending lifespan.

Altogether, these studies suggest that p16, which is commonly used as a marker for cellular senescence, has broader functions beyond its role in senescence, and highlights additional roles and implications of p16 in various biological processes (**Figure 10 and Table 1**).

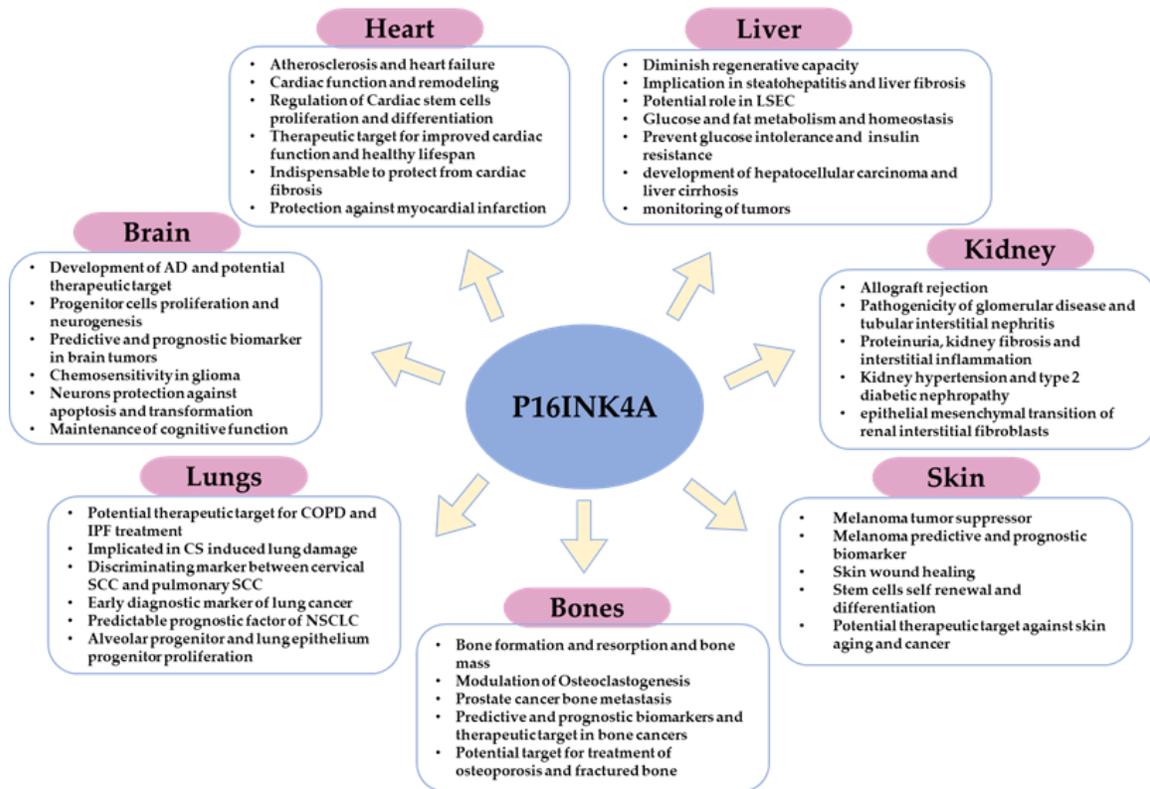


Figure 10. Schematic illustration that summarizes the major functions or implications of p16 in homeostasis, pathophysiology, and cancer of different organs [169].

Table 1. Major p16 functions in the variety of biological processes observed in different models and tissues [169].

| Process | Model and/or Tissue | Potential Role/Function | References |
|---|---|---|--|
| Intervertebral disc damage | Mouse and human intervertebral disc tissues | P16 is a potential therapeutic target for intervertebral disc damage relief. | [48] |
| Wound healing | P16-3MR model (fibroblasts, endothelial cells, and keratinocytes) | Accelerate wound closure and re-epithelialization | [209] |
| Tumorigenesis and tumor suppression | Skin, bone, lung, liver, and brain cancer patients tissues and immunohistopathology | Implicated in tumor development, progression, and metastasis Predictive and prognostic marker Therapeutic target Increases chemosensitivity | [228–231], [177,239–241], [244], [200], [245], [268], [269], [270], [284], [285], [286,287], [302], [303], [304], [339], [340] |
| Stem cell self-renewal and differentiation | Skin, lung, bone, brain, and heart stem cells | Balanced expression of p16 is a prerequisite for stem cells proliferation and differentiation. Therapeutic approach for maintenance of regenerative capacity | [249–253], [255], [235], [272], [219], [313], [289], [283], [290] |
| Cellular senescence | Primary mouse fibroblasts and melanocytes | Target for oncogene-induced senescence bypass and aging | [224,227] |
| Bone homeostasis | P16-3MR and p16-INK-ATTAC mouse model | Maintenance of bone mass Orchestration of osteoblast and osteoclast function | [257], [266], [267] |
| Bone fracture healing | Geriatric Mouse model (p16 ^{-/-} and WT) | P16-deletion stimulated osteoblastogenesis and vascularization and accelerated bone fracture healing | [272] |
| Muscle injury | Acute muscle injury (AIM) mouse model | Tissue regeneration | [341] |
| Osteoporosis | Ovariectomized p16 ^{-/-} and WT mice | Potential therapeutic target to prevent estrogen-induced osteoporosis | [258–261], [271] |
| COPD | Lung alveolar and lung epithelial cells in mice and human | Implicated in COPD severity Potential therapeutic target | [274–276], [283] |
| Cervical SCC and pulmonary SCC | Human cancer patients | Discriminating biomarker | [284] |
| Oxidative stress | Fibroblasts, keratinocytes, and melanocytes | P16 regulates oxidative stress and ROS production as pRB-independent tumor suppression mechanism | [200], [338] |
| Mitochondrial biogenesis | Primary mouse fibroblasts, human melanocytes, A375 melanoma cells | P16 balances mitochondrial structure and function | [244] |
| Alzheimer's disease (AD) | Alzheimer's disease patients and mouse model | Implicated in AD severity and development Therapeutic target | [293], [294,295], [300] |

| | | | |
|---|---|---|---------------------|
| Lung injury | P16 ^{-/-} and WT mouse model (lung epithelium) | P16 protects against lungs injury | [288] |
| Cardiac fibrosis | p16-CreERT2-tdTomato mouse model | P16-positive cells removal induces cardiac fibrosis | [210] |
| Myocardial Infarction | Mice | Indispensable for maintenance of cardiac function and cardiac remodeling after infarction | [315] |
| Glucose metabolism and homeostasis | Super-INK4A/ARF mice model | Prevented the development of glucose intolerance with aging Protective role against age-induced insulin resistance | [332] |
| Liver fibrosis | INK-ATTAC mouse model | Therapeutic approach for treatment of liver fibrosis | [215], [338], [210] |
| Fat metabolism | Mouse model and primary hepatocytes | Regulate fasting-induced fatty acid oxidation and lipid droplet accumulation in the liver | [335] |

PART II: The Senescence Markers p16Ink4a, p19ARF and p21 in Development

I. Mouse Embryonic Development

The development of a mouse embryo is a complex and highly orchestrated process that includes distinct stages that lead to the formation and differentiation of various organs. The mouse embryo goes through a series of remarkable transformations from the earliest stages of fertilization to the development of mature organism.

The process begins with the preimplantation stage, which occurs from approximately embryonic day 0.5 (E0.5) to E4.5. Following fertilization, the zygote undergoes rapid cell divisions that transform it into a solid ball of cells known as a morula. As the morula continues to divide, it develops a fluid-filled cavity, becoming a blastocyst. The blastocyst consists of an outer layer called the trophectoderm and an inner cell mass (ICM), which will contribute to the future embryo [342].

At around E4.5, implantation and gastrulation take place. The blastocyst attaches to the uterine wall, facilitating nutrient exchange. Gastrulation is marked by the formation of the primitive streak, which establishes the body axis. Cells from the ICM migrate and spread across the epiblast, giving rise to the three germ layers. The outermost layer, the ectoderm, will contribute to the nervous system, epidermis, and other tissues. The middle layer, the mesoderm, will give rise to muscles, bones, blood, and various organs. The innermost layer, the endoderm, will differentiate into the gut, liver, and respiratory system [343].

Organogenesis occurs from approximately E7.5 to E18 and involves the intricate formation and differentiation of specific organs. During this stage, multiple processes shape the developing organs. For instance, the neural plate folds and fuses to form the neural tube, which gives rise to the brain and spinal cord. In addition, the heart begins as a primitive tube and undergoes complex processes of looping and chamber formation. Differentiated cardiac cells generate the myocardium, valves, and major blood vessels. Moreover, in the kidney, the nephric mesenchyme and ureteric bud interact to form nephrons, the functional units of the kidney, including glomeruli, tubules, and collecting ducts. In the liver, the liver bud forms and differentiates into hepatocytes, bile duct cells, and other hepatic cell types [344–347].

1. Brain Development

Mouse brain embryonic development is a complex process that involves precise spatial and temporal regulation of gene expression, cell proliferation, migration, and differentiation. This

process results in the diverse cell types and intricate neural circuitry that characterizes the mature brain. The neural plate, which eventually forms the central nervous system, is formed during early embryogenesis by a flat sheet of cells called the ectoderm. [348]. The neural plate then folds inward to form the neural tube, from which the brain and spinal cord emerge. The neural tube closes from rostral to caudal, with the anterior part forming the brain (prosencephalon) and the posterior part developing into the spinal cord [349,350]. The neural tube undergoes regionalization, leading to the formation of distinct brain regions. This process is mediated by the expression of specific transcription factors and morphogens [351,352].

Neural progenitor cells (NPCs) emerge from the neural tube and are capable of self-renewal and multipotency. These cells proliferate and give rise to the various cell types found in the brain. NPCs are initially multipotent, but as they develop, they become limited to specific lineages. [353]. Neurons generated in the developing brain's ventricular zone migrate to their final destinations. Migrating neurons are guided along radial processes by radial glial cells, which act as both progenitors and scaffolds [354]. Neuronal migration is required for the formation of layered structures within the brain, such as the cerebral cortex's cortical layers [355]. The Reelin signaling pathway is critical for neuronal migration, and its disruption causes severe migration defects [356]. Neurons differentiate as they reach their final positions, extending axons and dendrites to form connections and establish neural circuits. This process entails precise gene expression regulation, which includes the activation of cell type-specific genes and the suppression of alternative cell fate programs. [357,358] (**Figure 11**).

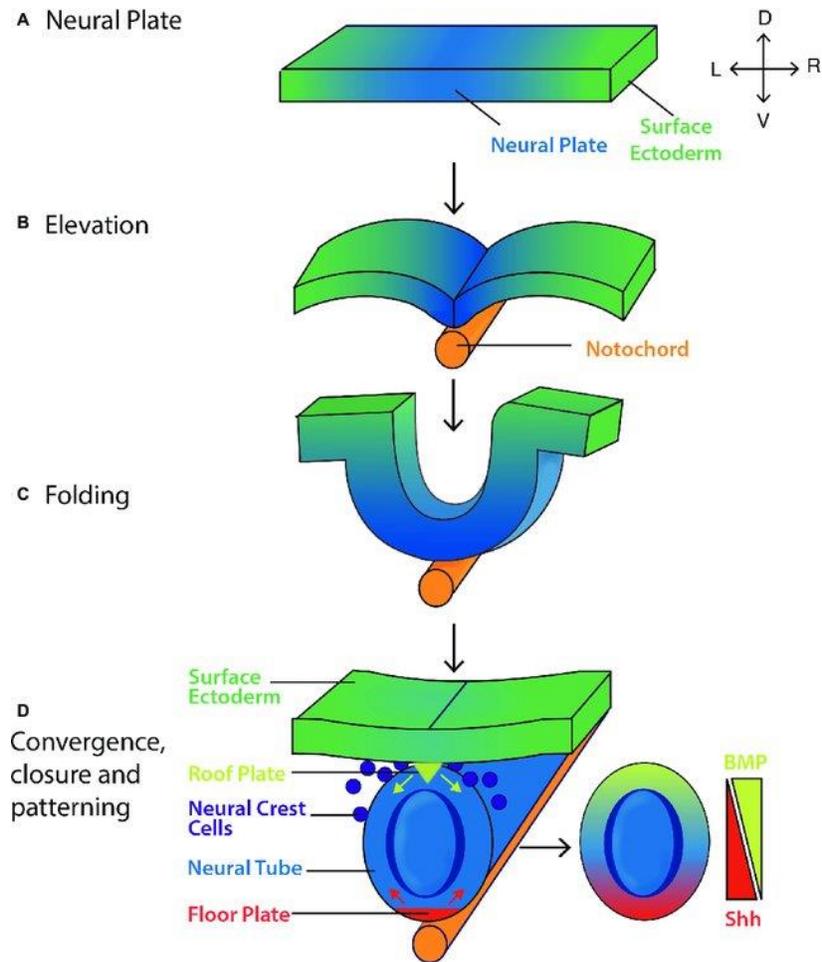


Figure 11. Development and organization of the mouse neural tube. (A) The neural plate takes shape as a pseudostratified columnar epithelium by E7.5. Subsequently, the **(B)** lateral edges of the neural plate undergo elevation and **(C)** folding around E8, followed by **(D)** convergence at the midline and closure by approximately E8.5. Signaling molecules such as Shh (depicted by red arrows) and BMP inhibitors released from the floor plate, along with BMP4/7 (depicted by green arrows) from the roof plate, contribute to the ventro-dorsal patterning of the neural tube. This intricate process gives rise to the distinct layers of the spinal cord. Key: V, ventral; D, dorsal; L, left; R, right.

Rachel A Shparberg et al. *Front. Physiol.* 2019

2. Heart Development

Early heart tube development is a crucial process in the formation of the heart during embryonic development. Starting from the formation of the heart tube, cardiac precursor cells migrate laterally from the anterior regions of the primitive streak to give rise to the anterior lateral mesoderm. The specification and differentiation of these cells into the heart occur through intercellular signaling events, including positive signals from underlying pharyngeal or foregut endoderm (such as BMP and FGF signals) and negative signals from midline structures (such as β -catenin/WNT signaling). Afterwards, the heart tube undergoes morphogenetic movements and transforms from a cardiac crescent into a linear structure. The heart tube consists of an outer myocardial layer and an inner endocardial tube. It has an anterior outflow (arterial pole) and a posterior inflow (venous pole) proportion. The heart tube initially remains open dorsally to ventral pharyngeal endoderm but eventually closes and becomes isolated in the ventral region of the embryo. The elongation of the heart tube is driven by the addition of cardiac progenitor cells from the second heart field. These cells, originating from splanchnic mesoderm, contribute to the arterial and venous poles of the heart. They play a crucial role in the development of various cardiac structures, including the right ventricle, outflow tract, atrial septum, and inflow tract myocardium [359–362].

Cardiac cushions, derived from endocardium and myocardium, form in the atrioventricular and outflow tract regions. These cushions undergo epithelial to mesenchymal transition, triggered by myocardial-derived BMP signals, and subsequently give rise to the definitive cardiac valves. The septation of the heart involves the formation of various septa to separate the systemic and pulmonary circulatory systems. The ventricular septum is formed by the interface between the linear heart tube and second heart field-derived myocardium. The outflow tract and atrioventricular cushions contribute to the muscular and membranous components of the ventricular septum. The aorticopulmonary septum separates the ascending aorta and pulmonary trunk. Atrial septation involves the formation of the primary and secondary atrial septa, closing the communication between the atria and completing the separation of systemic and pulmonary blood flow [359,360,362,363] (**Figure 12**).

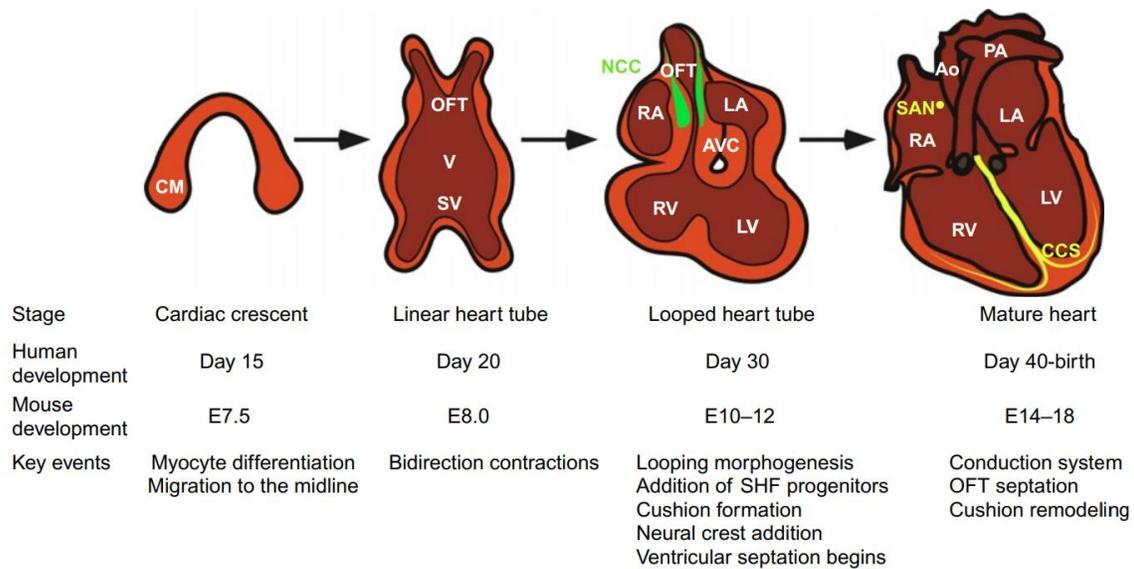


Figure 12. Illustrations representing various stages of mammalian heart development, highlighting key events in both mouse and human cardiac development. Mouse developmental stages are indicated by embryonic days (E). Key anatomical features include Ao (aorta), AVC (atrioventricular canal), CCS (cardiac conduction system), CM (cardiac mesoderm), LA (left atrium), LV (left ventricle), NCC (neural crest cells), OFT (outflow tract), PA (pulmonary artery), RA (right atrium), RV (right ventricle), SAN (sinoatrial node), SV (sinus venosus), and V (ventricle).

McCulley DJ et al. *Current Topics in Developmental Biology*. 2012

3. Kidney Development

The mammalian kidney is a complex organ made up of numerous nephrons and a network of branching collecting ducts. The development of the kidney involves a number of processes and interactions among various cell populations. The nephric duct (ND), also known as the Wolffian duct, and the metanephric mesenchyme (MM) are both essential in kidney development. The ND develops from the intermediate mesoderm (IM) during embryogenesis, whereas the MM develops at the hindlimb level. The ND grows caudally as the body axis lengthens and goes through a series of developmental events. ND extension is primarily driven by cell migration and rearrangements in lower vertebrates, whereas in mice, ND cell migration and/or proliferation contribute to its elongation [364–368].

Inductive signals secreted by the axial and paraxial mesoderm are at the basis to specify the ND from the IM. These signals induce the expression of specific transcription factors such as Pax2, Pax8, Lhx1, and Gata3. In mice, genetic studies revealed a regulatory network involving these transcription factors as well as other genes such as β -catenin and RET, which are required for

normal ND development. Mutations in these genes can cause a variety of ND formation abnormalities. The elongated ND eventually fuses with the cloaca and undergoes changes that result in the formation of a pseudostratified epithelium. Signals from the ectoderm and the MM help to convert the ND into an epithelial tube. The ND's caudal portion, from which the ureteric bud (UB) will emerge later, swells and exhibits increased cell proliferation. Pseudostratified domains are also found in other developing epithelia and may aid in the UB's rapid outgrowth and branching [364,368–372].

The formation of the UB is a crucial step in kidney development, and its proper positioning is critical. The UB develops from the pseudostratified caudal ND and is activated by MM signals, specifically the secreted protein GDNF (glial cell-derived neurotrophic factor). Many other genes and signaling pathways, such as transcription factors, receptors, and inhibitory signals like BMP4, influence UB formation and branching. Proper signal regulation ensures the formation of a single, discrete UB [364,366,373]

The UB undergoes approximately 10 generations of branching during kidney development, followed by a period of collecting duct (CD) elongation with limited branching and 1-2 additional rounds of branching before birth. The branching of the UB is required for nephron induction because a decrease in branching results in a decrease in the number of nephrons. [364,374] (**Figure 13**).

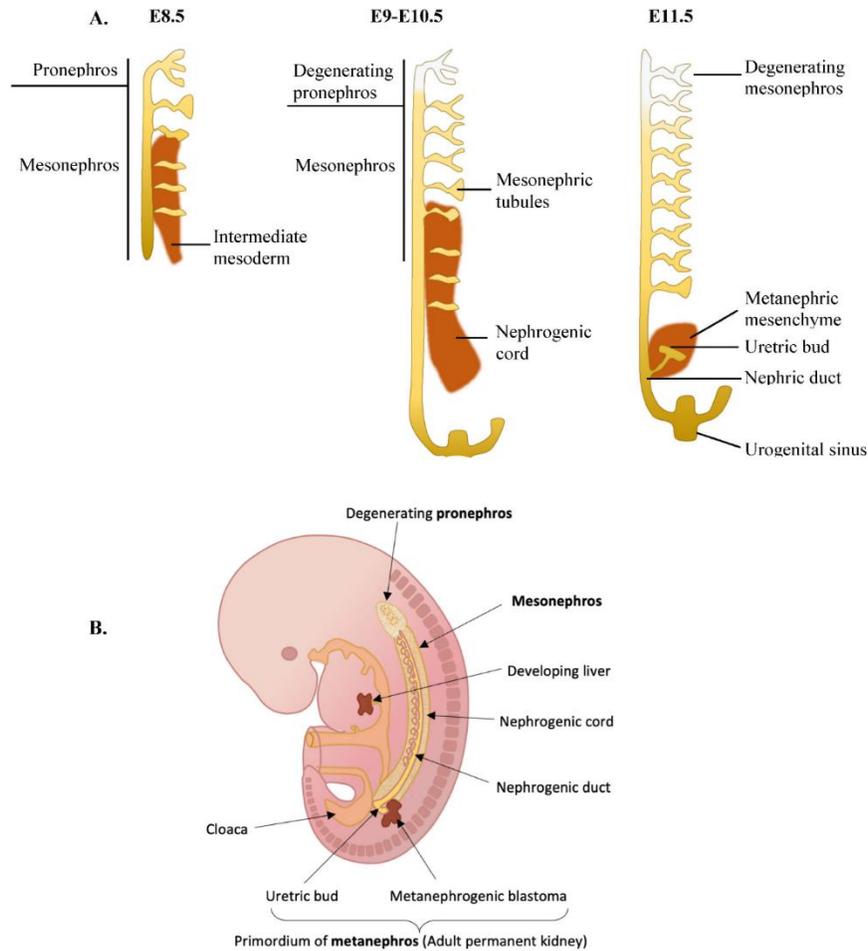


Figure 13. Illustration of embryonic kidney development in mice. (A) Illustration presenting the timeline of kidney development in mice. (B) Visual representation of mouse kidney development on embryonic day 11, situated within the overall context of the entire mouse embryo. Kakun RR et al. International Journal of Molecular Science. 2022

4. Liver Development

The development of the mouse liver is a well-studied process that can be divided into several key stages. The formation of liver progenitor cells, their proliferation and migration, the establishment of liver architecture, and the differentiation of various hepatic cell types are all part of this process. Endodermal cells in the foregut region are designated as liver progenitor cells, also known as hepatoblasts, during early embryogenesis. This specification necessitates the expression of specific transcription factors [375]. Hepatoblasts proliferate rapidly and migrate ventrally, forming a liver bud. The septum transversum, as well as the vitelline and umbilical veins, interact with the liver

bud [376]. Afterwards, the liver bud goes through branching morphogenesis as it grows, resulting in the formation of a complex three-dimensional structure.

Hepatoblasts divide into two types of cells: hepatocytes and cholangiocytes. Hepatocytes are in charge of the metabolic functions of the liver, while cholangiocytes form the bile ducts [376,377]. The activation of transcription factors, including hepatocyte nuclear factors (HNFs) such as HNF4, HNF1, and HNF6, induces the hepatocyte lineage. These transcription factors are critical in the regulation of hepatocyte-specific gene expression and function [378]. Similarly, cholangiocytes, which line the bile ducts, differentiate from hepatoblasts or bipotential liver progenitor cells. However, cholangiocyte specification and differentiation are mediated by different signaling pathways, including Notch, Wnt/ β -catenin, and Sonic hedgehog (Shh) [379,380].

Concomitant with hepatocyte and bile duct development, blood vessels develop and integrate into the liver. The hepatic artery and portal vein provide the liver with oxygenated blood and nutrient-rich blood, respectively. Vascular development and remodeling are regulated by factors such as vascular endothelial growth factor (VEGF) and angiopoietin signaling [381–383] (**Figure 14**).

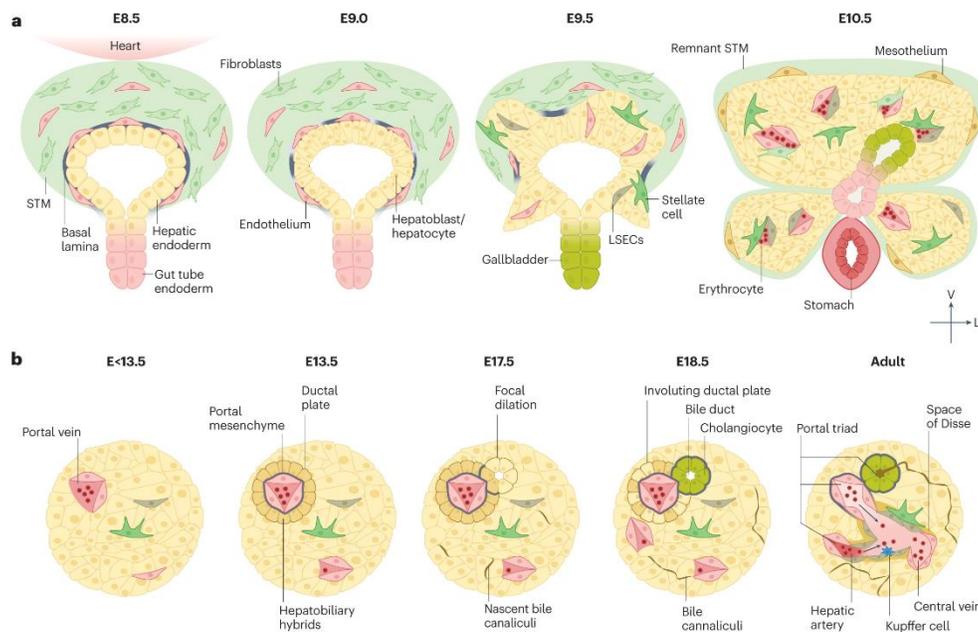


Figure 14. (a) The figure illustrates the transverse section of the developing mouse liver, highlighting key events in embryonic liver development. Hepatic identity is specified by signals from the cardiac mesoderm and septum transversum mesenchyme, guiding the migration of hepatoblasts. (b) The ductal plate formation, initiating around embryonic day 13.5, involves a monolayer of cells interacting with portal mesenchyme, leading to the emergence of focal dilations by E17.5. These dilations contribute to the development of the liver's bile duct network, integrating with the growing bile canaliculi network. Concurrently, the liver vasculature, including the sinusoid, forms to facilitate nutrient exchange. Lotto J et al. Fetal liver development and implications for liver disease pathogenesis. Nature reviews. Gastroenterology & hepatology. 2023.

II. Cell Cycle Control in Development

During early embryonic stages, cell proliferation is essential for the formation of different germ layers and tissue types. The precise control of cell division ensures the proper allocation of cells for the development of specific structures. For instance, the formation of the neural tube, which gives rise to the central nervous system, requires the rapid and coordinated proliferation of neural progenitor cells. Therefore, the cell cycle checkpoints play a crucial role in monitoring the fidelity of DNA replication and preventing the propagation of damaged DNA to daughter cells during this rapid proliferation phase. As embryonic development progresses, cells become more specialized through the process of cell differentiation. The cell cycle is intricately linked to cell differentiation, as specific cues regulate cell cycle exit and induce differentiation. For example, the exit from the cell cycle is necessary for cells to undergo terminal differentiation into specific cell types, such as neurons or muscle cells. This exit is often associated with the downregulation of cyclins and CDKs, leading to cell cycle arrest and the activation of differentiation-promoting factors [384,385].

Organogenesis, the process of organ formation, relies on the precise coordination of cell cycle regulation. Proper cell division, proliferation, and differentiation are required to generate the correct number of cells and establish the appropriate tissue architecture. During organogenesis, cells undergo complex morphogenetic movements, cell rearrangements, and tissue interactions. The cell cycle dynamics, including the timing and duration of cell division and the synchronization of cell cycles within tissues, play a crucial role in achieving the correct tissue patterning and organ morphogenesis [386].

The cell cycle can influence cell fate determination, which involves the decision between self-renewal and differentiation. The length of the cell cycle, the duration of specific phases, and the expression of cell cycle regulators can affect cell fate decisions during embryonic development. For instance, asymmetric cell divisions, where one daughter cell retains stemness while the other differentiates, rely on precise control of the cell cycle. Differential distribution of cell cycle regulators or asymmetric inheritance of cell fate determinants during cell division can bias the fate of daughter cells. Additionally, the integration of extracellular signals, such as growth factors and morphogens, with the cell cycle machinery further regulates cell fate determination during embryonic development [385,387].

III. P16, p19 and p21 in Development

Earlier studies [388,389] found no evidence of p16 expression during mouse embryonic development. These studies, however, did not rule out the possibility that p16 was expressed in different developing organs and at different time points, implying that the lack of detection could be attributed to technical limitations [388]. In contrast to the initial reports of p16 expression being absent during mouse development [388,389], its expression during rat brain development was described shortly afterwards. p16 expression was found to colocalize with p53 in the ventricular and subventricular zones during embryonic and early postnatal stages, with p53 primarily observed in postmitotic cells of the cerebral cortex and hippocampus [390]. In the olfactory epithelium, around birth, detectable levels of p16 and p21 were found, with p16 marking differentiating neurons and p21 marking mature neurons [391]. P16 expression was also found in hematopoietic progenitor cells derived from adult mouse bone marrow [392], epiphyseal growth plate chondrocytes, and growing mouse osteoblasts [393]. In these cases, higher p16 expression was associated with decreased cell proliferation, however cellular senescence was not reported. Increased expression of p16 and p21 in male germ cells has also been observed in conjunction with mitotic arrest but not senescence [394]. During postnatal life, these male germ cells undergo meiosis [395]. Between days 2 and 5 of pregnancy, p16 expression was found to be elevated in the endometrium of mice. When compared to a saline-injected group, p16 antibody injection resulted in a lower number of implanted blastocysts, indicating a potential role for p16 in blastocyst implantation [396]. During pregnancy, p16 expression has also been observed in the human endometrium [397].

Moreover, p16 expression was found in the gut during mouse embryonic development, specifically in intestinal stem cells and progenitor compartments. Loss of Bmi1, a regulatory protein, led to the accumulation of p16, p14/p19, and reduced proliferation of intestinal stem cells. This was accompanied by increased differentiation into post-mitotic goblet cells [398]. Bmi-1 also plays important roles in maintaining the self-renewal of neural stem cells [399–401], mesenchymal stem cell renewal and bone formation [402], immature retinal stem cells and retinal development [403], and hepatic stem cell expansion [404]. These functions are mediated through the negative regulation of p16, p14/p19, and p21. A significant number of p16-expressing cardiomyocytes, mostly bi- and multinucleated cells, were found in three-month-old mice [405]. The authors used p16 expression as a senescence marker without further investigation to confirm cellular senescence

[405]. Another study used *ex vivo* PCNA staining to look at cardiomyocyte proliferation in p16/p14/p19 knockout mice. Surprisingly, the authors found 70% of proliferating cardiomyocytes in mice around 8 weeks old, a finding that contradicts previous research [406]. Specific p16 knockout mice with p14/p19 function had a higher incidence of spontaneous and carcinogen-induced cancers [407], as well as melanomas [408] and thymus hyperplasia [407]. Thymus hyperplasia was associated with an increase in the number of CD4 and CD8 lymphocytes, which was surprisingly due to decreased apoptosis rather than increased proliferation [409]. On an FVBN genetic background, mice lacking both p16 and p14/p19 developed cataracts and microphthalmia. Beginning at E15.5, these mice displayed defects in the developmental regression of the hyaloid vascular system, retinal dysplasia, abnormal lens differentiation, and cataracts [410]. Interestingly, the absence of p16 and p14/p19 partially rescued the microphthalmia phenotype in Task1 knockout mice, indicating a role for these proteins in neuronal and eye development [411]. Furthermore, p14/p19 is involved in postnatal perivascular cell accumulation in the mouse eye before complete eye development [410,412–414]. p14/p19 inhibited Pdgfr- β expression and Pdgfr- β -driven proliferation independently of Mdm2 and p53, preventing the accumulation of perivascular cells and allowing regression of the developing eye's hyaloid vascular system [415,416]. Tgf- β 2 is required for the transcription of p14/p19 in the hyaloid vascular system, as well as the cornea and umbilical arteries [417,418]. In addition, p14/p19 is detectable in developing hepatoblasts [419]. Tbx3 deficiency causes upregulation of p14/p19 and p21 in the developing liver, which is associated with severe defects in proliferation and hepatobiliary lineage segregation, including promotion of cholangiocyte differentiation and abnormal liver development [419]. This study did not demonstrate whether Tbx3 could directly regulate p14/p19 and p21 expression.

Initially, p21 knockout mice were reported to develop normally, despite defective G1 checkpoint control observed in isolated knockout embryonic fibroblasts [420]. Interestingly, p21 expression was detected by Western blot analysis in human fetal atrial tissue but not in adult hearts [421]. p21 expression was also found in developing rat ventricular myocytes [422], but these studies did not compare the expression levels in older ages. Some p21-expressing cardiomyocytes were identified in developing mouse embryos at E15.5 [423] and in the trabecular myocardium at E18.5 [424]. The number of p21-expressing cardiomyocytes was significantly increased in Foxm1 knockout embryos and Tbx20 overexpressing hearts during early development, which correlated with reduced proliferation and cardiac hypoplasia [423,425,426]. Fog-2 was identified as a direct

transcriptional repressor of p21 in cardiac development. Fog-2 mutant embryos exhibited multiple cardiac malformations, upregulation of p21, and thin-walled myocardium [427]. p21 expression has also been reported in developing skeletal muscle, bones, lung, and spinal cord [428–432]. Furthermore, using p21 and P57 double-mutant mice, it was demonstrated that both proteins redundantly control the differentiation of skeletal muscle, bones, and alveoli in the lungs. Mice lacking both p21 and P57 failed to form myotubes and displayed enhanced proliferation and apoptosis of myoblasts, indicating the essential roles of p21 and P57 in normal muscle development. Skeletal defects were more pronounced in embryos lacking p21 [433]. This study highlights the redundancy among different cell cycle regulators in controlling embryonic development, which may explain the limited phenotypes observed in single knockout animals despite the significance of these cell cycle regulators.

In addition to the studies primarily implicating p21 in embryonic development and differentiation, there have been several reports suggesting that senescence may also play a role in normal embryonic development. Munoz-Espin *et al.* [155], conducted whole-mount staining for SA- β -gal in mouse embryos and observed SA- β -gal activity in various structures such as the endolymphatic sacs of the developing ear, the closing neural tube, the apical ectodermal ridge (AER) of the limbs, regressing interdigital webs, vibrissae, and the mesonephros of dissected gonad-mesonephros complexes [155]. This SA- β -gal activity in the regressing mesonephros had previously been reported in chicken embryos [158]. Furthermore, SA- β -gal activity was detected in the mesonephros and endolymphatic sacs of human embryos around 9 weeks of development [155]. Additionally, Munoz-Espin *et al.*, investigated potential developmental defects in p21-deficient embryos and found that these embryos exhibited abnormal endolymphatic sacs with infoldings at late stages of development (E18.5), which subsequently disappeared after birth, likely due to macrophage clearance. Interestingly, the developmental program to remove the abnormal cells in this context was independent of SA- β -gal positive cells or p21.

Another study by Storer *et al.* used a similar approach and detected SA- β -gal positive cells in various structures including the AER, optic vesicle, eye, branchial arches, gut endoderm, neural tube, tail, gall bladder, and interdigital tissue [156]. In this study as well, it appeared that p16 and p14/p19 were not involved in embryonic senescence, but p21 knockout embryos showed a lower number of SA- β -gal positive cells.

All combined, these data suggest that the expression and role of p16 in embryonic development is controversial. Earlier studies did not consistently detect the expression of p16 during embryonic development. More recent investigations have identified SnCs in various tissues and at different time points during development. These cells often showed expression of p21 but the presence of p16 and p19 was not observed. However, there are specific instances where p16 expression has been detected and linked to developmental processes, but a clear-cut function remained ambiguous. In some cases, p16 expression has been associated with reduced cell proliferation and increased differentiation. Therefore, these data provide evidence for a possible presence of p16 in other tissues and organs during development and implies that p16 might have a potential role in the developmental process.



CHAPTER II





THESIS PROJECT

**The Senescence Marker p16Ink4a in Development,
Adulthood and Aging**

I. Project Aims and Description

Aim 1. To know whether p16 is expressed in development and the expression pattern during the developmental process.

We conducted a developmental study of p16 RNA expression in the brain, heart, kidney, and liver. The organs were isolated from mice at different time points of developmental stages including, embryonic day 10.5, 12.5, 14.5, 16.5, and 18.5; postnatal days 1, 7, 21, 3 months, and 16–18 months (n=4) (for E10.5 and E12.5 n=7). We limited the current study to the investigation of brain, heart, kidney, and liver as these organs already develop at the embryonic time points chosen [59-62]. Afterwards, total RNA was isolated from all organs, and the mRNA expression level of p16, p19 and p21 was assessed using RT-qPCR.

Aim 2. We wanted to know which cell types express p16 protein during development

In addition to quantitative p16 assessment on the mRNA level, we investigated its expression in the brain, heart, kidneys, and liver, at the same time points chosen, by immunohistochemistry (IHC). Collections of paraffin-embedded whole embryos were used up to E18.5; for later stages, hearts, livers, kidneys, and brains were dissected. Samples from at least three different animals per time point were analyzed.

Aim 3. Based on the results observed from IHC, we wanted to compare p16 RNA and protein expression level in ECs versus non-ECs.

We isolated brain, heart kidney and liver from 4 adult (3month) and 4 old (16-18 months) mice. Afterwards, we separated ECs from non-ECs using Magnetic-activated cell sorting (MACS) via magnetic microbead-associated anti-CD31 antibody. Total RNA and protein were extracted from both ECs and non-ECs, then p16 RNA and protein level was assessed by RT-qPCR and Western blot respectively.

II. Schematic of the project

1. *p16* expression in development

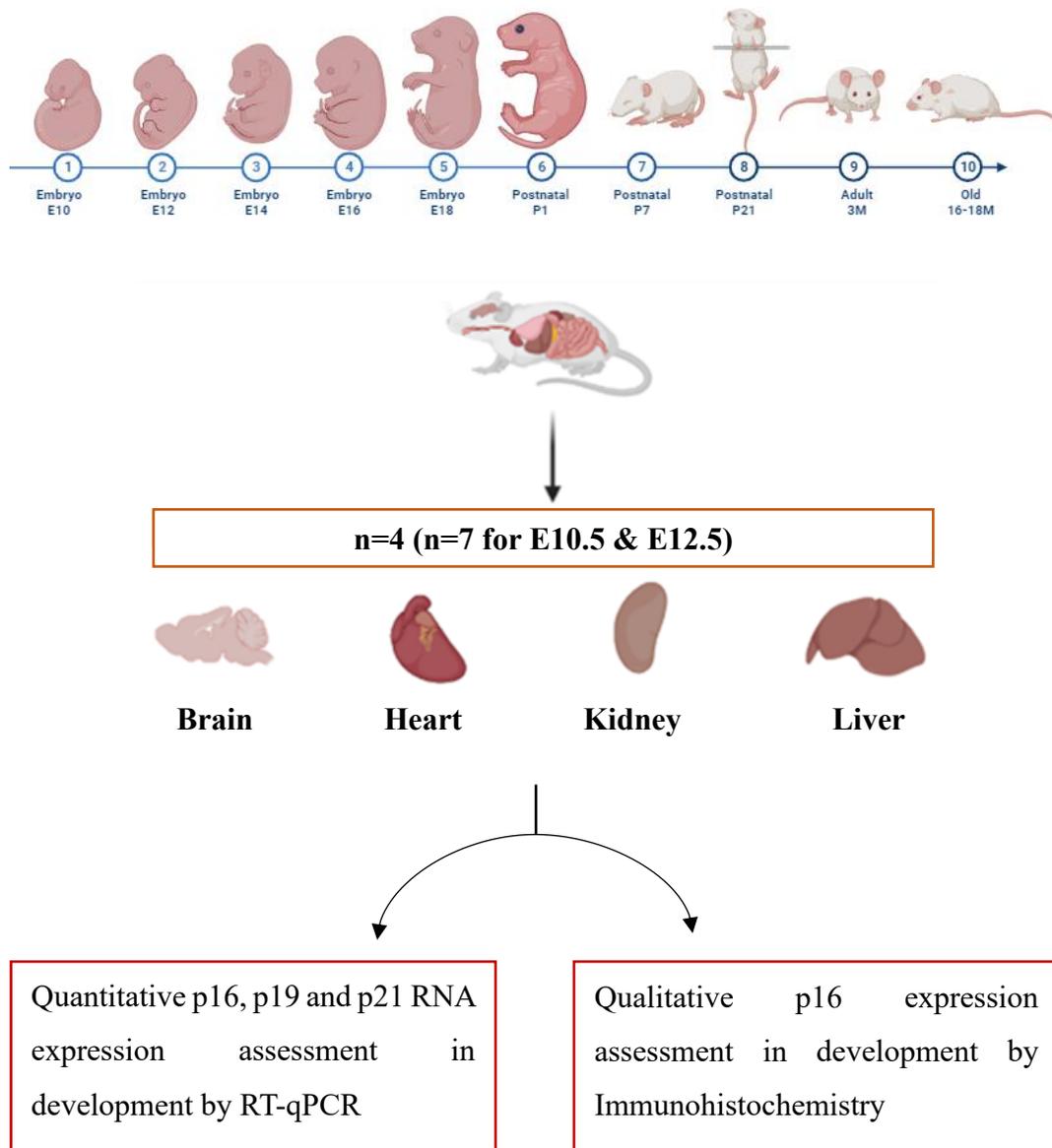


Figure 15. Organs were extracted from mice at various stages of development, spanning embryonic day 10.5, 12.5, 14.5, 16.5, and 18.5, as well as postnatal days 1, 7, 21, 3 months, and 16-18 months. The number of samples (n) for each time point was 4, except for embryonic days 10.5 and 12.5, which had 7 samples each. Total RNA was extracted from these organs, and RT-qPCR was employed to analyze the mRNA expression levels of p16, p19, and p21. Additionally, we utilized immunohistochemistry (IHC) to examine p16 expression in the brain, heart, kidneys, and liver across the same selected time points.

2. P16 Expression in Endothelial versus non-Endothelial Cells

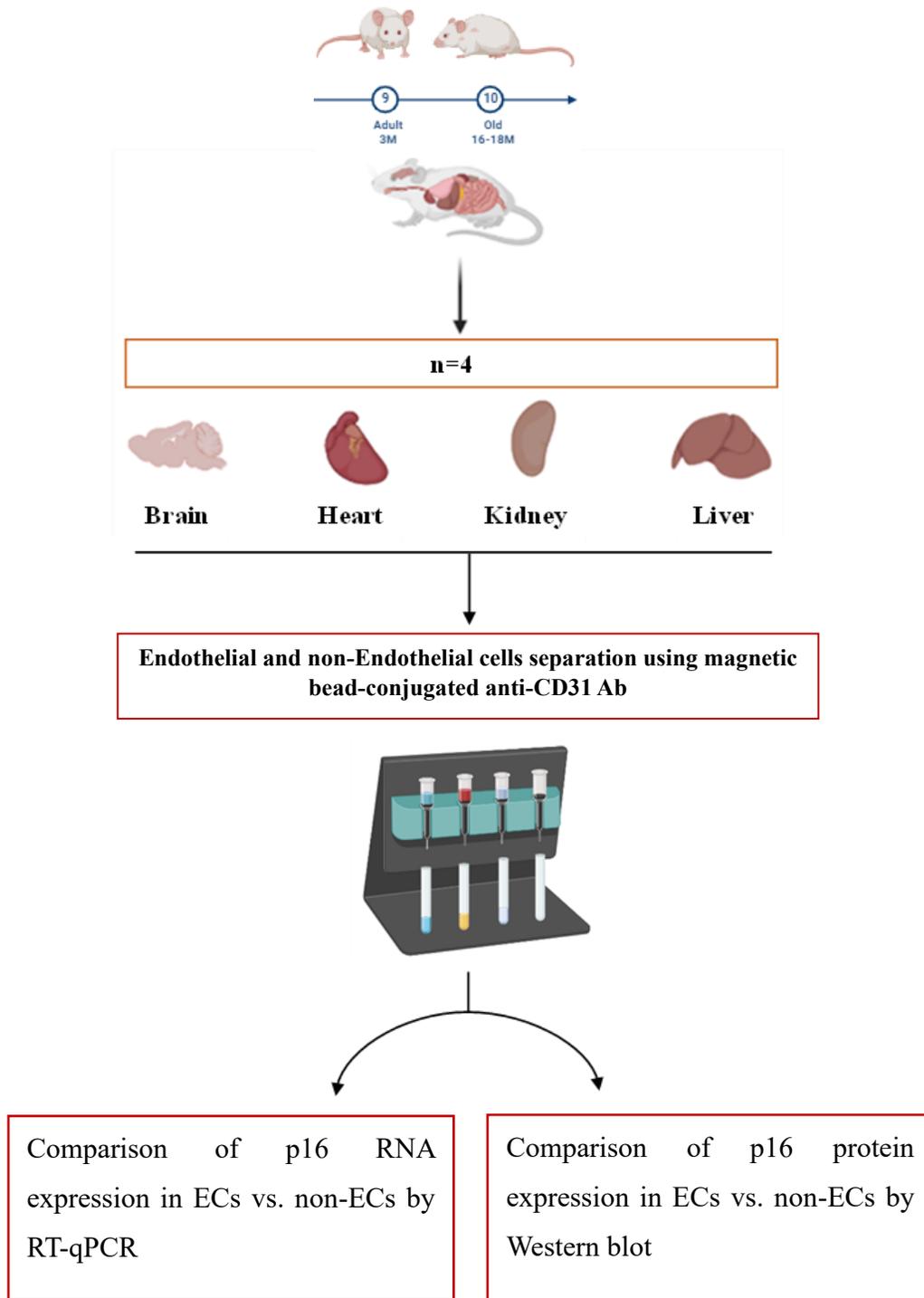


Figure 16. Brain, heart kidney and liver were isolated from 4 adult (3month) and 4 old (16-18 months) mice. separated ECs from non-ECs using Magnetic-Activated Cell Sorting (MACS) via magnetic microbead-associated anti-CD31 antibody. Total RNA and protein were extracted from both ECs and non-ECs and p16 RNA and protein level was assessed by RT-qPCR and Western blot respectively.

III. Manuscript entitled «Dynamic Spatiotemporal Expression Pattern of the Senescence-Associated Factor p16Ink4a in Development and Aging»

Article

Dynamic Spatiotemporal Expression Pattern of the Senescence-Associated Factor p16Ink4a in Development and Aging

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Abstract: A plethora of factors have been attributed to underlying aging, including oxidative stress, telomere shortening and cellular senescence. Several studies have shown a significant role of the cyclin-dependent kinase inhibitor p16Ink4a in senescence and aging. However, its expression in development has been less well documented. Therefore, to further clarify a potential role of p16 in development and aging, we conducted a developmental expression study of p16, as well as of p19ARF and p21, and investigated their expression on the RNA level in brain, heart, liver, and kidney of mice at embryonic, postnatal, adult, and old ages. P16 expression was further assessed on the protein level by immunohistochemistry. Expression of p16 was highly dynamic in all organs in embryonic and postnatal stages and increased dramatically in old mice. Expression of p19 and p21 was less variable and increased to a moderate extent at old age. In addition, we observed a predominant expression of p16 mRNA and protein in liver endothelial cells versus non-endothelial cells of old mice, which suggests a functional role specifically in liver endothelium of old subjects. Thus, p16 dynamic spatiotemporal expression might implicate p16 in developmental and physiological processes in addition to its well-known function in the build-up of senescence.

Keywords: aging; endothelial cells; development; liver; heart; brain; kidney; senescence; SASP



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1. Introduction

Aging is characterized by the gradual continuous decline of functions of cells, tissues, and the whole organism [1]. This age-related functional degeneration affects each organism that passes through developmental phases up to aging, as it is experienced by single cellular and multicellular organisms [2]. In mammals, aging is associated with a variety of pathologies and has been classified as the leading predictive factor of many chronic diseases that account for the majority of morbidity and mortality worldwide [3]. These diseases include neurodegenerative (Alzheimer's and Parkinson), cardiovascular, pulmonary, renal, and bone disorders, and cancers [4–9]. What makes aging a common risk factor is the fact that it arises from molecular mechanisms and pathological pathways that are cornerstones for the development of all these diseases. This includes oxidative stress and overproduction of reactive oxygen species, overproduction of inflammatory cytokines, activation of oncogenes, DNA damage, telomere shortening, and, consequently, accumulation of senescent cells [10–15].

Cellular senescence is a stress response defined as an irreversible arrest of cellular proliferation that results from experiencing potentially oncogenic stress [16]. Senescence was first discovered in primary cell culture in which cells exhibited a replicative senescence after extended period of growth which was termed the Hayflick's limit [17,18]. Senescent cells are usually characterized by phenotypic changes, morphological and biochemical,

and adopt a secretory phenotype known as the senescence-associated secretory phenotype (SASP) [3,19–21]. Morphologically, senescent cells are usually larger than normal ones and exhibit a flattened shape, sometimes with multi-nuclei. However biochemically, these cells show a differential expression profile especially for some genes which rendered them as senescence fingerprints. Senescence-associated β -galactosidase, is an enzyme that is upregulated in senescent cells, and which acts as senescence biomarker [22]. Moreover, ectopic expression or upregulation of several genes has been identified, which includes augmented secretion of proinflammatory cytokines, proteases, and growth factors, which are all together termed the SASP [23–25]. A variety of causes underly the induction of cellular senescence; this includes oncogenic stress, telomere shortening, mitogenic signals, genomic DNA damage, epigenomic modifications, and tumor suppressor gene dysregulation [26–33].

Two major pathways have been identified to generate and maintain senescence, representing the intrinsic arm of cellular senescence. The key regulatory proteins of these pathways are the cell cycle regulators p16Ink4a (afterwards termed p16), p19Arf (afterwards p19), and p21 in addition to p53 and retinoblastoma protein (pRB). p21 acts mainly as a downstream effector of p53, and p16 is an upstream regulator of pRB via inhibition of cyclin-dependent kinases Cdk4 and Cdk6 [34–38]. Based on their action in regulating the cell cycle, p16, p19, and p21 were associated with cancer, aging, senescence, regeneration, and tumor suppression [21,35,39]. Expression of p19 and p21 in embryonic development has been described [21,40–43], while little is known about the expression of p16 during development [44–46]. Therefore, we investigated p16, p19, and p21 RNA expression and p16 protein localization in several organs during embryonic and postnatal development as well as in adult and old mice.

2. Materials and Methods

2.1. Mice and Tissue Preparation

All animal work was conducted according to national and international guidelines and was approved by the local ethics committee (PEA-NCE/2013/106).

Timed pregnant mice (NMRI and C57BL/6) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). The day of vaginal plug was considered embryonic day (E) 0.5. Pregnant mice were sacrificed by cervical dislocation at the indicated time points. Embryonic tissues were dissected, and tissues were used to prepare RNA. The day of birth was considered postnatal day (P) 0.

2.2. Mouse Tissue Samples, Histology, and Immunohistology

For immunohistochemistry, collections of paraffin-embedded whole embryos were used up to E18.5; for later stages, hearts, livers, kidneys, and brains were dissected. Samples from at least three different animals per time point were analyzed. Three-micrometer paraffin sections were used for histological and immunohistological procedures. For p16 immunohistology, after heat-mediated antigen retrieval and quenching of endogenous peroxidase activity, the antigen was detected after antibody application (1:500 dilution, p16 mouse monoclonal antibody, clone 2D9A12; ab54210, Abcam, Cambridge, UK; additionally for some samples, a p16 mouse monoclonal antibody, clone 1E12E10, MA5-17142, Thermo Scientific, Courtaboeuf, France) using the M.O.M peroxidase kit from Vector (Vector Laboratories, PK-2200, Burlingame, CA, USA.) following the manufacturer's instructions. Avidin/Biotin blocking was performed using a kit from Vector (SP-2001). Diaminobenzidine (DAB) served as substrate (Sigma, Saint-Quentin-Fallavier, France). Sections were counterstained with hematoxylin (Dako, Trappes, France) [47,48]. Omission of the first antibody served as a negative control, and additional controls were livers from p16 knock-out mice. Slides were photographed using a slide scanner (Leica Microsystems, Nanterre, France) or an epifluorescence microscope (DMLB, Leica, Germany) connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, MI, USA). For immunofluorescence double-labelling of mouse livers, anti-CD31 rabbit monoclonal antibody (1:2000

dilution, clone EPR17259, Ref: ab225883) from Abcam was combined with the mouse monoclonal anti-p16 antibody (Abcam) using Alexa Fluor 594 donkey anti rabbit and Alexa-Fluor 488 donkey anti mouse secondary antibodies (Jackson ImmunoResearch, Newmarket, Suffolk, UK) [49]. Negative controls were obtained by omission of first antibodies. Images were taken using a confocal ZEISS LSM Exciter microscope (Zeiss, Jena, Germany).

2.3. RNA Isolation, Reverse Transcription, and Quantitative PCR

Using the Trizol reagent (Thermo Scientific, Courtaboeuf, France), total RNA was isolated from brain, heart, liver, and kidneys of four different samples each at different stages of development (embryonic day 10.5, 12.5, 14.5, 16.5, and 18.5; postnatal days 1, 7, 21, 3 months, and 16–18 months) [50]. For E10.5 and E12.5, tissues from 7 embryos each were pooled per sample. For E14.5 and E16.5, organs from 4 embryos were used per sample. First-strand cDNA synthesis was performed with 500 ng of total RNA using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit (#K1672, Thermo Scientific, Courtaboeuf, France), which contains DNase I, RNase inhibitor, oligo (DT) and random hexamer primers. The cDNAs were diluted 10 times in nuclease free water. Two microliters of the diluted reaction product were taken for real-time RT-PCR amplification which was performed using a StepOne Plus thermocycler (Thermo Scientific) and the PowerUp SYBR[®] Green Master Mix (#A25742, Thermo Scientific) or EurobioGreen Mix (GAEMMX02H, Eurobio, Les Ulis, France). For each sample, expression of the housekeeping genes *Gapdh*, *Rplp0*, and β -actin was determined. Three independent housekeeping genes were used as expression for each gene might vary under different experimental conditions [51,52]. Expression for each sample was calculated by subtracting the mean value of housekeeping gene Ct's from the gene of interest Ct using the Δ Ct method [47,48,50,52–58]. Afterward, relative gene expression values were obtained by normalization of each sample against the mean value of all samples at E10.5 to determine differences between the organs and time points investigated. The mean value of all samples at E10.5 was set to 1 for easier illustration as described [50]. Primer sequences are listed in Table 1.

Table 1. Primers used for quantitative RT-PCR.

| Gene of Interest | Oligonucleotide Sequences | References |
|------------------|---|---------------|
| <i>p16ink4</i> | F: AGGGCCGTGTCATGACGTG R: GCACCGGGCGGAGAAGGTA | [59] |
| <i>p19arf</i> | F: CGCTCTGGCTTTCGTGAAC R: GTGCGGCCCTCTCTCAA | [60] |
| <i>p21</i> | F: AATTGGAGTCAGGCGCAGAT R: CATGAGCGCATCGCAATCAC | [61] |
| <i>Tgf-b1</i> | F: AGCTGGTGAAACGGAAGCG R: GCGAGCCTTAGTTGGACAGG | This study |
| <i>Vegfa</i> | F: CTCACCAAAGCCAGCACATA R: AATGCTTCTCCGCTCTGAA | [54] |
| <i>Il-6</i> | F: CACTTCACAAGTCGGAGGCT R: TGCCATGCAACTCTTTTCT | [54] |
| <i>Mmp9</i> | F: CCATGCACTGGGCTTAGATCA B: GGCCTTGGGTCAGGCTTAGA | [54] |
| <i>Gapdh</i> | F: AGGTCGGTGTGAACGGATTG R: TGTAGACCATGTAGTTGAGGTCA | [47,48,54,58] |
| β -actin | F: CTTCTCCCTGGAGAAGAGC R: ATGCCACAGGATTCCATACC | [47,48,54,58] |
| <i>Rplp0</i> | F: CACTGGTCTAGGACCCGAGAAG R: GGTGCCTCTGGAGATTTTCG | [47,48,54,58] |

2.4. Endothelial Cell Magnetic-Activated Cell Sorting (MACS)

Kidneys, livers, hearts, and brains were isolated from four adult (3 months) mice and four old (18 months) mice each. Organs were minced and afterward digested with 0.1 mg/mL of DNase I (10104159001, Roche Diagnostics, Mannheim, Germany) and 1 mg/mL of Collagenase A (11088793001, Roche) in 10 mL of DMEM culture media (ThermoScientific) for 1 h at 37 °C. Digested samples were passed through 70- μ m filters (Smart-Strainers, 130-098-462, Miltenyi Biotec, Paris, France), centrifuged, and washed twice with PBS containing 2% fetal calf serum (FCS) and 0.5 mM of EDTA (ThermoScientific). Cells were re-suspended in 90 μ L of the same buffer (PBS + FCS + EDTA)/ 10^7 cells. Endothelial cells were labelled by adding 10 μ L/ 10^7 cells of magnetic microbead-associated anti-CD31 antibody (130-097-418, Miltenyi) at 4 °C for 15–30 min. Cells were separated via LS column (130-042-401, Miltenyi) pre-washed with 3 mL of PBS + FCS + EDTA and attached to a MidiMACS separator magnet (130-042-302, Miltenyi). Non-endothelial cells were eluted by washes with 3x 3 mL of PBS + FCS + EDTA. Afterward, endothelial cells were eluted by removing the LS columns from the magnetic field and flushing with 6 mL of PBS + FCS + EDTA. Eluted cells were separated as 1/3 for RNA extraction (see above) and 2/3 for protein extraction and quantification.

2.5. Protein Isolation, Quantification, and Western Blot

After endothelial cell sorting as described above, 2/3 of each endothelial and organ cells were taken from the total cell suspension. Cells were centrifuged at 3000 rpm for 10 min at 4 °C. Then, cells were incubated with 100 μ L and 150 μ L of RIPA buffer (Sigma) for endothelial and organ cells, respectively, and kept on ice for 30 min. Afterwards, samples were agitated overnight at 4 °C. The next day, the tubes were centrifuged at 16,000 rpm for 30 min at 4 °C. The total protein containing supernatant was recovered and stored at –80 °C.

Proteins were quantified by colorimetric BCA assay according to manufacturer's instructions (Uptima, Montluçon, France). Samples were diluted 20 times in distilled water and loaded in triplicates of 10 μ L each, in transparent 96-well plates. In addition, BSA standards ranging from 0 to 2 mg were loaded in triplicates (10 μ L). Absorbance was measured at a wavelength of 560 nm in a plate spectrophotometer (Biorad, Marnes-la-Coquette, France).

For Western blotting, 60 μ g of protein in Laemmli buffer was denatured at 95 °C for 5–10 min. Samples were loaded on acrylamide gels (acrylamide/bisacrylamide 37.5/1) and set for electrophoresis. Afterward, proteins were transferred to PVDF membranes (162-0177, Biorad), and the membranes were blocked with 5% milk for 1 h (232100, Difco Skim Milk). p16 was detected using a rabbit monoclonal anti-p16 (Abcam; ab211542) diluted 1:2000 in PBS + 0.05% Tween 20 + 2.5% milk powder (overnight, 4 °C), followed by anti-rabbit peroxidase-labeled secondary antibody addition (Vector Laboratories) diluted 1:2000 in PBS + 0.05% Tween 20 + 2.5% milk powder for 1 h. Then, the chemiluminescence signal was obtained by incubation with the enzyme-specific substrate (RPN2235, Amersham, ECL Select Western blotting detection reagent). Afterward, the membrane was stripped by application of 10 mL of stripping buffer for 15 min (ST010, Gene Bio-Application L.T.D., Kfar-Hanagid, Israel) and washed 5x 5 min with distilled water before a second identical blocking step with milk for the detection of Gapdh as housekeeping protein. A rabbit monoclonal anti-Gapdh antibody (Abcam; ab181602) was used, and the signal was generated with same secondary antibody and substrate mentioned above.

2.6. Statistics

Data are expressed as means \pm standard error of the mean (S.E.M.). Statistical differences were assessed by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test (Graph Pad InStat, GraphPad Software, Inc., San Diego, CA, USA). A *p*-value < 0.05 was considered to reflect statistical significance.

3. Results

3.1. *p16Ink4a*, *p19*, and *p21* mRNA Expression during Embryonic Development and Postnatal Stages in Different Organs

Expression of the mRNAs of the three genes *p16*, *p19* and *p21* was assessed at different ages (E10.5, E12.5, E14.5, E16.5, E18.5, P1, P7, P21, 3 months (adult), and 16–18 months (old)). Experiments were conducted on brain, heart, kidney, and liver tissues from which RNA samples were extracted and quantified by reverse transcription-quantitative PCR, normalized to the respective means of *Rplp0*, *Gapdh*, and β -*actin* housekeeping genes. The results below show the comparison of relative expression levels at all investigated ages in each organ for the three genes of interest (Figure 1) and the comparison of the expression levels of each gene in the different organs at each age (Figure 2).

In the brain, we observed a significant upregulation of *p16* expression beginning at E14.5 until P7 compared to E10.5. Surprisingly, *p16* expression dropped significantly at P21 compared to P7 ($p < 0.05$) to reach the highest levels in old animals. *p21* levels increased significantly around E16.5 during embryonic development and remained at stable levels during further development, increasing less than *p16* in brains from old mice. *p19* expression became upregulated around E14.5 and remained more or less stable during further brain development, showing an increase only in brains of old subjects.

Also in the heart, kidney, and liver *p16* expression increased constantly over time with higher expression levels than *p19* and *p21*, which both showed rather low, fluctuating expression during embryonic and postnatal development. Interestingly, in the heart, *p16* tended to drop between P7 and P21 ($p = 0.070$) comparable to the time course in the developing brain. In old stages, *p16* expression in brain, heart, kidney, and liver was largely increased. Also, *p19* and *p21* levels were upregulated in the respective organs, but to a much lesser extent than *p16* (Figure 1).

To further analyze the relative mRNA expression data for *p16*, *p19*, and *p21* in embryonic development and in postnatal stages, we compared the expression of each gene in the brain, heart, kidneys, and liver at each time point (Figure 2). Expression levels of *p16* were significantly higher at E10.5 in the brain compared to the developing heart, kidney, and liver. At E14.5, E18.5, and P21, the liver displayed the highest *p16* expression compared to the other investigated organs. At adult and old life stages, *p16* expression was high, but not significantly different in the four organ systems studied. *p19* expression did not vary much between brain, heart, kidneys, and liver. An increase of *p19* could be observed in the kidney during development at embryonic day E12.5. Therefore, although *p16* and *p19* are situated in the same chromosomal region, spatiotemporal expression patterns seem to be unrelated. Expression of *p21* increased mostly in the brain during embryonic and postnatal development beginning at E16.5 compared to E10.5, while in the other organs, only temporary very restricted significant alterations were observed. Only in old animals, in all organs a significant increase in *p21* expression was noted (Figure 1). At embryonic day 12.5, *p21* expression was highest in the kidneys, while at E16.5 and P21, it was highest in the brain, compared to heart, kidneys, and liver. Even in old animals, *p21* mRNA levels were elevated in brains compared to the kidneys (Figure 2).

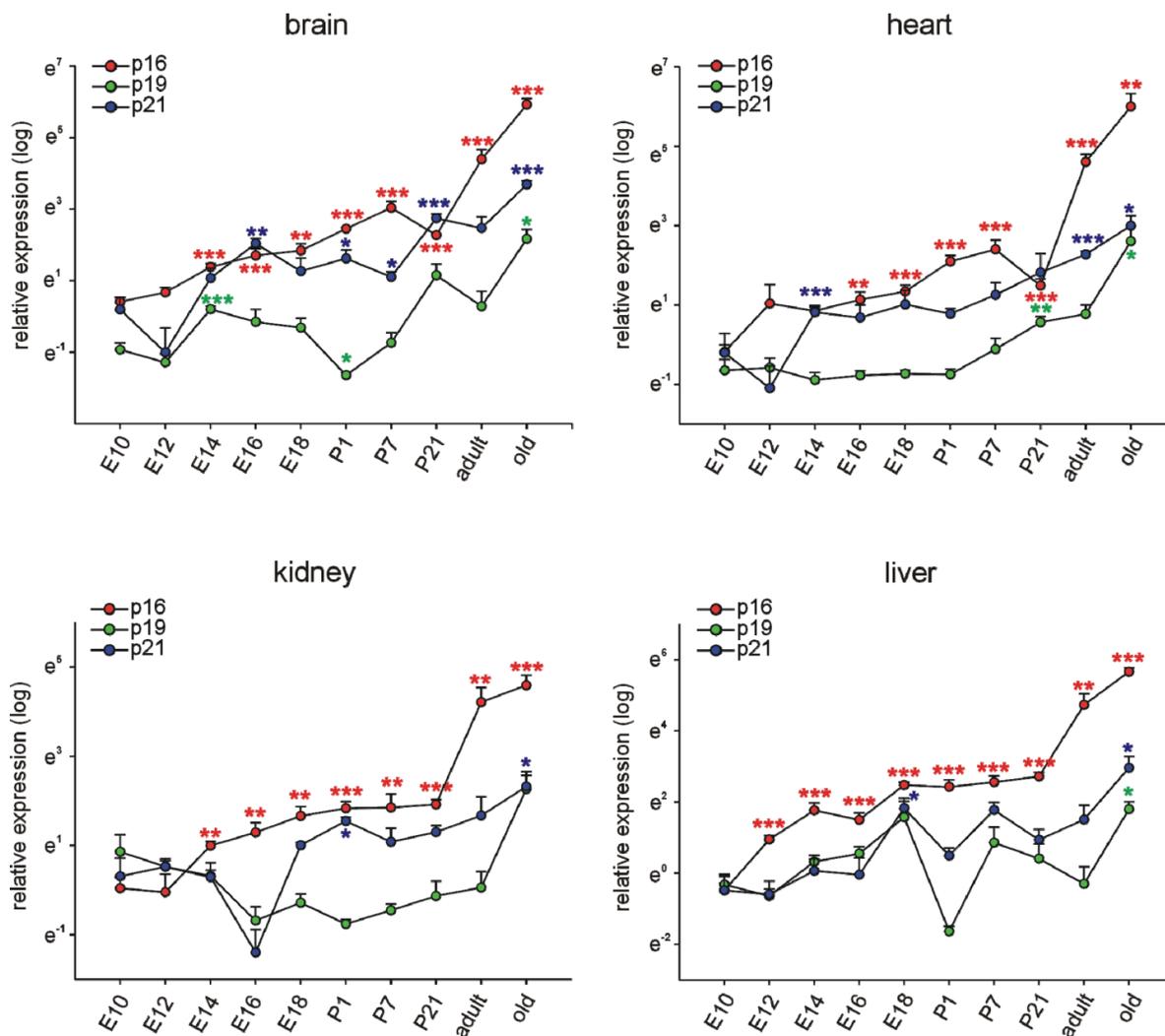


Figure 1. p16, p19, and p21 are differentially expressed during development and adulthood. Quantitative RT-PCRs for p16, p19, and p21 in mouse brains, hearts, kidneys, and livers at different time-points of development and in adulthood ($n = 4$ each, the four samples for E10.5 were each pooled from 7 organs, at E12.5, and 14.5 the four samples were pooled from four organs each). E: embryonic day, P: postnatal day, adult: 3 months of age, old: 16–18 months of age. Expression of each gene was normalized to the respective *Gapdh*, *actin*, and *Rplp0* expression. Next, the average of all organs and samples at E10.5 was calculated. Individual samples were then normalized against this average value (see Materials and Methods for details). Significance was tested for all time points between E10.5 and 18 months. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

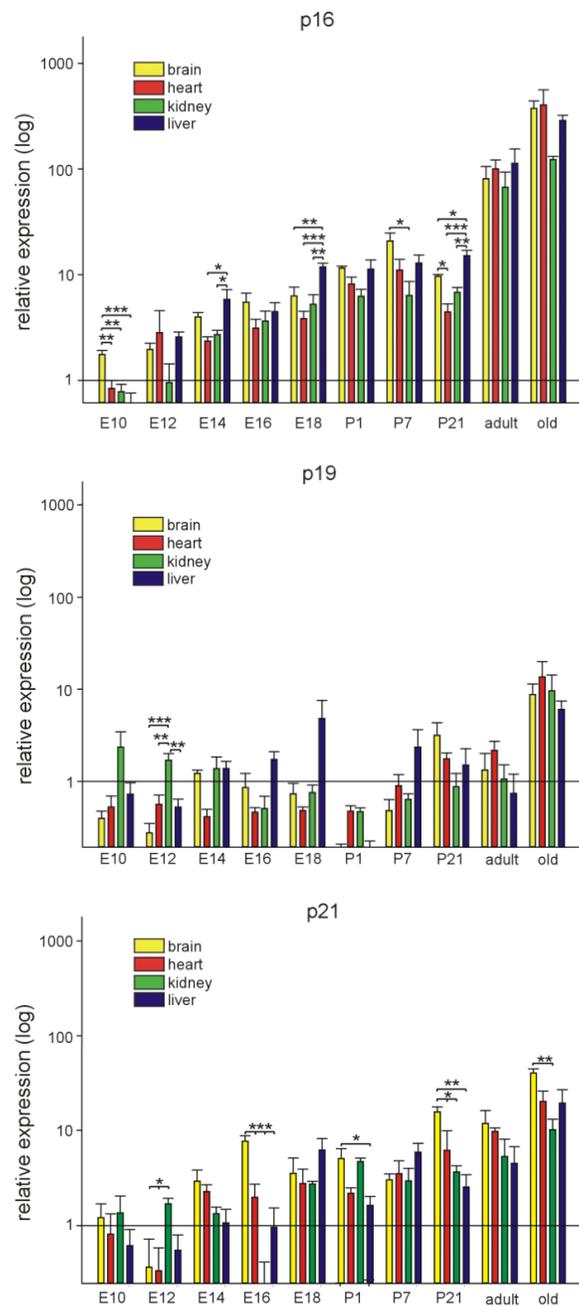


Figure 2. Differential spatiotemporal expression of p16, p19, and p21. Quantitative RT-PCRs for p16, p19, and p21 in mouse brains, hearts, kidneys, and livers at different time points of development and in adulthood ($n = 4$ each, the four samples for E10.5 were each pooled from 7 organs, at E12.5 and 14.5 the four samples were pooled from four organs each). E: embryonic day, P: postnatal day, adult: 3 months of age, old: 16–18 months of age. Expression of each gene was normalized to the respective *Gapdh*, *actin*, and *Rplp0* expression. The average of all organs and samples at E10.5 was calculated and set to 1. Individual samples were then normalized against this average value (see Materials and Methods for details). Significance was tested between the different organs for each time point. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2. Immunohistochemical Investigation of p16 Expression

In addition to quantitative *p16* assessment on the mRNA level, we investigated its expression in the brain, heart, kidneys, and liver at the different time points by immunohistochemistry. In the developing brain, we detected p16 in neuronal cells of the cephalic mesenchyme (E10.5) and the neopallial cortex (E12.5–E18.5). The number of p16-positive neurons increased with differentiation of the brain up to E18.5. (Figures 3 and 4). Neurons of the cortex of old animals displayed a high p16 reactivity. Endothelial cells of the cortex occasionally showed a faint p16 signal (Figure 5), which increased at adult (3 months) and old (16–18 months) stages (Figure 6). Some cardiomyocytes showed p16 expression at early embryonic stages E10.5–E12.5 (Figure 3). With compaction of the myocardium, the number of p16 expressing cardiomyocytes increased from E14.5 to P1. From P7 on, the frequency of p16 expressing cardiomyocytes decreased (Figures 3–5). Endothelial cardiac cells frequently showed p16 expression, with a strong increase in old animals (Figure 6). Similarly to the brain and the heart, more p16-positive cells were found upon differentiation of the kidney. Whereas only faint expression of p16 could be detected in the ureteric bud at E10.5 and E12.5, during formation of the metanephric nephrons and interstitial mesenchyme, a high number of cells expressed p16. The number of p16 expressing cells in the kidney decreased postnatally (Figures 3–5). In old mice, p16 was highly expressed in glomerular structures, composed of podocytes, fibroblasts, and endothelial cells, and in vessels of the kidney (Figure 6). In the hepatic primordium (E10.5–E12.5), very few cells exhibited p16 expression. With the onset of hepatic hematopoiesis, the number of p16 expressing cells in the embryonic liver increased (Figure 3). From P1 on, when the bone marrow becomes the dominant hematopoietic organ, very few cells in the liver expressed p16 (Figure 5). In livers of old mice, we detected a strong signal in endothelial cells compared to hepatocytes (Figure 6). Liver sections with omission of the primary antibody and sections from p16 knockout mice were used as negative controls for the immunostaining (Figure S1). Embryonic p16 expression was confirmed using a different monoclonal antibody (Figure S2).

3.3. Selected SASP Factor Expression

To gain additional insights into the potential relevance and function of *p16* expression during embryonic and postnatal development and in adult and old mice, we measured mRNA expression of *Il-6*, *Mmp9*, *Tgfb1*, and *Vegfa* as selected SASP (senescence associated secretory phenotype) factors [21,62,63] during the time points and in the organs mentioned before. As each of these genes has individual functions at different time points and in different organs, we considered only a concomitant modification of the four genes as indicative of SASP. In agreement with the literature, we observed an increase in the measured SASP factors in all organs in old mice (Figure 7). *Mmp9* was transiently upregulated during late embryonic and early postnatal development in the liver, but as the other investigated genes did not follow the same time course, it might not be indicative for SASP, and the increase in *p16* expression during embryonic and postnatal development alone is not indicative for senescence.

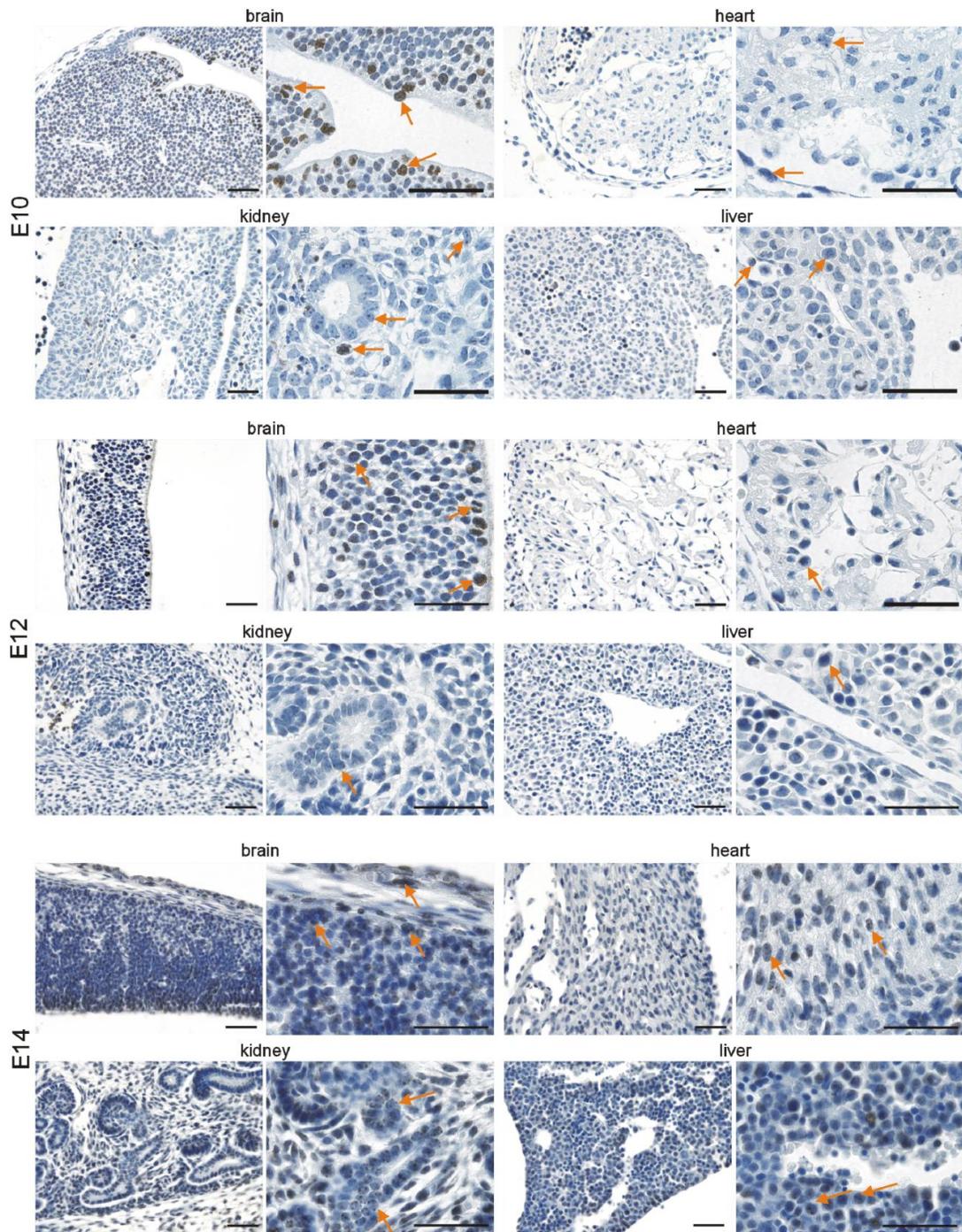


Figure 3. p16 is expressed in the brain, heart, kidneys, and liver during embryonic development (E10–E14). Representative photomicrographs of p16 immunostaining on sections of mouse embryos (3,3' diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining) at different stages before birth. Arrows indicate exemplary p16-positive cells. Scale bars represent 50 μ m.

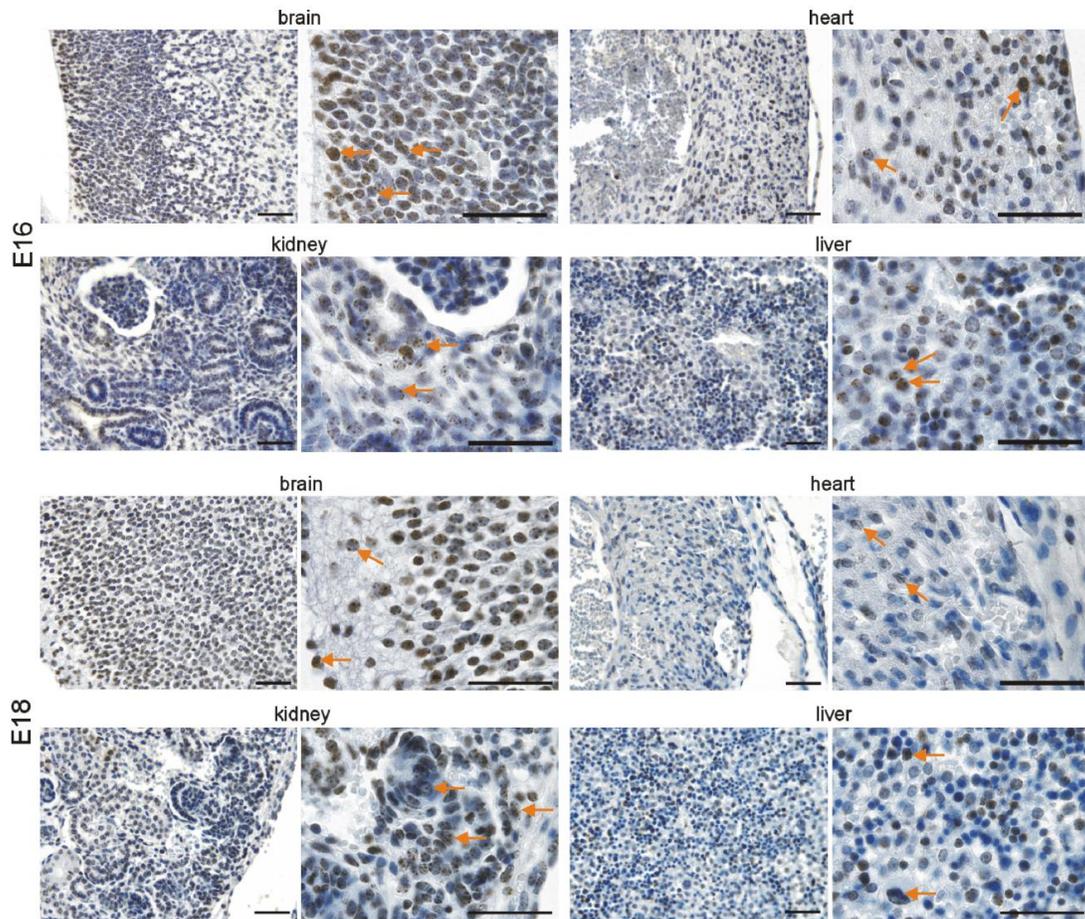


Figure 4. p16 is expressed in the brain, heart, kidneys, and liver during embryonic development (E16–E18). Representative photomicrographs of p16 immunostaining on sections of mouse embryos (3,3′ diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining) at different stages before birth. Arrows indicate exemplary p16-positive cells. Scale bars represent 50 μ m.

3.4. Higher p16 Expression in Endothelial versus Non-Endothelial Cells in the Liver

As our immunohistochemistry approach suggested higher p16 expression in endothelial versus non-endothelial cells in the liver, we confirmed colocalization of p16 with Cd31 by double-labelling and confocal imaging of wild-type mice livers at 3 and 18 months of age (Figure 8a). For quantitative determinations, endothelial and organ liver cells were isolated from adult (3 months) and old (18 months) mice. In adult livers, endothelial vs. non-endothelial p16 mRNA levels tended to be higher, which became highly significant in old livers (Figure 8b). A comparable result was obtained in Western Blot analyses, showing slightly higher p16 expression in endothelial vs. non-endothelial cells in adult livers, and a dramatic increase of p16 expression in endothelial cells from livers of old animals (Figure 8c). Expression levels for p16 mRNA in endothelial versus non-endothelial cells did not differ for the other adult and old organs, except for the hearts of old mice, where organ cells showed higher p16 expression than endothelial cells. Interestingly, although we observed high p16 expression in old liver endothelial cells versus non-endothelial cells, this was not correlated with an increase in the expression of SASP genes except for *Tgfb1*, while *Vegfa* expression was even lower in endothelial cells (Figure S3). This is consistent

with the previous observation that liver endothelial cells in aged mice are highly metabolic active and functional despite high p16 expression [64].

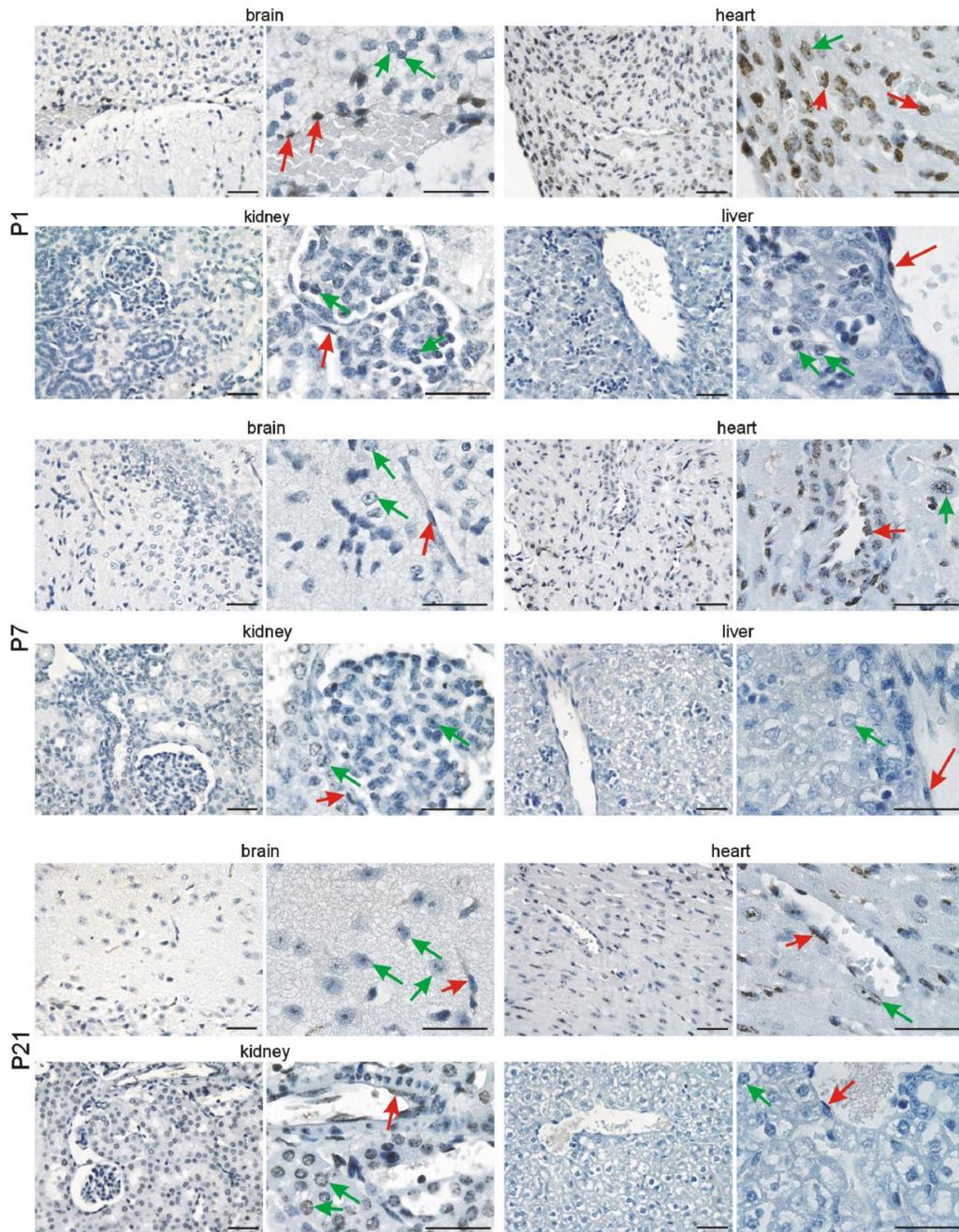


Figure 5. p16 is continuously expressed after birth in vascular and organ cells. Representative photomicrographs of p16 immunostaining for the brain, heart, kidneys, and liver (3,3' diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining) at different stages after birth. Note the persistent expression of p16 in neuronal cells of the brain, cardiomyocytes, tubular and glomerular kidney cells, and hepatocytes (green arrows) and endothelial cells (red arrows). Scale bars indicate 50 μ m.

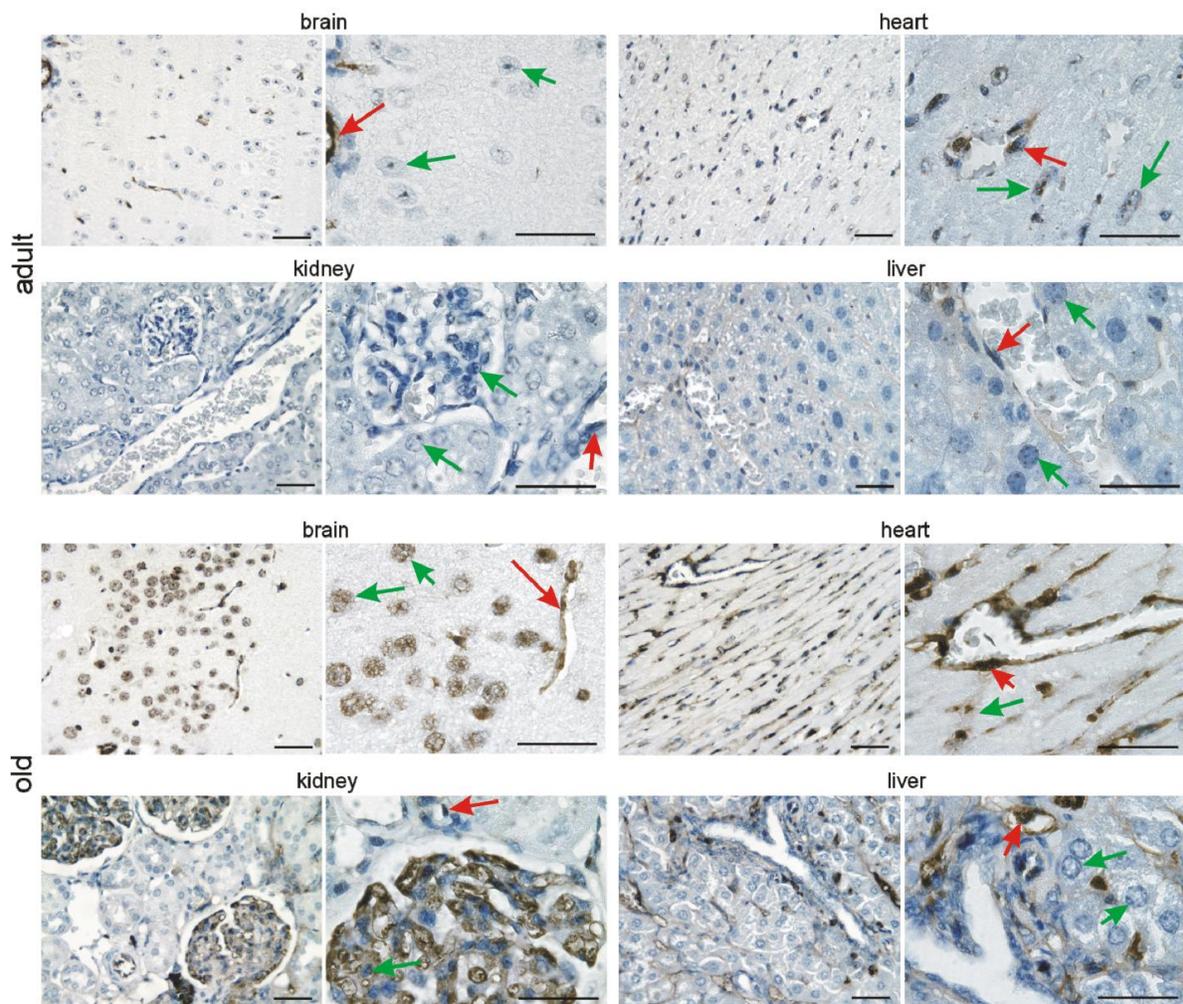


Figure 6. p16 is continuously expressed in adults and increases in old animals. Representative photomicrographs of p16 immunostaining for the brain, heart, kidneys, and liver (3,3′ diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining). Note the persistent expression of p16 in neuronal cells of the brain, cardiomyocytes, tubular and glomerular kidney cells, and hepatocytes (green arrows) and endothelial cells (red arrows), which increases with age. Scale bars indicate 50 μ m.

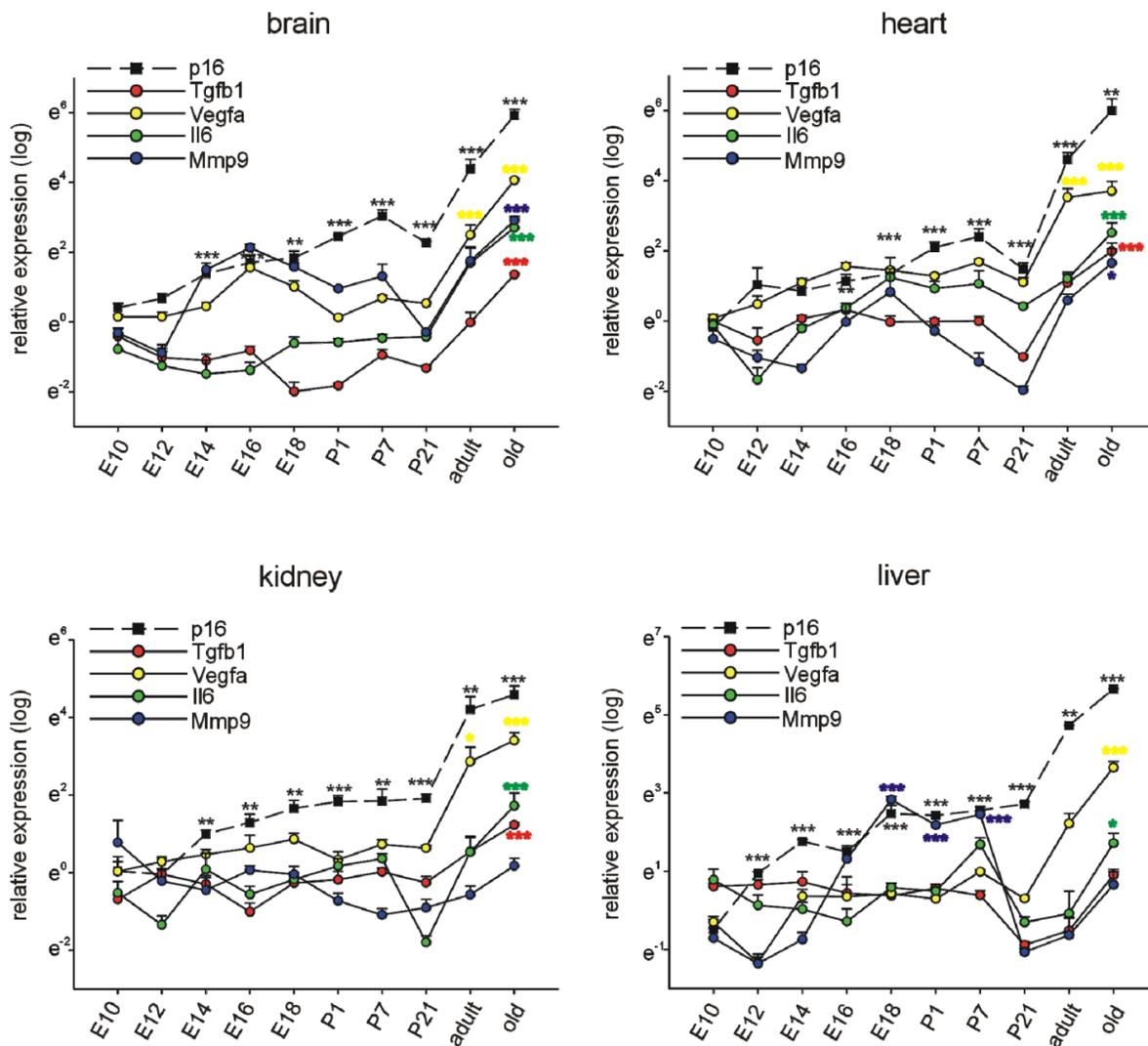


Figure 7. Differential spatiotemporal expression of SASP factors in comparison to *p16*. Quantitative RT-PCRs for *p16*, *Tgfb1*, *Vegfa*, *Il6*, and *Mmp9* in mouse brains, hearts, kidneys, and livers at different time points of development and in adulthood ($n = 4$ each, the four samples for E10.5 were each pooled from 7 organs, at E12.5 and 14.5 the four samples were pooled from four organs each). Expression of each gene was normalized to the respective *Gapdh*, *actin*, and *Rplp0* expression. Next, the average of all organs and samples at E10.5 was calculated. Individual samples were then normalized against this average value. Significance was tested for all time points between E10.5 and 18 months. E: embryonic day, P: postnatal day, adult: 3 months of age, old: 16–18 months of age. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

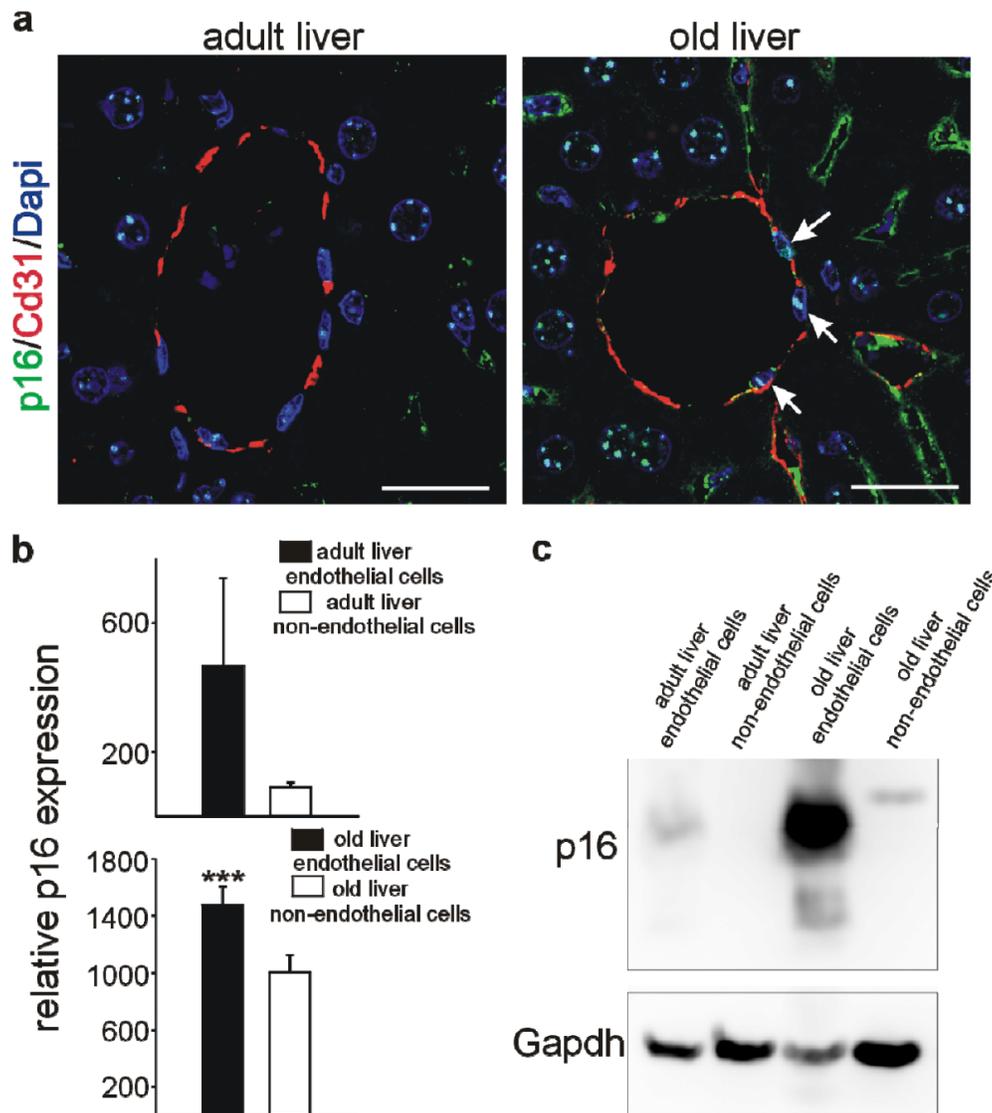


Figure 8. Liver vascular cells express higher levels of p16 than liver cells with aging. **(a)** Confocal images of Cd31 (red)/p16 (green) double-labeling on adult (3 months) (left image) and old (18 months) (right image) liver tissues. Arrows indicate p16/Cd31-positive vascular cells. Scale bars represent 50 μ m. **(b)** Quantitative RT-PCRs for *p16* of sorted liver endothelial cells (black bar) and liver organ cells (white bar) at 3 months (upper panel) and 18 months (lower panel). Expression of p16 was normalized to the respective *Gapdh*, *actin*, and *Rplp0* expression. Data are mean \pm SEM. *** $p < 0.001$. **(c)** Western Blot for p16 in 3- or 18-month-old liver endothelial cells and 3- or 18-month-old liver cells. Gapdh served as standard.

4. Discussion

Our results have shown dynamic and differential expression of *p16* during embryonic and postnatal development as well as in adult and old mice in the brain, heart, kidneys, and liver. Expression of *p16* was varying significantly within each organ during embryonic development in a matter of days. At the same time, *p19* and *p21* did not show such a remarkable variation of expression. We limited the current study to the investigation

of brain, heart, kidney, and liver as these organs already develop at the embryonic time points chosen [65–68] and are relatively easy to isolate. Nevertheless, it is possible that *p16*, *p19*, and *p21* might be expressed in a variety of developing organs. For example, *p21* expression has already been described during embryonic development, i.e., in muscle, nasal epithelium, tongue muscles, hair follicles, epidermis, and cartilage, and was related in part to growth arrest and senescence [42,69,70]. Expression of *p19* has been described in the developing nervous system [44], while *p16* has not been detected during embryonic development in earlier studies [45]. However, the authors of this study did not exclude that *p16* mRNA might be expressed at low levels or restricted sites in embryos. The authors reported an upregulation of *p16* transcripts in organs from 15 month-old mice; however, the original PCR data do not show a specific *p16* signal [45]. Using highly sensitive quantitative RT-PCR and antibody staining methods [48,54–58,71], our finding of a relatively high *p16* expression during development and especially at old stages might be more accurate.

Upregulation of *p16/p19* and *p21* is widely accepted as a marker of aging and senescence [16,72–74]. In human tissue samples, P16 was detected in endocrine and exocrine pancreas, skin, kidneys, liver, intestine, spleen, brain, and lung. Its expression increased in all investigated organs except for the lung with increasing age [75]. We demonstrate here that murine *p16* expression highly increased in all organs investigated between 3 months and 16 months of age. We observed a less pronounced increase in *p19* and *p21* compared to *p16* in old versus adult mice, which is consistent with previous reports in mice and humans [76]. We could not detect organ-specific differences in *p16* expression at 16 months of age, which contrasts with a recent study from Yousefzadeh et al. [77]. This might be explained by the age difference of the animals used in their study which compares mice aged from 15–19 weeks with 120-week-old subjects. A study comparing P16 protein expression by immunohistochemistry in human tissues from young, middle-aged, and old donors confirmed a significant increase of P16 in the liver, kidneys, and brain in old subjects. However, no P16 expression could be detected at all ages investigated in the heart, which might be due to species differences [75].

Regarding expression of *p16*, *p19*, and *p21* and the role of senescence during embryonic development, the literature is more controversial. Unlike *p21*, *p16* and *p19* were reported to be absent in early studies as discussed above [45,69]. Senescence, however, has been detected based on SA- β -galactosidase staining during embryonic development, which seems to depend on *p21* expression [42,43,78]. Interestingly, absence of *p21* was compensated by apoptosis, but still slight developmental abnormalities were detectable [43]. Although these studies focused mainly on *p21*, *p16* loss has also been shown to result in developmental defects in the eye [79]; inactivation of *p16* and *p19* induced cardiomyocyte proliferation [80]; *p16* has been detected in the ventricular and subventricular zones at embryonic and early postnatal stages in the rat brain; SA- β -galactosidase activity and *p16* expression has been detected in regressing mesonephros of quails [81]; *p16* expression in mouse embryos has been detected in motoneurons and the senolytic ABT-263 decreased the number of these cells [82]. Nevertheless, not all highly *p16*-positive cells are necessarily senescent [83,84]. For example, overexpression of *p16* slowed cell cycle progression in the G0/G1 phase and induced erythroid lineage differentiation [85], which might correspond to the early *p16* expression in embryonic mouse livers [86]. Lack of *p16* is linked to increased cardiomyocyte proliferation [80], while lower cardiomyocyte proliferation, differentiation, and specification are required for myocardial compaction [87,88], which coincides with our observed cardiac *p16* expression.

Furthermore, the notion of senescence as an irreversible form of cell cycle arrest, leading to death of the cell [18] has been recently questioned by the observation that cancer cells can escape from the senescence induced cell cycle arrest and gain a highly aggressive growth potential [89]. Highly interesting, it has also been demonstrated that embryonic senescent cells re-enter the cell cycle and contribute later to tissue formation [40]. We observed organ specific variations of *p16* expression, especially by immunohistochemical

localization of p16 protein during development. Expression of p16 in development might reflect its function of slowing down cell cycle progression, a process essential for cell type specific differentiation. Knockout mice for *p16/p19* and selectively for *p16* are prone to tumor development [90–92], but potential developmental defects have not been investigated as the mice are viable and fertile. Thus, re-evaluation of potential developmental defects in mice with inactivation of *p16* or elimination of p16 expressing cells remains an interesting challenge for further studies.

In postnatal livers, p16 has been intensively studied. p16 has protective effects in non-alcoholic steatohepatitis and liver fibrosis through the regulation of reactive oxygen species (ROS) and oxidative stress [93,94]. Specific removal of liver endothelial cells expressing high levels of p16 resulted in fibrosis and liver deterioration, indicating that these cells are required for the maintenance of liver physiology [64]. However, detailed future studies using conditional cell type-specific knockout approaches will be needed to determine the specific function of p16 in liver endothelial cells.

5. Conclusions

Taken together, p16 expression in embryonic stages might reflect an implication in developmental differentiation processes. Further elucidation of the characteristics of p16 expressing cells, using embryos with inactivation or specific elimination of p16 expressing cells will hopefully shed light on the possible functions of p16 in differentiation, in addition to its implication in senescence and aging. Moreover, in aged mice, the significant upregulation of p16 expression in liver endothelial cells points to a selective role in liver endothelial physiology.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cells11030541/s1>, Figure S1: Representative photomicrographs of p16 immunostaining on sections of mouse livers, Figure S2: Representative photomicrographs of p16 immunostaining using a different p16 antibody (clone 1E12E10) on sections of mouse embryos, Figure S3: Expression of selected senescence-associated secretory phenotype (SASP) factors and p16 in endothelial and non-endothelial cells.

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IV. Conclusion and Future Perspective

The Role of p16 in Development and Endothelial Cell Physiology.

In our study, we investigated the expression patterns and potential roles of p16 during embryonic and postnatal development, in adults, and aging in various organs including brain, heart, kidney and liver. We demonstrated that p16 expression varied significantly within each organ during embryonic development, changing rapidly within a matter of days. On the other hand, the expression levels of p19 and p21 showed less remarkable variation during the same developmental stages. While our study was limited to specific organs, it is worth noting that p16, p19, and p21 may be expressed in other developing organs as well.

Moreover, we noticed a significant increase in p16 expression in all organs as mice aged from 3 to 16 months, consistent with the notion that p16 is a marker of aging and senescence. P19 and p21 showed a less pronounced upregulation compared to p16 in older mice.

The role of p16, p19, and p21 in embryonic development senescence has been debated. While early studies suggested that p16 and p19 were absent, senescence has been observed using SA- β -gal staining, which is dependent on p21 expression. Our findings add to this debate by demonstrating the presence of p16 in various tissues during embryonic development. However, the increase in p16 expression during embryonic and postnatal development was not indicative of senescence, as our findings revealed an overall increase in SASP factors only in old mice, indicating other roles of p16 than only its implication in the senescence process.

Moreover, in the liver, a notable finding was the higher expression of p16 in ECs compared to non-ECs, particularly in old animals. This observation suggests a selective role of p16 in liver ECs physiology, which may have implications for understanding the aging process in the liver.

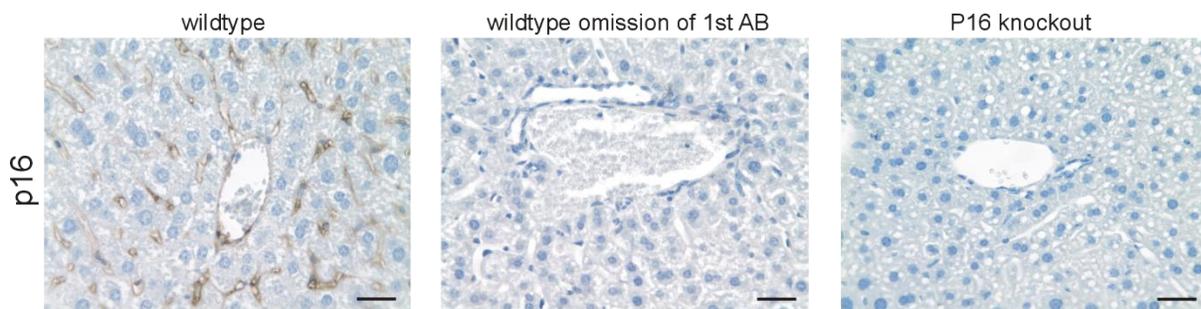
Combined, these results suggest dynamic p16 expression and a potential role in embryonic development. A p16-knockout model or the p16 ablator mouse strains for phenotype observations might be a promising strategy for unveiling the role of p16 in development. In addition, p16 is specifically upregulated in ECs in the liver of aged animals, suggesting a selective role of p16 in ECs of the liver. Therefore, further elucidation of the p16 mechanism in development and liver physiology is required. Most of the different roles and pathological conditions previously described are in general agreement with the basic function of p16 as a negative regulator of the cell cycle. Elevated P16 expression in cells during development coincides with the beginning of

differentiation. For this, lower proliferation is a prerequisite. Additionally, tumor suppression is a logical consequence of cell cycle inhibition. Senescence in old age with dramatically increased p16 expression could be viewed as an extreme case of cell cycle inhibition. In many preclinical models, removal of SnCs or modification of the SASP showed beneficial effects, and first clinical trials with senolytics are promising (reviewed in [434,435]). Nevertheless, different p16 ablator mouse models also showed opposite effects on health span. Thus, it would be highly interesting to directly compare the SASPs of these different models, which might open the way to target the divergent SASP factors for improved health outcome in old people.

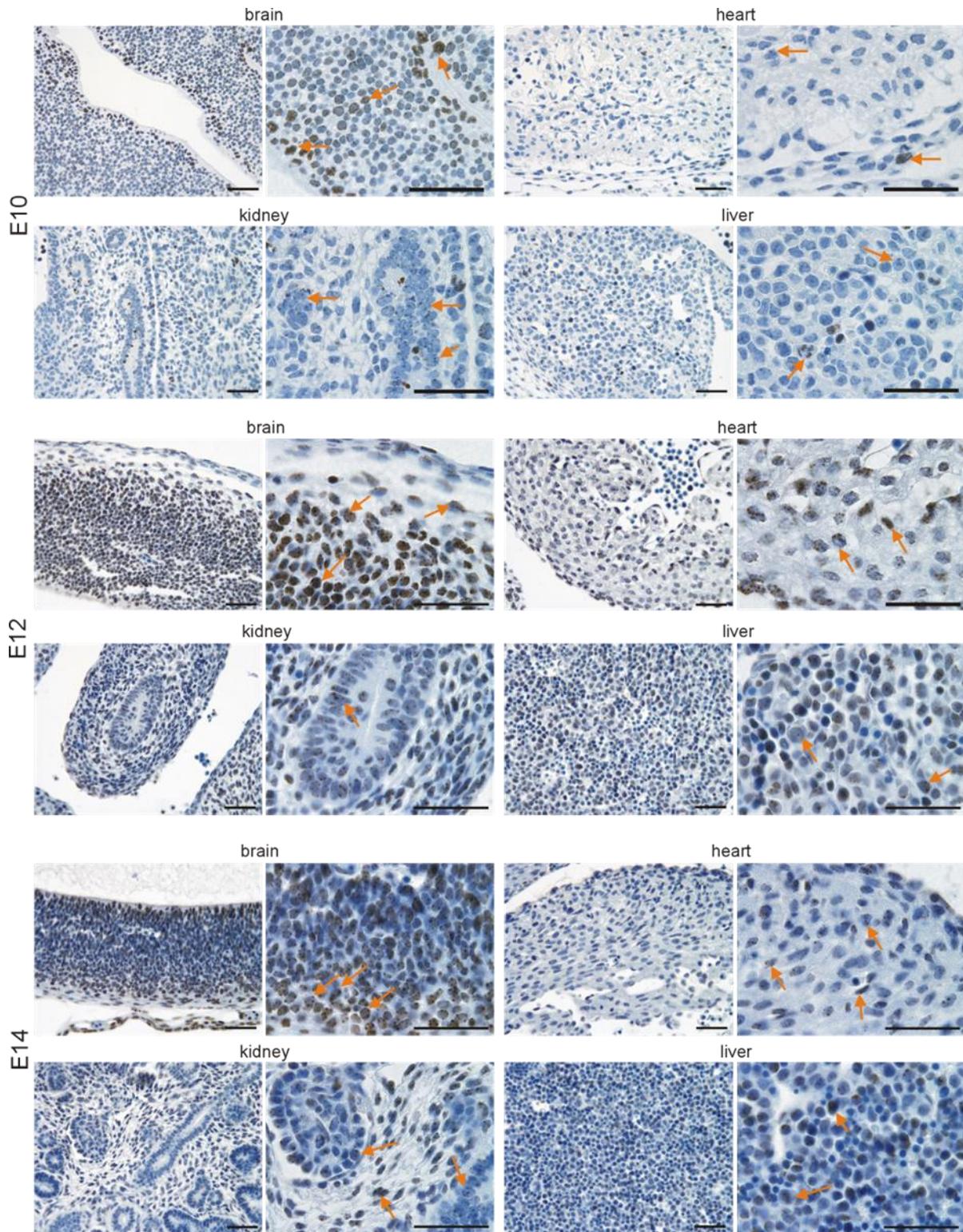
Finally, the high p16 expression, especially in ECs in old age, and the fact that elimination of these p16high cells causes liver damage needs further functional elucidation. One approach would involve conducting single-cell RNA sequencing to compare the genetic profiles of young and old liver ECs. This analysis would help identify differences beyond the augmented p16 expression and provide insights into the broader molecular changes associated with aging in these cells.

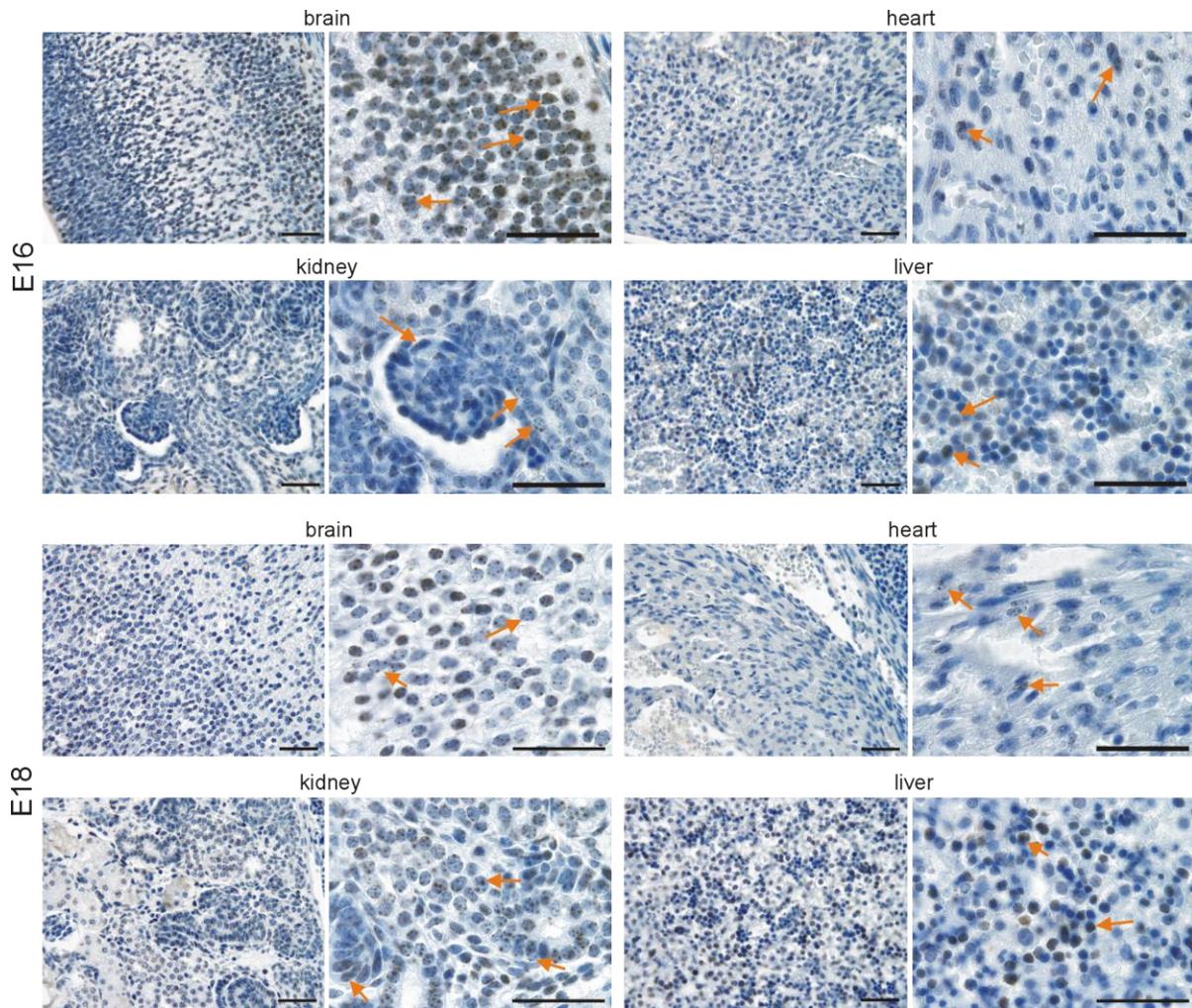
Furthermore, it would be valuable to experimentally interfere with p16 expression levels in ECs. Techniques such as p16 knockdown and overexpression could be employed to precisely determine the implications of p16 loss of function and gain of function in endothelial cell physiology. These manipulations would shed light on the specific role of p16 in regulating endothelial cell function and provide valuable insights for the development of potential targeted anti-aging therapies.

V. Supplementary Material

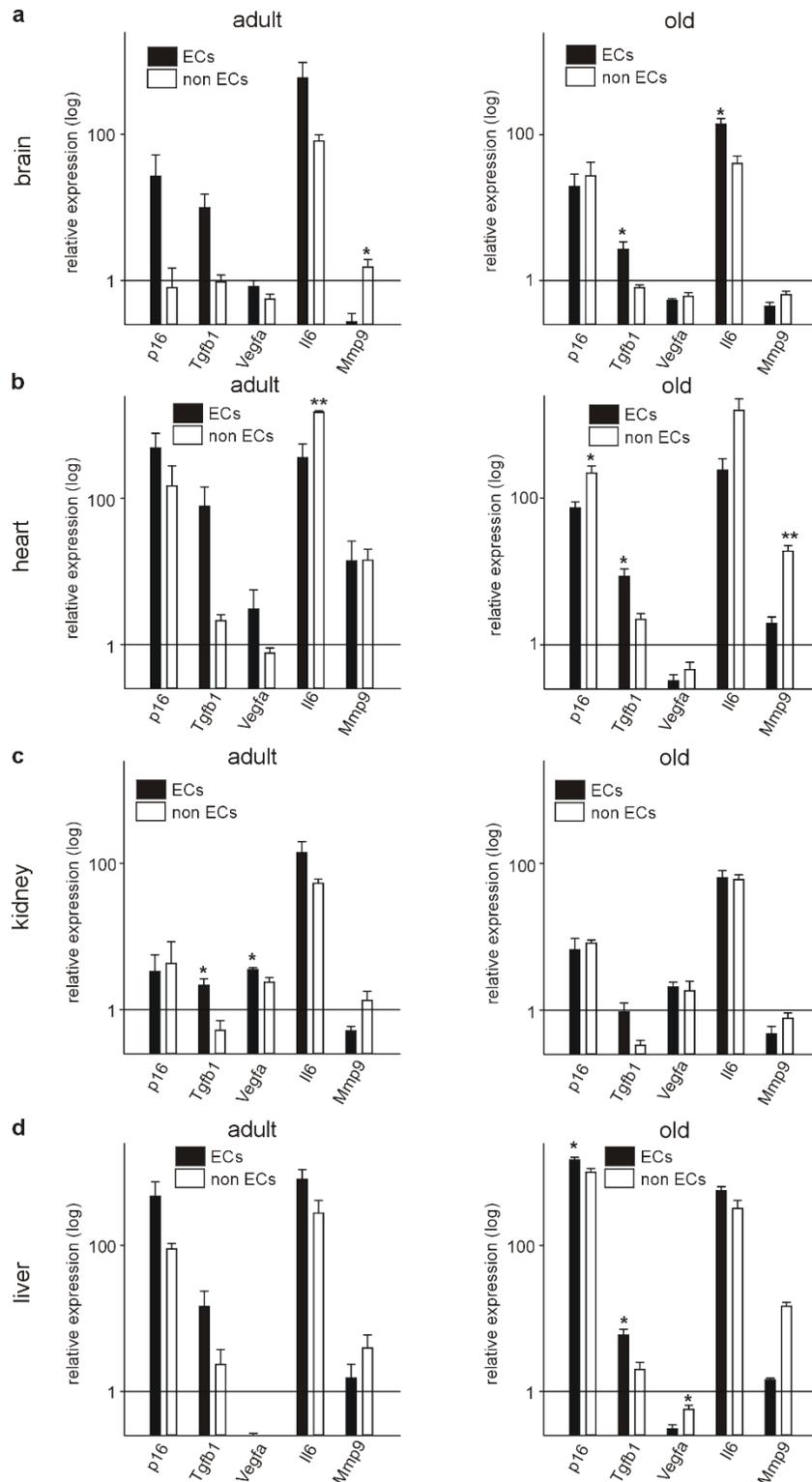


Supplementary Figure 1: Representative photomicrographs of p16 immunostaining on sections of mouse livers (3,3' diaminobenzidine (DAB) substrate, brown, hematoxylin, blue, nuclear counterstaining) showing examples for the staining of a wildtype liver, a section with omission of the first antibody (1st AB), and a p16 knockout liver. Scale bars represent 50 μ m.





Supplementary Figure 2: Representative photomicrographs of p16 immunostaining using a different p16 antibody (clone 1E12E10) on sections of mouse embryos (3,3' diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining) at different stages before birth. Arrows indicate examples of p16 positive cells. Scale bars represent 50 μ m.



Supplementary Figure 3: Expression of selected senescence-associated secretory phenotype (SASP) factors and p16 in endothelial and non-ECs of different organs from adult (3 months) and old (18 months) mice. Quantitative RT-PCRs for p16, Tgfb, Vegfa, Il6, and Mmp9 in mouse brains, hearts, kidneys, and livers. Expression of each gene was normalized to the respective *Gapdh*, *actin*, and *Rplp0* expression. Significance was tested for each gene between endothelial and non-ECs. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.



CHAPTER III





THESIS PROJECT

**The Senescence Marker p16Ink4a - a Player in
Liver Endothelial Cell Physiology
(Outlook – Ongoing Experiments)**



INTRODUCTION



I. Endothelial Cells

1. Definition

Endothelial cells (ECs) are specialized cells that line the inner surface of blood vessels, lymphatic vessels, and the heart, which places them in direct contact with the blood, lymph and circulating cells. They form a monolayer of thin, flat cells, called the endothelium, that traverses every tissue and acts as a barrier between the bloodstream and the surrounding tissues [436–439]. The endothelium has the ability to control the exchange of nutrients, gases, and waste products between the blood and the surrounding cells. It acts as a selectively permeable barrier, regulating the movement of molecules and cells into and out of the bloodstream. ECs also play a variety of important roles in the body, including maintaining vascular integrity and homeostasis, regulating blood flow, mediating immune responses, and promoting angiogenesis [438–442]. Previously, ECs were thought to be a homogeneous cell population. Recent investigations, however, have revealed significant heterogeneity among ECs meaning that they exhibit differential structure, function, and molecular characteristics across different vascular beds, tissue types and organs. This heterogeneity contributes to the specialized roles of ECs in specific tissues and underscores their versatility in responding to local microenvironmental cues, and it is important in a variety of physiological and pathological processes. Over fifty years ago, electron microscopy already revealed the structural heterogeneity of the endothelium, with different types distinguished by their connections, fenestrae (holes), and basement membrane properties. Furthermore, immunohistochemistry studies revealed that different vascular beds express different proteins, emphasizing the ultrastructural and molecular heterogeneity of intact endothelium. It is now recognized that there are numerous types of ECs that differ significantly in structure and, to some extent, function. Furthermore, understanding endothelial cell heterogeneity is critical for understanding their tissue-specific functions, stimuli responses, and contributions to vascular pathologies [436,438,443–446].

There are only a few protein/mRNA markers in the endothelium that show both specific and uniform expression. Some of the leading candidates, such as platelet/endothelial cell adhesion molecule (PECAM)-1 (also known as CD31), are found in monocytes. Thrombomodulin is also found in keratinocytes, trophoblasts, and leukocytes, while VE-cadherin is found in trophoblasts and fetal stem cells [447]. ECs are considered "quiescent" because they do not actively proliferate

and have a lifespan of more than a year. The endothelium in the corpus luteum and uterus, on the other hand, goes through cyclic periods of intense physiological proliferation [448].

Overall, endothelial cell biology has progressed from a simplistic viewpoint to recognizing the heterogeneity in structure and function among different types of ECs in different vascular beds. Therefore, they remain in short for precise definition that would capture their structural and functional complexity (**Figure 17**).

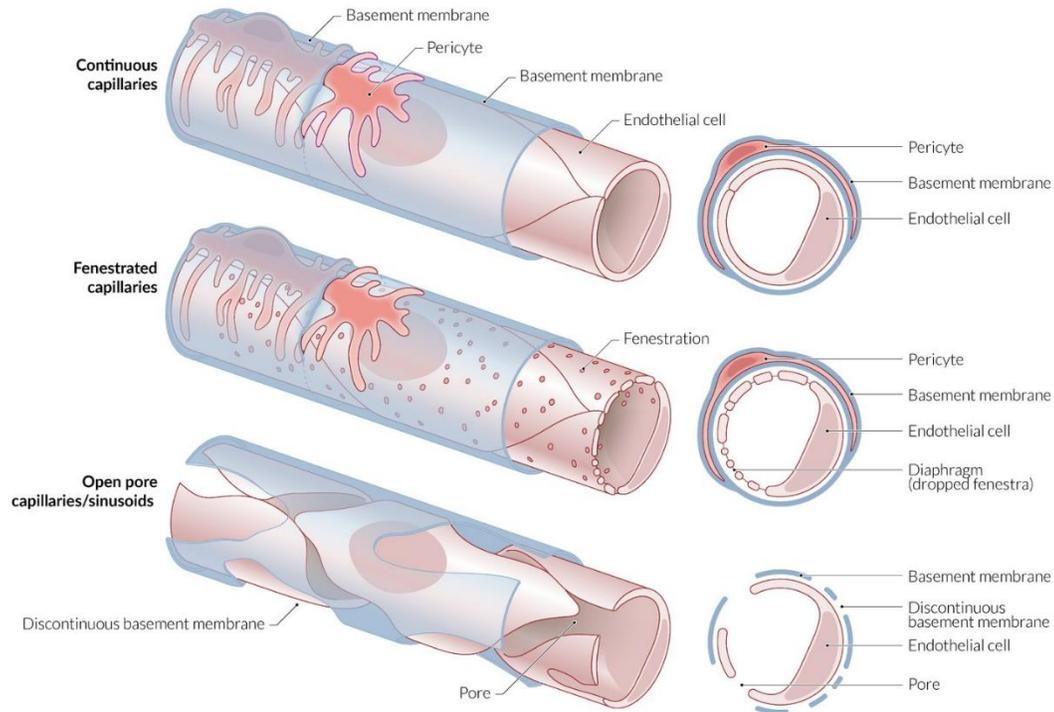


Figure 17. Illustration showing three main types of capillaries. Continuous capillaries, exhibits a seamless barrier formed by a basement membrane and endothelial cells connected through tight junctions. Selectively permeable for small molecules like water, gases, and ions. Fenestrated capillaries, characterized by continuous basement membrane and tightly attached endothelial cells, however they feature existence of pores, allowing passage of larger molecules. Sinusoids are the least common type of capillaries, characterized by intercellular gaps in addition to fenestrations, which render them highly permeable to largest molecules such as proteins and even cells.

Figure taken from www.amboss.com/us/knowledge/blood-vessels.

2. Endothelial Cells Heterogeneity

Endothelial cell heterogeneity has been extensively described in terms of cell morphology, function, gene expression, and antigen composition [438,446]. This heterogeneity is observed not only between different organs but also within the same organ and blood vessel type. Single-cell RNA sequencing (scRNA-seq) studies have revealed important information about endothelial cell

heterogeneity [449–453]. Precisely, single-cell RNA sequencing revealed that, within the same organ, blood vessels differ in gene expression and function; however, the same vascular bed has a common transcriptional signature across tissues [454]. In human lung ECs, for example, distinct subpopulations with diverse gene expression patterns were revealed, implying their functional specialization [453]. Another scRNA-seq study of the mouse brain vasculature discovered multiple endothelial subtypes, each with its own function [449]. Moreover, ECs heterogeneity has been also demonstrated in vascular inflammation and disease. In atherosclerosis, different subsets of ECs are implicated in the regulation of leukocyte adhesion, vascular permeability, and the secretion of inflammatory mediators [455]. In addition to that, vascular development and embryogenesis are highly dependent on endothelial cell heterogeneity. Studies have shown that ECs within developing blood vessels acquire diverse phenotypes, contributing to the establishment of arterial, venous, and lymphatic vascular beds [456]

These examples demonstrate a growing understanding for endothelial cell heterogeneity and its functional importance in a variety of biological processes. Therefore, recognizing the heterogeneity of vascular physiology and pathology is critical for unraveling the complex mechanisms underlying vascular physiology and pathology.

i. Structural Heterogeneity

ECs vary in shape throughout the vascular system. ECs are typically flat, but in high endothelial venules, they can be plump or cuboidal [457,458]. ECs and their nuclei are aligned in the direction of blood flow in straight segments of arteries, but this alignment is not observed at branch points. When arterial vessel segments are rotated and reimplanted, the ECs realign with the new blood flow direction within 10 days. This reversible structural remodeling of ECs in response to hemodynamic shear stress is represented by flow-dependent EC alignment [459–461].

Different structural components of ECs are involved in endocytosis and transcytosis [462,463]. The endocytic pathway includes clathrin-coated pits, clathrin-coated vesicles, multivesicular bodies, and lysosomes. Endocytosis is in charge of directing macromolecules to be degraded or recycled. Scavenger receptors are responsible for receptor-dependent endocytosis of substances such as LDL and transferrin. Clathrin-mediated endocytosis is abundant in LSECs [462,464]. Transcytosis, the transfer of molecules across the endothelium, is facilitated by caveolae and vesiculo-vacuolar organelles (VVOs). Caveolae are flask-shaped vesicles that can be found in the

cytoplasm, while VVOs are collections of vesicles and vacuoles. Caveolae are more numerous in ECs compared to clathrin-coated pits, particularly in continuous non-fenestrated endothelium [463,465,466].

Nonetheless, intercellular junction composition and organization differ across the vascular tree, with large artery ECs having well-developed tight junctions and post-capillary venules having disorganized tight junctions to facilitate inflammation-induced extravasation. Endothelium can be continuous or interrupted. Non-fenestrated continuous endothelium is found in certain organs' arteries, veins, and capillaries. Fenestrated continuous endothelium is found in exocrine and endocrine glands, gastric and intestinal mucosa, glomeruli, and specific renal tubules that require increased filtration or transport. Discontinuous endothelium, as distinguished from liver sinusoidal endothelium, has larger fenestrations and gaps within individual cells [467–469] (**Figure 17 & 18**).

ii. Functional Heterogeneity

ECs also demonstrate functional heterogeneity. Basal and inducible permeability, which involve the transfer of fluids, solutes, and macromolecules, are differentially regulated across the vascular tree. The mechanisms of permeability include paracellular, and transcellular routes mediated by tight junctions, caveolae, VVOs, and trans-endothelial pores. The site-specific expression of adhesion molecules and receptors contributes to leukocyte trafficking, primarily occurring in postcapillary venules. The transmigration of lymphocytes occurs across specialized postcapillary venules known as high endothelial venules [438].

Heterogeneity is also observed in the hemostatic functions of ECs. The regulatable production of procoagulant and anticoagulant molecules varies across the vascular tree, contributing to site-specific hemostatic balance. Changes in the expression of these molecules during inflammation differ between vascular beds [470,471]. Functional heterogeneity among ECs has been also demonstrated in the context of angiogenesis. Endothelial tip cells, found at the leading edge of sprouting blood vessels, exhibit distinct morphological and molecular features compared to stalk cells behind them. These tip cells are responsible for guiding the formation of new blood vessels [472].

ECs demonstrate functional diversity, reflecting their adaptation to specific tissue requirements as well [473]. For instance, ECs in the microvasculature of the lungs play a crucial role in gas

exchange, while those in the kidneys are involved in filtration and reabsorption [474]. ECs in lymphatic vessels are specialized in facilitating lymphatic drainage and immune surveillance [475]. Moreover, ECs in pathological conditions, such as inflammation or tumor angiogenesis, can acquire unique functions, such as increased expression of adhesion molecules or enhanced angiogenic capacity [444]. Furthermore, the hemodynamic forces experienced by ECs vary across different vascular beds, and this heterogeneity can influence their phenotype and function. Shear stress, the frictional force exerted by blood flow, is a key hemodynamic force that impacts endothelial cell behavior. High shear stress areas, like arterial regions, promote the production of nitric oxide (NO) and inhibit inflammatory responses. In contrast, low shear stress areas, such as curvatures or branch points, may be prone to endothelial dysfunction and atherosclerosis development [476]. Additionally, the epigenetic modifications, such as DNA methylation and histone modifications, can contribute to the heterogeneity of ECs. These modifications can influence gene expression patterns and cellular responses. For example, differences in DNA methylation patterns have been observed between arterial and venous ECs, contributing to their distinct functional properties [477,478] (**Figure 18**).

ECs express a diverse array of molecules and receptors that are involved in mediating various functions. This molecular heterogeneity is observed in the differential expression of adhesion molecules, receptors, and signaling molecules. For example, ECs in different vascular beds express distinct combinations of adhesion molecules, such as selectins, integrins, and immunoglobulin superfamily proteins, which enable tissue-specific interactions with circulating cells. Gene expression profiles further contribute to endothelial cell heterogeneity. While there are few endothelial-specific genes constitutively expressed throughout the vascular tree, many genes are limited to subsets of ECs or expressed in a spatially and temporally distinct manner. Proteomic approaches have revealed vascular bed-specific phenotypes, highlighting the existence of site-specific molecular profiles [447,479,480] (**Figure 18**).

Overall, endothelial cell heterogeneity is a fundamental aspect of vascular biology, playing crucial roles in various physiological processes and disease pathogenesis.

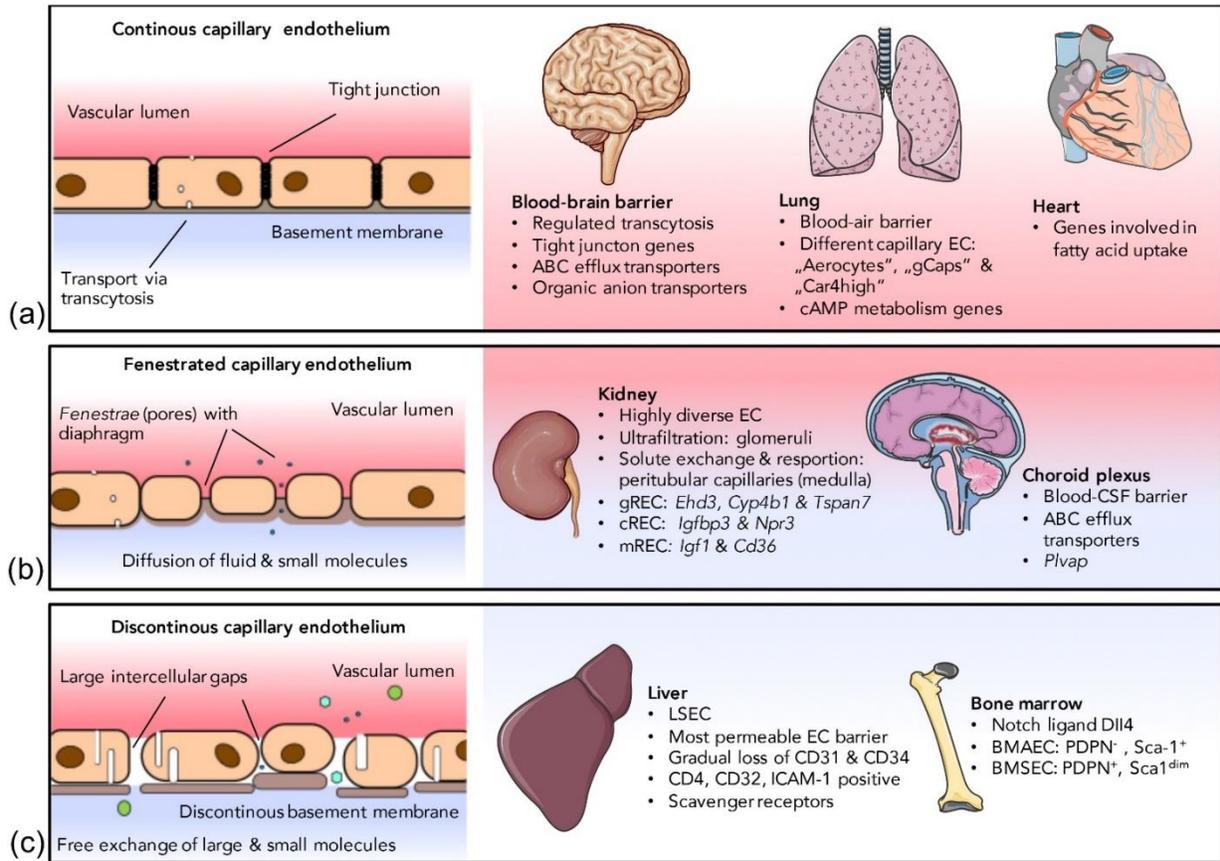


Figure 18. Illustration representing the structural, molecular, and functional heterogeneity of endothelial cells, in different tissues. (a) Capillaries in organs like the brain, lung, and heart feature a tightly continuous endothelium along with a continuous basement membrane. The regulated process of transcytosis allows molecules to traverse this continuous endothelium in a controlled manner. (b) In the kidney and choroid plexus, the endothelium is fenestrated, facilitating the diffusion of fluids and small molecules. (c) The capillary endothelium in the liver and bone marrow is discontinuous, characterized by intercellular gaps and a disjointed basement membrane, promoting unrestricted exchange of molecules.

Hennigs JK et al. *Vascular Endothelial Cells: Heterogeneity and Targeting Approaches*. Cells. 2021.

3. Functions

i. Physical Barrier and Permeability

ECs form a continuous layer along the inner surface of blood vessels, which acts as a physical barrier that separates the blood compartment from the surrounding tissues and prevents the direct contact of blood components on one side of the endothelium, such as red blood cells and platelets, with underlying tissues on the other side [438,441,445]. This barrier is a critical key for regulating the exchange of substances between the blood and tissues. It exhibits selective permeability of specific molecules and cells while restricting others based on the size and charge of molecules. Small molecules like oxygen, carbon dioxide, and nutrients can diffuse through the ECs, facilitating the exchange of gases and nutrients between blood and tissues. However, larger molecules and cells, such as white blood cells, are tightly regulated and require specific mechanisms to pass through the endothelium. ECs facilitate the transport of essential nutrients, such as glucose and amino acids, from the blood into the surrounding tissues [481]. Conversely, they aid in removing waste products, such as carbon dioxide and metabolic byproducts, from the tissues into the bloodstream for elimination from the body [482]. Therefore intact endothelial physical barrier under normal conditions is governed by several mechanisms that control its integrity and function to maintain homeostasis [483]. However, dysregulated barrier and subsequently increased permeability due to several factors such as inflammation, oxidative stress or environmental cues, underlie several pathological condition and contribute to increased morbidity [441,444,483–487].

ii. Hemostasis

Besides their role in adjusting vascular permeability, ECs are also critical in orchestrating the balance between preventing excessive bleeding and avoiding inappropriate blood clot formation and therefore maintaining vascular hemostasis. To maintain an anticoagulant state under normal conditions and promote procoagulant activity when needed, ECs express and release various molecules that control the coagulation cascade [437–439,441,443,488].

ECs actively regulate the coagulation cascade, platelet function, and vascular tone for adequate blood flow and clotting through several anticoagulant factors, such as tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), antithrombin (AT) and heparin-like molecules. ECs synthesize and release tissue factor pathway inhibitor (TFPI), which is a potent inhibitor of the

tissue factor (TF)-dependent coagulation pathway. TFPI blocks the activation of factor Xa and the subsequent coagulation cascade, thereby regulating clot formation [489]. On the other hand, Thrombomodulin binds to thrombin and converts it from a procoagulant to an anticoagulant enzyme, activating protein C, which in turn inhibits clotting factors and limits the coagulation response. Moreover, ECs also produce and release antithrombin III (ATIII), a serine protease inhibitor that inactivates several coagulation factors, including thrombin (factor IIa) and factor Xa. ATIII is key regulator of clot formation, it initiates a counter mechanism to prevent excessive coagulation that could be detrimental [490,491].

However, in case of damaged endothelium, ECs release tissue factor (TF), which is a key activator of the coagulation cascade's extrinsic pathway. This pathway produces thrombin, which converts fibrinogen to fibrin, the primary component of blood clots [491]. Furthermore, ECs also mediate adhesion and aggregation of platelets via the secretion of the von Willebrand factor (vWF) which binds to the glycoprotein (Gp) Ib-IX-V adhesion receptor on platelets surface leading to thrombus formation [492,493]. In the absence of injury, ECs act to maintain platelet function and prevent inappropriate platelet aggregation and adhesion, through the secretion of nitric oxide (NO) and prostacyclin (PGI₂), which act as vasodilators and inhibit platelet activation and aggregation [494,495]. Additionally, these molecules support the preservation of vascular tone and inhibit platelet activation on endothelial surfaces [437–439,441,443,488].

Finally, when the clot had met its purpose and no longer needed, ECs promote fibrinolysis, the process of clot resolve, by the synthesis and release of tissue-type plasmin activator (tPA), which converts plasminogen into plasmin. Plasmin is a serine protease that in turn degrades fibrin clot promoting fibrinolysis [496–498] (**Figure 19**).

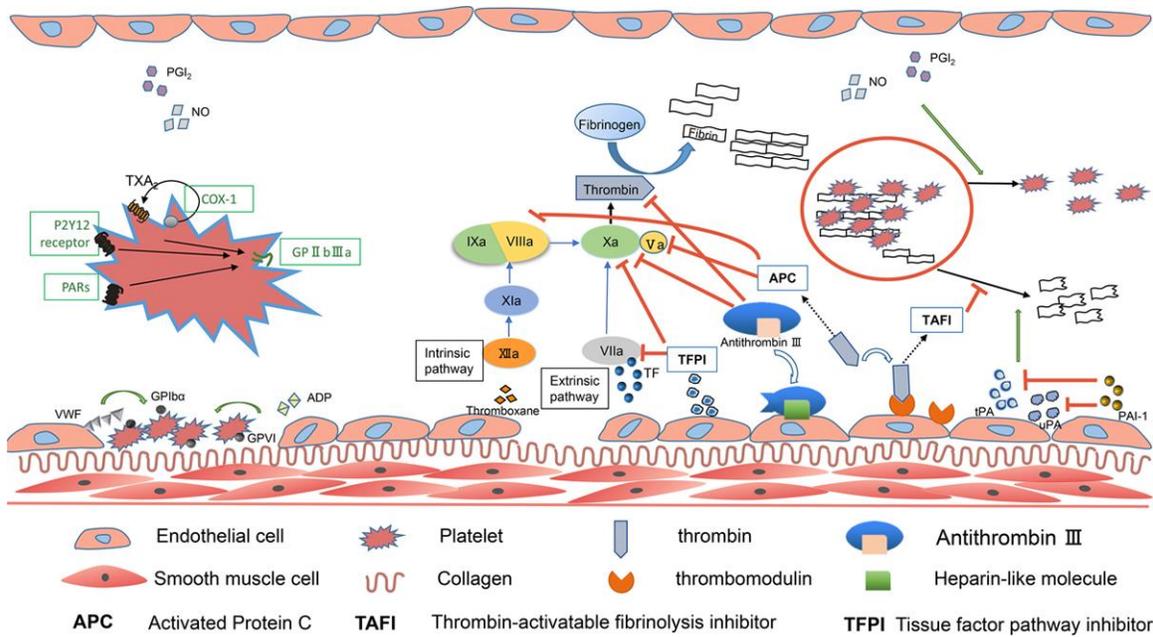


Figure 19. The endothelium plays a central role in the regulation of thrombosis, influencing the activation of platelets, coagulation, and thrombolysis. Key components of these processes, including activated protein C (APC), glycoproteins (GP), nitric oxide (NO), plasminogen activator inhibitor (PAI), tissue factor (TF), tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and Von Willebrand factor (VWF), converge at the endothelial interface. The endothelium actively modulates these pathways, highlighting its crucial involvement in the finely tuned orchestration of thrombotic events.

Wang M et al. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2018.

iii. Vascular Tone

Vascular tone, which describes the extent of blood vessel constriction or dilation, has a direct impact on blood flow, blood pressure, and tissue perfusion [499]. Blood vessel diameter and blood flow are ultimately determined by various vasoactive substances that affect the relaxation or contraction of vascular smooth muscle cells, and which are actively produced and released by ECs. Endothelial nitric oxide (NO) is one of the most prevalent vasodilators produced by ECs nitric oxide synthase (eNOS) activity. eNos catalyzes the redox reaction of the amino acid L-arginine and molecular oxygen. In this reaction, L-arginine is oxidized by molecular oxygen into NO and L-citrulline [500–503]. Once released, NO diffuses into adjacent vascular smooth muscle cells and stimulates guanylate cyclase-mediated production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP, in turn, induces smooth muscle relaxation and consequent vasodilation [501,502]. Prostacyclin, also known as PGI₂, is another vasodilator produced by ECs and it increases cAMP levels in vascular smooth muscle cells, leading to relaxation and vasodilation [504,505]. Moreover, ECs produce other factors known as endothelium-derived hyperpolarizing factors (EDHF) in addition to NO and prostacyclin that causes vascular smooth muscle cells to hyperpolarize, resulting in relaxation and vasodilation [506]. On the contrary, endothelin-1 is a vasoconstrictor produced by ECs, which acts on smooth muscle cells to increase intracellular calcium levels, leading to contraction and vasoconstriction [507]. In addition, through the production and release of various vasoactive substances ECs influence the diameter of blood vessels, thereby determining blood flow and tissue perfusion [499]. ECs are also able to respond to mechanical forces, such as shear stress, generated by blood flow. Shear stress leads to the release of vasodilators like NO, promoting vascular dilation and regulating blood flow [508]. This characteristic function of ECs has implicated them in several physiological and pathological conditions. For instance, vasodilation mediated by ECs helps to reduce vascular resistance, which in turn lowers blood pressure. Conversely, diminished vasodilation due to ECs impairment can lead to increased vascular tone, and lead to hypertension. Moreover, endothelial dysfunction induced low NO bioavailability is a common feature of several cardiovascular diseases, such as atherosclerosis, hypertension, and diabetes [509].

iv. Vasculogenesis and Angiogenesis

Vasculogenesis and angiogenesis are two distinct biological processes which are at the basis of new vessel formation. Vasculogenesis is characterized by the emergence of blood vessels in early embryonic development. During initial stages of development, vasculogenesis entails the differentiation and proliferation of ECs, called angioblasts, within previously non-vascularized tissues. These ECs then gather and fuse to form a primitive tubular network, which further develops into vascular plexus. This nascent network encompasses major embryonic vessels like the aorta and major veins, along with an intricate network connecting these vessels, resembling a honeycomb structure [445,510–512]. Vasculogenesis begins by recruiting specialized mesodermal precursors at specific locations in the mesoderm. In amniotes, the first blood vessels arise in the extra-embryonic mesoderm of the yolk sac, where mesenchymal cells aggregate to create blood islands. These islands give rise to primitive blood cells and endothelial cell precursors. Other vascular progenitors originating from intra-embryonic mesoderm contribute to the formation of key embryonic vessels such as the dorsal aorta, cardinal veins, and vitelline plexus [510–512]. Studies on quail and chick embryos demonstrated two distinct lineages of endothelial precursor cells. The first one is derived from the paraxial mesoderm, which gives rise to angioblasts, while the second bipotential haemangioblastic lineage originates from the splanchnopleural mesoderm and is able to differentiate into both endothelial and hematopoietic cells [510,512,513]. The two lineages are molecularly associated by shared gene expression such as CD34, CD133, PECAM-1, c-Kit, and Sca-1. Various molecular factors orchestrate the vasculogenesis process. Among these, the vascular endothelial growth factor receptor-2 (VEGFR-2 or Flk-1) that is essential for lineage differentiation from mesodermal precursor. Embryos lacking VEGFR-2 exhibit defects in both hematopoietic and angioblastic lineages and its loss was also associated with severe vascular defects. The fibroblast growth factors, VEGF, and other signaling molecules play paracrine roles, with endoderm producing VEGF, and mesoderm-derived angioblasts expressing VEGF receptors [510,512,513]. The endoderm plays a role in regulating vasculogenesis in adjacent mesoderm. Signaling molecules like Sonic hedgehog (Shh) are implicated in the interaction between endoderm and angioblasts. While the endoderm seems to influence the assembly of angioblasts into vascular tubes, its role may vary between different species. As vasculogenesis progresses, additional processes like angiogenesis contribute to the expansion and refinement of the vascular network [510,512] (**Figure 20a**).

Angiogenesis is the process of formation of new blood vessels from preexisting ones. Angiogenesis is a fundamental physiological process that is involved in developmental stages, tissue repair, wound healing, and other biological processes [514–516]. Although the development of functional blood vessel demands a meticulous coordination and signal exchange between different cell types, ECs are the primary cellular constituents responsible for constructing and organizing a new blood vessel [517–519]. Angiogenesis is initiated by pro-angiogenic stimuli such as hypoxia or inflammation, which induces the release of growth factors responsible for ECs activation. The vascular endothelial growth factor (VEGF) is one of the most prevalent factors that is produced by tumor cells, stromal cells, or macrophages. Vegf binds to its receptor (VEGFR-2) on ECs' surface and activates their proliferation and migration [514,520]. Once activated, ECs secrete proteolytic enzymes termed matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, into the surrounding extracellular matrix (ECM), which aim to degrade ECM components and creating space, allowing ECs sprouting and migration through tissue [521]. Proliferating ECs form an elongated structure called “sprout”. Sprouts with continuous proliferation of ECs extend into the tissue and are guided by gradients of pro-angiogenic factors, such as VEGF, and endothelial tip cells at the sprout edge, specialized for sensing this gradient and directing the migration process. Afterwards, sprouting ECs form the tubular structure of the vessel, which is termed lumen formation or vessel maturation through which the blood flows, followed by recruitment of pericytes and smooth muscle cells to stabilize the blood vessel. The newly formed vessel integrates into the circulation allowing to support tissue with blood, oxygen, and nutrients exchange. Once oxygen levels are elevated and tissue oxygen demand is met, this marks the termination of angiogenesis [520,522–525] (**Figure 20b**).

Therefore, vasculogenesis indicates a complex tightly regulated process that involves differentiation, proliferation, migration, and arrangement of ECs from distinct avascular mesodermal sites to form a primitive blood during early development. On the other hand, angiogenesis encompasses the arousal of new capillaries and blood vessels from pre-existing ones. Angiogenesis is a crucial event for several physiological processes such as reproduction, development, and tissue repair, but it can also be aberrant in many pathological conditions. Whether physiological or pathological both essentially require ECs involvement [510,511,514].

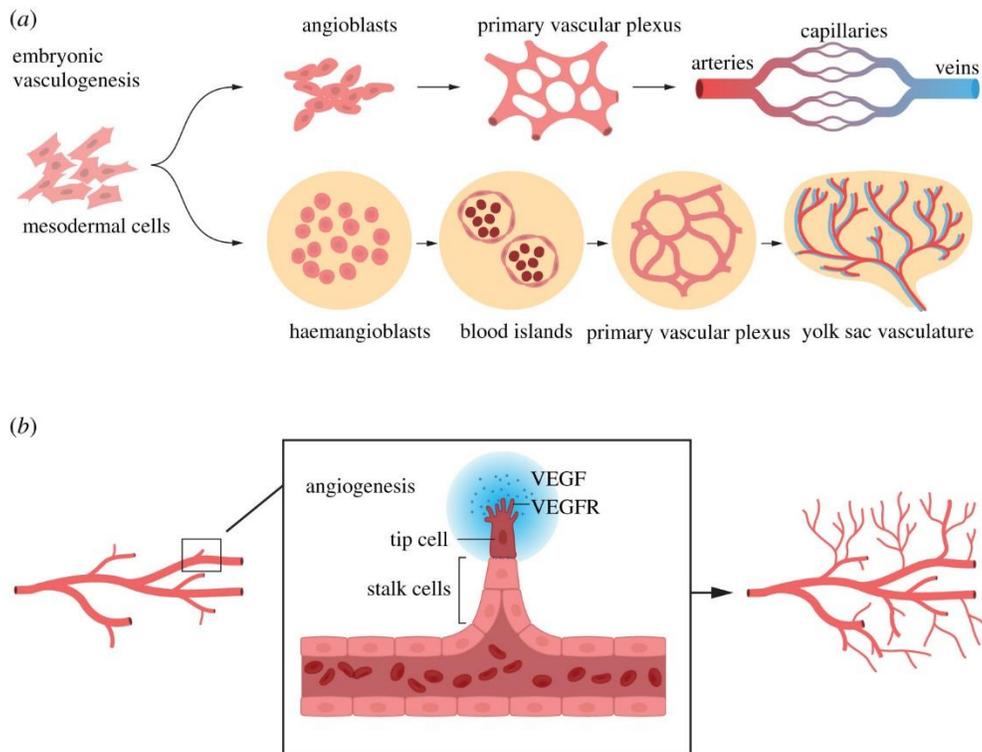


Figure 20. Vasculogenesis and Angiogenesis. (a) Vasculogenesis: Mesodermal cells differentiate into angioblasts during embryonic and extraembryonic vasculogenesis, forming primary vascular plexuses. In embryonic vasculogenesis, vessels acquire arterial and venous identities along an axis. Extraembryonic vasculogenesis in the yolk sac involves the initial formation of blood islands from haemangioblasts, contributing to a primary vascular plexus. (b) Angiogenesis: New blood vessels sprout from an initial vascular tree, with a leading tip cell responding to VEGF via VEGFRs. Stalk cells follow, forming the lumen of the sprouting vessel.

Del Gaudio F et al. Open Biology. 2022.

v. Immune and inflammatory regulation

Evolving studies have demonstrated ECs role in the immune system and inflammatory response beside their functions in regulating vascular permeability, tonicity, hemostasis, and angiogenesis. The fact that they form a dynamic interface between the circulating leukocytes and tissues, implicates them in regulation of immune response and control of immune cells and regulation of inflammatory response [442].

A wide range of immune receptors, chemokine, adhesion molecule and cytokines that are involved in triggering innate immune response were found to be expressed by ECs. Toll like receptors (TLRs) are one notable group of receptors expressed by ECs, and they are specialized for recognition of conserved pathogen-associated molecular patterns (PAMPs) present on the surface of various pathogens including bacteria, viruses, and fungi. Among them, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9 that were found in various tissue-specific ECs. On the contrary, TLR7, TLR8, and TLR10 are not present in resting ECs but can be induced under inflammatory conditions [526–528]. In addition to immune receptors, ECs were also found to express various adhesion molecules that facilitate immune cell recruitment and extravasation during inflammation. For instance, E-selectin, is one of the genes that are highly specific to ECs, and it is primarily found in postcapillary venules in activated endothelium. However, P-selectin expression is induced in different tissues in response to inflammatory mediators like histamine and lipopolysaccharide. Moreover, numerous vascular and nonvascular cell types express ICAM-1 and VCAM-1, which are involved in leukocyte adhesion and transmigration, can both be upregulated in response to lipopolysaccharide [529–531]. Additionally, ECs secrete chemokines like monocyte chemoattractant protein-1 (MCP-1/CCL2) and interleukin-8 (IL-8/CXCL8), which recruit immune cells to the sites of inflammation. They also release cytokines that contribute to inflammation and immune cell activation, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) [442,529,532] (**Figure 21**).

When ECs encounter pathogens or PAMPs and TLRs are activated by their respective agonists, they induce a proinflammatory response in ECs mediated by NF- κ B and MAPK signaling. TLRs activated ECs undergo structural changes in their adhesion molecules such that E selectin, P selectin, ICAM and VCAM to increase vascular permeability, and produce proinflammatory cytokines such as IL 1 β , IL 6, IL 8 and TNF α which are essential for triggering innate immunity and recruitment and activation of leukocytes [442,526,527,529,533,534]. Specific TLRs, such as

TLR1/2, TLR3, and TLR4, elicit a strong pro-inflammatory response when activated by their respective agonists, leading to the secretion of various cytokines and factors that promote inflammation and coagulation [527,533,534]. It's worth noting that TLR responses in monocytes and ECs function differently because they use different NF- κ B and MAPK signaling pathways [535]. TLR2 is expressed at a lower baseline level in ECs but is strongly upregulated in response to its ligand, possibly acting as a safeguard against excessive endothelial activation and subsequent complications [533]. Furthermore, vascular TLR2 and TLR4 are not only activated by PAMPs but also in response to tissue damage, such as the presence of extracellular histones, which results in increased tissue factor production and may affect the equilibrium between the risk for sepsis and the formation of advantageous local microthrombus [536]. They are also involved ECs activation in hyperglycemia-mediated proinflammatory stimulus, which promote leukocyte adhesion and ROS production [537]. Furthermore, ECs possess other pattern recognition receptors that possess a nucleotide-binding oligomerization domain NOD1, NOD2, and the retinoic acid-inducible gene RIG-I, which play roles in detecting bacterial components and viral RNA structures, triggering NF- κ B signaling [538,539]. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which is also expressed in ECs, is a member of a lectin-like receptor family, encoded in the natural killer (NK) gene complex along with dectin-1 and CLEC-1. Endothelial LOX-1 promotes uptake of oxidized LDL, which is generated under oxidative stress during inflammatory events. It's activation is related to exacerbated inflammatory response, endothelial dysfunction, and atherosclerosis [540,541] (**Figure 21**).

Overall, these pattern recognition receptors in ECs play critical roles in immune responses, inflammation, and vascular homeostasis, and their dysregulation can contribute to various pathological conditions.

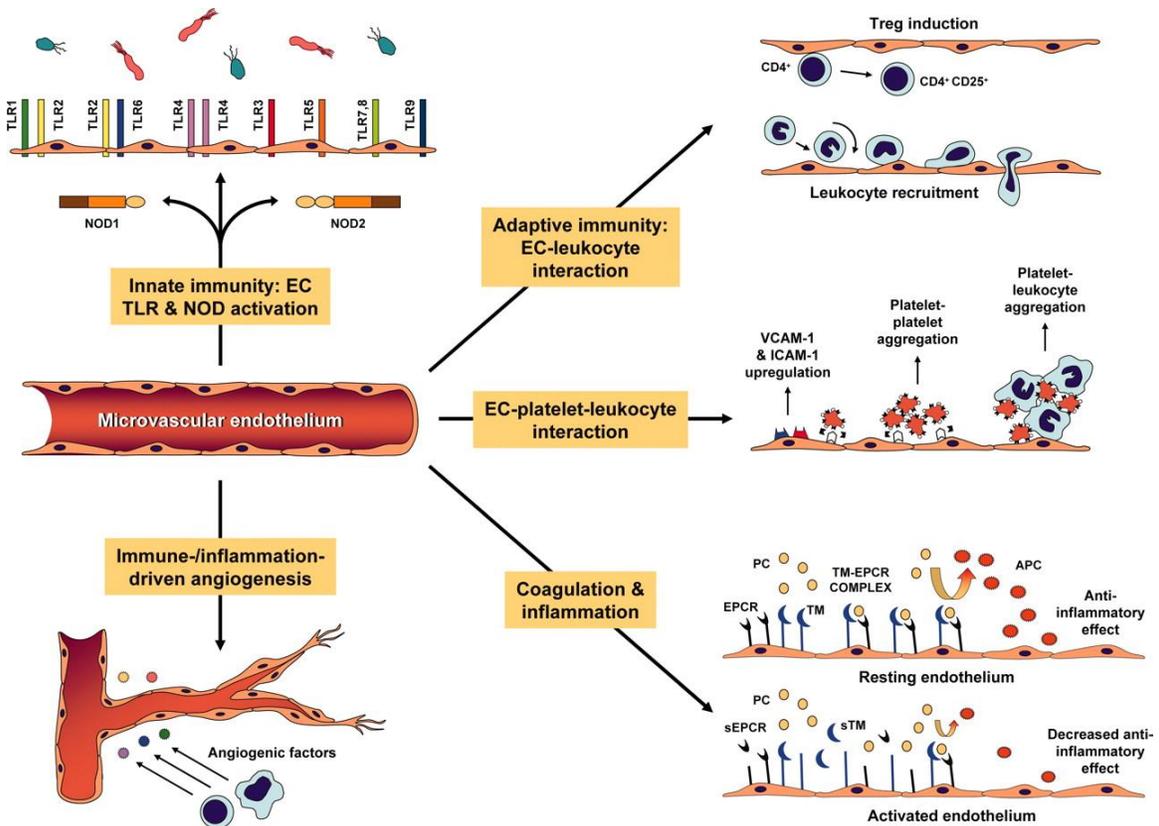


Figure 21. Schematic overview illustrating the diverse functions and cellular interactions orchestrated by microvascular endothelial cells (ECs) that influence innate and adaptive immunity, coagulation, and inflammation. Protein C (PC), activated Protein C (APC), soluble Endothelial Protein C Receptor (sEPCR), and soluble Thrombomodulin (sTM).

Danese S. et al. *The Journal of Immunology*. May 2007.

II. Liver Endothelial Cells

Aside from general vascular cells, the liver contains a subset of ECs with distinct morphology that form the inner lining of the liver sinusoids. These cells are known as liver sinusoidal endothelial cells (LSEC) which represent around 15 to 20% of total liver cells and 3% of liver volume. LSEC are a unique type of ECs due to their specific characteristics and localization within the liver sinusoids. Liver sinusoids are specialized capillaries that allow a direct contact between blood stream and hepatocytes in the liver parenchyma. The key feature of facilitated blood-parenchymal exchange is the traversing open pores system, or fenestrations, with approximate diameter of 100-150 nm. These pores give rise to a discontinuous sinusoidal endothelium and act as an open channel between sinusoidal blood and the underlying space of Disse. Besides that, LSEC lack diaphragm and basal lamina underneath, which allows a bidirectional transfer of substances to and from parenchymal hepatocytes [542–547] (**Figure 22**).

Moreover, unlike other ECs, LSECs are characterized by a highly active endocytic capacity, with a uniquely efficient clathrin-mediated endocytosis, reflected by the numerous endocytic vesicles and specialized endocytic receptors. This particular endocytic capacity enables them to effectively uptake various substances from blood such as macromolecules and waste products. Together, the fenestrae and the endocytic characteristics and the absence of basal lamina, makes LSECs a distinguished type of ECs as a selective sieve of blood-parenchymal components exchange and an effective scavenger that clears bloodborne waste macromolecules [543,544,547].

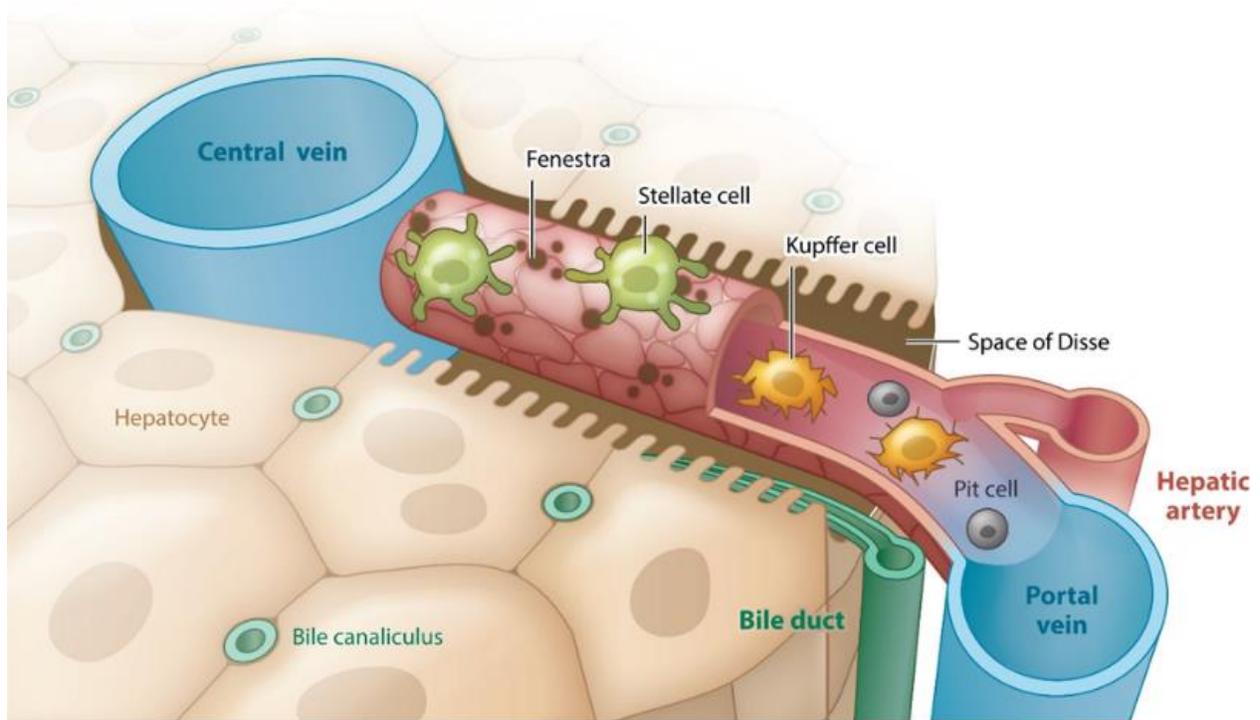
LSECs are deficient of a specific characteristic marker on their surface, however they share common surface makers with other ECs such as CD31, ICAM-1, VCAM-1 and VEGFR2. Therefore, a combination of different markers is required for their identification. ICAM-1 is found on LSECs surface and increased upon inflammatory conditions, while CD31 and VCAM-1 are induced [542]. CD146, also known as Melanoma Cell Adhesion Molecule (MCAM), is a cell adhesion molecule that is expressed on the surface of various cell types, including ECs. In mice, CD146 has been found to be strongly expressed in LSECs compared to other cells and it can be efficiently used as a specific marker to label and isolate LSECs by magnetic cell sorting [542,548]. Liver ECs are a vital component of the liver's microvasculature and play essential roles in maintaining normal liver function under physiological condition [542]. Through their fenestrations, liver ECs are able to exchange nutrients, oxygen, waste products, and other substances, essential for liver function and maintenance between the blood and hepatocytes.

Therefore, they act as an efficient semipermeable biofilter and scavenger system in the liver. In addition, liver ECs are also implicated in the detoxification and clearance function of the liver. They interfere in the clearance of harmful toxin, such as endotoxins released by bacteria in addition to their role in drug detoxification and metabolism through expressing various drug-metabolizing enzymes and transporters. Moreover, LSEC are key regulators of blood flow and consequently of fibrogenesis, via controlling hepatic stellate cells in the space of Disse [542,544–547,549–551]. Besides that, LSECs are considered as gatekeepers of hepatic immunity. LSECs have both innate and adaptive immunological functions, including antigen presentation and the critical task of balancing tolerance and effector immune responses. During inflammatory liver diseases, these cells have a significant impact on the composition of hepatic immune populations, facilitating the movement of specific leukocyte subsets through the use of various adhesion molecules and chemokines. Furthermore, LSECs play an important role in regulating the cellular crosstalk that drives the progression of chronic liver diseases, eventually leading to fibrosis and carcinogenesis. Targeting LSECs may hold promise as a potential therapeutic approach for managing inflammatory liver conditions due to their role in initiating immune responses and contributing to the progression of liver disease [552].

On the contrary, dysfunction of LSECs contributes to plenty of liver pathologies. For instance, impaired LSECs were associated with inflammation in nonalcoholic fatty liver disease (NAFLD) due to an abnormal balance between vascular endothelium-derived relaxing and contracting factors, leading to impaired vasodilation during increased blood flow [553]. Moreover, LSEC endothelial dysfunction promotes inflammation by activating Kupffer cells (KCs) in NAFLD [554]. KCs, the resident macrophages in liver sinusoids, can be activated by various factors, including lipopolysaccharide (LPS), damage-associated molecular patterns, and lipids such as free fatty acids (FFAs) and oxidized lipoproteins [555–557]. Under physiological conditions, LSECs maintain KCs quiescence through NO-dependent pathways. However, reduced endothelial NO bioavailability under pathological conditions promotes KCs activation, resulting in the upregulation of pro-inflammatory factors TNF- α and IL-6 via NF- κ B activation [558]. Furthermore, autophagy dysregulation in LSECs under pathological conditions contributes to inflammation. Impaired autophagy in LSECs has been observed in NASH patients, leading to decreased fenestration and increased expression of pro-inflammatory chemokines in mice fed with a HFD [559,560]. Additionally, dysfunction of LSECs contributes to steatosis by increasing

intrahepatic vascular resistance [558,561–563]. NO plays a crucial role in regulating liver lipid content and fatty acid synthesis [558,564–566]. NO-deficient eNOS knockout mice exhibit elevated liver triglyceride levels and lipid droplets, which can be improved by increasing NO availability treatment [558,566]. Dysfunctional LSECs facilitate bone marrow-derived macrophages (BMM) recruitment through promoting KC activation and the release of CCL2, crucial for macrophage recruitment [558,567]. Additionally, LSECs increase the expression of adhesion molecules, such as ICAM-1, VCAM-1, VAP-1, E-selectin, and CD31, directly promoting leukocyte recruitment [568–570]. LSECs also produce pro-inflammatory mediators, including IL-1, IL-6, TNF- α , and CCL2, further promoting inflammatory cell activation, adhesion, and migration [570–572] (**Figure 23**).

Therefore, it is important to note that liver ECs are key regulators of liver function and maintenance under physiological condition. However, like other liver cells, they can be affected by various liver diseases, such as cirrhosis, viral hepatitis, and fatty liver disease. Disruptions in their normal functions can contribute to liver dysfunction and pathology. Therefore, understanding the roles of liver ECs is crucial for develop therapies and interventions to treat liver diseases effectively.



AR Ehrlich A, et al. 2019.
Annu. Rev. Biomed. Eng. 21:219–39

Figure 22. Diagram of the hepatic sinusoid. This fenestrated capillary, measuring 275 μm in length, consists of sinusoidal endothelial cells (in red). Enveloping the capillary are liver-resident pericytes known as stellate cells (in green). Meanwhile, the interior of the vessel is patrolled by resident macrophages, referred to as Kupffer cells (in yellow), and natural killer cells, known as pit cells (in gray).

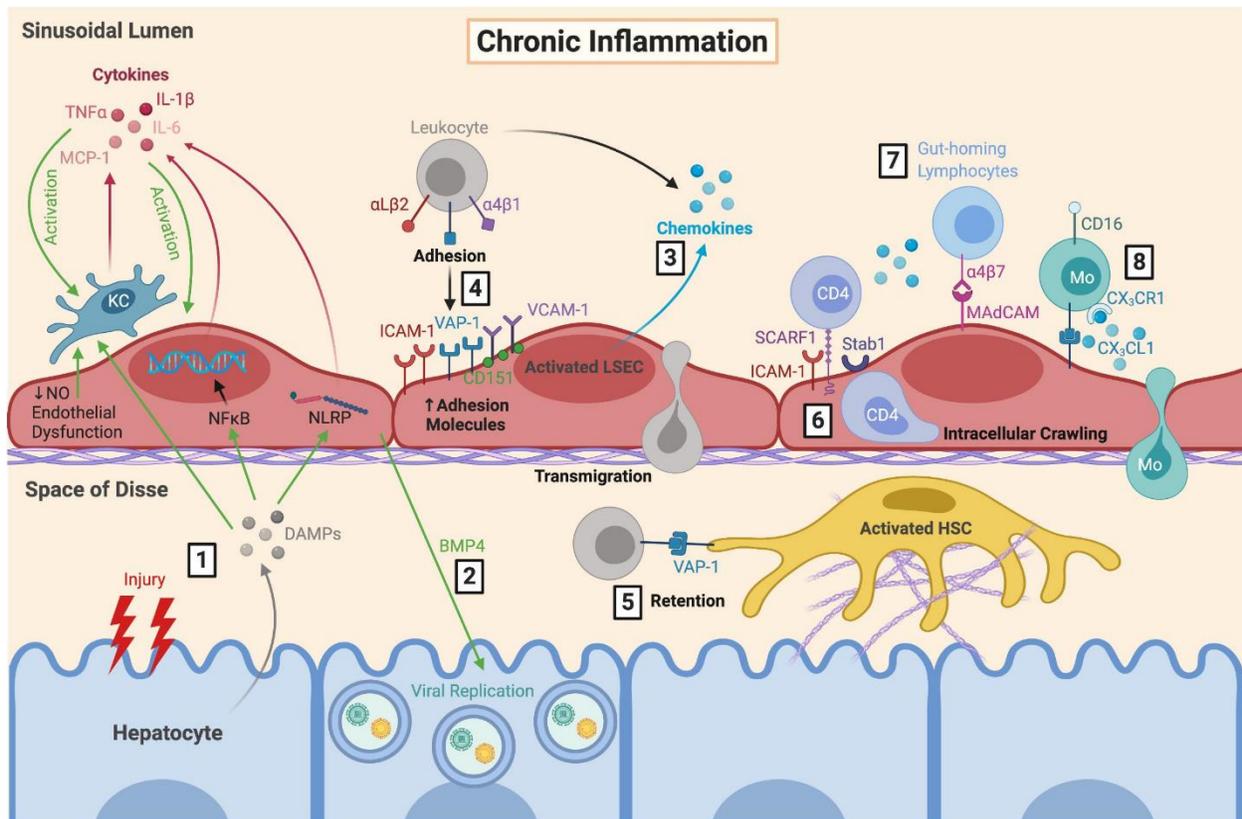


Figure 23. Liver sinusoidal endothelial cells (LSECs) play a pivotal role in orchestrating the immune microenvironment during chronic inflammation. (1) Persistent hepatocyte injury leads to the release of damage-associated molecular patterns (DAMPs), sensed by Kupffer cells (KCs) and activating them to produce pro-inflammatory cytokines. DAMPs also trigger cytokine release from LSECs via NFκB and inflammasome signaling, exacerbating LSEC and KC activation, especially in the presence of endothelial dysfunction. (2) LSEC-produced BMP4 may enhance viral replication, worsening hepatocyte damage in chronic viral infections. Activated LSECs (3) secrete chemokines and (4) upregulate adhesion molecules, promoting leukocyte recruitment, adhesion, and transmigration. (5) Vascular adhesion protein 1 (VAP-1) expression by hepatic stellate cells (HSCs) retains leukocytes in the space of Disse. (6) CD4+ T cells perform lateral intracellular crawling between LSECs via SCARF1-mediated adhesion, involving ICAM-1 and Stabilin-1. LSECs also recruit specific pro-inflammatory leukocyte subsets during disease states, including (7) gut-homing lymphocytes through $\alpha4\beta7$ -MAdCAM interactions and (8) CD16+ monocytes via CX3CL1 secretion. LSECs actively contribute to the intricate immune landscape in the liver during chronic inflammation.

Wilkinson AL et al. *Frontiers in Physiology*. August 2020

III. P16Ink4a and Aging of Liver Endothelial Cells

As individuals age, LSECs undergo distinct functional and phenotypic changes which consequently affect liver function. In humans [573], baboons [574], mice [575,576], and rats [577–579], LSECs aging is associated with pseudocapillarization, which is thickening of the sinusoidal endothelium, reduced porosity of LSECs, and increased deposition of basal lamina and collagens in the space of Disse. Additionally, von Willebrand factor expression is observed in LSECs of old livers, which is not typically found in young individuals [573,574,577,578,580,581]. This age-related pseudocapillarization refers to the specific changes in the liver sinusoids due to aging, distinct from capillarization seen in liver diseases [578]. In aging, hepatic stellate cells become fat-engorged but remain quiescent [574,582,583], contributing to the narrowing of the sinusoidal lumen and reduced liver blood flow seen in old livers [577,583,584]. Moreover, decreased LSECs porosity can also be implicated in vascular disease of other organs. Decreased LSEC porosity for instance is linked to conditions like postprandial hypertriglyceridemia, a risk factor for atherosclerosis [547,585–587]. Moreover, the endocytic capacity of LSECs decreases with age, leading to reduced uptake of certain substances. This impaired endocytic capacity may become evident during episodes of increased blood levels of waste macromolecules, as of the attenuated scavenging capacity of LSECs in older individuals compared to young ones [575,579,588,589]. The aging process may be linked to various factors, such as exposure to gut-derived toxins, nicotine, and oxidants, which can cause defenestration of LSECs [544,590]. Additionally, chronic low-grade inflammation associated with aging affects the liver and other organs, potentially contributing to the changes observed in the hepatic sinusoids [591–593]. Oxidative stress and accumulation of degradable material, such as the advanced glycation end-products (AGEs)-metabolites, may also play a role in cell aging and changes of LSECs [580,594].

Recent research has highlighted significant findings regarding the aging process in the liver. Specifically, there is evidence of increased expression of the p16 senescence marker, elevated oxidative stress in mitochondria, and higher levels of inflammatory genes in LSECs [330,575]. In an aged rat model, upregulation of p16 was also observed in liver tissue and LSECs [330].

To delve further into the role of senescent cells in liver aging, a mouse model using p16 CreERT2–tdTomato was utilized. This model revealed detectable p16 high cells in various organs, including the liver, and their prevalence increased with age. Notably, the majority of these p16 high cells in the liver were vascular ECs, which are crucial for liver function and structure. Removing these

cells led to adverse effects like steatohepatitis and perivascular tissue fibrosis, indicating their significance in healthy aging control and potential influence on lifespan [210,331]. An interesting discovery from our recent study is that p16 RNA and protein expression in the livers of old mice mainly occurs in ECs compared to liver parenchymal cells [595]. All combined, these data suggest a particular role of p16 in liver endothelial physiology, which may hold key insights into the liver's aging process. Understanding the mechanisms behind p16 in liver ECs could provide valuable information for developing targeted anti-aging therapies aimed at endothelial cell function, ultimately contributing to healthier aging. Therefore, further research is necessary to fully elucidate the specific role of p16 in these cells.



PROJECT DESCRIPTION



I. Project Aims and Description

Aim: we aimed at better understanding the role of p16 in biological processes and aging of liver vascular ECs (CD31+) and LSEC (CD146+).

1. p16 loss of function and gain of function in liver endothelial cell physiology

We used small hairpin (shRNA) constructs and a p16 cDNA-GFP vector transduced via a lentivirus, to knock-down and over-express p16 in-vitro, in order to assess p16 loss and gain of function in both types of liver ECs' biological processes such as proliferation, senescence, migration, and apoptosis. Cells were isolated from the liver through magnetic activated cells sorting (MACS) assay using a magnetic bead-attached monoclonal antibodies against CD31 and CD146 surface markers. Non-coding shRNA sequence and an empty-GFP vector transduced cells were used as a control for p16shRNA and p16-GFP transduced cells, respectively.

II. Schematic Project Description

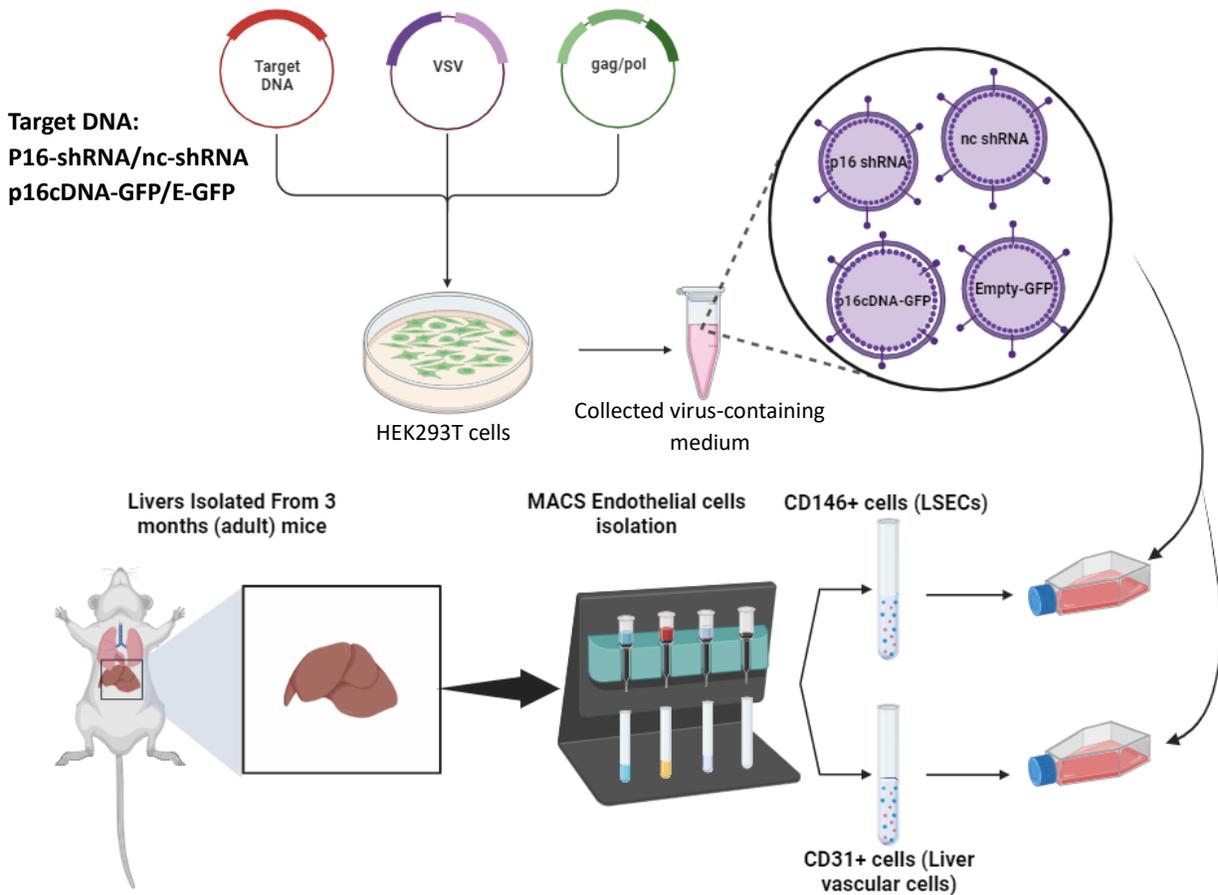


Figure 24. This schematic depicts an experimental approach involving the genetic modulation of p16 in liver endothelial cells (ECs). Utilizing p16shRNA and p16cDNA-GFP vector, p16 expression was both knocked down and overexpressed in vitro through lentiviral transduction. The liver ECs were isolated through a Magnetic Activated Cell Sorting (MACS) assay using CD31 and CD146 surface markers. Two control groups included cells transduced with non-coding shRNA and an empty-GFP vector.



MATERIALS AND METHODS



I. Animals

All animal work was conducted according to national and international guidelines. Timed pregnant mice (NMRI and C57BL/6) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice were sacrificed by cervical dislocation and liver ECs were isolated from livers of adult mice around the age of 3 months.

II. Magnetic Activated Cell Sorting (MACS)

Livers were isolated from 24 adult (3 months; n=24) mice. Organs were minced and afterward digested with 0.1 mg/mL of DNase I (10104159001, Roche Diagnostics, Mannheim, Germany) and 1 mg/mL of Collagenase A (11088793001, Roche) in 10 mL of DMEM culture media (ThermoScientific) for 1 h at 37 °C. Digested samples were passed through 70- μ m filters (SmartStrainers, 130-098-462, Miltenyi Biotec, Paris, France), centrifuged, and washed twice with PBS containing 2% fetal calf serum (FCS) and 0.5 mM of EDTA (ThermoScientific). Cells were re-suspended in 90 μ L of the same buffer (PBS + FCS + EDTA)/ 10^7 cells. Vascular ECs and LSEC were labelled separately by adding 10 μ L/ 10^7 cells of magnetic microbead-associated anti-CD31 (130-097-418, Miltenyi) and anti-CD146 antibody (130-092-007, Miltenyi) respectively, at 4 °C for 15–30 min. Cells were separated via LS column (130-042-401, Miltenyi) pre-washed with 3 mL of PBS + FCS + EDTA and attached to a MidiMACS separator magnet (130-042-302, Miltenyi). Non-ECs were eluted by washes with 3x 3 mL of PBS + FCS + EDTA. Afterward, ECs were eluted by removing the LS columns from the magnetic field and flushing with 6 mL of PBS + FCS + EDTA.

III. Cell culture

Isolated primary CD31⁺ vascular cells and CD146⁺ LSEC were purified and seeded each cell type in 75m³ falcon flask coated with collagen (Sigma-Aldrich C3867-1VL) diluted 1:100 in PBS. Cells were grown in 10 ml RPMI medium supplemented with 10% FCS, 1 % penicillin-streptomycin (Gibco 15140-122) and 1% Glutamine (Gibco 25030-024) incubated at 37°C, 5% CO₂. For all experimental procedures, the cells used were pooled from up to 6 mice donors (n=6) which did not exceed passage 4.

Human embryonic kidney (HEK) 293 cells (ATCC CRL-1573) were grown in DMEM medium (Lonza, Levallois- Perret, France) supplemented with 10% fetal calf serum (FCS), 100IU ml⁻¹ penicillin, and 100µg ml⁻¹ streptomycin (Invitrogen, Cergy Pontoise, France)

IV. Generation of transduction constructs (sh, nc, OE, E)

1. p16 plasmids

p16 was knocked down using small hairpin construct RNA (shRNA- sh) with ampicillin antibiotic resistance. Several shRNAs were used in attempts of knocking down p16 which were labeled as follows: Sh15 (Sigma Aldrich; Ref: TRCN0000077815), sh16 (Sigma Aldrich; Ref: TRCN0000077816), shMix which is a mixture of three different shRNAs from (Santa Cruz; sc-36144-SH). A mouse non-coding sequence shRNA (nc) was used as a control of p16 knock- down cells. On the other hand, p16 overexpression (OE) was assessed through a p16 cDNA-GFP vector from (ORIGENE – Catalog No. MR227284L2) with chloramphenicol antibiotic resistance. Empty-GFP vector achieved by excision of the p16 cDNA from the overexpression vector was used as overexpression control (E) for OE transduced cells.

2. Empty - GFP Vector

First we started with the digestion of 2.8 µg of p16 cDNA-GFP vector for 2hrs at 37°C, using 1µl of each of the restriction enzymes BamHI (NewEnglandBioLabs – R01336S) and NotI-HF (NewEnglandBioLabs – R3189L) in 2µl NEBuffer4 (NewEnglandBioLabs – B7004S) and 0.2µl BSA and complementary volume of H₂O to 20µl. Digestion step was followed by heat inactivation of the enzymes for 20 minute at 60°C. Afterwards, the DNA gap was filled using dNTPs (Invitrogen – ref: 10297-018) and T4 DNA polymerase (NewEnglandBioLabs – M0203S) for 15 minutes at 12°C. The complete volume of the reaction solution was then loaded on a 1% agarose gel with 4µl 10x loading buffer for validation of the digestion. Gel DNA extraction was then assessed using the quick gel extraction kit from Invitrogen (Ref: K21002 – 32584 Lohne, Germany) according to manufacturer's instructions. The quantity of DNA was then estimated to be around 20ng/µl by running 1µl of our DNA on a 1% agarose gel for 30 minutes in parallel with 1µl λ DNA Hind III marker (NewEnglandBioLabs – R0104S) in a 1µl of 10x loading buffer. This was followed by ligation of the linear plasmid using 1µl T4 DNA ligase (NewEnglandBioLabs – M0202S) with 8µl of our plasmid DNA in 1µl 10x buffer. Ligated DNA was then transformed into

bacteria and grown overnight at 37°C on a LB agar plate with 1:1000 chloramphenicol. 12 clones were picked and grown in 1ml LB agar with 1:1000 chloramphenicol overnight shaking at 180 rpm, 37°C. Then bacterial DNA extraction was assessed using Quick Plasmid Miniprep Kit (Invitrogen – K210011 - 32584 Lohne, Germany) according to manufacturer's instructions. After that, to confirm p16 cDNA excision, the DNA from the 12 samples in addition to complete vector with p16 cDNA, as a positive control, were digested using 0.5µl of each of EcoRI (Promega – R601J) and XhoI (NewEnglandBioLabs – R0146S) for 1hr at 37°C. Second digestion enzymes should have restriction sites at the exterior of the first ones to avoid polymerase closure imperfection. Afterwards, load on 1% agarose gel with 2µl 10x loading buffer and run for 30 minutes at 130 volts.

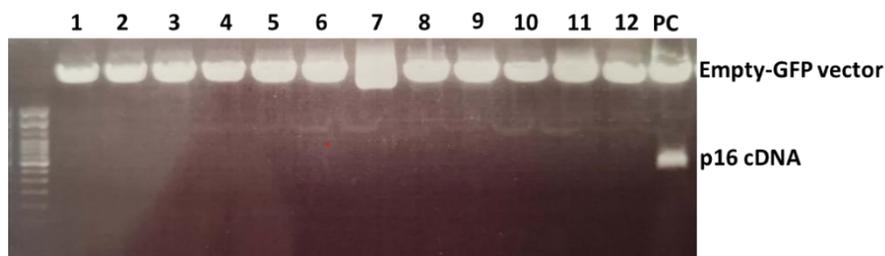


Figure 25. 1% agarose gel electrophoresis of the 12 DNA samples extracted from DH5α bacteria selected colonies, transformed with p16cDNA excised-vector (lanes 1-12). The DNA sample extracted from DH5α bacteria, transformed with p16cDNA-vector acts as a positive control (lane PC). Empty-vector band obtained at 7 kbp and p16cDNA insert obtained at 504 bp.

3. Bacterial transformation and Plasmid DNA amplification

DH5α bacteria were thawed in ice for 30 minutes. Then 100 µl of the bacteria were mixed with 100 ng of the plasmid DNA and kept on ice for 30 minutes. A heat shock uptake of the plasmid was achieved by placing the tube at 42 °C for 45 seconds then on ice for 2 mins. 250 µl of SOC medium (Sigma Aldrich – S1797) was added to the DNA/cells mixture and then incubated by shaking at 180 rpm, 37°C for 1 hr. Afterwards, the DNA containing bacteria were seeded on an agar plate with 1:1000 antibiotic and incubated at 37°C open for 10 mins to dry. Plates were closed, twisted upside down and incubated overnight at 37°C. One colony of bacteria was picked and grown in 1ml LB agar medium over night at 37°C shaking at 180-200 rpm with 1:1000 antibiotic. Afterwards 100 µL of the grown bacteria were moved to an Erlenmeyer flask containing 200ml LB agar medium with 1:1000 antibiotic. The total plasmid DNA extraction was assessed used

HiPure plasmid Maxiprep Kit from Invitrogen (K210007 – Lohne, Germany) according to manufacturer's instructions.

4. Generation of transduction viruses

For virus production, we needed three plasmids. The VSV plasmid which encodes the viral envelope, the gag/pol plasmid which encodes the core particle protein, and the target plasmids to be transduced in our cells. A total amount of 20 µg of plasmid DNA was transfected in 5×10^6 HEK293T adjusted as 2.8µg VSV, 8.6µg gag/pol and 8.6 µg plasmid of interest, through calcium phosphate mediated transfection. HEK293T cells were fed with DMEM medium, supplemented with 10% Fetal Calf Serum at 37° in 5% CO₂ in 10 cm dishes. The virus containing medium was collected 48 and 72 hours after HEK293T cells transfection.

5. Endothelial cell transduction

CD31+ and CD146+ ECs were passed and grown in 100 mm plates prior to transduction. Cells were freshly fed with 3 ml RPMI complete medium then treated with additional 3 ml of the virus containing medium. Different shRNAs were used to knock down p16, sh15, sh16, shMix (mix of 3 different shRNAs) and ALL (sh15 + sh16 + shMix) and non-coding (nc) sequence as a control. Cells were exposed for 1 week, 2 weeks, and for 4 days. P16 silencing and overexpression verification and quantification was assessed at the protein level by Western Blot.

V. Western blot

After endothelial cell transduction (similarly for p16 baseline expression without transduction), cells were washed twice with 10 ml 1x PBS, and then scraped off manually, using a scraper. Cells were centrifuged at 2000 rpm for 5 min at 4 °C. Then, cells were incubated with 150 µL of RIPA buffer (Sigma) on ice for 30 min. Afterwards, samples were agitated overnight at 4 °C. The next day, the tubes were centrifuged at 16,000 rpm for 30 min at 4 °C. The total protein containing supernatant was recovered and stored at –80 °C.

Proteins were quantified by colorimetric BCA assay according to manufacturer's instructions (Uptima, Montluçon, France). Samples were diluted 5 times in distilled water and loaded in triplicates of 10 µL each, in transparent 96-well plates. In addition, BSA standards ranging from 0 to 2 mg were loaded in triplicates (10 µL). Absorbance was measured at a wavelength of 560 nm in a plate spectrophotometer (Biorad, Marnes-la-Coquette, France).

For Western blotting, 30 µg of protein in Laemmli buffer was denatured at 95 °C for 5–10 min. Samples were loaded on acrylamide gels (acrylamide/bisacrylamide 37.5/1) and set for electrophoresis. Afterward, proteins were transferred to PVDF membranes (162-0177, Biorad), and the membranes were blocked with 5% milk for 1 h (232100, Difco Skim Milk). p16 was detected using a rabbit monoclonal anti-p16 (Abcam; ab211542) diluted 1:2000 in PBS + 0.05% Tween 20 + 2.5% milk powder (overnight, 4 °C), followed by anti-rabbit peroxidase-labeled secondary antibody addition (Vector Laboratories) diluted 1:2000 in PBS + 0.05% Tween 20 + 2.5% milk powder for 1 h. Then, the chemiluminescence signal was obtained by incubation with the enzyme-specific substrate (RPN2235, Amersham, ECL Select Western blotting detection reagent). Afterwards, the membrane was stripped by application of 10 mL of stripping buffer for 20 min (ST010, Gene Bio-Application L.T.D., Kfar-Hanagid, Israel) and washed 5x 5 min with distilled water before a second identical blocking step with milk for the detection of Gapdh as housekeeping protein. A rabbit monoclonal anti-Gapdh antibody (Abcam; ab181602) was used, and the signal was generated with same secondary antibody and substrate mentioned above.

VI. RNA Isolation, Reverse Transcription, and Quantitative PCR

For baseline p16 expression both CD31 and CD146 ECs isolated from 4 mice (n=4) were passed to 6 wells plates and grown until confluent. While for p16 knockdown and overexpression condition, CD31 and CD146 ECs isolated from 6 mice (n=6) were passed to 6 wells plate, then transduced with lentivirus of the four conditions 1:1 in RPMI complete medium. Using the Trizol reagent (Thermo Scientific, Courtaboeuf, France), total RNA was isolated. First-strand cDNA synthesis was performed with 500 ng of total RNA using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit (#K1672, Thermo Scientific, Courtaboeuf, France), which contains DNase I, RNase inhibitor, oligo (DT) and random hexamer primers. The cDNAs were diluted 10 times in nuclease free water. Two microliters of the diluted reaction product were taken for real-time RT-PCR amplification which was performed using a StepOne Plus thermocycler (Thermo Scientific) and the PowerUp SYBR[®] Green Master Mix (#A25742, Thermo Scientific) or EurobioGreen Mix (GAEMMX02H, Eurobio, Les Ulis, France). For each sample, expression of the housekeeping genes *Gapdh*, *Rplp0*, and *β-actin* was determined. Three independent housekeeping genes were used as expression for each gene might vary under different experimental conditions [596,597]. Expression for each sample was calculated by subtracting the

mean value of housekeeping gene Ct's from the gene of interest Ct using the Δ Ct method [597–606]. Afterwards, relative gene expression values were obtained by normalization of each sample against the mean value of all samples at nc and E to determine differences between the organs and time points investigated. Primer sequences are in table below (**Table 2**).

Table 2. Primers used for quantitative RT-PCR

| Gene of interest | Oligonucleotide sequences | References |
|------------------|---|------------|
| <i>p16ink4</i> | F: AGGGCCGTGTGCATGACGTG R: GCACCGGGCGGGAGAAGGTA | [607] |
| <i>Cd31</i> | F: CTGCCAGTCCGAAA ATGGAAC R: CTTCATCCACCGGG GCTATC | [608] |
| <i>Cd146</i> | F: CCCAAACTGGTGTG CGTCTT R: GGAAAATCAGTATCT GCCTCTCC | [609] |
| <i>Tgf-b1</i> | F: AGCTGGTGAAACGGAAGC G R: GCGAGCCTTAGTTTGGACAGG | |
| <i>Tgf-b2</i> | F: CCATCCCGCCCACTTTCTAC B: CATCAAAGCGGACGATTCTGA | |
| <i>Tgf-b3</i> | F: GGACTTCGGCCACATCAAGAA B: TAGGGGACGTGGGTCATCAC | |
| <i>Igf1</i> | F: GTGAGCCAAAGACA CACCCA B: ACCTCTGATTTTCC GAGTTGC | |
| <i>Igf1R</i> | F: GTGGGGGCTCGTGTTTCT B: GATCTCCGTGCAGTTTCCA | |
| <i>Vegfa</i> | F: CTCACCAAAGCCAGCACATA R: AATGCTTTCTCCGCTCTGAA | [606] |
| <i>Vegfb</i> | F: CTGTGAAGCCAGACAGGGTTG B: GATGGATGATGTCAGCTGGGG | |
| <i>Vegfc</i> | F: ACCTCAGCAAGACGTTGTTTG B: AGTTTAGACATGCACCGGCA | |
| <i>Vegfd</i> | F: GCCTGGGACAGAAGACCACT B: GCAGCAGCTCTCCAGACTTT | |
| <i>VegfR1</i> | F: TACCTCACCGTGCAAGGAAC B: AAGGAGCCAAAAGAGGGTTCG | |
| <i>VegfR2</i> | F: AGTGGTACCAGCTAGAAG B: ACAAGCATACGGGCTTGTTT | |

| | | |
|---------------|--|-------|
| <i>VegfR3</i> | F: ACGCAGAGTGATGTGTGGTC B: ATGATGTGGCGTATGGCAGG | |
| <i>Cdh5</i> | F: GCAGAGTCCATCGCAGAGT B: TCAGCCAGCATCTTGAACCT | |
| <i>Angpt1</i> | F: AGCGCCGAAGTCCAGAAAAC B: TTGCCCATGTTGAATCCGGTT | |
| <i>Angpt2</i> | F: CTCGAATACGATGACTCGGTG B: TCATTAGCCACTGAGTGTGTTT | |
| <i>Pdgfa</i> | F: GAGGAAGCCGAGATACCCC B: TGTGGATCTGACTTCGAGCCA | |
| <i>PdgfRa</i> | F: GGAACCTCAGAGAGAATCGGC B: CATAGCTCCTGAGACCCGCTG | |
| <i>Pdgfb</i> | F: TGCTGCACAGAGACTCCGTA B: GTGCTCGGGTCATGTTCAAGT | |
| <i>PdgfRb</i> | F: CCAGCACCTTTGTTCTGACCT B: TGCCGTCCTGATTCATGGC | |
| <i>VhL</i> | F: CAGCTACCGAGGTCATCTTTG B: CTGTCCATCGACATTGAGGGA | |
| <i>Vwf</i> | F: TGTGACACATGTGAGGAGCC B: CTTTGCTGGCACACTTTCCC | |
| <i>Il-6</i> | F: CACTTCACAAGTCGGAGGCT R: TGCCATTGCACAACCTTTTTCT | [606] |
| <i>Il-8</i> | F: CAAGGCTGGTCCAT GCTCC B: TGCTATCACTTCCTT TCTGTTGC | |
| <i>Il-10</i> | F: GCTCTTACTGACTG GCATGAG B: CGCAGCTCTAGGAG CATGTG | |
| <i>Il-18</i> | F: CAAAGTGCCAGTGAACCCCA B: TTCACAGAGAGGGTCACAGC | |
| <i>Inf</i> | F: ACCTGAGCAACCTA CCAAGAA B: TGGACATGGGGTTT TGGAAATTC | |
| <i>Tnfa</i> | F: GTAGCCCACGTCGTAGCAA B: ACAAGGTACAACCCATCGGC | |
| <i>NF-κB</i> | F: ATGGCAGACGATGA TCCCTAC B: TGTTGACAGTGGTAT TTCTGGTG | |
| <i>Stat1</i> | F: TCACAGTGGTTCGAGCTTCAG B: GCAAACGAGACATCATAGGCA | |

| | | |
|---------------|--|-------|
| <i>Stat2</i> | F: TCCTGCCAATGGACGTTTCG B: GTCCCACTGGTTCAGTTGGT | |
| <i>Ccl2</i> | F: AGCTGTAGTTTTTGTACCAAGC B: GTGCTGAAGACCTTAGGGCA | |
| <i>Ccl3</i> | F: TTCTCTGTACCATGA CACTCTGC B: CGTGGAATCTTCCG GCTGTAG | |
| <i>Ccl5</i> | F: GCTGCTTTGCCTAC CTCTCC B: TCGAGTGACAAACA CGACTGC | |
| <i>Ccl9</i> | F: CCCTCTCCTTCCTC ATTCTTACA B: AGTCTTGAAAGCCC ATGTGAAA | |
| <i>Ccl12</i> | F: ATTTCCACACTTCTA TGCCTCCT B: ATCCAGTATGGTCC TGAAGATCA | |
| <i>Ccl20</i> | F: ACTGTTGCCTCTCG TACATACA B: GAGGAGGTTACAG CCCTTTT | |
| <i>Cxcl10</i> | F: CCAAGTGCTGCCGTCATTTTC B: GGCTCGCAGGGATGATTTCAA | |
| <i>Mmp1</i> | F: GGCCAGAACTTCCCAACCAT B: AGCCAGAAATTTTCTCCCTCT | |
| <i>Mmp2</i> | F: CAAGTTCCCCGGCGATGTC B: TTCTGGTCAAGGTCACCTGTC | |
| <i>Mmp7</i> | F: CTGCCACTGTCCCAGGAAG B: GGGAGAGTTTTCCAGTCATGG | |
| <i>Mmp8</i> | F: CCTGCAGGACTCCTTCTTCCT B: CCTCATAGGGTGCGTGCAA | |
| <i>Mmp9</i> | F: CCATGCACTGGGCTTAGATCA B: GGCCTTGGGTCAGGCTTAGA | [606] |
| <i>Mmp12</i> | F: GAGTCCAGCCACCAACATTAC B: GCGAAGTGGGTCAAAGACAG | |
| <i>Timp1</i> | F: GCAACTCGGACCTGGTCATAA B: CGGCCCGTGATGAGAACT | |
| <i>Timp2</i> | F: TCAGAGCCAAAGCAGTGAGC B: GCCGTGTAGATAAACTCGATGTC | |
| <i>Timp3</i> | F: CTTCTGCAACTCCGACATCGT B: GGGGCATCTTACTGAAGCCTC | |
| <i>Timp4</i> | F: TGTGGCTGCCAAATCACCA B: TCATGCAGACATAGTGCTGGG | |

| | | |
|-------------------|--|-------------------|
| <i>Caspase3</i> | F: ATGGAGAACAACAAAACCTCAGT B: TTGCTCCCATGTATGGTCTTTAC | |
| <i>Caspase4</i> | F: ACAAACACCCTGACAAACCAC B: CACTGCGTTCAGCATTGTATAA | |
| <i>Caspase8</i> | F: CAACTTCCTAGACTGCAACCG B: TCCAACCTCGCTCACTTCTTCT | |
| <i>Caspase9</i> | F: GGCTGTAAACCCCTAGACCA B: CTTTGCTGTGAGTCCCATTGG | |
| <i>Bcl2</i> | F: ATGCCTTTGTGGAACCTATATGGC B: GGTATGCACCCAGAGTGATGC | |
| <i>Bax</i> | F: AGACAGGGGCCTTTTTGCTAC B: AATTCGCCGGAGACTCG | |
| <i>Bak</i> | F: GCCCTGTACGTCTACCAGC B: ATCAGCCAAAAGCAGGTCAC | |
| <i>V-cam1</i> | F: TATGTCAACGTTGCCCCAA B: CAGGACTGCCCTCCTCTAGT | |
| <i>I-cam1</i> | F: CACGTGCTGTATGGTCCCTCG B: TAGGAGATGGGTCCCCAG | |
| <i>E-selectin</i> | F: ATGCCTCGCGCTTTCTCTC B: GTAGTCCCGCTGACAGTATGC | |
| <i>P-selectin</i> | F: CATCTGGTTCAGTGCTTTGATCT B: ACCCGTGAGTTATTCCATGAGT | |
| <i>Mastl</i> | F: TCGGCAAGTGAGGAGAATGAA B: CACCACGGCTAATGGGCTT | |
| <i>Mafk</i> | F: ATGACGACTAATCCCAAGCCC B: CGTAGCCTCTGTTCTTGAGTGT | |
| <i>Telo</i> | F: CGGTTTGTGGGTTTGGGTTTGG GTTTGGGTTTGGGTT B: GGCTTGCCCTACCCTTACCCTTAC CCTTACCCTTACCCT | |
| <i>Telo36B4</i> | F: ACTGGTCTAGGACCCGAGAAG B: TCAATGGTGCCTCTGGAGATT | |
| <i>Gapdh</i> | F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA | [598,599,603,606] |
| <i>β-actin</i> | F: CTTCTCCCTGGAGAAGAGC R: ATGCCACAGGATTCCATACC | [598,599,603,606] |

VII. BrdU incorporation assay

Cell proliferation was assessed using a colorimetric BrdU cell proliferation kit according to the manufacturer's instructions (Cat. no. 11647229001; Roche, Indianapolis, IN, USA). CD31 and CD146 ECs were seeded at 50×10^3 /well in 12 well plates and transduced with lentivirus ratio 1:1 in complete RPMI medium. The transduced cells were labeled with BrdU for 3 to 4 h. The genomic DNA was fixed and denatured, followed by incubation with peroxidase-conjugated anti-BrdU antibody for 90 min. A substrate for the conjugated peroxidase was then added and the reaction product was quantified by measuring the absorbance (performed and analyzed by Dr. K. and N. Wagner).

VIII. Manual cell counting

Manual cell counting was assessed using KOVA plastic slides 10 with grid (REF: 87144F - Pennsylvania, 17084 USA). Transduced cells were seeded in a concentration of 5×10^3 in 96 wells plate with 12 wells /condition (12 days counting). Counting was achieved according to the chamber's instructions. Cells were washed twice with PBS then detached by 50 μ l trypsin and cellular clumps were resolved by pipetting inside the well. Afterwards, 6.6 μ l of cells were filled inside each chamber by capillarity. Cells were counted at x100 magnification (performed and analyzed by Dr. K. and N. Wagner).

IX. Cellular senescence assay

Cellular senescence of ECs was assessed by the cellular senescence assay kit from (Sigma Aldrich – KAA002). CD31 and CD146 ECs were seeded at 50×10^3 /well in 12 well plates and transduced with lentivirus ratio 1:1 in complete RPMI medium for 1 week. Medium was aspirated and cells were washed once with 2ml 1X PBS. 1ml/well of fixing solution was added and cell were incubated at room temperature for 15 mins. The fixing solution was removed, and the cells were washed again twice with 1X PBS. 1ml of freshly prepared 1X SA- β -gal detection solution was added. Then cells were incubated at 37°C in dark for minimum 4 hours. SA- β -gal detection

solution was removed, stained cells were washed twice with PBS and then counted under light microscopy (performed and analyzed by Dr. K. and N. Wagner).

X. Telomeres Shortening

Telomere shortening was assessed in transduced cells isolated from 3 mice (n=3). CD31 and CD146 ECs were seeded at 100×10^3 /well in 6 well plates and transduced with lentivirus (nc, sh, E and OE) ratio 1:1 in complete RPMI medium and kept growing for 1 week. Afterwards, cells were trypsinised, a part was taken for DNA extraction, while the other part was kept growing for another week. DNA was extracted from the cells using DNeasy Blood & tissue Kit (QIAGEN, Germany – Cat. No. 69506) according to manufacturer's instructions. Telomere length was measured by qPCR using oligos listed in **Table 2** (Telo and Telo36B4) and 10 ng of DNA. The mean CT value obtained from Telo amplification, refers to the abundance of telomeres in the input genome, was normalized to the amplification mean CT value obtained from a single copy gene (Telo36B4). The ratio of $CT_{Telo}/CT_{Telo36B4}$ represent the average length of telomere [610].

XI. Migration: Scratch assay

One week after transduction of ECs in 12 wells plates, scratch migration assays were performed. Straight and gentle scratches to the cellular monolayer was done using a sterile 200 μ l pipette tip. Detached cells were washed with serum free medium and cell were fed back with RPMI medium. Images were captured after 0, 3, 6, 9, 12, and 24. Scratch diameters were measured by Image J.

XII. Migration: Trans-well assay

Alternatively, transduced ECs were seeded in the upper compartment of a modified Boyden chamber (5×10^4 cells/chamber, pore size 8 μ m, Corning Costar, SchipholRijk, Netherlands). After 24h, cells remaining in the upper chamber were scraped off and the migrated cells at the bottom side fixed with PFA, followed by staining with DAPI. Quantification was done by counting five random fields of eight independent experiments (performed and analyzed by Dr. K. and N. Wagner).

XIII. Apoptosis Assay

One week after transduction of ECs in 6 well plates, apoptosis of the cells was assessed using Annexin V-FITC Apoptosis Detection Kit from (Sigma Aldrich – APOAF) according to manufacturer’s instructions. Briefly cells were scraped off in 1X PBS using a scraper then centrifuged at 4°C, 15000 rpm, 5 mins. Afterwards cells were resuspended in 100 µl 1X binding buffer then moved to a glass apoptosis tube. 2.5 µl Annexin V and 5µl of propidium iodide (PI) were added to each sample followed by FACS analysis using Cytoflex machine from (Beckman Coulter Life Sciences – United States).

XIV. Statistical Analysis

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical differences were assessed by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test (Graph Pad Instat, GraphPad Software, Inc., San Diego, CA, USA). A p-value < 0.05 was considered to reflect statistical significance.



UNPUBLISHED RESULTS



I. P16 expression in CD31⁺ and CD146⁺ cells

There are two different types of ECs in the liver, which are vascular ECs that have predominantly a CD31 surface marker, and the LSECs which are CD146-positive (in mice). To verify that both cells are similar in terms of p16 expression, both types of cell were magnetically sorted, from 2 different animals (3months old), using anti-CD31 and anti-CD146 monoclonal antibodies, grown in a cell culture, then lysed for RNA and protein extraction and quantification by RT-qPCR and western blot respectively. Our results show that both types of ECs have similar level of p16 RNA (Figure 26A) and protein expression (Figure 26B).

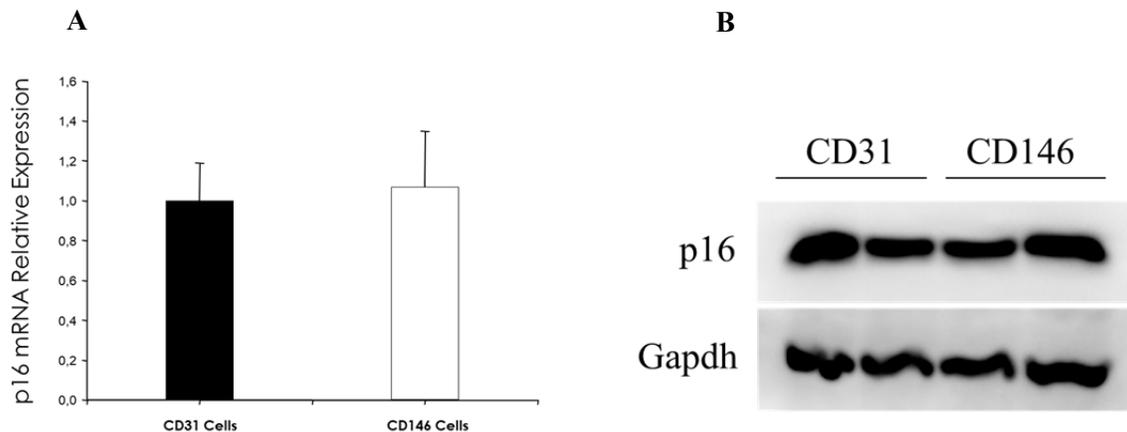


Figure 26. Both CD31 and CD146 cells have same expression of p16 at the RNA and protein level. (A) p16 mRNA relative expression in CD31 cell (black bar) and CD146 (white bar) endothelial cells normalized to the expression level of *Gapdh*, *β -actin*, and *Rplp0*. (B) Western Blot for p16 in CD31 and CD146 liver endothelial cells of 3 months old mice liver. *Gapdh* served as standard.

II. P16 silencing and overexpression

For p16 silencing, cells were treated with different shRNAs (sh15, sh16, and ALL) and for 3 different transduction periods (1 week-7days, 2 weeks-14days, then 4 days) as explained in the materials and methods. Afterwards, p16 silencing was verified at the protein level by western blot with Gapdh used as a standard. First, among the different shRNAs used, sh16 was the most efficient for silencing p16 in 1 week and 2 weeks exposure period in both CD31 and CD146 cells (**Figure 27A**). Afterwards, to verify if we could reach the same efficiency of knockdown in a shorter period, we transduced the cell with the virus for 4 days with sh16 only. The results have shown that 4 days were not enough to reach the same efficiency obtained with 1 week transduction (**Figure 27B**). Therefore, p16 silencing (sh) was optimized for 1 week transduction with sh16 in both cell types for the rest of the experiments and non-coding shRNA (nc) served as control for p16 silenced cells.

P16 overexpression (OE) was achieved by transduction of the cells by p16cDNA-GFP vector. Then p16 overexpression and silencing were verified by RT-qPCR and western blot at the RNA and protein level and compared to the (nc) and Empty-GFP (E) vector transduced cells as a control for silencing and overexpression respectively. RT-qPCR results have shown significant down regulation of p16 in sh transduced cells compared to nc in both cell types, while a significant upregulation of p16 expression was observed in OE condition compared to E (**Figure 27C**). at the protein level, we observed p16 overexpression (OE) as a single band at the 16 KD as in E, in addition to a strong band obtained at 43 kD, due to the fusion of p16 with the GFP (16+27). While the silencing was obtained as a slight band compared to nc transduced cells in both cell types, all normalized to α -tubulin expression (**Figure 27D**)

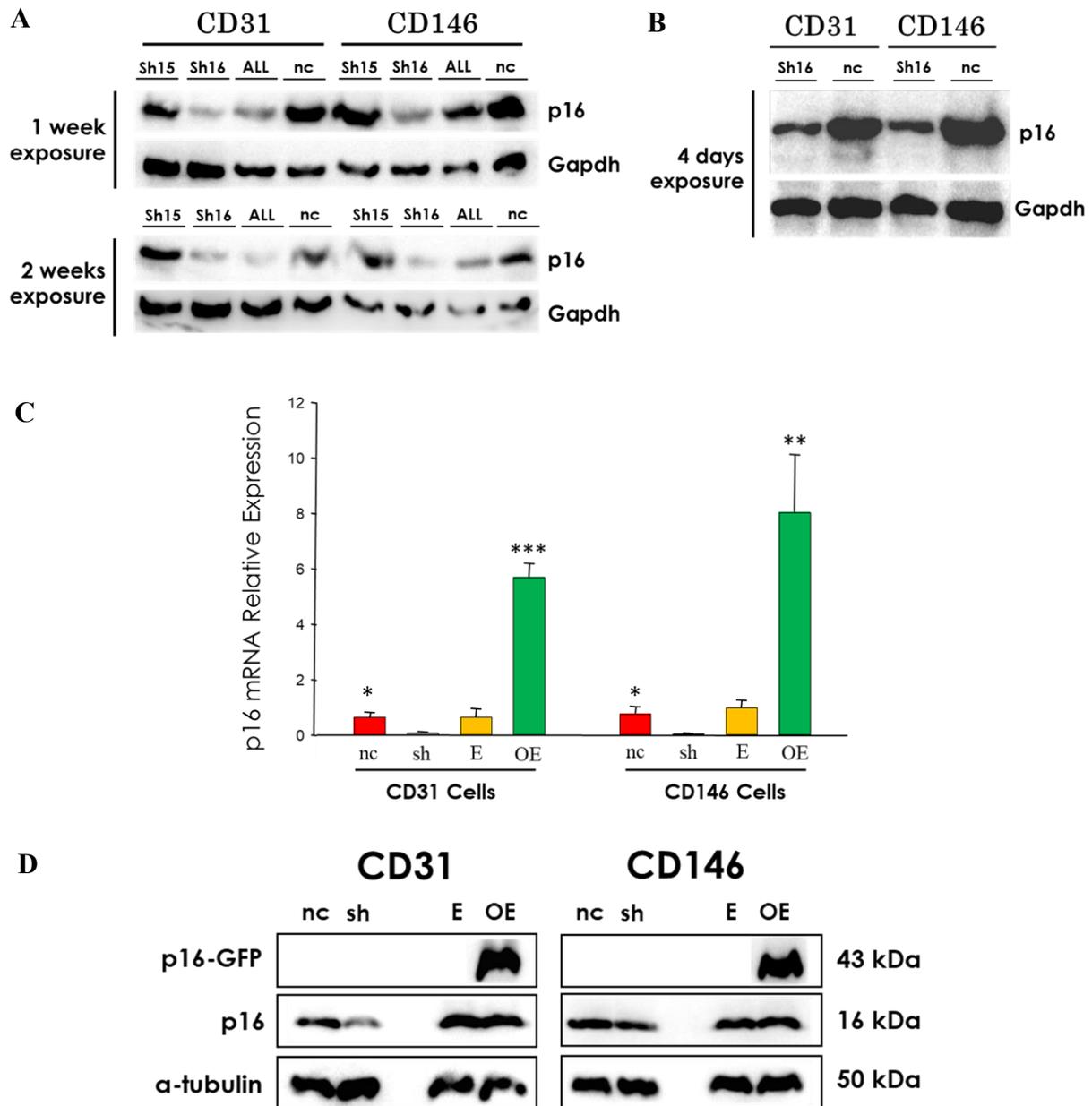


Figure 27. Optimization of p16 silencing and overexpression in CD31 and CD146 cells. (A) Western blot result of p16 expression after transduction of the CD31 and CD146 cells by different shRNAs for 1 week and 2 weeks. nc transduced cells served as control and Gapdh as standard for p16 expression. (B) Western blot results of p16 expression after transduction of the CD31 and CD146 cells with sh16 and nc as a control for 4 days. (C) Quantitative RT-PCR of CD31 and CD146 cells p16 expression after transduction with nc, sh16 (sh), E (control of OE) and OE (overexpression vector) for 1 week. Expression of p16 was normalized to the respective *Gapdh*, *actin*, and *Rplp0* expression. Data are mean \pm SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (D) Western blot of p16 expression after transduction of the CD31 and CD146 cells with the four different conditions nc, sh, E (all band at 16 kDa) and OE (1 band at 16kDa for p16 and 1 band at 43kDa for p16-Gfp fusion) Data provided from N. Wagner. P16 expression was normalized to α -tubulin expression (50kDa).

III. P16 and Liver Endothelial Cell Proliferation

Effects of p16 silencing and overexpression on CD31 and CD146 endothelial cell proliferation was assessed by daily manual cell counting (for 12 day) and BrdU incorporation assay. In manual cell counting, no change in the cell numbers was obtained in sh compared to nc, neither in p16 OE compared to empty vector (E) transduced both cell types, in the first week. However significantly higher cell numbers were obtained starting at day 7 (time needed for p16 knockdown and overexpression) then days 8, 9, 12 and 14 in sh compared to nc and in E compared to OE in CD31 (**Figure 28A**). Similarly, in CD146 cells, significantly higher cell counts were obtained at days 7, 8, 9, and 12 in sh compared to nc, while at days 7, 9, 12, and 14 in E compared to OE (**Figure 28C**).

BrdU incorporation assay demonstrated significantly higher proliferation, measured by the relative absorbance at 450nm of BrdU incorporation, in sh compared to nc control, and lower proliferation in OE compared to E control in both CD31 and CD146 cell (**Figure 28B & 28D**). Therefore, p16 knockdown was associated with higher proliferation while p16 overexpression was associated with lower proliferation.

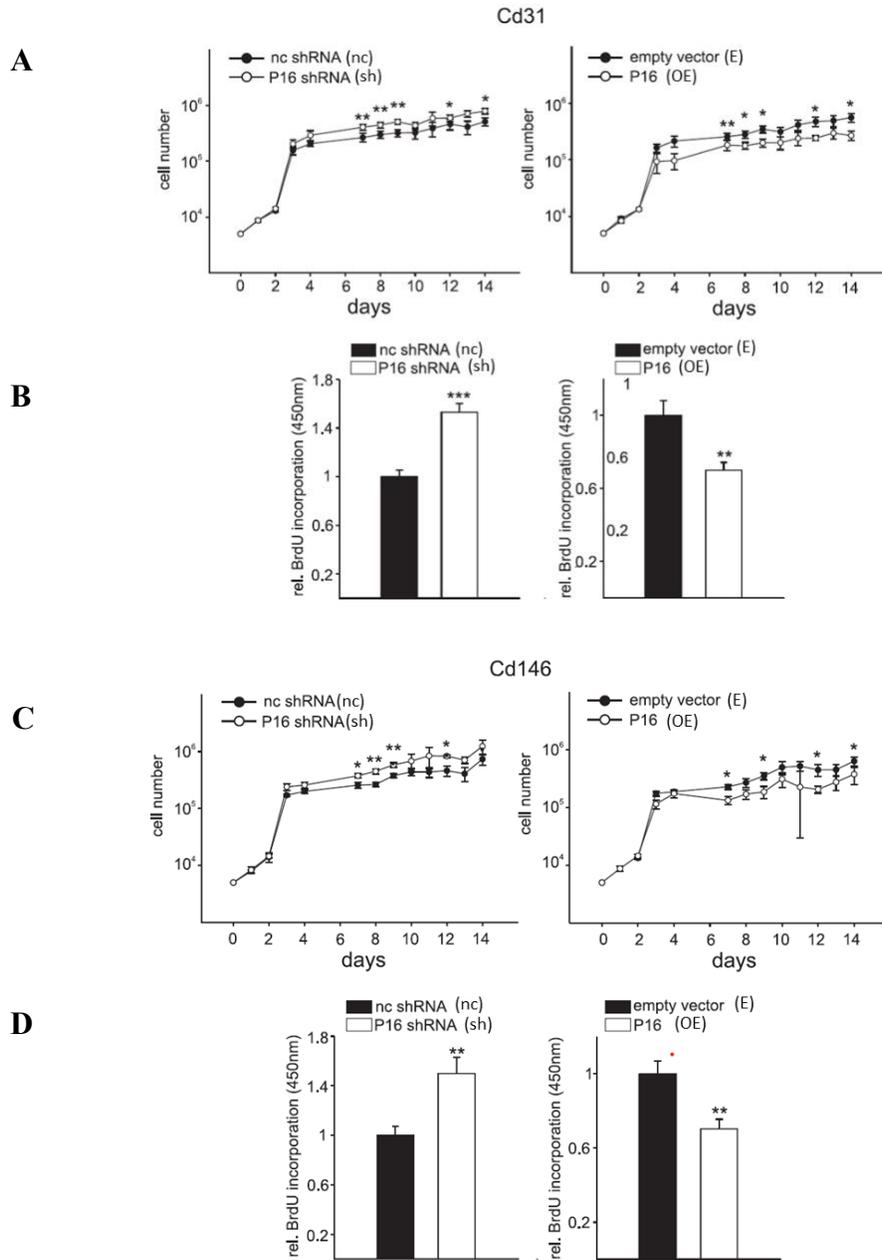


Figure 28. p16 knockdown was associated with higher proliferation while p16 overexpression was associated with lower proliferation. (A & C) Cell counts plots as function of time in days, starting at day 0 with 5×10^3 cells in (A) CD31 and (C) CD146, on the left sh (white dotted line) versus nc (black dotted line), on the right OE (white dotted curve) versus E (black dotted curve). (B & D) BrdU incorporation assay result, relative BrdU incorporation absorbance at 450nm of (B) CD31 and (D) and CD146, on the left nc (black bar) vs sh (white bar), on the right E (black bar) vs OE (white bar). . Data are mean \pm SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Data provided by K. and N.Wagner

IV. P16 and Liver Endothelial Cells Senescence

As p16 is known to be a senescence marker, we wanted to know the effect of p16 silencing and overexpression on CD31 and CD146 senescence, and this was assessed by β -gal staining. P16 knockdown was correlated with lower number of β -gal-stained cells compared to their nc controls in CD31 and CD146 cells. The amount of stained cell was lower by almost 2 folds in both cell types (**Figure 29A and 29C**). However, p16 upregulation led to increased number of β -gal-stained cells by approximately 2 folds compare to empty vector (E) transduced cells in both cell types (**Figure 29B and 29D**). Quantitative assessment by RT-qPCR, of selected SASPs, such as *Mmp9*, *Tgf β 1*, *Il-6* and *Vegfa*, did not show any change among p16 silencing or overexpression neither correlated with p16 overexpression-induced senescence, except for *Il-6* which was significantly upregulated with p16 silencing (sh) compared to nc transduced cells, in both cell types. Taken together, p16 overexpression induced senescence in CD31 and CD146 cells as indicated by β -gal staining, while lower senescence was obtained upon its silencing. This, however, did not correspond to the expression of selected SASP markers.

Moreover, given the variation in telomere length, within the same species, several studies suggested that the rate of telomere shortening rather than the telomere length is a more reliable variable for assessing telomeres shortening [611–614]. Therefore, we have measured the length of telomeres in CD31 and CD146 by qPCR (as described in materials and methods) at 1 week and 2 weeks after p16 silencing and overexpression besides the expression level of mouse telomerase catalytic subunits (*mtert* and *mterc*). The results obtained were subtracted from each other (at 1 week – 2 weeks) to obtain the rate of telomere shortening difference in 1 week time span. Our result has revealed a higher rate of telomere shortening in p16 overexpressed compared to E control cell while no difference in sh compared to nc was observed, however this result was not significant. Moreover, the catalytic subunits expression level was not affected by p16 expression.

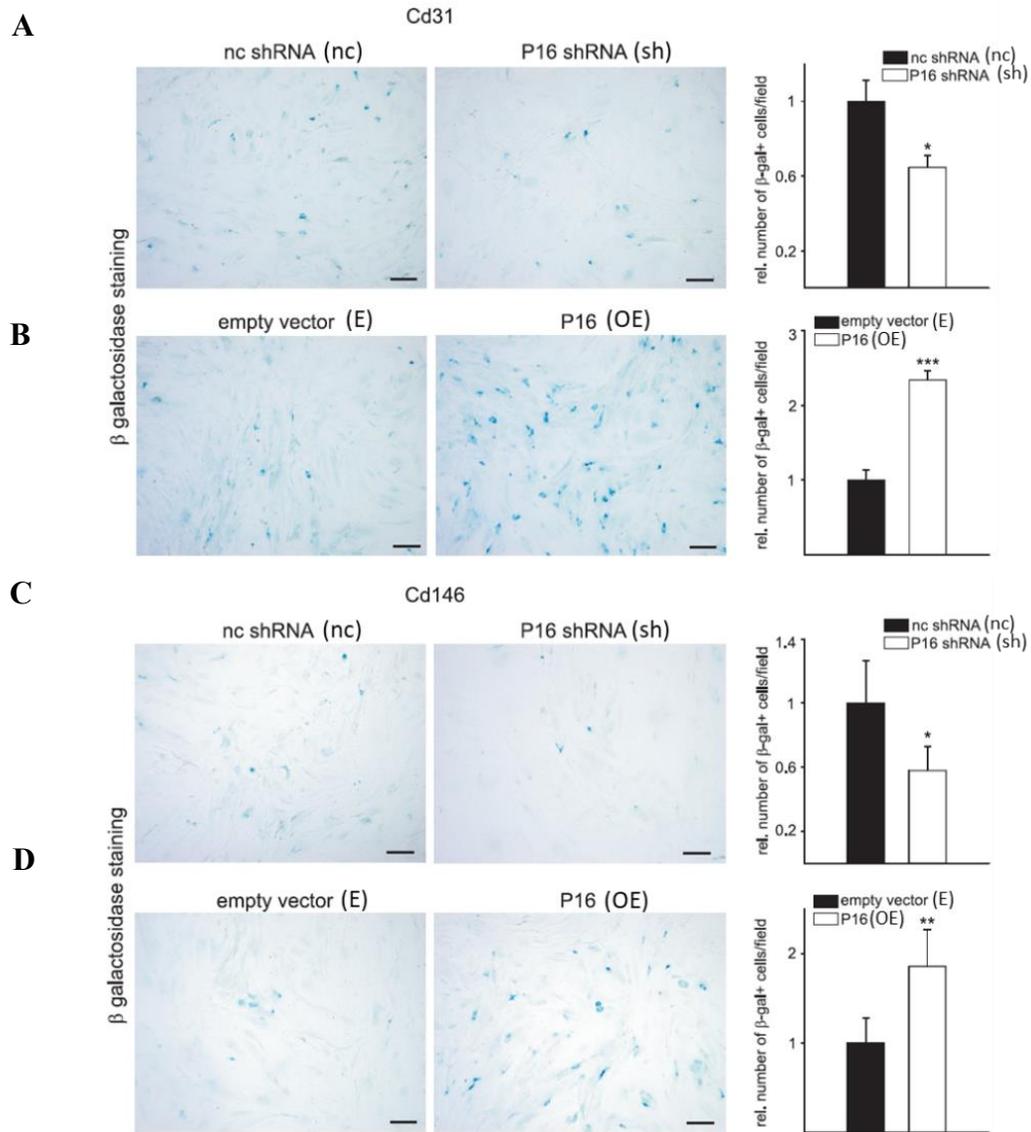


Figure 29. CD31 and CD146 cells senescence as function of p16 expression. (A & C) CD31 and CD146 sh versus nc. On the left images obtained under light microscope with their respective cell counts on the right. nc (black bar) and sh (white bar). (B & D) CD31 and CD146 OE versus E. On the left images obtained under light microscope with their respective cell counts on the right. E (black bar) and OE (white bar). Data are mean \pm SEM. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Data provided by K. and N.Wagner**

V. P16 and Liver Endothelial Cells Migration

The effect of p16 knockdown and overexpression on liver CD31 and CD146 ECs migration was assessed by scratch assay and trans-well migration assay. Initial scratches were made and normalized for average of approximately 570 μm , at time 0 in both CD31 and CD146 cells. Then the distance was measured every 3 hour at t 3, 6, 9, 12 and finally at t 24h. for both cell types no difference was observed in cellular migration between p16 knocked-down cells and their control (nc), we obtained full closure of the scar after 24 hrs. Nevertheless, no difference of the measured distance was observed at each time point. However, significantly attenuated migration was observed with p16 upregulation in both CD146 and CD31 at each time of scar measurement in OE cell compared to E control cell. In addition, full closure of the scar was obtained in E transduced cell while a scar distance of more than 200 μm remained after 24h in p16 induced cell (**Figure 30 and 31**).

On the contrary to scratch assay, trans-well migration assay has shown ameliorated migration with higher number of cells passed through the trans-well in sh transduced cells compared to nc in CD31 and CD146 cells. However, the difference in the number of migrated cells was slightly higher with low significance in CD31 and insignificant in CD146. While p16 overexpression in both cell types was correlated with significantly diminished migration, measured by 4 folds for CD31 and 5 folds for CD146, lower cell numbers passing down the trans-well in OE compared to E control (**Figure 32**). All these results show, that p16 increased expression has an anti-migratory effect on liver ECs. while its downregulation might slightly improve liver ECs migration.

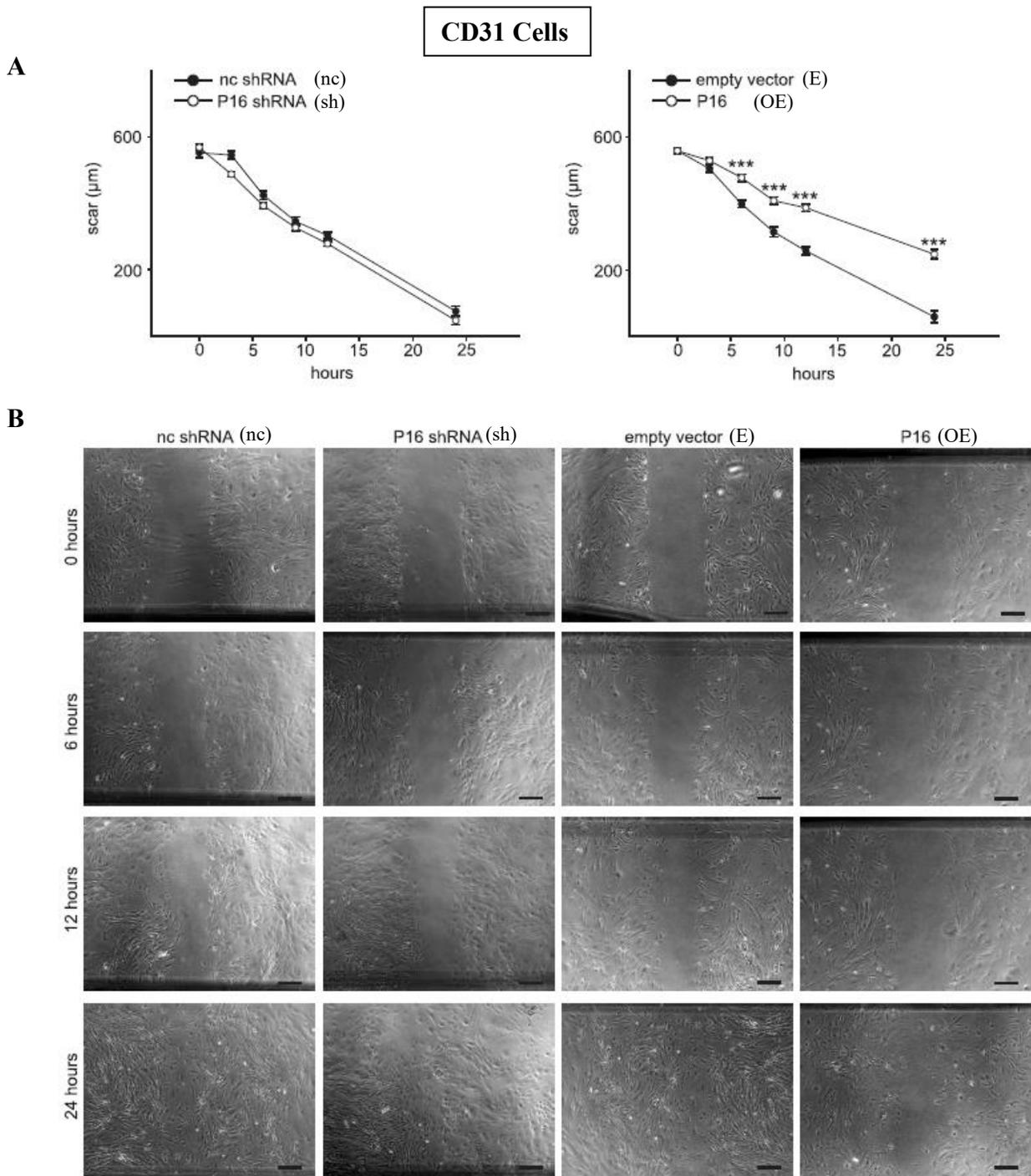


Figure 30. p16 silencing had no effect on CD31 migration however p16 overexpression attenuated their migration. (A) plot of the scar size in μm as function of time in CD31 cells. the left plot is for sh (white dotted curve) versus nc (black dotted curve). The right plot is for OE (white dotted curve) versus E (black dotted curve). The distance between the edges of the scratch was measure using ImageJ software. (B) image of the scratches at time 0, 6, 12, and 24 hours of the four different conditions nc, sh, E, and OE showing the gradual closure of the scratch with time. Images were captured at 10x magnification with phase contrast mode. Data are mean \pm SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

CD146 Cells

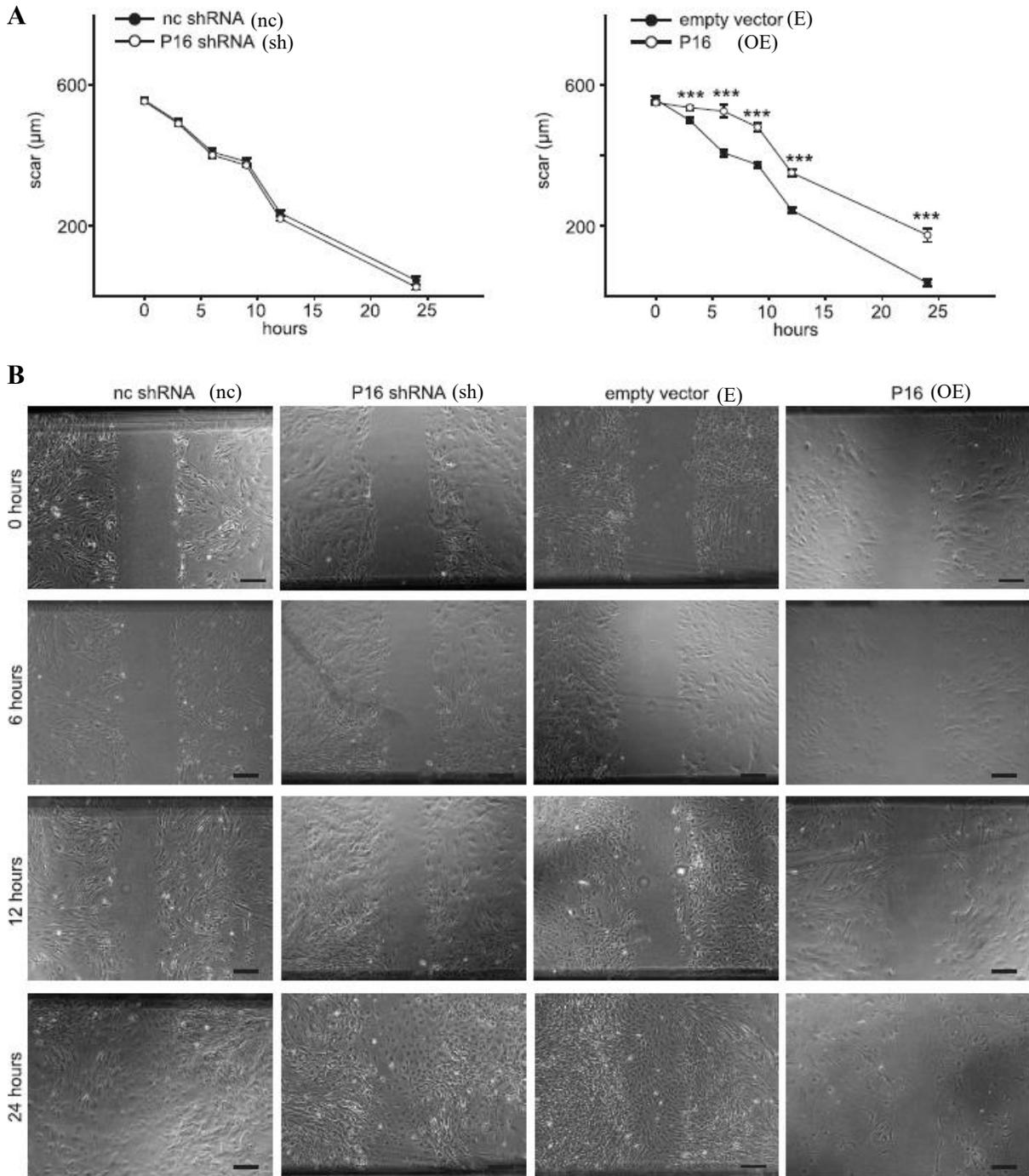


Figure 31. p16 silencing had no effect on CD31 scratch migration however p16 overexpression attenuated their migration. (A) plot of the scar size in μm as function of time in CD146 cells. the left plot is for sh (white dotted curve) versus nc (black dotted curve). The right plot is for OE (white dotted curve) versus E (black dotted curve). The distance between the edges of the scratch was measure using ImageJ software. (B) image of the scratches at time 0, 6, 12, and 24 hours of the four different conditions nc, sh, E, and OE showing the gradual closure of the scratch with time. Images were captured at 10x magnification with phase contrast mode. Data are mean \pm SEM. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.**

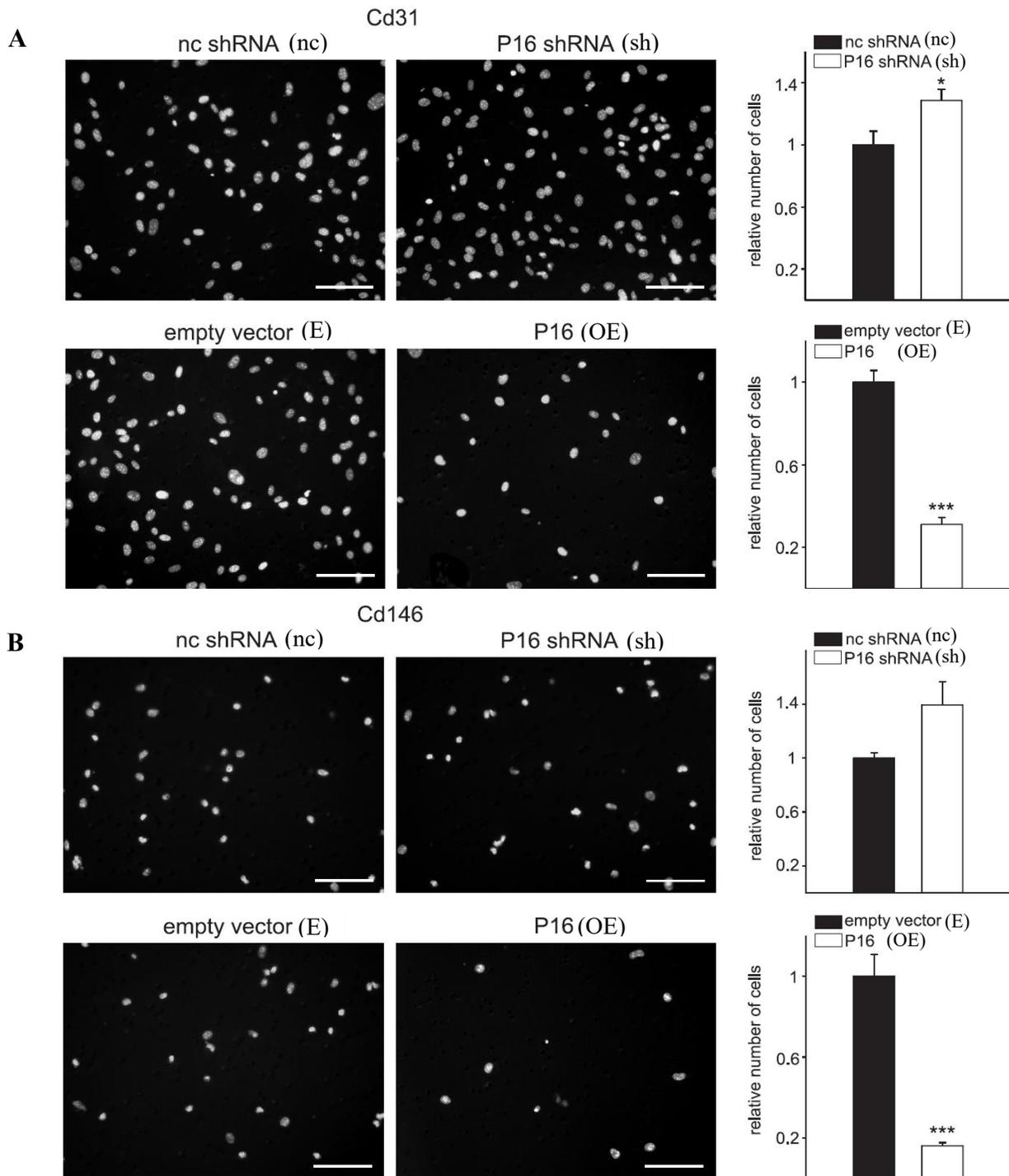


Figure 32. CD31 and CD146 cells migration is inhibited upon p16 upregulation, while their migration is slightly ameliorated when p16 is silence. (A) CD31 transwell migration assay results, upper images with their respective cellular counting represent nc control cells (black bar) vs sh p16 silenced cells (white bar). Lower images with their respective cellular counting represent E control cells (black bar) vs OE p16 overexpressed cells (white bar). (B) CD146 transwell migration assay results, upper images with their respective cellular counting represent nc control cells (black bar) vs sh p16 silenced cells (white bar). Lower images with their respective cellular counting represent E control cells (black bar) vs OE p16 overexpressed cells (white bar). Data are mean \pm SEM. * $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Data provided by K. and N.Wagner**

VI. P16 and Liver Endothelial Cells Apoptosis

The implication of p16 in CD31 and CD146 endothelial cell apoptosis was assessed by Annexin V/PI double labeling followed by FACS analysis and quantification of Annexin V single labeled population (green-early apoptosis). the result demonstrated that p16 silencing (sh) was correlated with lower percentage of apoptotic cells, ~2% for CD31 and ~3% for CD146 compared to the nc transduced control cells, ~3.5 for CD31 and ~5% for CD146. Whereas p16 overexpression (OE) was correlated with higher percentage of apoptotic cells, ~11% for CD31 and ~8% for CD146, compared to E transduced control cells ~5% for both CD31 and CD146 cells (**Figure 33**). However, the results obtained have shown only a tendency of p16 overexpression to induce apoptosis, or p16 silencing to inhibit apoptosis in both cell types, while the difference between sh and nc and between OE and E was not significant.

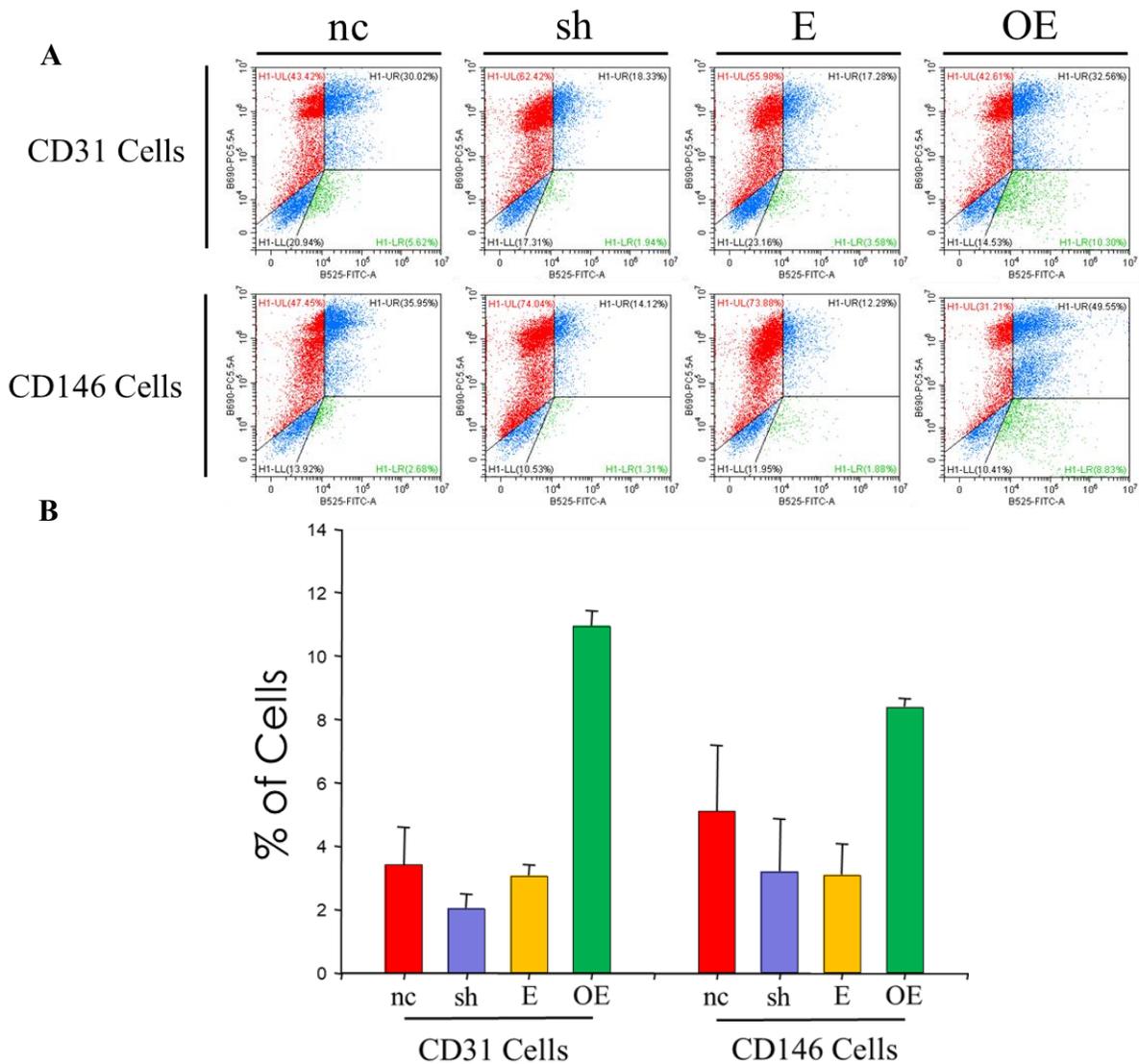


Figure 33. p16 silencing has a tendency to inhibit apoptosis, whereas p16 over expression has the tendency to induce apoptosis in CD31 and CD146 cells. (A) Flow cytometry results in 2D dot plot diagram. Green population labeled with FITC-A only where only considered to assess p16 role implication in early apoptosis. (B) cellular quantification of the green population in percentage of the total cell. nc (red), sh (blue), E (yellow), OE (Green).

VII. P16 Potential Anti-inflammatory Role

Given that Il-6 plays a crucial role as a major inflammatory cytokine and a key immune response regulator during infections and injuries [615], and based on the significant upregulation of Il-6 obtained in CD31 and CD146 cells upon p16 silencing during SASPs assessment, we aimed to investigate whether silencing and overexpressing p16 could impact the mRNA expression of other inflammatory cytokines in CD31 and CD146 cells. For this purpose, we selected a panel of inflammatory cytokines (**listed in Table 2**) based on their established roles in inflammation from the existing literature. We conducted quantitative RT-qPCR analysis, comparing their mRNA expression outcomes in p16-silenced cells (sh) relative to their expression in nc control cells, as well in p16 overexpressing cells (OE) relative to control cells (E)

Showing only the genes with significant variations, our findings revealed several other inflammatory cytokines and chemokine that were significantly upregulated beside Il-6 when p16 was silenced. Among them, caspase-4, Ccl2, Ccl3, Ccl5, Ccl9, Ccl12, Cxcl10, Stat1 and Stat2 were upregulated in sh condition of CD31 cell relative to their expression in nc CD31 cells (**Figure 34A**). Similarly, in CD146 cells, except for Ccl3, Ccl9 and Ccl12 which didn't show significant variation in sh CD146 cells relative to theirs in nc CD146 cells (**Figure 34B**). On the other hand, p16 over expression (OE) only induced Ccl5 and Stat2 significant downregulation in OE CD31 and OE CD146 cells respectively, relative to their expression in E control cells (**Figure 34A and B, OE vs. E**).

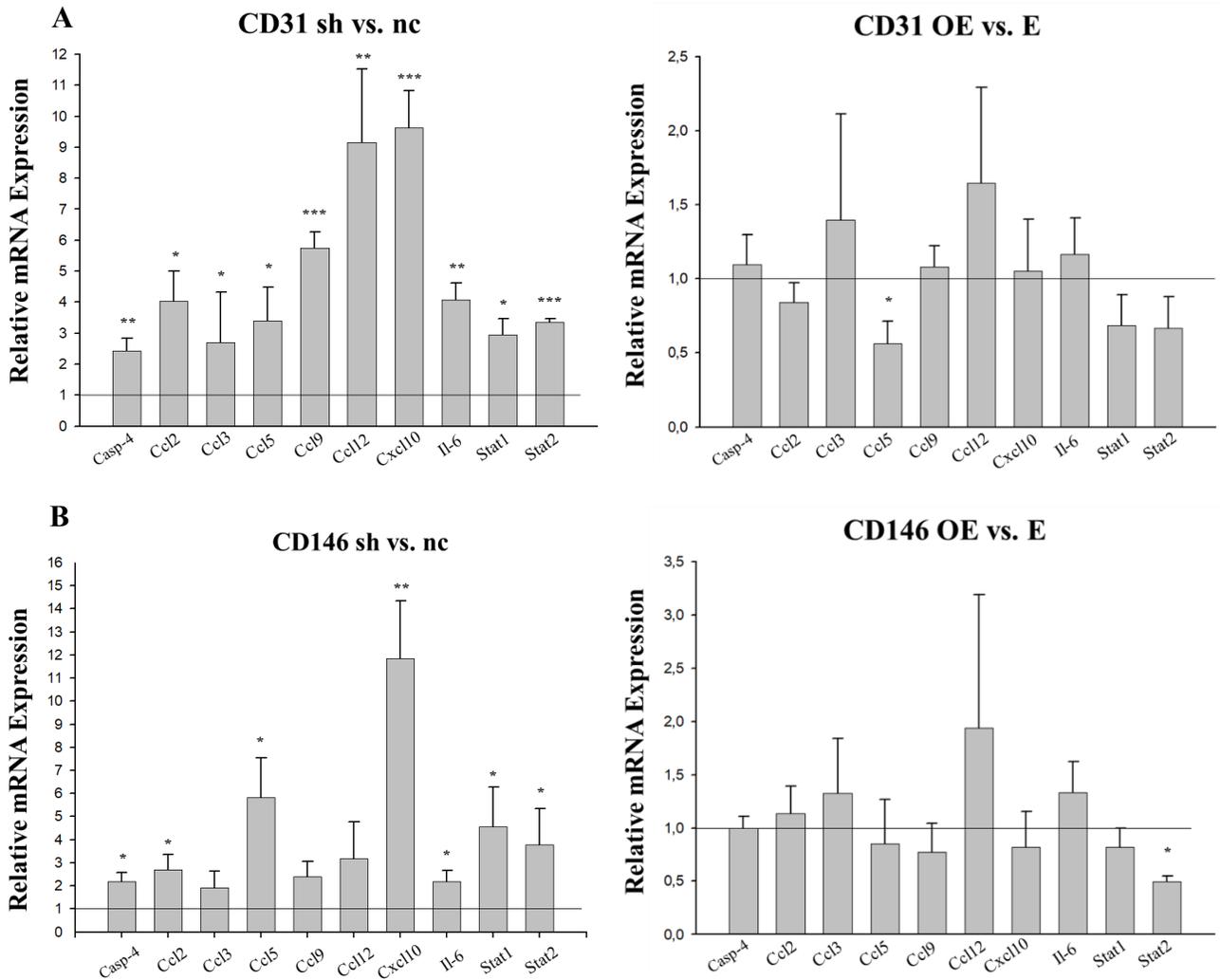


Figure 34. Quantitative RT-PCR analysis of mRNA expression of inflammatory cytokine and chemokine of CD31 and CD146 cell upon p16 silencing (sh) and overexpression (OE). (A) Relative mRNA expression of the assessed inflammatory cytokines in CD31 cells on the left mRNA expression in sh relative nc, on the right mRNA expression in OE relative E. (B) Relative mRNA expression of the assessed inflammatory cytokines in CD146 cell. on the left mRNA expression in sh relative nc, on the right mRNA expression in OE relative E. Data are mean \pm SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.



DISCUSSION
CONCLUSION
FUTURE PERSPECTIVES



I. Discussion

The results obtained from our study have demonstrated a dynamic and distinct pattern of expression for the p16 gene during various stages of embryonic and postnatal development, as well as in adult and aging mice, specifically in the brain, heart, kidneys, and liver. Notably, p16 expression exhibited significant fluctuations within each organ during embryonic development, occurring rapidly over the span of a few days. Conversely, the genes p19 and p21 did not exhibit such dramatic changes in expression levels. For the purposes of our investigation, we chose to focus on the brain, heart, kidney, and liver due to their development during the selected embryonic time points and their relative ease of isolation [364,616–618]. However, we acknowledge that p16, p19, and p21 might also be expressed in various other developing organs. For instance, previous studies have reported the presence of p21 expression during embryonic development in tissues like muscle, nasal epithelium, tongue muscles, hair follicles, epidermis, and cartilage, which was linked to growth arrest and senescence [156,428,619]. Additionally, p19 expression has been observed in the developing nervous system. In contrast, p16 had not been previously detected during embryonic development, although the possibility of low-level or restricted expression was not excluded [388,389]. Our study employed sensitive techniques such as quantitative RT-PCR and antibody staining [599,601–604,606], leading to the identification of relatively high p16 expression during development and particularly in advanced stages of aging.

The upregulation of p16, p19, and p21 is widely recognized as an indicator of aging and senescence [20,49,179,620]. In human tissue samples, p16 was found in various organs including the endocrine and exocrine pancreas, skin, kidneys, liver, intestine, spleen, brain, and lung [621]. Its expression increased in most investigated organs with advancing age. Our research on mice revealed a substantial increase in p16 expression across all the organs studied between 3 and 16 months of age. Comparatively, the increase in p19 and p21 expression was less pronounced in old mice when contrasted with adult mice, aligning with earlier reports in both mice and humans. Notably, we did not observe any organ-specific differences in p16 expression at 16 months of age, which contradicted a recent study by Yousefzadeh et al. [622]. This discrepancy could potentially be attributed to the age disparity among the animals used in their study, where they compared mice aged 15 to 19 weeks with those aged 120 weeks. Additionally, a study analyzing p16 protein expression in human tissues of young, middle-aged, and old donors confirmed a significant

increase in p16 in the liver, kidneys, and brain of older subjects. However, unlike our findings, they did not detect any p16 expression across all age groups in the heart [621].

The understanding of p16, p19, and p21 expression and their roles during embryonic development is complex and often contentious within the existing literature. While p21's role in embryonic senescence has been extensively examined, the presence of p16 and p19 during this phase has been inconsistent in earlier studies [388,619]. Senescence have been identified through SA- β -galactosidase staining during embryonic development, with p21 playing a significant role. Interestingly, the absence of p21 was compensated by apoptosis, but still resulted in minor developmental irregularities [156,159]. Despite the focus on p21, previous research has indicated that p16 loss can lead to developmental defects in the eye and that p16 and p19 inactivation can induce cardiomyocyte proliferation [406,410]. Which explains that p16 expression found in our study is crucial for lower proliferation and differentiation which are essential requirements for myocardial compaction [623,624]. During rat embryonic and early postnatal stages, there was an observed co-localization of p16 expression with p53 in the ventricular and subventricular zones [390]. In mouse embryos, noticeable levels of p16 were also identified in the olfactory epithelium as well as in motoneurons [391,625]. Additionally, p16 expression was detected in hematopoietic progenitor cells originating from the bone marrow of adult mice [392]. Elevated levels of p16 expression in mice were observed in the endometrium between days 2 and 5 of pregnancy [396]. Furthermore, during mouse embryonic development, p16 expression was identified in the gut, specifically within intestinal stem cells and the progenitor compartment [398].

The notion of senescence as an irreversible form of cell cycle arrest resulting in cell death has been challenged by recent findings indicating that cancer cells can escape senescence-induced cell cycle arrest and gain aggressive growth potential [22,626]. Intriguingly, embryonic senescent cells have been shown to re-enter the cell cycle and participate in tissue development [627]. Our study has revealed variations in p16 expression, particularly through the immunohistochemical localization of p16 protein, during the developmental process. The expression of p16 during development may reflect its function in slowing down cell cycle progression, a crucial process for cell differentiation. While knockout mice lacking p16/p19 and specifically p16 are susceptible to tumor development [407,628], potential developmental abnormalities have not been extensively explored due to the viability and fertility of these mice. Consequently, further investigations are necessary to reassess

potential developmental defects in mice with p16 inactivation or elimination of p16-expressing cells.

Furthermore, a significant finding was the higher expression of p16 in liver ECs compared to non-ECs, particularly in old animals, which aligns with previous study, that indicate p16-high cells are predominantly liver ECs and are enriched with aging [210]. This distinct increased p16 expression in liver ECs suggests a potentially selective role of in liver endothelial physiology, which could have implications for understanding the aging mechanism in the liver. Regarding postnatal livers, p16 has been extensively studied, revealing its protective effects in non-alcoholic steatohepatitis and liver fibrosis by regulating reactive oxygen species (ROS) and oxidative stress [338,629]. The specific removal of liver ECs expressing high levels of p16 led to fibrosis and liver deterioration, highlighting their role in maintaining liver physiology [210]. However, detailed studies utilizing conditional cell type-specific knockout strategies will be imperative to elucidate the specific functions of p16 in liver ECs.

Therefore, to probe the functional consequences of p16 expression in liver ECs, experimental manipulation of p16 expression levels in liver ECs, CD31 and CD146, was undertaken via viral transduction. P16 knockdown and overexpression would allow for a precise investigation into the consequences of both p16 loss-of-function and gain-of-function in the physiology of ECs.

The results obtained from this study have demonstrated that p16 has a significant effect on the proliferation, senescence, migration, and apoptosis of both types of liver ECs, the CD31 and CD146. P16 overexpression diminished liver ECs proliferation, whereas p16 silencing was associated with ameliorated proliferation. In addition to that, p16 overexpression in liver ECs induced demonstrated higher β -gal staining and consequently more senescent cells in culture compared to control cell. Reversely, p16 silencing decreased the number of stained cells compared to their control cells. In addition to that, p16 overexpression has attenuated CD31 and CD146 cells migration in scratch assay and transwell assays, while silencing didn't have an effect on migration. Finally, the upregulation of p16 has shown a tendency to induce apoptosis in CD31 and CD146 liver ECs, while p16 repression led to a lower percentage of apoptotic cells.

The attenuated proliferation and increased senescence observed upon p16 overexpression, emphasizes the role of p16 as a cell cycle regulator, and therefore its upregulation would certainly induce the cell cycle arrest and causes senescence [172,182,190]. This result aligns with several previous results in the context of p16 and ECs senescence. For instance, investigations in bovine

capillary ECs [630] and HUVECs [631] revealed elevated expressions of both p21 and p16, accompanied by predominantly hypo-phosphorylated RB. In contrast, one of these studies demonstrated that ectopic expression of hTERT suppressed p21 and p16 expression while maintaining RB in a hyperphosphorylated state. Consequently, this allowed cells to bypass senescence, despite ongoing telomere shortening [630]. Concurring with these findings, Tang et al. (2002) [632] demonstrated that the helix-loop-helix protein Id-1 delayed the onset of replicative senescence in human ECs by repressing p16 expression. This effect may be mediated by Id-1's ability to downregulate the p16 trans-activator Ets1, as observed in fibroblasts [81]. Additionally, in human aortic ECs, both p21 and p16 were upregulated when senescence was induced by introducing a dominant negative mutant of TRF-2 [633]. Collectively, these findings suggest that in ECs, both the p53/p21 and p16 pathways contribute to the senescence response triggered by telomere integrity loss. In addition, although the expression of p16 increases in cell culture compared to in vivo, its overexpression further inhibited the cellular proliferation and induced senescence. This implies that the expression of p16 is not inhibited by negative feedback loop of hypophosphorylated pRB as suggested in previous reports [194].

Migration is a key process in tissue homeostasis, repair and angiogenesis [634]. The overexpression of p16 diminished the migratory capacity of both CD31 and CD146 liver ECs, as evidenced by scratch and transwell assays. This implies that p16 might play a role in modulating cell motility, potentially affecting tissue repair mechanisms during aging [209,406,515]. This result also agrees with a previous study that showed anti-migratory and antiangiogenic effect of p16 on ECs [635]. However, another study showed that p16 repression was associated with attenuated migration and angiogenic capacity in human ECs [636].

Nevertheless, apoptosis was also influenced by p16 expression levels, however, with no significant differences observed. Upregulation of p16 exhibited a tendency to induce apoptosis in both CD31 and CD146 liver ECs. Conversely, p16 silencing was linked to a lower percentage of apoptotic cells. This highlights the intricate involvement of p16 in regulating cell survival achieved by cellular senescence and apoptosis in liver ECs, although it has been long time believed that senescent cells are resistant to apoptosis [637]. On the contrary, senescence-associated apoptosis was observed in human ECs [631]. Another study has shown that increased expression of p16 has the capability to maintain the Retinoblastoma protein (pRb) in a growth-inhibitory state with reduced phosphorylation. This effect is achieved by its capacity to dampen the activity of cyclin-

dependent kinases 4 and 6 (CDK4/6). Meanwhile, heightened levels of p14 protein can interact with and sequester MDM2, preventing the MDM2-mediated degradation of p53. Consequently, this leads to the activation of several genes, with p21 being among them. The activation of p21 orchestrates the arrest of cell growth and triggers cellular senescence. Furthermore, the activation of the p53/p21 pathway can initiate a controlled program of apoptosis in HUVECs. Moreover, it's worth noting that the activation or functional integrity of p53 plays a facilitating role in allowing the regulation of the p16/Rb senescent pathway [638].

The upregulated expression of inflammatory cytokine upon p16 silencing in CD31 and CD146 cells provide an intricate insight into the role of p16 in modulating inflammatory response. Il-6, a well-established major inflammatory cytokine, was significantly upregulated upon p16 silencing, aligning with its recognized role as a key regulator in immune responses [615]. This observation underscores the complexity of p16's involvement in immune processes and prompted us to explore its broader impact on other inflammatory cytokines. Our findings unveiled a significant upregulation of several other inflammatory cytokines and chemokines when p16 was silenced in liver ECs. This suggests that p16 could act as a pivotal regulator not only for Il-6 but also for a broader range of immune-related factors. On the contrary, p16 overexpression in (OE) CD31 and CD146 didn't inverse the expression outcome of most of these cytokines. Several reports mentioned p16 interference in inflammation, however their results have shown a pro-inflammatory function of p16 [639–642]. However our results provide an opposing scenario showing an anti-inflammatory role of p16 to add another beneficial function to the previously reported ones [169,210].

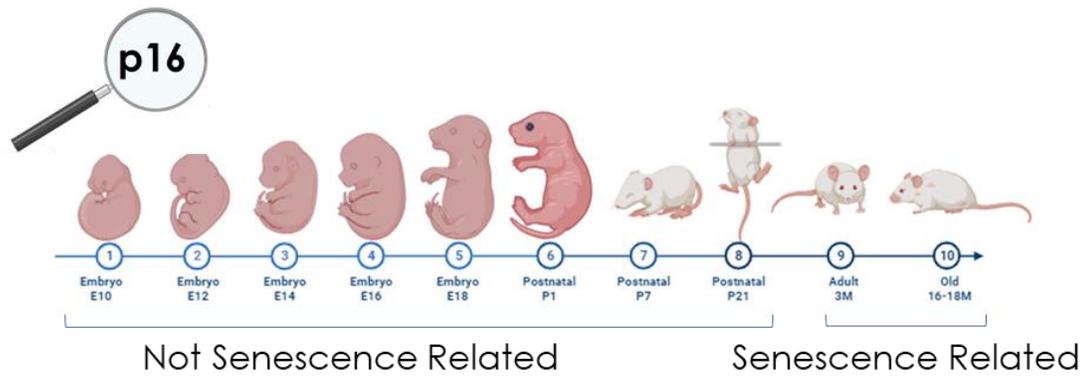
Aging in LSECs is characterized by pseudocapillarization, involving thickening of the sinusoidal endothelium, reduced LSEC porosity, increased basal lamina and collagen deposition in Disse's space, and von Willebrand factor expression in aged LSECs. This phenomenon, known as age-related pseudocapillarization, is distinct from capillarization seen in liver diseases. Reduced LSEC porosity is linked to conditions like postprandial hypertriglyceridemia, a risk factor for atherosclerosis. Additionally, aging leads to a decline in LSECs' endocytic capacity, affecting substance uptake, especially evident during elevated blood levels of waste macromolecules. Factors contributing to LSEC aging include exposure to gut-derived toxins, nicotine, and oxidants, which can induce LSEC defenestration [573,574,576,578,580,590]. In addition, before this study, previous research had demonstrated that elevating p16Ink4a expression in human umbilical vein

and microvascular ECs (ECs) led to decreased proliferation, impaired migration, and disrupted vessel formation in vitro [635]. The findings reported in this context indicated how p16Ink4a overexpression induced senescence in ECs, resulting in their impaired function. Similar outcomes were reported by other researchers, who also highlighted the correlation between high levels of endogenous p16Ink4a and dysfunctional ECs in cases of stress-induced or replicative senescence [631,633,643,644].

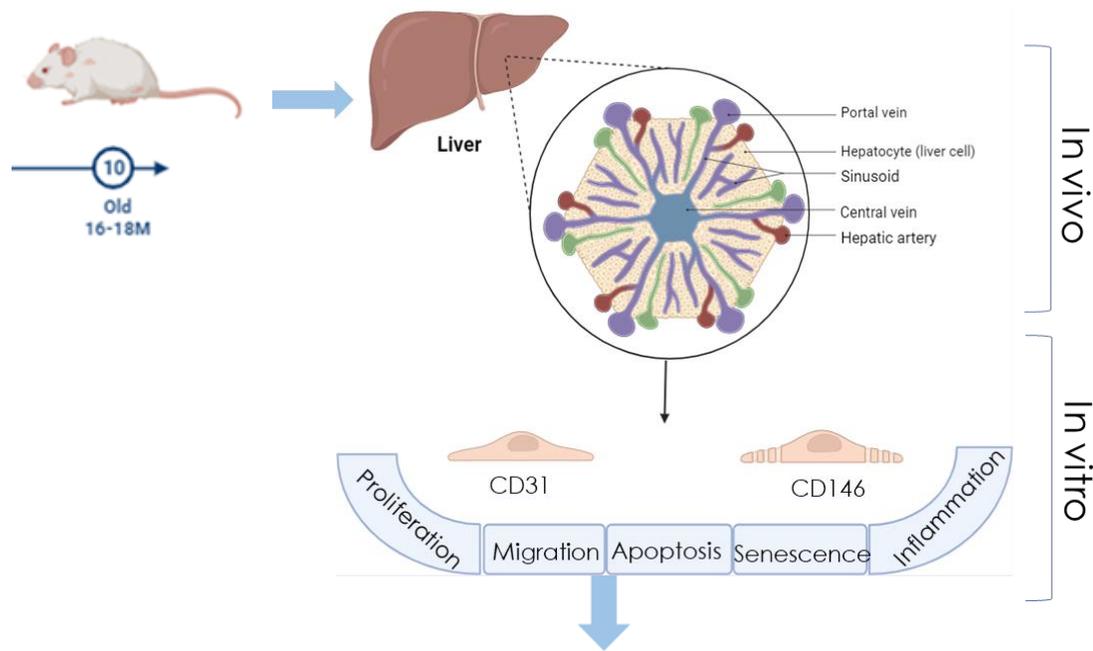
II. Conclusion and Future Perspectives

This study delved into the functional significance of p16 expression in liver ECs, shedding light on its impact on various aspects of cell behavior. P16 differential expression in liver ECs compared to non-ECs, in aging, underscores its potential role in age-related processes within the liver. Through experimental interferences in p16 expression, the study revealed its effects on cell proliferation, senescence, migration, and apoptosis, providing a more comprehensive understanding of its role in endothelial cell physiology, mechanism of ECs dysfunction and general liver aging. This knowledge not only contributes to our understanding of liver aging but also potentially paves the way for targeted interventions to modulate the effects of p16 in age-related liver conditions.

However, this study marks just the beginning of unraveling the complexities of p16-induced EC dysfunction and its contributions to liver aging. To provide a more comprehensive understanding, further investigations are warranted. For instance, exploring additional physiological processes like tube formation, which gauges ECs' angiogenic capacity under p16 overexpression, could offer more insights. Given that oxidative stress is a hallmark of senescence and aging, assessing ROS levels in p16-silenced or p16-overexpressing cells could provide a clearer picture. Moreover, considering that liver EC dysfunction is associated with defenestration, evaluating the permeability of liver endothelial cell monolayers would be valuable. Moreover, RNA seq of the different condition (sh vs nc) and (OE vs E) would reveal several other differentially expressed genes that contribute beside p16 in liver ECs aging mechanism. Moreover, further investigation into the specific mechanisms through which p16 influences these cytokines could provide valuable insights into immune regulation and potential therapeutic avenues. Therefore, a deeper comprehension of the intricate mechanisms through which p16 induces liver EC dysfunction and contributes to liver aging could potentially promising for p16 targeted anti-aging therapies (**Figure 35**).



Implication in Embryonic Development



Implication in Liver Aging & immune Regulation



➤ Significance

- Insights into embryonic development and Liver Aging

Figure 35. Conclusive schematic summary of key findings from the study investigating p16 expression during embryonic development and in liver endothelial cells, specifically focusing on CD31 and CD146. The observed correlation between p16 expression and embryonic development, along with its association with liver endothelial cells marked by CD31 and CD146, hints at potential implications of p16 in both embryonic development and liver aging. These insights pave the way for future investigations into the role of p16 in these processes, promising valuable perspectives for understanding embryonic development and liver aging dynamics.



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