Università degli Studi di Cagliari

DOTTORATO DI RICERCA

SVILUPPO E SPERIMENTAZIONE DEI FARMACI ANTIVIRALI

Ciclo XXIV

CELL SENESCENCE AND AGING

IN CARCINOGENESIS AND LIVER REPOPULATION

Settore/i scientifico disciplinari di afferenza

BIO/19 MED/04

Presentata da:	**MARIA PAOLA SERRA**
Coordinatore Dottorato	ALESSANDRA PANI
Tutor/Relatore	EZIO LACONI

Esame finale anno accademico 2010 - 2011

Table of Contents

1 Summary

Aging is the strongest risk factor for neoplastic disease. The hypotheses that have been proposed to explain this association can be grouped into two broad categories.

One is a “cell-orientated” hypothesis, and focuses on alterations (mainly genetic) taking place in rare cells in a tissue such that they eventually acquire a full malignant phenotype. Within this framework, aging can increase the risk of cancer by various mechanisms, such as (i) simply allowing more time for random mutations to occur in a single cell, or (ii) by increasing the generation of DNA damaging metabolites (e.g. through chronic inflammation), or (iii) by decreasing the efficiency of DNA repair processes and/or antioxidant defences.

A second, more holistic hypothesis is instead “tissue-orientated”, and considers overall changes taking place at tissue and possibly at a systemic level as critical factors that contribute to the increased risk of cancer with aging. While this proposition has long been centred on the idea that the link between aging and cancer relates to a decreased immune-surveillance in older age, allowing for the immune-escape of putative cancer cells, during the last decade the role of age-related, specific alterations in the tissue microenvironment is emerging has a major determinant in the pathogenesis of neoplastic disease.

These two hypotheses, “cell-orientated” and “tissue-orientated”, need not to be mutually exclusive and may in fact complement each other towards our understanding of the origins of cancer, both in general terms and as it relates to the specifics of the links between cancer and aging. However, it is important to point out

that they are conceptually distinct and they often run in parallel, with little or no sharing of research approaches and scientific experience. There is also little doubt that the “cell-orientated” hypothesis is still largely prevalent, although the more holistic approach is gaining ground.

The studies presented in my thesis delve into the complex relationship between cancer and aging using analytical approaches shared with the field of regenerative medicine. We took advantage of a model of orthotopic cell transplantation to explore the basic question as to whether the contribution of aging to the risk of cancer is, at least in part, related to alterations in the tissue microenvironment. Previous investigations conducted by our research group had revealed that isolated normal hepatocytes form larger clones of daughter cells when transplanted into the liver of old animals as compared to those formed in young recipients. In a relatively simple experiment we then utilized a similar approach to transplant preneoplastic cells isolated from hepatic nodules into either young or old animals of a syngeneic strain. Results were striking: very limited expansion of transplanted nodular hepatocytes was observed in the liver of young animals, while the same cell preparations grew significantly upon injection into older hosts, with a few cases of progression to hepatocellular carcinoma (HCC).

The relevance of these findings is twofold. Firstly, they clearly indicate that preneoplastic cell populations isolated from hepatic nodules are not endowed with any measurable degree of growth autonomy, in that they were unable to form nodules when transferred into the liver of young hosts. Thus, their focal growth is dependent on stimuli originating from the local microenvironment. In this respect they behave like normal hepatocytes. Secondly, it is evident from these results that the microenvironment of the aged liver is able to foster the growth of transplanted

syngeneic nodular hepatocytes, again reproducing analogous findings already obtained with normal cells.

Having established this fundamental fact, it became important to explore possible biological and underlying molecular mechanisms explaining the growth-promoting nature of the aged liver microenvironment. To this end, intriguing insights came again from the field of regenerative medicine.

Our research group is involved in the characterization of an experimental model of massive liver repopulation through hepatocyte transplantation. This approach was set up in our laboratory and is based on the administration of retrorsine (RS), a naturally occurring alkaloid, as a preconditioning treatment.

Rat liver exposed to RS and then transplanted with isolated normal hepatocytes is entirely replaced by donor-derived cells within a few months. Importantly, transplanted nodular hepatocytes can also selectively expand in the RS-treated liver, rapidly evolving to HCC. Again, neither normal, nor nodular hepatocytes are able to proliferate when injected into the liver of untreated recipients, as already noted above. This pattern of findings is similar to that observed in the aged liver, although the magnitude of the observed phenomena is far greater following exposure to RS. Moreover, it is apparent from the foregoing discussion that the growth of normal and nodular hepatocytes is controlled by fundamentally similar biological mechanisms, such that carcinogenesis and liver repopulation represent in fact “two sides of the same coin”, as it has been proposed.

Given the similarities of biological responses between the RS-treated and the aged liver, we then probed into possible cellular and molecular analogies shared by the two systems. Much to our surprise, it was found that exposure to RS is a powerful inducer of cell senescence in hepatocytes *in vivo*. As reported in the preceding

section of this report, several markers of cell senescence were expressed by RS-exposed hepatocytes, including senescence-associated β-galactosidase (SA-β-gal), cell hypertrophy, a persistent block in the cell cycle coupled with activation of cell growth pathways.

While cell senescence is primarily considered as an important biological barrier against the emergence of potentially neoplastic cells, it has now become clear that the presence of senescent cells can also foster neoplastic progression, possibly through effects related to the senescence-associated secretory phenotype (SASP). The latter includes a growing list of cytokines, growth factors and matrix-remodelling products which are released by senescent cells in the local microenvironment and exert their action through autocrine and/or paracrine mechanisms. A main component of SASP is IL-6, a pro-inflammatory cytokine which is also known for its important role in hepatocyte proliferation. Interestingly, IL-6 was found increased in the rat liver exposed to RS, suggesting that it might be involved in the stimulation of transplanted hepatocytes in this system.

The picture emerging from these findings is that RS treatment, which sets the stage for the selective growth of both normal and nodular transplanted hepatocytes, leading to massive liver repopulation and emergence of HCC, respectively, is also associated with the induction of a senescent phenotype in resident hepatocytes.

Such a senescence phenotype is commonly found in aged tissues including the liver, as also shown in our studies. This phenotype is likely to explain, at least in part, the similarities observed between the microenvironments of the aged liver and that of RS-exposed liver.

The last section of this thesis describes studies aimed at reversing the RS-induced alterations in the liver microenvironment and, in doing so, at verifying whether this

would modulate the growth of preneoplastic lesions promoted by RS. Animals given the genotoxic agent diethylnitrosamine (DENA) followed by RS developed large hepatocyte nodules within a few months. However, the growth of preneoplastic nodules was significantly delayed in animals receiving hepatocyte transplantation following the DENA+RS regimen. These results represent a proof of principle that an altered microenvironment is indeed a powerful driving force in neoplastic progression; most importantly, they clearly indicate that strategies aimed at “normalizing” such an altered microenvironment might impact on the rate of progression of the neoplastic process.

2 Introduction

2.1 Foreword

This dissertation delves into the intriguing analogies between the relatively new and expanding field of regenerative medicine and the old, but still highly relevant field of neoplastic disease. One shared theme between these two fields that comes to mind, almost immediately, is stem cells: it goes without saying that stem cells represent the cornerstone of regenerative medicine, but they have also gained enormous attention and stimulated a very active debate within the cancer researcher's community. However, stem cells are not the topic of this thesis. Rather, the studies presented here highlight common pathogenetic mechanisms rooted in the local microenvironment which appear to modulate both tissue repair and regeneration and the emergence of the neoplastic phenotype. A major finding of these studies is that cell senescence stands out as a dominant component of such a microenvironment. More specifically, the senescence-associated secretory phenotype, with its complex composition of inflammatory cytokines and growth factors, has the potential to impact on both tissue regeneration and carcinogenesis. The role of cell senescence is just beginning to be unraveled. However, it is already evident that this phenotype, far from being a merely passive end result of a cell life cycle, emerges as a new state of differentiation, which in turn can exert profound effects on chronic disease processes related to cell and tissue turnover. This topic is explored using a novel experimental system that bridges regenerative

medicine and carcinogenesis: the retrorsine-based model for massive liver repopulation *via* hepatocyte transplantation; however, reference will be also made to normal physiological aging. The essential background information is provided in the following introductory sections.

2.2 Cell Senescence

According to Greek mythology, the Goddess Eos asked Zeus to grant immortality to the Trojan Tithonus, her mortal lover. But Eos had forgotten to ask for eternal youth, so Tithonus received the ‘‘gift” of immortality and continued to age, withering beyond recognition and begging eternally for death.

In similar fashion, improved sanitation and better nutrition, with the contribution of modern medicine, have increased the life expectancy of individuals in the developed world, but this has not been matched by equal progress in our ability to alleviate the ill health associated with increased age. Thus, aging has become a major research topic over the past few decades[1].

Interest in the role of aging within the sphere of hepatology has increased, especially with the recent recognition of the importance of age in determining the clinical outcome in common livers diseases such as chronic hepatitis C virus infection[2] and the influence of donor age on graft survival after liver transplantation[3]. It is also well established that the ability of the liver to withstand the consequences of injury falls with each decade. Thus, liver-related death in older persons is increased substantially when compared to younger individuals with the same condition[4]. The cellular and sub-cellular changes that underlie this predisposition are the subject of much scrutiny. There is undisputable evidence that the aging

process takes place, at least in part, at the cellular level and that these changes can be stimulated ‘prematurely’ if affected by chronic inflammation. Within this context, the role of cell senescence is receiving increasing attention[5].

Cellular senescence was formally described more than four decades ago when Hayflick and colleagues showed that normal cells had a limited ability to proliferate in culture[6]. This fundamental finding carried two main conceptual implications. Given the fact that many cancer cells proliferate indefinitely in culture, the senescence response was interpreted as one mechanism that protected tissues and organisms from cancer. On the other hand, considering that the ability to perform tissue repair deteriorates with age, cellular senescence was seen as the possible basis for the aging process at organ and organismal levels, as exemplified by the loss of regenerative capacity of tissues *in vivo*.

The idea that a biological process such as cellular senescence can be both beneficial (tumor suppressive) and deleterious (limit tissue renewal) is consistent with a significant evolutionary theory of aging named antagonistic pleiotropy.[7]

In agreement with the antagonistic pleiotropic theory of aging, natural selection has favored genes providing advantages to the organism during the reproductive years, at the expenses of deterioration in the distant future. The ‘disposable soma’ theory expresses this as a life-history strategy in which somatic maintenance is below the level required to prevent aging, hence allowing increased prompt fertility.

However, the interpretation of cell senescence within the scheme of antagonistic pleiotropy is not universally accepted. Recently, Blagosklonny *et al.* have argued that cell senescence is the end result of two opposing signals: one that reads for cell growth and the other imposing a persistent block in the cell cycle. As such, it is perfectly functional to the biology of both young and older tissues and organisms.

Furthermore, the discovery and characterization of the SASP has clearly indicated that cell senescence leads to profound changes in the tissue microenvironment, potentially modulating tissue repair and regeneration as well as the progression of chronic disease processes such as neoplasia.

2.2.1 Characteristics of senescent cells

Cellular senescence is an important stress related response to injury that induces stable cell-cycle arrest with important implications for the survival and function of the cell, the organ, and the organism.

Cellular senescence is confined to mitotically capable cells. Although these cells can proliferate, they can also spend long periods in a reversibly switched off mode named quiescence or G_0. Quiescent cells resume division in response to appropriate signals, comprising the need for tissue repair or regeneration. On the other hand, post-mitotic cells permanently lose the ability to proliferate owing to differentiation[8]. Mitotic cells can senesce when they come across potentially oncogenic circumstances. When this happens, the cells cease proliferation (known as growth arrest), and cannot be reversed by known physiological stimuli. They frequently become resistant to cell-death stimuli (apoptosis resistance) and they acquire widespread changes in gene expression. Together, these features comprise the senescent phenotype.

One of the main characteristics of cellular senescence is an inability to progress through the cell cycle. Senescent cells arrest growth, usually with a DNA content that is typical of the G_1 phase, yet they remain viable with low levels of cellular metabolism[9-12]. Once arrested, initiation of DNA replication is unsuccessful

regardless of ideal growth conditions. This replication failure is in fact induced by an activated DNA damage response (see next section).

As opposed to quiescence, the senescence growth arrest is essentially permanent (at least in the absence of experimental manipulation), since senescent cells cannot be induced to resume the cell cycle by known physiological stimuli.

2.2.2 The DNA damage response and cell senescence

The senescence response is caused by many potentially oncogenic signals, comprising dysfunctional telomeres, DNA damage and strong mitogenic stimuli including those transmitted by some oncogenes. Many of these signals, directly or indirectly cause DNA damage and activate the DNA damage response (DDR) [5,13].

Historically, telomere shortening was suggested to be the main mechanism causing replicative cellular senescence (Hayflick's limit). The inability of standard DNA polymerases to fully replicate linear DNA molecules causes the estimated loss of around 50 to 200 base pairs during each cycle of replication at the 5′ end of the lagging strand. Therefore, the end replication problem leads to gradual shortening of chromosomes at every DNA replication round and subsequent cell division, eventually leading to DDR activation at critically short telomeres. Yet, the precise critical length of such dysfunctional telomeres is still unclear[14].

The DDR is mediated by DNA damage protein sensors such as the MRE-RAD50-NBS-1 (MRN) complex, which triggers the switch of a signal transduction system comprising the protein kinases ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), CHK1 and CHK2 (checkpoint kinase 1 and 2). According to the prevailing view, the DDR then activates p53, which promotes either apoptosis or

senescence *via* transactivation of its downstream targets, including the cyclin dependent kinase inhibitor, p21 [Cip1 15].

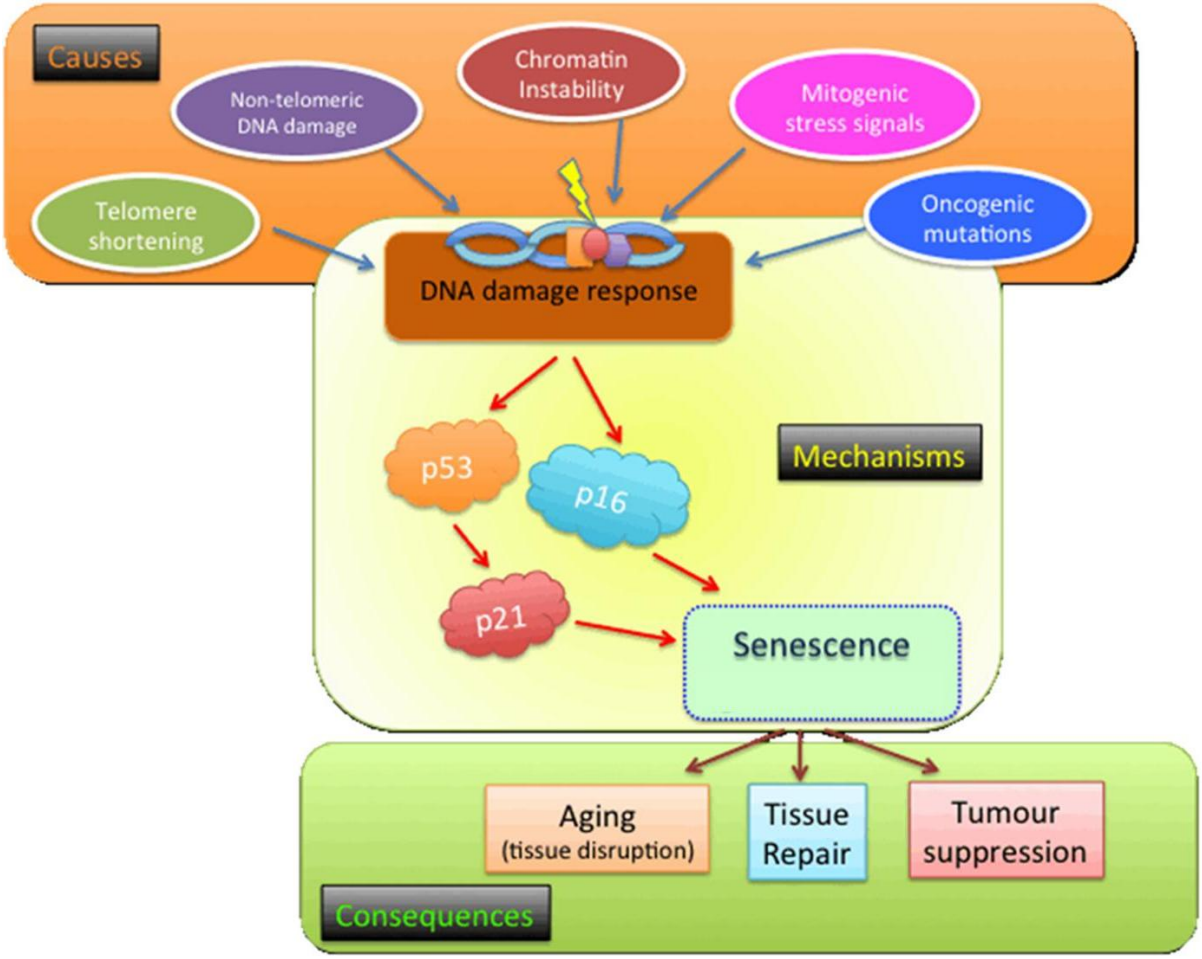


Figure 1: DNA damage response: causes-mechanisms-consequences.

Cells that undergo senescence due to continuous DDR signaling harbor persistent nuclear foci, termed DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS)[16]. These foci contain activated DDR proteins, including phospho-ATM and phosphorylated ATM/ataxia telangiectasia and Rad3 related (ATR) substrates[9,17-19], and are distinguishable from transient damage foci[16] .

The DDR might lead to senescence in two ways. First, it can switch on p53, which inhibits cell growth *via* p21[CIP1/WAF1 20].

It has been suggested that at low levels of damage, the DDR is transient, while high levels would cause chronic DDR signaling and p53 activation, which would then maintain a senescence growth arrest [20-21.]

Second, the DDR activates a subset of the senescence-associated secretory phenotype (SASP), independent of p53 activity; accordingly, loss of p53 function amplifies the SASP, suggesting that p53 restrains this phenotype[22].

2.2.3 Factors inducing cellular senescence

Besides the continuous passaging of cells in culture described by Hayflick, cellular senescence can be induced *in vitro* by multiple stimuli (figure 2). Methods of induction include:

2.2.3.1 DNA damage by genotoxic agents

Genotoxic agents such as radiation (X or γ-rays, UV) or DNA-interacting drugs (e.g. bleomycin, doxorubicin, mitomycin)[23-26], cause DNA double-strand breaks (DSBs). Short or dysfunctional telomeres are also thought to form a structure that resembles DSBs[27-31]. The response of the cell to such damage is the assembly of large protection complexes around the breaks that stabilize the structure and prepare for potential repair[32-35].

DSB is a type of DNA damage which occurs when two nearby complementary strands of the double helix of DNA get damaged simultaneously. DSB is the most dangerous type of DNA damage, because it has been suggested that a single unrepaired DSB is sufficient for the initiation of the cell death procedure[36-37].

The factors leading to the formation of DSBs comprise also endogenous factors, that are associated with physiological processes that take place in the cell, and the exogenous ones[38-39] in living cells. DNA is constantly subject to a process of oxidative damage by oxygen free radicals (reactive oxygen species—ROS) that are produced inside the cell as a consequence of metabolic processes[40].

It has been estimated that in a single cell cycle at least 5000 single-stranded DNA breaks can occur as a result of ROS production. About 1% of these DNA lesions deteriorate into DSBs, mainly during DNA replication, whereas the remaining 99% are fixed. Hence, during the cell cycle in a single nucleus, around 50 “endogenous” DSBs are produced. Accumulation of unrepaired DNA damage induced by ROS leads to cellular aging and might be accountable for the initiation of neoplastic transformation[39-41]. Is it accepted that the main cellular response to DSBs include cell-cycle regulation, DSB repair, transcriptional activation of relevant genes (including those associated with repair), increase in cellular levels of deoxyribonucleotides and in certain cells induction of apoptosis[42-43].

In eukaryotic cells, the effective repair of DSBs is essential for survival. Two major pathways have evolved to counteract these breaks, homologous recombination (HR), and non-homologous end-joining (NHEJ)[44]. The mechanism of HR relates to the use the genetic information from a corresponding undamaged region present on the second DNA molecule or homologous chromosomes and hence is active mainly during S and G2 phases of cell cycle. NHEJ is based on a direct ligation of the two ends of damaged DNA molecules and repairs DSBs mainly in G1 phase[45].

2.2.3.1.1 H2AX, ATM and SCARS

H2AX phosphorylation is an important step in the DDR, having a role in signaling and initiating the repair of DSBs[46].

It was reported several years ago, that in mammalian cells the phosphorylation of the subtype of histone H2A, called H2AX, in the position of Ser139 occurs in response to DSB formation. The phosphorylated form of H2AX is referred to as *γ*-H2AX[47-48]. Histone H2AX is a substrate of several phosphoinositide 3-kinase-related protein kinases (PIKKs), such as ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), or DNA-dependent protein kinase (DNA-PK). These processes are aimed to stop the progression of the cell cycle and to activate proteins responsible for DNA repair[49-51]. Several lines of evidence suggest the critical role of H2AX phosphorylation at DSB sites for nuclear foci formation and induction of DSB repair:

- H2AX-knockout cells manifested impaired recruitment of NBS1, 53BP1, and BRCA1 to irradiation-induced foci [52].
- Both H2AX+/− and H2AX−/− mouse thymocytes show an increase in chromosomal aberrations[53-55].
- Mouse embryonic stem (ES) cells deficient in H2AXphosphorylation have alterations in efficiency of DNA repair by NHEJ or HR. As a result of these defects in DNA damage repair, such cells have increased sensitivity to DNA damage[54-58].
- H2AX knock-out mice show male-specific infertility and reduced levels of secondary immunoglobulin isotypes, suggesting defects in class switch recombination (CSR)[52]. It was shown that efficient resolution of DSBs

induced during CSR in lymphocytes requires H2AX[54-56], and its absence is associated with chromosome abnormalities involving the immunoglobulin locus[54].

Moreover, this specific “anchoring” function of γ-H2AX at sites containing DSBs, would inhibit the irreversible disassociation of broken DNA ends and promote chromatin compaction to facilitate error-free repair, thereby suppressing inappropriate translocations of chromatin fragments. In this way, γ-H2AX-mediated mechanisms prevent DNA ends from drifting apart, inappropriate rejoining of chromatin fragments, resulting in genetic translocations and other abnormalities.[44]

The contribution of γ-H2AX to signaling is thought to lie in the ability of this chromatin modification to increase DNA accessibility, leading to the recruitment and accumulation of specific DDR proteins at DNA ends. Discrete nuclear foci that form as a result of H2AX phosphorylation are now widely used as a quantitative marker of individual DSBs[59]. Studies have found that each DSB corresponds to one γ-H2AX focus but the reverse is not applicable as γ-H2AX may persist even after DSBs are rejoined[60-61]. High numbers of small foci are formed at the early stages of the DDR, decreasing in number and increasing in size as the DDR progresses[62] . The loss of γ-H2AX at DSB sites is thought to reflect the completion of repair of DNA at break sites[63]. It is unclear when a DSB is completely repaired. Some studies suggest that this is on rejoining of both DNA strands whereas others propose that chromatin has to be returned to its original state of compaction before a DSB is truly repaired[64-65].

One of the most critical kinase-activating cell cycle checkpoints following DNA damage is ATM. Mutations in the *ATM* gene results in the genomic instability and cancer predisposition syndrome Ataxia-Telangiectasia (AT) syndrome. In response

to DSBs, ATM phosphorylates many cell cycle checkpoint-related factors such as p53, Chk2 ,SMC1 and NBS1[66].

ATM kinase is considered as a major physiological mediator of H2AX phosphorylation in response to DSB formation[50-51]. ATM is activated by its auto phosphorylation at Ser1981 position, which leads to dissociation of the inactive ATM dimers into single protein molecules with increased kinase activity[47-48]. A tri-protein complex called MRN complex (MRE11-RAD50-NBS1) recognizes DNA damage, recruits ATM to the site of damage and also functions in targeting ATM to initiate phosphorylation of the respective substrates[67-68]. It is also reported that ATM activation requires prior ATM acetylation, mediated by Tip60 histone acetyltransferase[68-69].

When DNA lesions are repairable, DNA damage foci are transient. They typically resolve within 24 hours, during which time cells transiently arrest growth, presumably to allow time for repair[15]. However, severe or irreparable DNA damage, such as complex breaks or uncapped telomeres, causes many cells to senesce with persistent DNA damage foci, constitutive DDR signaling and chronic p53 activation. These persistent changes precede establishment of senescence-associated phenotypes, including growth arrest[9-16-20] and SASP[70] .

It is not known whether the persistent foci associated with senescence contain components that are distinct from those in transient foci. In addition, while the persistent foci clearly contain active DDR components, it is not known whether these structures maintain the DDR activity that is required for the senescence-associated growth arrest or cytokine secretion. Because markers of persistent foci are recruited to locally modified chromatin, these persistent foci are termed “DNA

segments with chromatin alterations reinforcing senescence" or DNA–SCARS, that are relatively stable structures distinct from transient damage foci and functionally important for maintaining the senescence-associated growth arrest and secretion of IL-6[15].

Individual persistent foci were stable for several hours in living cells, and, in fixed senescent cells, were detected for days and weeks after their formation. The repair system HR and NHEJ are likely either inactive or incapable of resolving these foci after 48h. Formation of DNA-SCARS was accelerated in cells that are deficient in certain DNA repair proteins supporting the idea that ineffective or defective repair initiates the formation of these structures. DNA –SCARS include also foci generated by senescence-inducing DNA damage inflicted by H_2O_2 or oncogenic RAS. The formation of DNA-SCARS is a robust phenomenon that is conserved between mouse and human, as are many other features of cellular senescence[71] , that occur in culture and *in vivo* .

2.2.3.2 DNA damage by oxidative stress

Oxidative stress results in formation/accumulation of reactive oxygen species (ROS)[56,72-73]. The generation of reactive oxygen species (ROS) and free radicals is considered as one of the mechanisms of aging, acting through oxidative stress inflicted on DNA and other cellular constituents[74]. ROS can also activate transcription of genes implicated in inflammatory and other chronic disease-processes[75]. According to recent data oxygen radicals can also directly participate in the induction of cell senescence[11] .

2.2.3.3 DNA damage by oncogenic or hyper-proliferative signals

Oncogenes are mutant versions of normal genes that have the potential to transform cells in conjunction with additional events. Normal cells respond to the activation of some oncogenes by eliciting a senescent phenotype[76].

Because oncogenes that induce senescence stimulate cell division, the senescent response could be aimed at counteracting excessive activation of the cell cycle, which would put cells at risk of oncogenic transformation[5].

The pathways mediating 'oncogene-induced senescence' (OIS) are not completely elucidated but, the proliferative arrest involves activation of both Rb and p53 pathways[77]. The normal endogenous expression level of an activated oncogene is not sufficient to trigger senescence .

OIS is often accompanied by the upregulation of the CDK inhibitors $p15^{INK4B}$ $p16^{INK4A}$, and $p21^{CIP1}$ [78].

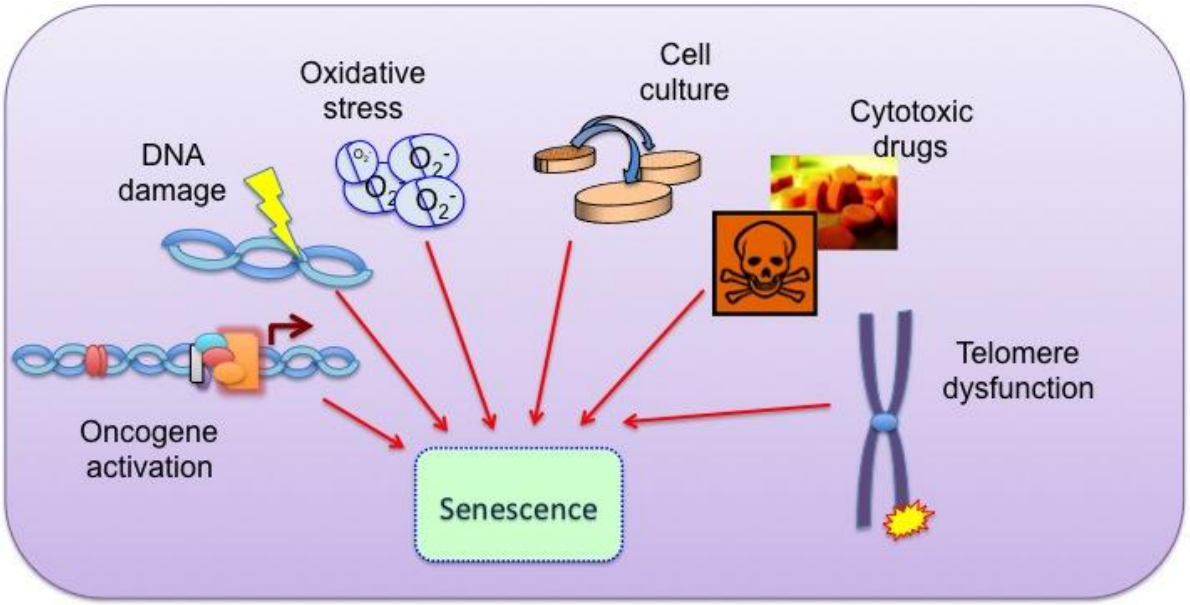


Figure 2: stimuli inducing cellular senescence

2.2.4 The secretory phenotype of senescent cells

Cells undergo cellular “reprogramming” during senescence, result in permanent growth arrest and in changed morphology and function[5].

A significant trait of this reprogramming is the gain of a secretory phenotype, which involves a striking enhancement in the secretion of growth factors and cytokines by senescent cells, hence exerting a broad range of effects on the local tissue environment. This secretory pattern has been referred to as the “senescence-associated secretory phenotype” (SASP)[22], and includes changes in the secreted levels of >40 factors involved in the intercellular signaling. For the induction of several of these SASP factors are required persistent DNA damage.

A significant features of the SASP is that it occurs *in vivo* and remains conserved in both human and mouse cells[79] , and in numerous proliferative cell types (fibroblasts, epithelial cells, endothelial cells, astrocytes, etc..)[20,80-81]. SASP factors can globally be divided into the following major categories: soluble signaling factors (interleukins, chemokines, and growth factors), secreted proteases, and secreted insoluble components. SASP proteases can have three main effects:

- shedding of membrane-associated proteins resulting in soluble forms of membrane-bound receptors
- cleavage/degradation of signaling molecules
- degradation or processing of the extracellular matrix.

These roles provide potent mechanisms by which senescent cells can modify the tissue microenvironment[82].

Proteins that are known to stimulate inflammation, including IL-6, IL-8, IL-1, granulocyte macrophage colony stimulating factor (GM-CSF), growth regulated

oncogene (GRO)α, monocyte chemotactic protein (MCP)-2, MCP-3, MMP-1, MMP-3, and many of the Insulin-like growth factor (IGF)-binding proteins[20,83] are among the most robustly induced and secreted factors in the SASP response.

The SASP inflammatory cytokines are of particular interest because they can play a role in many age-related pathologies[84]. In addition to being inflammatory mediators, IL-6 and IL-8 were recently shown to belong to a minor number of secreted factors which also reinforce the senescence growth arrest *via* autocrine and paracrine mechanisms[85-89].

The mechanisms that initiate and maintain the SASP are unclear. IL-1α and IL-β are minor SASP components which, compared to IL-6 and IL-8, are secreted at low levels[90]. IL-1 (α/β forms) is a multifunctional cytokine that regulates inflammatory and immune responses mainly by originating a signal transduction cascade which lastly induces IL-6 and IL-8 expression[91]. Recombinant IL-1α and IL-1β interact with the same receptor (IL-1R) and mediate similar biological effects. However, IL-1β is active only as a mature secreted form, while IL-1α is rarely secreted at high levels and produces effects either intracellularly or as a cell surface-bound protein. In addition, IL-1α, differently from IL-1β, can function both as an uncleaved precursor protein (pIL-1α) or as a cleaved protein[92]. Once IL-1R binds its receptor, IL-1 initiates the formation of a complex containing IL-1R and its coreceptor (IL-1RAcP). This complex triggers a series of cytoplasmic events that ultimately activate the transcription factor NF-KB[93]. NF-kB then transactivates numerous genes, including those encoding IL-6 and IL-8[94].

The most prominent cytokine of the SASP is interleukin (IL)-6, a pleiotropic pro-inflammatory cytokine[22]. IL-6 secretion has been shown to increase markedly after

DNA damage- and oncogene-induced senescence of mouse and human keratinocytes, melanocytes, monocytes, fibroblasts and epithelial cells[86].

The senescence response, SASP in particular, may reduce health by stimulating both the low level inflammation associated with aging as well as the development of specific age-related diseases[95].

Interestingly, this secretory phenotype seems to behave as a double edged sword regarding tumor initiation and maintenance:

a. On the one hand, it has been shown that the SASP can have pro-tumorigenic effects; for instance, it has been shown that senescent mesenchymal cells have been documented to increase the tumorigenicity of nearby breast cancer cells[96]. Similarly, it has been proposed that loss of proliferative competition of non-transformed, senescent cells can accelerate progression of leukemic cells during aging[97]. It remains to be seen whether aberrant secretion of cytokines and growth factors by the SASP can accelerate this process in aged and chronically damaged organ systems.

b. Conversely to its pro-tumorigenic properties, the SASP presents anti-tumor effects. It has been reported that in a mosaic liver cancer mouse model the activation of p53 induced senescence, an up-regulation of inflammatory cytokines, and stimulation of innate immune responses causing tumor cell clearance[98]. Additionally, more studies reported that components of the SASP can stabilize the senescence cell cycle arrest via an autoregulatory feedback mechanism or it can induce apoptosis of tumor cells[85-86,88].

Many of SASP factors can contribute to normal tissue repair: IL-6, IL-8, MMP, growth factors and protease S participate in wound healing, attractants for immune cells kill pathogens, proteins mobilize stem or progenitor cells, thus, another function is to communicate cellular damage/dysfunction to the surrounding tissue and if needed, stimulate repair.

Besides its influence on tumorigenesis, the SASP could also affect tissue aging. Experiments on aging telomere dysfunction in mice show direct proof for an *in vivo* activation of the SASP[99].

Interestingly, *in vivo* SASP provoked alterations in stem cell differentiation that are also characteristic signs of human aging. Tissue specific or adult stem cells, which are capable to self-renewal and differentiation, are essential for the normal homeostatic maintenance and regenerative repair of tissues through the lifetime of an organism.

The self–renewal ability of stem cells is known to decline with advancing age, eventually leading to the accumulation of unrepaired, damaged tissues in old organisms. By limiting cell proliferation, senescence in stem cells is hypothesized to contribute to aging by reducing the renewal capacity of these cells.

It remains to be determined whether stem cells undergo senescence during aging and whether inhibition of senescence may slow aging.

The reason why senescent cells are found with increasing frequency in older tissues is still unclear[100-102].

It is not known whether this rise is caused by increased generation, decreased elimination, or both an *in vivo* activation of the SASP in response to telomere dysfunction. One theory could be that the aging immune system, which shows both decrements and derangements in function, becomes less capable of clearing

senescent cells[103]**.** Furthermore, the production of senescent cells may increment with age due to an age-dependent enhancement in tissue damage, e.g. by increasing oxidative stress caused by progressively more damaged and therefore less functional mitochondria[104]. Is also possible that a constant fraction of senescent cells escape immune clearance such that they steadily accumulate with advancing age. Whatever the case, the chronic presence of cells which secrete various proteins with significant biological activities may be predicted to considerably modify tissue structure and cause a decline in tissue repair and regeneration.

2.2.5 Biomarkers of cellular senescence

Cellular senescence has been well described in a wide variety of cells tissues *in vivo* and in vitro. Indeed, *in vivo* senescent cells are found in human, rodent vascular endothelial and smooth muscle cells, animal skeletal muscle, fat tissue and liver[105-106]. Several cellular markers are used to identify senescent cells in vitro and *in vivo* including:

a. **Cell enlargement**. Flatness and enlarged cell shape of senescent cells has been previously reported in the seminal work of Hayflick and Moorhead (1961), who discovered the phenomenon of replicative senescence of human fibroblasts. An obvious marker for senescent cells is the lack of DNA replication, which is typically detected by the incorporation of 5-bromodeoxyuridine or ^{3}H−thymidine, or by immunostaining for proteins such as PCNA and Ki-67. Unfortunately,

these markers do not discriminate between senescent cells and quiescent or differentiated post-mitotic cells.

b. **SA-β-gal activity**. Since it was first reported[107] , SA-β-gal activity has been the most widely utilized biomarker in detecting senescent cells because of the simplicity of the assay method and its apparent specificity for senescent cells. This histochemical stain identifies a variety of cell types at replicative senescence *in vitro* . The precise function of SA-β-gal in senescence remains unknown, but it is thought that it becomes elevated in senescent cells due to the expansion of lysosomes, which contain β-galactosidase as a lysosomal enzyme[107]. The technique derives from an observation that β-galactosidase activity measured at pH 6.0 selectively stains senescent cells, while the same procedure conducted at pH 4.0 uniformly stains cultures for lysosomal β-galactosidase. The pH 6.0 activity, now known as senescence-associated β-galactosidase, is extensively utilized to identify the presence of senescent cells in vitro.

SA-β-gal activity has been detected in organs of old individuals and animals, suggesting that cellular senescence is an aging trait of organisms and that senescent cells accumulate with age in various tissues[108].

The presence of the SA-β-gal biomarker is independent of DNA synthesis and generally distinguishes senescent cells from quiescent cells. SA-β-Gal activity is commonly accepted as a marker of senescence both *in vitro* and *in vivo*, although there is evidence for a positive SA-β-Gal

reaction in settings of cellular stress that are independent of senescence, including serum withdrawal or high confluence in cell culture.

A pathogenetic role of SA-β-Gal in cell senescence is unknown. A possibility is that SA-β-Gal activity is correlated to the increased lysosomal content of senescent cells, which allows the detection of the enzyme activity at suboptimal pH of 6.0 (the optimal pH being at 4.0)[107].

c. **DNA damage foci.** The use of DNA damage foci has been suggested as a marker for the detection of senescent cells[31-109]. Both DNA double strand breaks and telomere uncapping[16] are able to induce a DNA damage response. This reaction is characterized by activation of ATM/ATR which is recruited to the site of damage, causing the phosphorylation of Ser-139 of histone H2AX molecules (γ-H2AX) next to the site of DNA damage. The phosphorylation of histone H2AX facilitates the focal assembly of checkpoint and DNA repair factors such as 53BP1, MDC1/NFBD1 and NBS1, and also mediates the activation by phosphorylation of Chk1 and Chk2, which converge the signal on p53/p21[12].

d. **Inhibitors of cell cycle progression.** The cyclin-dependent kinase inhibitor p16 is an important regulator of senescence and is expressed by many senescent cells[70,110]. Investigation of the signaling pathways leading to OIS has shown that two main pathways are involved in cell cycle arrest, p16INK4a–RB (retinoblastoma) and ARF–p53[111]. These two pathways are thought to be crucial for tumor suppression and are often

mutated in tumors[112-113]. Among the important components of these pathways are cyclin-dependent kinase inhibitors (CDKIs). CDKIs inhibit critical cell cycle-regulatory phosphorylation events, such as those that inactivate the growth suppressive activity of pRB[112]. One such CDKI, $p21^{CIP1/WAF1}$ (CDKN1A), is a direct target of p53 transactivation, generally in response to genomic damage, and is crucial for establishing and maintaining the p53-mediated senescence growth arrest [114-115.]. Another CDKI, $p16^{INK4a}$ (CDKN2A), is a tumor suppressor in its own right, can be induced by stress that does not entail DNA damage[116-117], and acts upstream of pRB to establish the pRB-regulated growth arrest[118-120].

There are also additional markers of senescence that have been proposed, whose precise role is currently unclear. For example, an important feature of the aging phenotype is reduced capacity to activate autophagy, a highly conserved mechanism and very efficient cellular response to stress. However, an increase of autophagy activity during brain aging has been recently shown[121] and other groups have reported that autophagy/mTOR activity may be involved in the transition to the OIS state [122]. Although the latter observations indicate a similar role of autophagy and senescence in suppressing cancer development, the role of autophagy/senescence transition urgently needs to be verified in other cellular systems.

The information that is available regarding the different pathways implicated in OIS is being exploited to obtain new senescence markers. For example, modifications to protein-degradation pathways (through the proteasome and the lysosome), the

epigenetic status of the chromatin, cell-cycle arrest and others are being investigated[123].

Another possibility is the identification of soluble factors released by senescent cells that could, for example, be measured in body fluids such us blood, urine or sputum. Thanks to the analysis of the gene-expression profile of senescent cells, it has become clear that senescent cells have characteristic alterations in secreted growth factors, inflammatory cytokines, extracellular-matrix components and matrix-degrading enzymes, some of which could be easily detected[124].

2.2.6 Cellular Senescence and Inflammation

The link between inflammation and cellular senescence was initially proposed following experimental reports on the gene expression profiles of senescent cells. These findings demonstrate that senescence is connected with gene expression patterns analogously to those observed in inflammatory responses and wound healing processes. Enhanced expression of inflammation associated genes, comprising the chemokines monocyte chemotactic protein-1 (MCP-1) and Gro-α, cytokines IL-15 and IL-1β, Toll-like receptor 4 (TLR4) and intercellular adhesion molecule-1 (ICAM-1), was originally observed in senescent human dermal fibroblasts[125-126] and subsequently confirmed in other cell types, such as epithelial cells.

Chronic inflammation is linked to numerous age-related pathophysiologic processes and diseases, such as cancer, diabetes, Alzheimer's disease, atherosclerosis, osteoarthritis etc.[127-128]. Chronic inflammation is also associated with normal aging. For instance there is an average 2-4 fold enhancement in serum levels of pro-

inflammatory signals (e.g. interleukin (IL)-6 and tumor necrosis factor (TNF)-α in aged individuals (>50 years of age), compared to younger individuals[129-130]. Furthermore, individuals who experience unusually healthy aging (for instance, healthy centenarians) typically have a lower inflammatory profile than frail centenarians[131]. The inflammatory status of cellular senescence might have opposite effects on neoplastic disease. Secretion of inflammatory cytokines and chemokines is crucial for the induction and maintenance of the senescence phenotype, which prevents premalignant tumor cells from progressing into a malignant state. Meanwhile, these inflammatory cytokines and chemokines produced by the senescent cells also create a cancer-inducing microenvironment for their neighboring tumor cells. Consequently, the net effect of the inflammatory state of senescence on the initiation of neoplastic disease significantly depends on the tissue environment, the genetic makeup of the tumor cells as well as the stromal cells, and the extracellular signals occurring in the tumor microenvironment[132]. Inflammation is necessary to contrast damaging agents and is essential for survival, mainly to deal with acute inflammation during our reproductive years. But chronic exposure to a wide range of antigens, particularly to some viruses such as cytomegalovirus, a longer exposure than that predicted by evolution, induces a chronic low-grade inflammatory status that contributes to age-associated morbidity and mortality[136].This condition is defined as “inflammaging”[133].

“Inflamm-aging” indicates the up-regulation of inflammatory cytokines over the course of the aging process, and the connected damage and chronic diseases.

For the immune system, a typical consequence of aging is the progressive filling of the immunological system by activated lymphocytes, macrophages, and dendritic cells in response to chronic stress stimuli either from pathological or physiological

antigens and toxins[134]. Therefore, the state of inflammaging causes a constant mild antigenic challenge leading to a pro-inflammatory condition linked to the progressive stimulation of the immune system and other organismal systems[135-138]. Overall, immunosenescence can be referred as the proof that the beneficial effects of the immune system, aimed at the neutralization of harmful agents early in life, become detrimental as life progresses, as if evolution did not take into account old age[134] . This viewpoint is consistent with basic assumptions of evolutionarily antagonistic pleiotropy theory[7] in regard to aging, which suggests that a compromise between early beneficial effects and late negative outcomes can occur at the genetic and molecular level (Fig. 3).

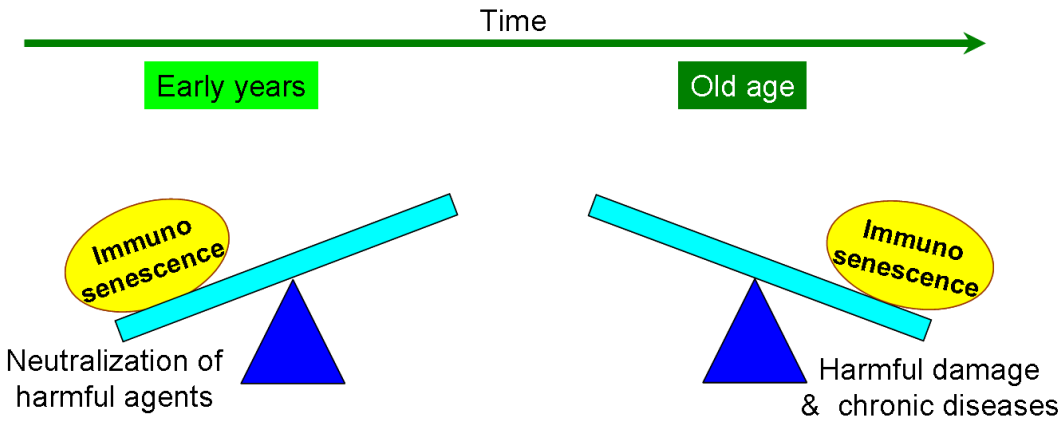


Fig. 3: Consequences of an inflammatory responses, a compromise in evolution: beneficial effects in early age and late negative outcomes.

2.2.7 Cellular senescence and aging.

The first studies of cellular senescence *in vivo* showed that the number of senescent cells increase with age[81,105,107]. The increase in senescent cells occurs primarily in tissues which have mitotically competent cells, and it is now reported in many rodent and human tissues. Furthermore, senescent cells have been identified in tissues of several age-related pathologies such as atherosclerosis and osteoarthritis[81,111].

Studies suggest two mechanisms by which cellular senescence might actively induce aging and age-related pathology.

First, the majority of adult stem cells are capable of undergoing senescence, and cellular senescence might account at least in part for the age-related diminishing number or function of certain stem cells in adult organisms[139-142].

Therefore, an accumulation of senescent stem cells may contribute to the reduced in tissue repair and regeneration that is hallmark of aging organisms.

Second, the senescence-associated secretory phenotype (SASP) referred to above comprises factors that control many vital processes within tissues and organs, including cell growth, motility and differentiation, as well as tissue structure and vascularization. This feature of the SASP is important because a common trait of aging tissues is low-level chronic inflammation; moreover, it is well documented that inflammation causes or contributes to virtually every major age-related disease[143-144]. It is then possible that senescent cells are a source of “sterile” inflammation that is hallmark of aging tissues and driver of multiple age-related pathologies.

2.3 Aging

Aging is a persistent process that affects all cells, tissues, organs and organisms, reducing homeostasis and enhancing organism vulnerability[145]. Aging progression leads to a decreased response to environmental stimuli, and is associated with an increased predisposition to illness[146-147]. Hence, mortality due to all causes increases exponentially with aging.

The comparison of the mortality rate in developed countries among individuals over 65 years, versus people in the age range between 25 and 44 years, shows an increase of 100-fold for stroke, and chronic lung disease, 92-fold for heart disease, 89-fold for pneumonia & influenza and 43-fold for cancer [148].

Aging can be defined as a continuous regression of tissue function that ultimately results in organ failure. Such functional decline can result from the loss or reduced function of post-mitotic cells or from failure to replace such cells by a malfunctioning ability of (stem) cells to carry out replication and cell divisions. Aging is not a disease, and the biology of aging, which varies between individuals, is best understood in the context of evolution[149]. On the cellular level, aging is thought to be caused by the accumulation of damage, induction of senescence, and loss of replicative capability. Nonetheless, aging takes place at different levels: the cellular, the organic specific, and at the level of the organism as a whole[150].

An essential mechanisms of aging is based on the production of reactive oxygen species (ROS) and free radicals[74] .

ROS and free radicals can be produced by environmental stimuli or can originate from intrinsic cellular causes as a consequence of cellular respiration, referred as oxidative stress. ROS mediates transcription of genes implicated in inflammatory

and other disease-associated processes, or can cause damage to DNA and protein species[75]. Preventing oxidative stress is therefore an important factor to slow down the aging process.

Genome instability is an important factor of aging in all eukaryotes. How age related genome instability occurs remains unclear[151].

The free radical theory of aging makes oxidative damage to DNA and other cellular constituents a major factor in aging. Studies in the model organism *Saccharomyces cerevisiae* (budding yeast) and in higher eukaryotes suggest that growth signaling also influences aging and age-related diseases -such as cancer and neurodegeneration- by inducing DNA replication stress, which causes DNA damage. Replication stress, which is not considered as a factor in aging, may be enhanced by ROS that signal growth.

Numerous potential mechanisms, often overlapping, have been proposed to explain age-dependent genome instability. These include the accumulation of oxidative damage to DNA, defects in mitochondrial function that promote oxidative stress and damage to DNA and other cellular components, mutations in proteins necessary for efficient DNA replication, DNA repair and checkpoints, telomere dysfunction and epigenetic effects on DNA repair and other genome maintenance programs. Although many of these mechanisms are likely to contribute to aging in a variety of eukaryotes, the specific contributions of each and their relative weight remain unclear.

There is convincing evidence now that in all eukaryotes, aging is regulated by conserved insulin/insulin-like growth factor (I-(IFG-1)) pathways and growth-signaling pathways regulated by the Target of Rapamycin (TOR) family of protein kinases[152]. In general, in experiments where these pathways are up-regulated aging

is promoted, whereas by down regulating these pathways through such as mutational inactivation extend life span and mitigate age-related pathologies. Down regulation of these pathways often leads to a reduction in oxidative stress and oxidative damage to DNA and other cellular components.

It is documented that key mechanisms of both aging and cancer are linked *via* endogenous stress-induced DNA damage caused by reactive oxygen species. These include oxidative nuclear and mitochondrial DNA damage and repair, telomere shortening and telomere-driven cellular senescence[153].

2.2.1. Aging and cancer

The incidence of malignant tumors is proportional to the increase of age in both animals and humans[154-158]. Three main hypotheses have been suggested to clarify the connection between cancer and age. The first proposes that this link is a consequence of the duration of carcinogenesis. In other words, the high prevalence of cancer in older individuals simply reflects a more prolonged exposure to carcinogens[159]. The second hypothesis supports the idea that age-related progressive changes in the internal milieu of the organism might provide an increasingly favorable environment for the initiation of new neoplasms and the proliferation of already occurring but latent malignant cells[154,158,160-161].

Frequent age related changes in tissue milieu comprise:

- An increasing proportion of senescent fibroblast cells that support a proteolytic microenvironment facilitating the growth of the transformed cells[162].
- Inefficiency of aerobic pathways[163] that are partially compensated by increased glycolysis.

- Local and systemic immune-deficits[164] that can maintain a tissue microenvironment that also favours survival of transformed cells.

- Dysfunctional cell-death programs in old age[165] that would be harmful in tissues with a retained growth capacity.

These mechanisms might also include proliferative senescence, as the senescent cells lose their ability to undergo apoptosis and produce some signals that stimulate epithelial cells with oncogenic mutations[30].

A third strategy for primary cancer prevention, which basically combines the two mentioned above, proposes that the cancer-prone phenotype of older animals, including humans, might reflect the combined effects of cumulative mutational load, enhanced epigenetic gene silencing, telomere dysfunction, and altered stromal milieu[166].

2.4 Regenerative medicine: the liver as a model.

The idea that isolated cells can be introduced into the body and directed to repair irreversible damage in solid organs is gaining increasing attention[167].

The liver has been among the first targets for strategies based on transplantation of isolated cells. Over the past 15 years, significant progress in this field has been made by development of several experimental models for extensive liver repopulation *via* transplantation of isolated hepatocytes[168-170]. The urokinase-plasminogen-activator (u-Pa) transgenic mouse model was the first to demonstrate the biological possibility of massive replacement of a disease liver *via* exogenously provided normal cells[171-174]. In this system, endogenous hepatocytes expressing the targeted u-PA transgene are selectively deleted and replaced by normal cells.

Based on this finding, hepatocytes isolated from a congenic donor where then transplanted into the u-PA mouse and tested for their ability to selectively proliferate in the host liver. Four to five weeks later, up to 80% of hepatocytes in the recipient liver were found to be of donor origin[171], thereby confirming that the constitutive expression of the uPA transgene in resident hepatocytes generates a selective environment which favors the growth of cells with a normal phenotype. A similar principle of selective survival advantage seems to form the basis for the massive proliferation of transplanted cells in the liver of the fumaryl-acetoacetate-hydroxylase- (Fah)-null mouse, a model for human hereditary tyrosinemia type I[172]. In both human and mice, the lack of Fah enzyme, involved in the tyrosine catabolic pathway, leads to accumulation of its substrate, fumaryl-acetoacetate (FAA) and its precursor maleyl-acetoacetate (MAA). Both FAA and MAA cause liver toxicity, which is found in tyrosinemia type I patients and includes progressive liver failure and development of hepatocellular carcinoma.

In both cases, the essential feature of the model resides in the inherent genetic defect of resident cells which causes toxicity and leads to their selective death and replacement by wild type cells.

An alternative means to create a differential in growth and/or survival capacity between the resident and the transplanted cell population is by targeting the latter, such that it acquires specific phenotypic properties which can be selected for in a normal background environment. In this case, the end result will be an organ repopulated with a genetically altered cell population. Based on this approach, hepatocytes over-expressing the human Bcl-2 transgene were introduced into normal recipient mice. Transplanted cells were then selected by differential killing of normal endogenous hepatocytes, *via* repeated systemic injections of a Fas/CD95

ligand, which caused single cell death in resident hepatocytes expressing only normal levels of the Bcl 2 protein [175].

In our laboratory, we have used an alternative approach to develop a more general strategy for effective liver repopulation *via* isolated cell transplantation. In this system, donor hepatocytes are transplanted into a normal host liver in which the endogenous growth capacity has been hampered by a preconditioning treatment[173,176].

However, in this case no “genetic” differential is present between the two cell types, i.e. both donor-derived cells and the recipient animal have a normal genetic background. The differential is imposed *via* external treatment of the host prior to cell transplantation.

This transplantation model is based on exposure of the recipient animal to retrorsine (RS), a naturally occurring pyrrolizidine alkaloid (PA), that is able to impose a persistent block on the endogenous hepatocyte cell cycle[177]. PAs were originally studied because of their toxicity in animals, particularly sheep and cattle, where they cause both acute and chronic liver injury[178]. These agents are natural plant substances that are selectively taken up and metabolized by the liver to bioactive compounds that bind to DNA[179]. They produce a block in the hepatocyte cell cycle with accumulation of cells in late S and/or G_2 phase[180-181]. Although pyrrolizidine alkaloids are metabolized rapidly, their effect on hepatocyte proliferation lasts for weeks to months[182].

However, the mechanism by which mature hepatocytes are blocked in their proliferative response after treatment with RS has yet to be clarified and is still the subject of debate. There are data suggesting that the mitotic process itself is impaired, hence that the block is located very late in the mitotic cycle, i.e. in late S

or early G_2 phase. This is supported by the observation of the induction of megalocytosis, which is thought to be a consequence of normal DNA, RNA and protein synthesis in the absence of cell division[183].

Under these conditions, normal hepatocytes, transplanted after the alkaloid has been metabolized, can selectively respond to growth stimuli and gradually repopulate the entire liver (>90% after 2 to 8 months post Tx, depending on the intensity of the growth stimulus applied)[184-185].

It was observed that normal hepatocytes undergo clonal proliferation when injected into the liver of RS-treated hosts. They integrate in the host liver and gradually repopulate the entire organ with a seemingly normal histology[186]. However, virtually no growth of transplanted hepatocytes occurs in the liver of syngeneic untreated recipients.

2.4.1 Association between Liver Repopulation and Carcinogenesis.

Interestingly, it was found that the original observations obtained using normal hepatocyte transplantation could be reproduced with hepatocytes isolated from preneoplastic liver nodules as the donor cell[187].

In a series of experiments, it was demonstrated that the growth-constrained microenvironment imposed by RS on endogenous hepatocytes could also sustain the selective expansion of transplanted nodular cells, resulting in the emergence of liver nodules and subsequent progression to hepatocellular carcinoma.

Thus, the experimental findings obtained with this model provide compelling evidence that the same liver microenvironment that is permissive for the selective growth of normal transplanted hepatocytes also has the capacity to stimulate the

clonal expansion of altered/nodular hepatocytes and set the stage for their progression toward neoplasia.

This association is intriguing and leads one to consider that these two processes may share common basic biological mechanisms; ie, the clonal growth of normal and altered/preneoplastic hepatocytes appears to be sustained by similar driving forces[188].

3 Aim

To explore possible biological and molecular mechanisms sustaining the growth of transplanted cells in a physiologically aged microenvironment and following a preconditioning treatment for massive liver repopulation.

4 Materials and Methods

4.1 Animals and treatments

A colony of DPPIV$^-$ Fischer 344 rats has been established in our laboratory. Donor DPPIV$^-$ F344 rats were purchased from Charles River (Milan, Italy).

Animals were maintained on an alternating 12hr light/dark cycle with food (Purina Rodent Chow diet) and water available *ad libitum*. All experiments were approved by the University of Cagliari Ethical Committee for Animal Experimentation; all animals received humane care in accordance with NIH Guidelines for the care and use of animals. Animals were killed by exsanguination from the abdominal aorta while under light ether anesthesia. Livers were excised and samples from each lobe were snap frozen (for cryostat sections, protein and gene expression analysis) or fixed in 10% buffered formalin and embedded in paraffin (for histology).

4.2 Retrorsine

Pyrrolizidine alkaloids are naturally occurring genotoxic chemicals produced by a large number of plants from *Symphytum* and *Senecio* species. Retrorsine is one of the main alkaloids present in the *Senecio* species (figure 4). This plant is poisonous and has been found to contain pyrrolizidine alkaloids which produce hepatic necrosis.

Fig.4 Senecio vulgaris

In animals, the major metabolic routes of pyrrolizidine alkaloids are:

(a) hydrolysis of the ester groups;

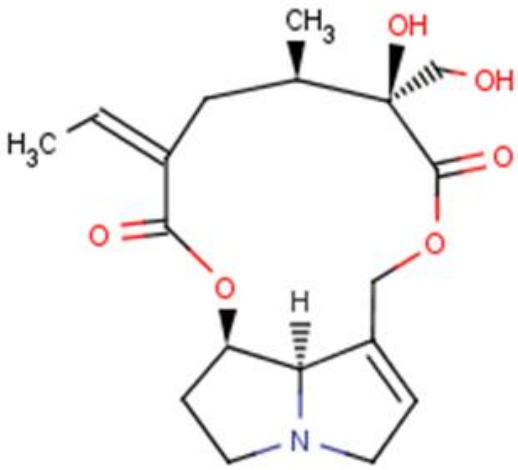
(b) N- oxidation;

(c) dehydrogenation of the pyrrolizidine nucleus to pyrrolic derivates.

Routes (a) and (b) are believed to be detoxification mechanisms. Route (c) leads to toxic metabolites. Route (a) occurs in liver and blood; routes (b) and (c) are brought about in the liver by the microsomal mixed function oxidase system[189].

Retrorsine is a 12-membered macrocyclic diester pyrrolizidine alkaloid with an α, β-unsaturated double bond linked to the ester group at C-7 position of the retronecine base. The toxicity of retrorsine is due to the metabolic formation of the reactive pyrrolic metabolite; however, the mechanism of retrorsine-induced tumorigenicity is not clear.

6 weeks DPPIV$^-$ old rats (with body mass 70-90g) were treated with 2 injections of retrorsine (RS, Sigma Chemical Co.), 30 mg/kg each, i.p., two weeks apart, or saline. RS was dissolved in diluted HCl (pH 2.5) followed by neutralization with 0.1N NaOH.



Formula: $C_{18}H_{25}NO_6$
Molecular weight: 351.4

Fig.5 RS structure

4.3 Generation of hepatic nodules

Hepatocyte nodules were induced according to a well characterized experimental model in the rat[190]. Two-month-old male Fischer 344 rats (Charles River Breeding Laboratories) were injected with a single initiating dose of diethylnitrosamine (200 mg/kg body weight i.p.; Sigma), followed, 2 weeks later, by administration of 3 consecutive daily doses of 2-acetyl-amino-fluorene (20 mg/kg) and coupled, on the 4^{th} day, by a single treatment with CCl_4.

4.4 The dipeptidyl-peptidase type IV-deficient rat model for hepatocyte transplantation

Normal Fischer (F344) rats express a specific exopeptidase, dipeptidyl peptidase IV (DPPIV), in a characteristic pattern in the liver, restricted to the apical domain of the plasma membrane[191]. To follow the fate of donor hepatocytes into the recipient liver, the dipeptidyl peptidase type IV-deficient ($DPPIV^-$) rat model can be used. In this system, cells isolated from a Fischer 344 rat expressing the enzyme DPPIV ($DPPIV^+$) are transplanted into a syngeneic DPPIV deficient ($DPPIV^-$) host, such that donor-derived cells can be detected in the recipient liver through simple histochemical and/or immunohistochemical techniques.

Histochemical determination of DPPIV enzyme activity was performed as described[176]: 5-µM thick cryostat sections from frozen tissue were cut. Fixation was for 5 min in 95% ethanol/5% glacial acetic acid (99:1 vol/vol) at 0°C to −10°C, followed by a 5-min wash in 95% ethanol at 4°C. Air-dried slides were incubated for 10–20 min at RT in the substrate reagent: 2.5 mg Gly-Pro-4-methoxy-β-

naphthylamide (Sigma) dissolved in 150 ml of dimethylformamide and mixed with a 5 ml solution of Fast blue BB salt (Sigma) in PBS.

4.5 Staining for senescence associated β-galactosidase activity

Staining for SA-β-gal was performed according to published procedures[107]. Immediately before staining, X-Gal stock solution was prepared by dissolving 20mg/ml X-Gal (Invitrogen, Carlsbed, CA) in dimetylformamide. SA-β-Gal staining solution was prepared as follows: 1 mg/ml of X-Gal stock solution were dissolved in 40 mM citric acid in sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM MgCl2. Frozen sections of 10-μm thickness were fixed for 5' in 4% formaldehyde/0.5% glutaraldehyde at 4°C, washed in PBS and incubated in fresh SA-β-Gal staining solution for 16h at 37°C. Sections were counterstained with Hematoxylin.

4.6 Histology, Immunohistochemistry and Immunoflorescence

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) according to standard procedures. Immunohistochemical staining for p21, p27 and mTOR was performed on paraffin embedded sections, following de-wax and antigen retrieval with sodium citrate buffer (pH 6, 0.01M). Slides were blocked for 30', incubated with primary antibodies (diluted in PBS) overnight at 4°C, and then incubated with AP-conjugated secondary antibodies. Detection of specific

signal was accomplished using the avidin/biotin alkaline phosphatase system (Vectastain ABC kit; Vector Lab, Burlingame, CA). Immunohistochemical staining for GSTP-P was performed on frozen sections fixed in acetic alcohol/ethylic alcohol, washed in PBS and blocked, incubated and detected with the same protocol for paraffin sections. Immunoflorescent staining for H2AX, 53BP1, ATM, Cyclin D1, KI67 and CD26 was performed on frozen sections, following fixation in cold acetone or methanol. Slides were blocked for 20'with goat or donkey serum, incubated with primary antibodies for 1 h at RT, followed by incubation (45') with fluorescent-conjugated secondary antibodies. Slides were counterstained with DAPI and images were acquired with an IX71 fluorescence microscope with CCD camera (Olympus, Tokyo, Japan). In table 1 the complete list of primary and secondary antibodies is reported.

4.7 Western Blot

Liver tissue samples were homogenized in RIPA lysis buffer containing protease Inhibitors and centrifuged at 12000 rpm for 30' at 4°C. Protein concentration in supernatants was measured using the BCA method[192]. Samples (20µg protein) were prepared in Laemmli buffer, boiled at 95°C for 5' then loaded into SDS-PAGE precast gels (Biorad, Hercules, CA) and run under denaturing conditions. Proteins were transferred onto nitrocellulose membranes (Amersham, UK), blocked with 5% non-fat milk for 1 h, followed by incubation with primary antibodies overnight at 4°C. Antibodies are listed in Table 1. Membranes were washed and incubated for 2 h

with the appropriate secondary antibody conjugated with HRP. Protein bands were detected using a chemoluminescent substrate (Biorad) and imaged onto Kodak film.

4.8 RNA Isolation, RT-PCR and Real-Time qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA integrity and purity were confirmed by 1% agarose gel electrophoresis and OD260/OD280 nm absorption ratio >1.8. Two grams of DNase-I treated RNA of each sample were reverse-transcribed by PCR using Promega reagents. The resulting cDNA was analysed by quantitative real-time PCR using specific TaqMan assays and TaqMan Gene Expression Master Mix on an StepOne System (all from Applied Biosystems, Carlbad, CA). The rat specific assays were: IL-6 (Rn01410330_m1); β2-microglobulin (Rn00560865_m1); IL-8 (Rn00578225_m1). For both assays the thermal profile was as follows: 50˚C for 2 minutes, 95˚C for 10 minutes, 45 cycles at 95˚C for 15 seconds and 60˚C for 1 minute. Fold change was calculated by the 2-ΔΔCT method[193].

Statistical analysis of results was performed with Student "t" test.

4.9 DNA liver content

The Diphenylamine (DPA) method is used for the determination of DNA content in tissue samples. This method is based on the colorimetric measurement of products formed from the reaction of o-hydroxylevulinylaldehyde (from the deoxyribose released after depurination of DNA) with diphenylamine in 1 N perchloric acid.

Sample preparation: 500 mg of liver per sample were homogenized in 1N perchloric acid (PCA) and centrifuged at 12.000 rpm for 15' at 4°C. The pellet was then resuspended in 0.5N PCA and incubated at 70°C for 1.3 h while stirring.

After incubation, samples were centrifuged at 12000 rpm for 15' at 4°C and the supernatant was transferred into a new tube and kept on ice. Sample were diluted in 1:10 in 1ml of 0.5N PCA and then added with 2ml of DPA. The samples were incubated in the dark for 16-18 hours at room temperature, and absorbance values at 600nm were read at the spectrophotometer. A standard curve with Calf Thymus DNA was used as a reference.

TABLE 1

Primary Antibody	Brand	Cat. No.
p21 (M-19)	Santa Cruz	sc-471
p27 (C-19)	Santa Cruz	sc-528
ATM [2C1(1A1)]	Abcam	ab78
H2AX (phosphor S139)	Abcam	ab2893
53BP1	Abcam	ab87097
mTOR	Abcam	ab2732
Cyclin D1 (DCS-6)	Sigma	C7464
IL-6	Abcam	ab6672
TNF-α	Abcam	ab66579
KI-67 (SP6)	Abcam	ab16667
CDK4 (C22)	Santa Cruz	sc-260
β-Actin	Abcam	ab8227
GSTP1-1	CalBiochem	354212
CD26	BD Pharm	559639
Secondary Antibody	**Brand**	**Cat. No.**
Anti-Rabbit IgG HRP-conj.	Abcam	ab16284
Anti-Mouse IgG HRP-conj.	Abcam	ab6808
Anti-Rabbit IgG Atto 550-conj.	Sigma	43328
Anti-Mouse IgG Atto 550-conj.	Sigma	43394
Anti-Rabbit IgG Dylight 488 conj.	Abcam	ab96899
Anti-Mouse IgG Dylight 488 conj.	Abcam	ab96879

5 RESULTS

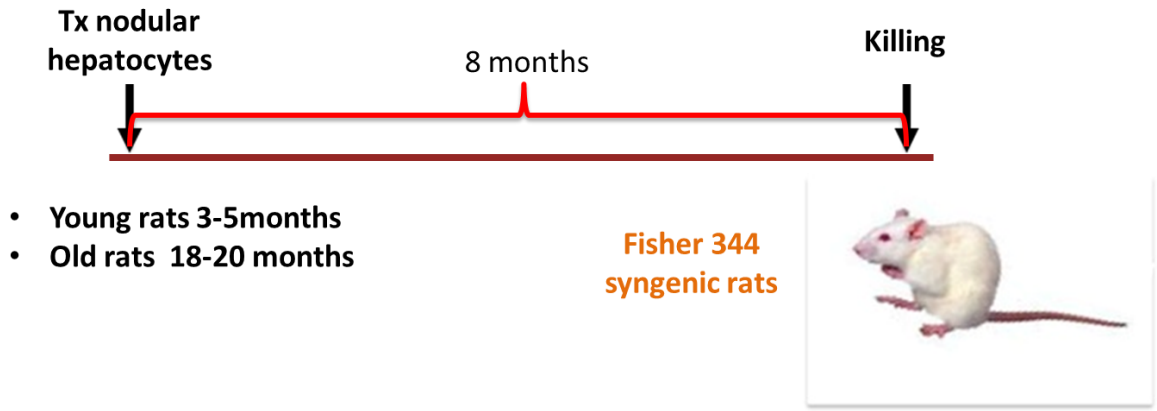
5.1 The microenvironment of the aged rat liver supports the growth of transplanted hepatocytes isolated from hepatic nodules.

Previous investigations conducted by our research group had revealed that isolated normal hepatocytes form larger clones of daughter cells when transplanted into the liver of old animals as compared to those formed in young recipients[194].

We then utilized a similar approach to verify whether the growth of transplanted pre-neoplastic cells isolated from hepatic nodules was also dependent on the age of the recipient animal.

Young (3-5 months old) or aged (18-20 months old) Fisher 344, $DPPIV^-$ rats were injected with $5x10^5$ cells freshly isolated from $DPPIV^+$ hepatocyte nodules.

Experimental strategy



Hepatocyte nodules were induced according to a well-characterized experimental model in the rat. Two-month-old male Fischer 344 rats were injected with a single initiating dose of diethylnitrosamine (200 mg/kg body weight i.p.; Sigma) followed, 3 weeks later, by exposure to a modified version of the Solt and Farber protocol to stimulate the growth of hepatocyte foci and nodules. Such a protocol consisted of

three consecutive daily doses of 2-acetylaminofluorene (20 mg/kg body weight, given by gavage tube; Sigma) followed, on the fourth day, by administration of CCl_4, as described in Materials and Methods. Six months after the initial treatment, livers were perfused according to a standard two-step collagenase perfusion technique[195]. Large (>5 mm in diameter) nodules were physically separated from surrounding tissue and cells isolated from nodular tissue were suspended in Williams E culture medium (Sigma, cat No. W-4125) and prepared for transplantation experiments. Cell viability, determined by trypan blue dye exclusion, was 80-85%. Cells were delivered via portal vein infusion, suspended in 0.3 ml of Williams E medium. Animals were killed 8months after the transplant.

Results are presented in Figures 6 and 7 and Tables 2 and 3.

On macroscopic examination, no visible lesion were seen in any of the recipient animals transplanted at young age (figure 6, panel A); by contrast, almost all animals transplanted at 18-20 months of age and killed up to 8 month later, developed visible hepatic nodules, ranging from 1 to 8 mm in size, including five large tumours measuring up 3 cm of diameter (figure 6, panel B).

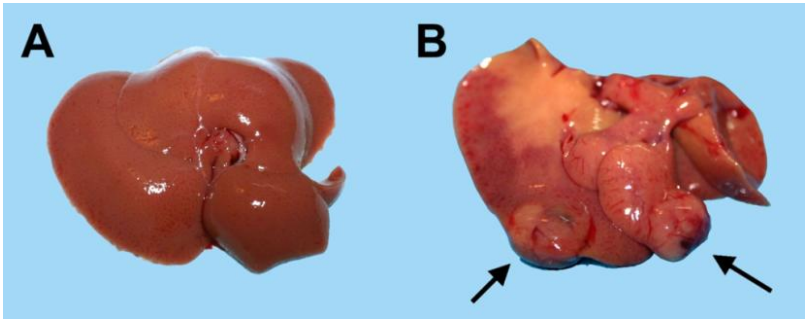


Fig.6: Macroscopic appearance of the liver 8 months after transplantation of nodular cells. (A) No macroscopic nodules are visible in the liver of animals transplanted at 3 month of age. (B) Large nodular lesions are evident in the liver of animals transplanted at 18 months of age (arrows).

These findings were then confirmed and extended through histological and histochemical analysis (figure 7 and tables 2 and 3). Only rare, very small clusters of transplanted nodular hepatocytes were discerned in the liver of animals transplanted at 3-5 months of age and killed as late as 8 months post-injection (Figure 7, panel A). However, the same cell preparations were able to form hepatic nodules when transplanted in the liver of aged rats; these nodules had similar histological appearance to those isolated at the primary site (figure 7, panel C and D).

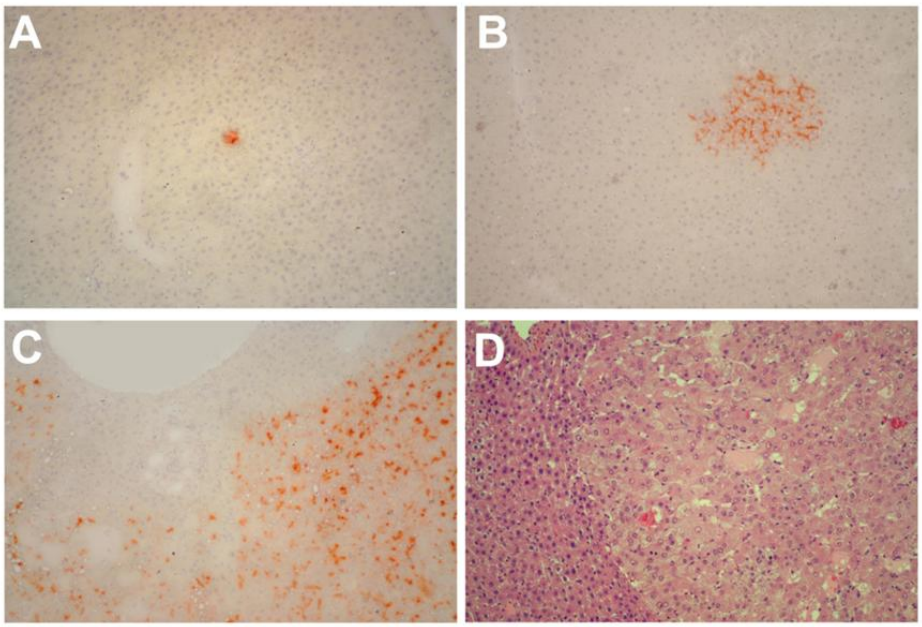


Fig 7 nodular hepatocytes followed by DPPIV staining: young rat (A), old animal (B) and old animals with HCC (C). Morphological analysis with H&E (D).

Tables 2 and 3 report a summary of the distribution of nodular lesions in animals transplanted at different ages, and the average number of cells in DPPIV-positive hepatocyte clusters.

Table 2

Months at Tx	Animals w/ lesions	Nodules (<1cm)	Size Range	Tumors (>1cm)	Size Range
3	0/3	none	-	none	-
5	0/4	none	-	none	-
18	7/7	33	1-5 mm	4	2-3 cm
20	10/11	23	1-8 mm	3	1-2 cm

Table 3

	Sample	No. of clusters	Average no. $DPPIV^+$ cells/cluster	Range no. $DPPIV^+$ cells/cluster
Young	1	—	—	—
	2	—	—	—
	3	—	—	—
	4	—	1	—
	5	—	1	—
	6	1	3	—
	7	3	6	3-15
	8	4	10	2-50
Old	1	—	1	—
	2	8	8	2-30
	3	3	9	4-20
	4	11	16	2-50
	5	14	23	2-100
	6	11	36	3-195
	7	Tumor	—	—
	8	Tumor	—	—

5.1.1 Beta-galactosidase

Senescence-associated β-galactosidase (SA-β-gal) is a marker enzyme described by Dimri et al.[105], whose activity can be detected at pH 6 only in senescent cells. Although it does not appear to play any specific role in the induction of the senescent phenotype, it is still considered a useful marker of cell senescence, possibly reflecting the expansion of the lysosomal compartment in senescent cells[107].

The figure shows the increase of SA-β-gal activity in the animals killed at 26 months of age (transplanted at 18 months) compared with animals of 11 months (transplanted at 3 months). As expected, enzyme activity was virtually absent in the liver of young rats, while it was very prominent in older animals, indicating the progressive accumulation of senescent cells in the liver with age.

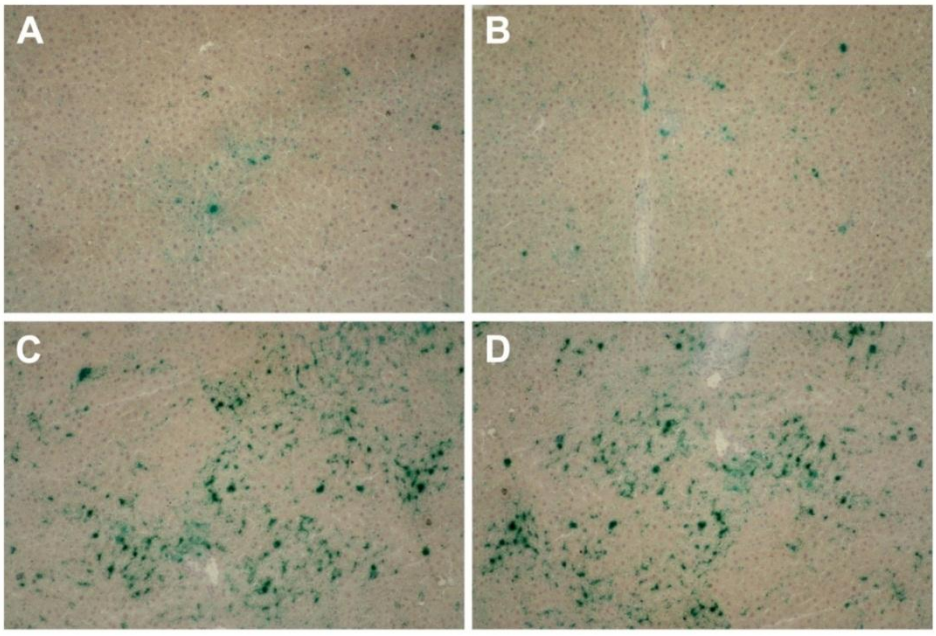


Figure 8: SA-β-gal staining in 2 animals transplanted at 3 months (A and B) and in 2 animals transplanted at 18 months (C and D) killed 8 months after.

5.1.2 Expression of IL-6 in animals of different age

IL-6 is one of the main signaling pathways modulating the complex relationship between aging and chronic morbidity. There is evidence that free radical damage may be responsible for changes in the genetic control of IL-6 regulation, which, in turn, causes elevated levels of IL-6 in the elderly and in diseased patients[196].

Western Blot analysis and real time PCR show an increase of IL-6 levels in old animals compared with young rats. Especially in the animals 26 months aged we can see the augmentation of IL-6 (figure 9).

Another component of SASP, IL-8 is analyzed by real time PCR but the IL-8 m-RNA didn't change in the 3 classes of samples like IL-6.

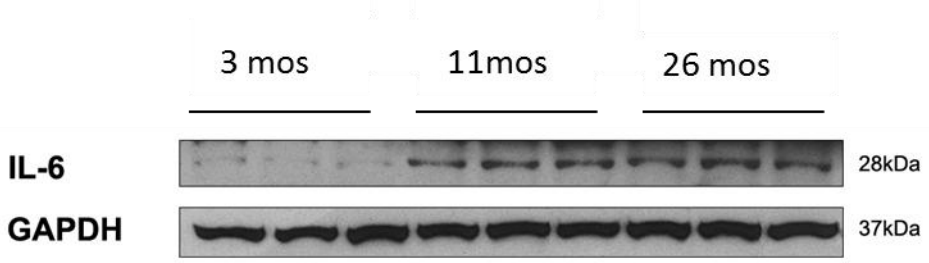


Fig.9 :Western Blot analysis on IL-6 in young animals of 3 months (ctrl) ,11 months and 26 months after transplant of nodular hepatocytes.

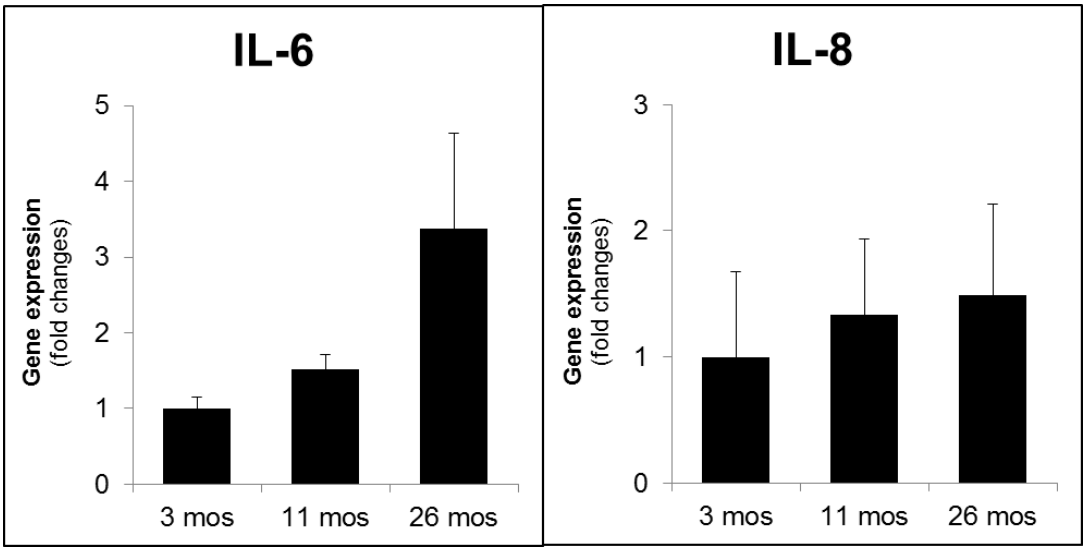


Fig.10: Gene expression for IL-6 (A) and IL-8 (B)

5.1.3 Inhibitors of cell cycle regulation

Since cell senescence is often associated with altered regulation of CDK-Is, such as p21 and p27, the levels of expression of these proteins were analysed in rats of different age. As indicated in figure 11 and 12, both IHC and Western Blot showed no significant changes in the levels of these proteins.

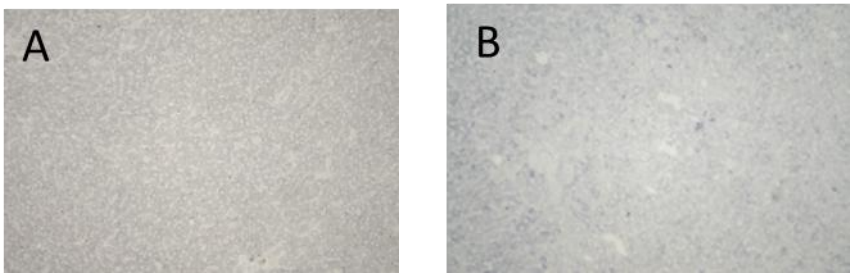


Fig.11 Immunohistochemical analysis of p21: A liver of animal transplanted at 3 months. B old liver transplanted at 18 months both killed 8 months after.

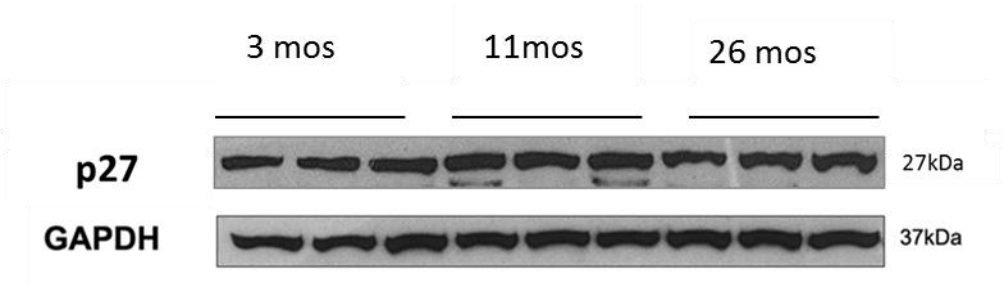


Fig. 12 Western Blot analysis of p27

5.2 Cell senescence in liver repopulation

The next series of experiments was aimed at probing into the hypothesis that cell senescence might be involved in the preconditioning effect for liver repopulation exerted by retrorsine[197]. After 1 week of acclimatization, rats were divided into two groups, of 12 animals each, and given either 2 injections of retrorsine 30 mg/kg each, i.p., two weeks apart, or saline. Four weeks after the last injection 6 control (untreated) and 6 RS-treated rats were killed, while the remaining 6 animals in each group underwent 2/3 partial hepatectomy (PH) and were killed 4 weeks after surgery.

5.2.1 RS-exposed hepatocytes express senescence-associated β-galactosidase.

Exposure to RS caused a prominent increase of SA-β-gal expression in hepatocytes, observed as late as 4 weeks after treatment. The enzyme activity was detected throughout the liver parenchyma, although hepatocytes in zone 3 of the liver acinus were relatively spared (figure 13).

Given our previous observations that PH is able to accelerate the kinetics of liver repopulation in this model, we analysed the expression of SA-β-gal in animals receiving RS followed by PH, according to the original protocol for hepatocyte transplantation[173]. Animals were killed 4 weeks after surgery. Results indicated that SA-β-gal was diffusely expressed in hepatocytes exposed to RS and PH, with no apparent zonal distribution, while very limited enzyme activity was detected in control animals receiving PH (panels c and d).

.

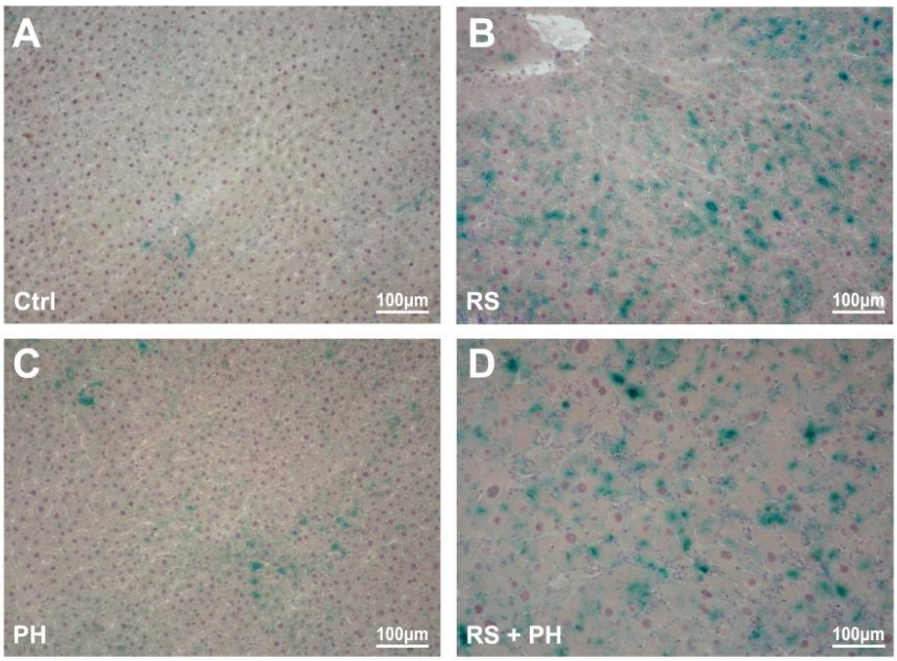


Fig. 13 RS-exposed hepatocytes express senescence-associate β-Galactosidase. Histochemical staining for SA-β-gal in representative samples for: (A) control group; (B) RS-treated group; (C) Control+PH group; (D) RS + PH treated group.

5.2.2 Hepatocyte megalocytosis induced by RS.

Cellular enlargement (hypertrophy) is considered as one of the hallmarks of senescent cells, being the result of cell growth stimulation under conditions of replicative arrest. The presence of enlarged hepatocytes in rat liver exposed to PAs, including RS, has long been reported in the literature as one of the typical effects of these naturally occurring compounds[109]. We've measured the size of hepatocytes in rat liver exposed to RS under conditions that are conducive to liver repopulation, both in the absence and in presence of the proliferative stimulus of PH.

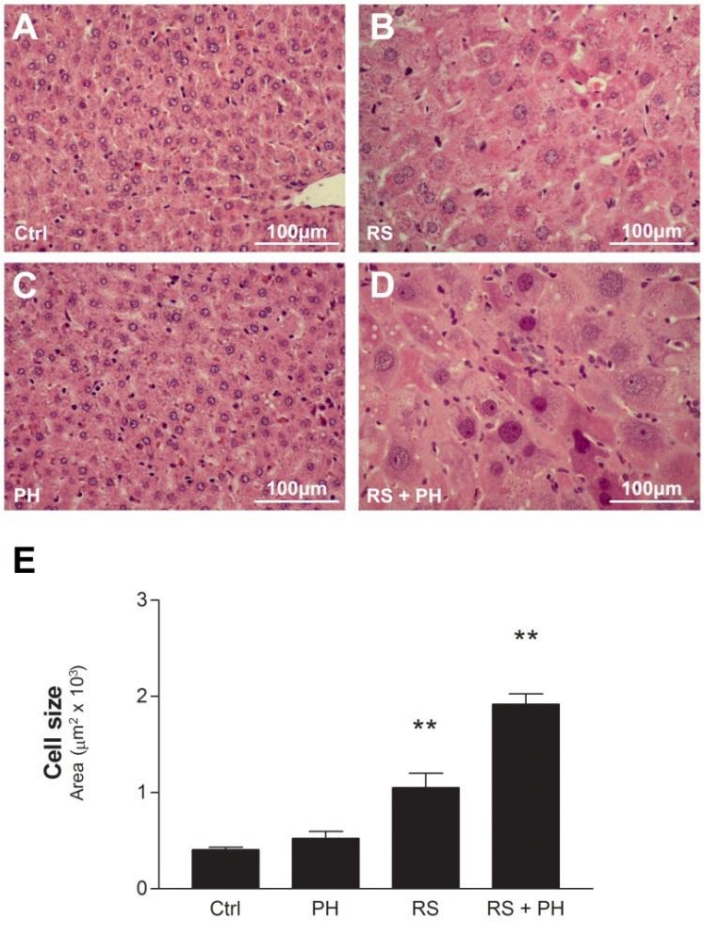


Fig. 14. RS treatment induces megalocytosis in hepatocytes. (A-D) Hematoxylin & Eosin staining in representative samples for: (A) control group; (B) RS-treated group; (C) Control+PH group; (D) RS + PH treated group. (E) To measure cell size, 10 high power fields/animal were counted in each group. *Significantly different from controls, $p < 0.001$; #Significantly different from RS only, $p < 0.001$;

In figure 14, the histological appearance of the liver is reported. Enlarged hepatocytes are readily evident in panel b (RS-treated) compared to panel a (untreated control). It is noteworthy that exposure to RS alone did not cause other prominent alterations in liver histology, e.g. proliferation of bile ductular cells, chronic inflammation or fibrosis.

In rats receiving RS+PH and killed 4 weeks after surgery hepatocyte megalocytosis was greatly enhanced (panel d), while no detectable change was seen in control animals after PH (panels a and c). To measure cell size, images from H&E-stained

tissue sections were uploaded into a computer and cell boundaries were outlined in 10 high power fields per animal. Computation was performed using Image Pro Plus program (Media Cybernetics, Inc. MD, USA). As shown in panel e of figure 14, highly significant differences were recorded between RS-treated and control groups, both prior to and post-PH, as well as between RS and RS+PH groups.

5.2.3 DNA damage foci in RS-treated rat liver.

One of the triggers of cell senescence is DNA damage. More specifically is a frequent association with the senescence phenotype[198]. Cells that senesce with persistent DDR signalling harbour typical nuclear foci, which have been referred to as DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS)[15]. These persistent foci contain proteins involved in chronic DDR, such as the ataxia telangiectasia-mutated (ATM) gene product. However, they are distinguishable from transient DDR complexes based on the presence of specific markers, including the H2AX and the p53 binding protein1 (53BP1). Based on the well- established genotoxic potential of RS, we tested whether its induced hepatocyte senescence was associated with signs of persistent DDR in rat liver *in vivo*.

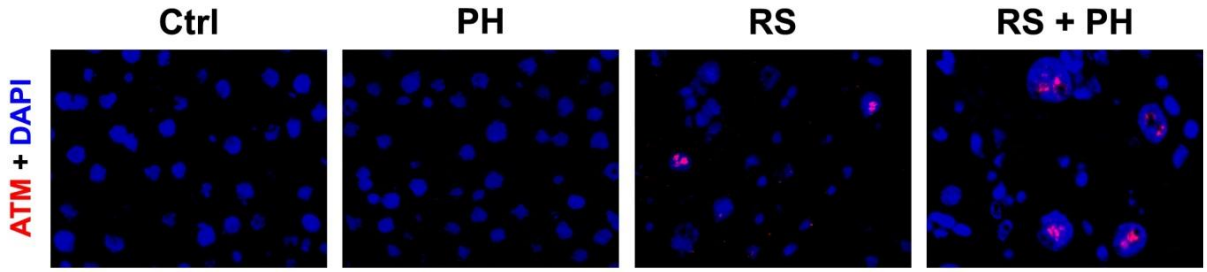


Fig.15: DNA damage foci in RS-treated rat liver. Immunofluorescence staining for ATM expression in control animals and RS-treated animals, prior to- or after PH.

As presented in figure 15, 30-40% of hepatocytes from rats exposed to RS and killed 4 weeks later expressed ATM-positive nuclear foci, and this finding was even more frequent (60-65%) in hepatocytes from animals treated with RS+PH and killed 4 weeks after surgery; by contrast, virtually no ATM-positive nuclear foci were found in control rats, either with no treatment or following PH .

It was also investigated if RS-treated hepatocytes express H2AX and 53BP1, which are considered as indicative markers of persistent DDR. Both proteins were detected in hepatocyte nuclei of rats treated either with RS alone or with RS+PH.

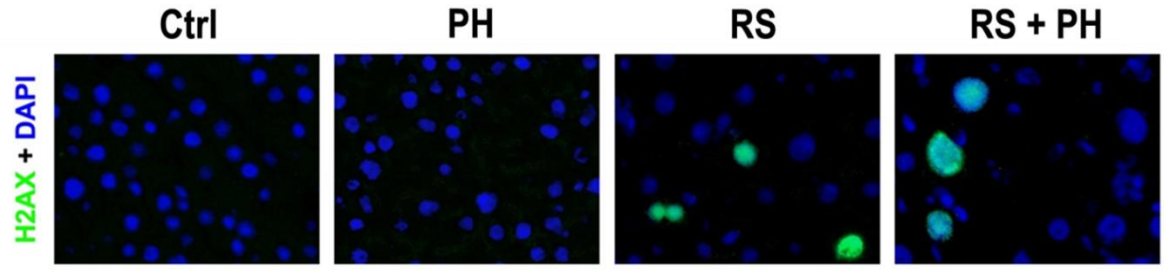


Fig. 16. DNA damage foci in RS-treated rat liver. Immunofluorescence staining for γ-H2AX expression in control animals and RS-treated animals, prior to- or after PH.

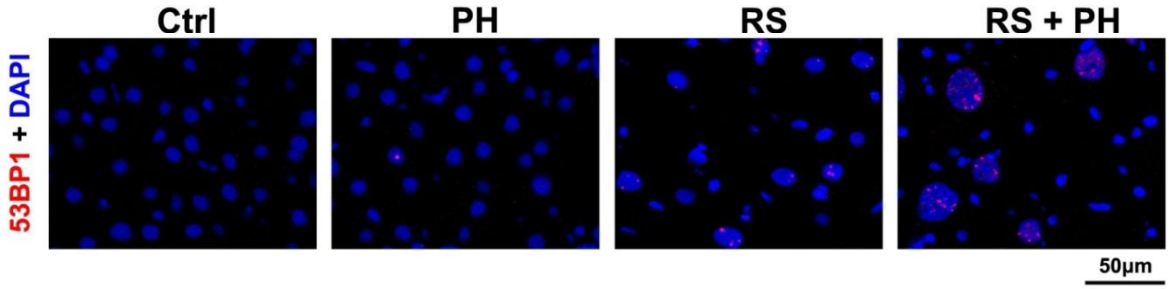


Fig.17:DNA damage foci in RS-treated rat liver. Immunofluorescence staining for p53BP1 expression in control animals and RS-treated animals, prior to- or after PH.

More specifically, H2AX-positive hepatocytes were about 10% in both RS and RS+PH groups (figure 16), while 53BP1-expressing nuclear foci were present in 40-45% of hepatocytes in rats given RS and increased to 65-70% in animals treated with RS+PH (figure 17). Both H2AX and 53BP1 were undetected (H2AX) or rarely detected (53BP1) in hepatocytes from control rat liver. These data are summarized in table 4.

Table 4: % DNA damage foci

	H2AX	53BP1	ATM
Ctrl	0%	0%	0%
PH	0%	0.9%	0%
RS	8.7%	42.5%	35.6%
RS+PH	9%	64.6%	62%

5.2.4 Up-regulation of cell cycle inhibitory proteins in RS-treated rat liver.

Progression of hepatocytes through the cell cycle is controlled by the activity of cyclin dependent kinases (CDKs), regulated by cyclins and by other proteins including CDK-inhibitors (CDK-Is)[199]. In order to investigate the molecular bases of the RS-induced, persistent replicative block, we analysed the expression two CDK-Is of the Cip/Kip family, p21 and p27, which are known to interact with complexes of cyclins D and E and their CDKs targets. Western blot analysis of total liver proteins

revealed over-expression of $p21^{Cip1}$ in animals exposed to RS compared to controls, and a further increase in p21 protein was seen in the group receiving RS+PH.

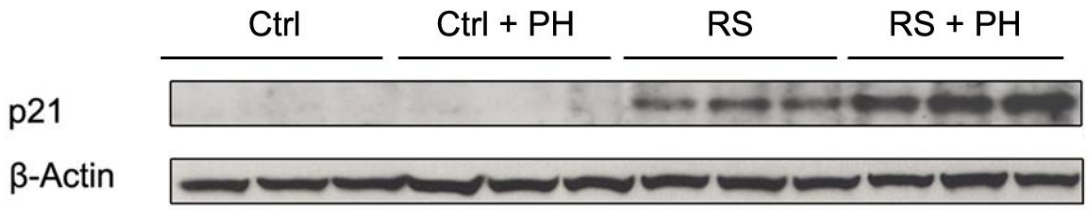


Fig 18. Cell cycle inhibitory proteins are up-regulated in RS-treated rat liver. Expression of p21 protein was measured in control animals and RS-treated animals, prior to or after PH.

This pattern of results was consistent with data obtained through immuno-histochemical staining of liver sections with an antibody specific to p21; the protein was found to be highly expressed in nuclei of RS-induced megalocytes, both in the absence of PH and, more so, in animals killed 4 weeks after PH, while it was rarely detected in control groups, either untreated or following PH (figure 19)

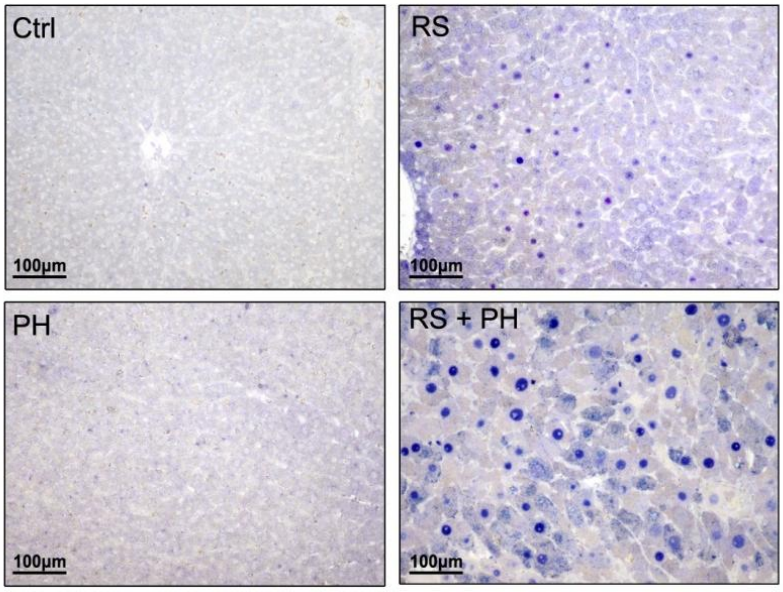


Fig.19: Immunohistochemical staining for p21

Results were similar when the expression of $p27^{Kip1}$ was analysed. Both western blotting and immunohistochemical staining revealed a prominent increase of p27 levels in the liver of rats exposed to RS, and the effect was further amplified following PH (figure 20 and 21). Interestingly, increased expression of p27 was also observed in control rats as late as 4 weeks after PH.

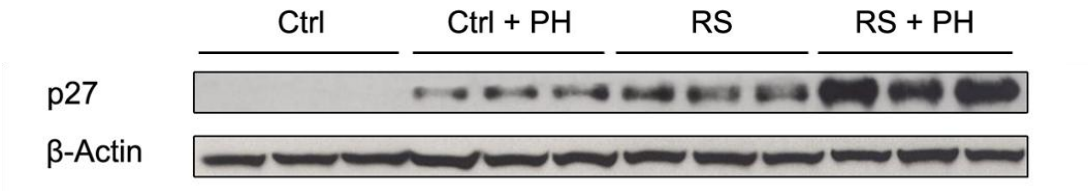


Fig.20: Western Blot analysis of p27 expression

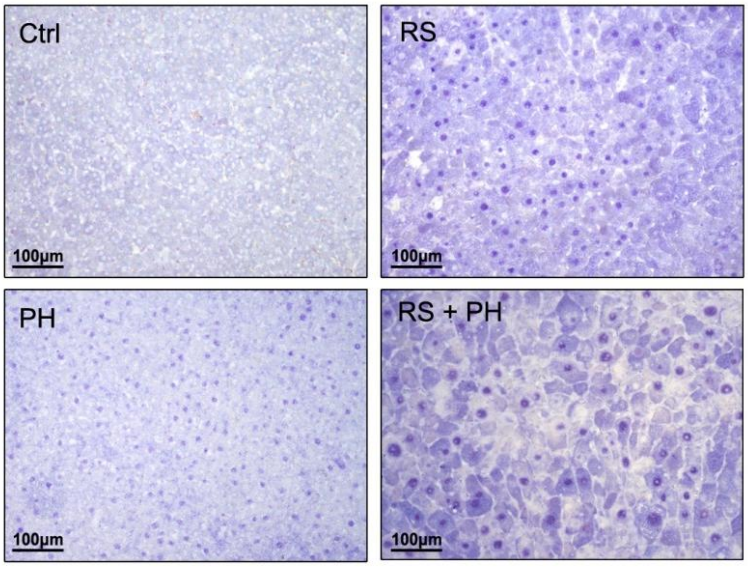


Fig.21: Immunohistochemical staining for p27

5.2.5 The increased expression of positive regulators of cell growth and cell-cycle progression in RS-exposed liver.

Stimulation of cell growth and cell cycle progression is considered as the other side of the coin, opposite to cell cycle arrest, sustaining the emergence of a senescence phenotype[200]. In an earlier report, we described the over-expression cell cycle related gene products, including cyclin D1, CDK4 and PCNA, in rat liver exposed to RS[201]. In the present study, we extended the analysis to the mammalian target of rapamycin (mTOR), which plays a pivotal role at the crossroads of cell metabolism, integrating energy balance and cell growth signals[202]. There is in fact evidence to indicate that activation of mTOR under conditions of cells cycle arrest leads to cell senescence[198]. Results are presented in figure 22: increased levels of mTOR protein were detected in the liver of rats exposed to RS compared to untreated controls (figure 22); such a difference was even more prominent in groups receiving PH and killed 4 weeks later, despite the fact that PH caused a decrease in mTOR levels in both RS and control groups. We also confirmed the accumulation of cyclin D1 in rat liver treated with RS, as already reported[199]; moreover, a further increase in this protein was seen in the group receiving RS+PH and killed 4 weeks later (figure 22).

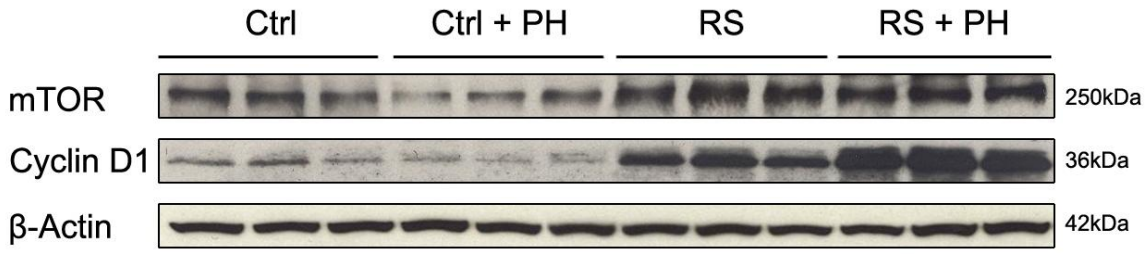


Fig.22. Increased expression of positive cell-cycle regulators in RS-treated liver. Western Blot analysis of mTOR and Cyclin D1 expression in control animals and RS treated animals, prior to- or post-PH

5.2.6 IL-6 is over-expressed in rat liver exposed to RS.

A most intriguing feature of cell senescence is the associated secretory phenotype (SASP)[22]. This pattern of gene expression includes a series of cytokines, growth factors and other products that are secreted by senescent cells and can exert a plethora of biological effects, mostly limited to the local tissue microenvironment[122]. The specific composition of SASP is likely to vary depending on cell and tissue type. However, a consistent finding is the presence of the inflammatory cytokine interleukin 6 (IL-6), which has also been implicated in reinforcing the senescence phenotype through autocrine and/or paracrine mechanism[86].

Based on this evidence, it appeared reasonable to explore the possibility that alterations in IL-6 expression might also be present along in association with other markers of cell senescence in rat liver exposed to RS. As documented in figure 23 and 24, both IL-6 mRNA and protein levels were found to be increased in animals treated with RS according to the protocol for liver repopulation; a further increase in both parameters was seen in RS+PH group, and this was particularly prominent for IL-6 mRNA.

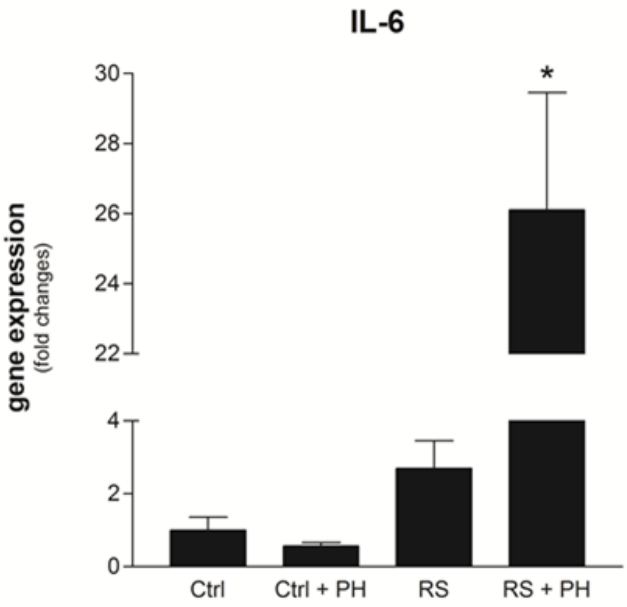


Fig.23 Quantitative mRNA expression of IL-6 in all four experimental groups. *Significantly different from control, $p < 0.001$

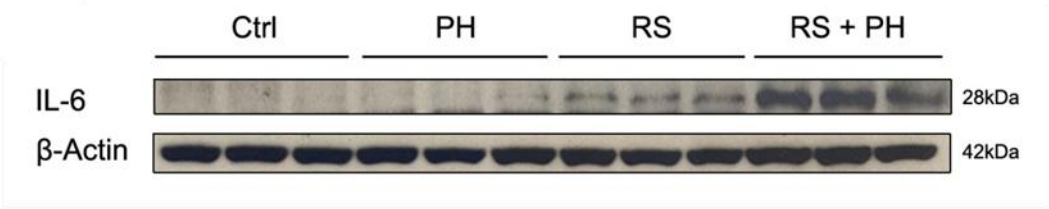


Fig.24: western blot protein analysis of IL-6 in all four experimental groups.

5.2.7 Regulation of IL-8 following exposure to RS

Rat cytokine-induced neutrophil chemoattractant 1 (CINC-1) belongs to the CXC chemochine family and is a counterpart a human growth-related oncogene (GRO) in the Interleuchin-8 (IL-8) family. In rats, CINC-1 plays a key role in neutrophil-mediated inflammatory disease by attracting neutrophils to the site of inflammation. Since IL-8 has also been reported to be a component of SASP in some cell systems, we investigated its expression profile during hepatocyte senescence induced by RS.

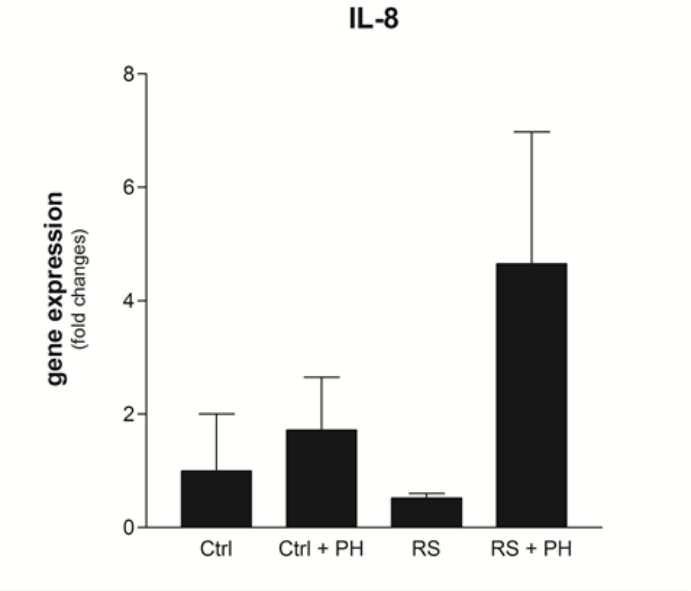


Fig.25: m-RNA analysis of IL-8

5.2.8 Up regulation of TNF-α

The pro-inflammatory cytokine TNF-α is an important mediator of the inflammatory responses with multiple biologic activities. It has been implicated in the pathogenesis of cancer, mainly in the context of chronic inflammation, because it

triggers DNA damage, angiogenesis, invasion and metastasis[203]. Western Blot analysis revealed an increase of this cytokine in livers treated with RS.

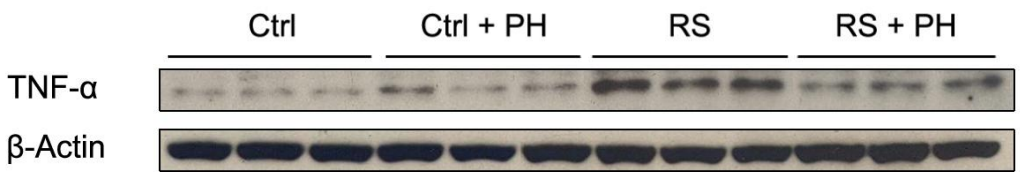


Fig.26: Western blot analysis on TNF-α

5.2.9 Regenerative nodules do not express markers of cell senescence.

Remarkably, no signs of phenotypic senescence were seen in regenerative nodules developing in rats given RS and PH. These nodules have long been described in the literature[204] and are composed of small hepatocytes which are able to withstand the cell cycle block imposed by RS on surrounding parenchymal cells. They slowly expand and replace the entire liver, in a process unfolding over several months that we have referred to as endogenous liver repopulation[205]. The cell of origin of small hepatocytes is still a matter of debate[206-207].

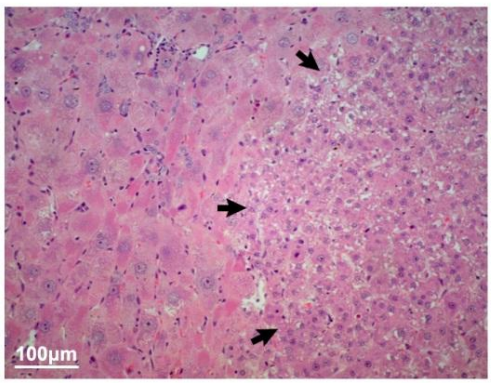


Fig.27: H&E of regenerative nodule (see arrows) in RS-treated liver.

When regenerative nodules were probed for the presence of markers of cell senescence, they were found to express a phenotype that was in sharp contrast to that of surrounding megalocytes. In fact, small hepatocytes composing regenerative nodule showed no significant SA-β-gal activity; in addition, mTOR expression was low in these nodule compared to surrounding liver (figure 28).

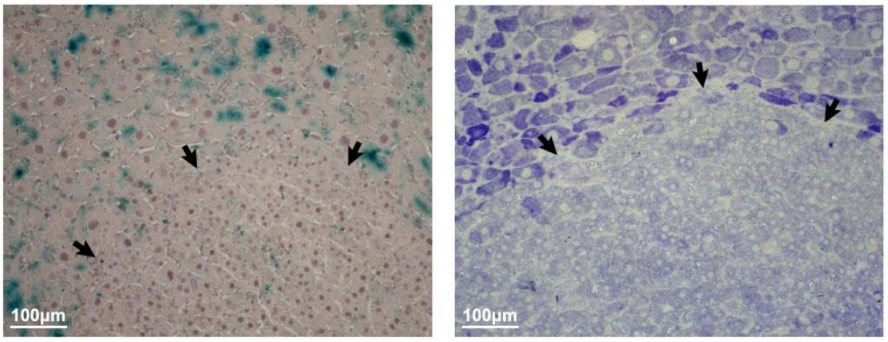


Fig 28 Histological analysis of liver tissue samples from animals treated with RS + PH and killed 4 weeks after surgery. Regenerative nodules are indicated by arrowheads. SA-β-gal histochemical staining (right); mTOR immunohistochemical staining (left).

Only rare nuclear staining for p21 or p27 CDK-Is (figure 29).

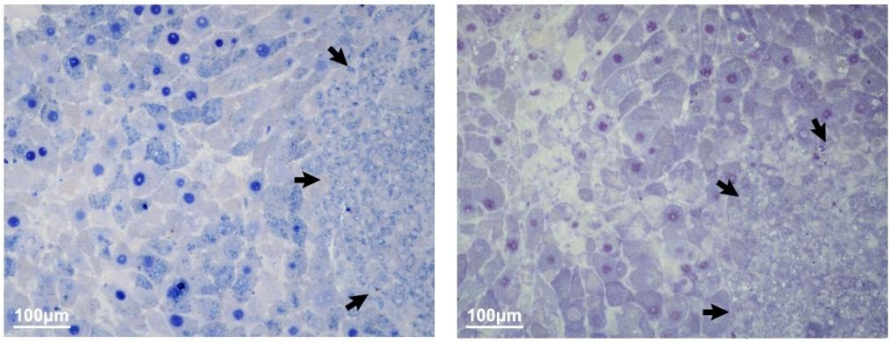


Fig.29 Immunohistochemical staining for p21 (right) and p27 (left) in regenerative nodule and surrounding liver

Furthermore, cyclin D1-positive nuclei were common in RS-exposed megalocytes, while they were rare in regenerative nodules; conversely, numerous Ki67-expressing hepatocytes were found inside regenerative nodules while they were rarely seen in the megalocytic surrounding liver (figure 30).

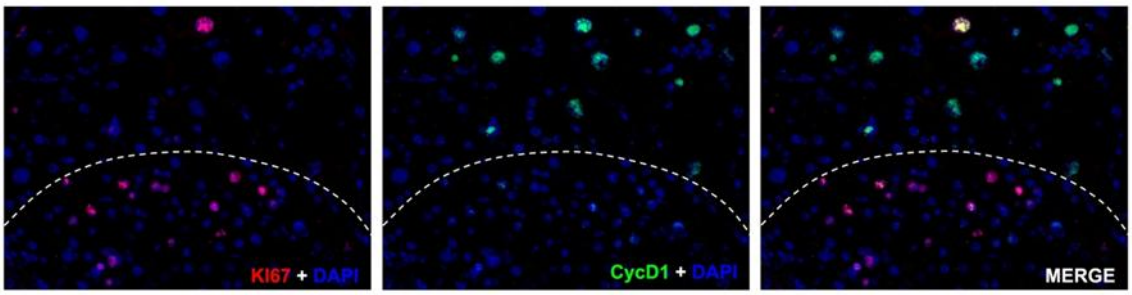


Fig.30 immunofluorescence staining for Cyclin D1 (green) and KI67 (red). Regenerative nodules are indicated by a dashed line.

Similarly, we found that CDK4 protein, which associates with cyclin D1, was frequently detected in the nuclei of enlarged hepatocytes, while it was virtually absent in small hepatocytes of regenerative nodules (figure 31).

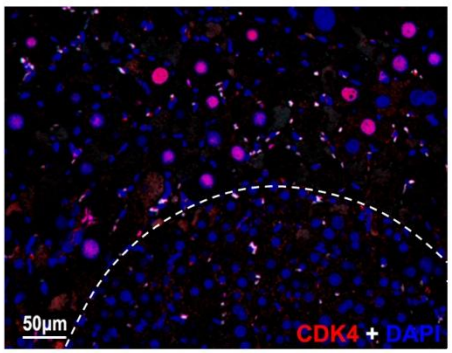


Fig.31 immunofluorescence staining for CDK4 (red).

5.3 Normal hepatocyte transplantation delays the emergence of chemically-induced pre-neoplastic nodules in rat liver

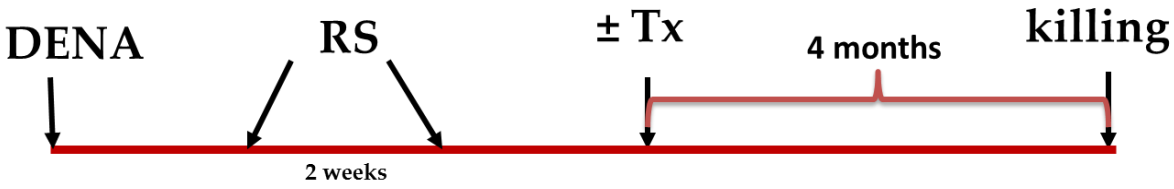
The intriguing analogies existing between liver repopulation and carcinogenesis, suggest that these two processes may share common pathogenetic mechanisms[188]. Within this conceptual framework, normal cells could possibly be used to competitively counteract the selective growth of altered cells during early stages of carcinogenesis. Thus, it was investigated whether strategies aimed at normalizing the microenvironment of a chronically and/or irreversibly injured liver tissue prone to cancer, may help reduce the risk of neoplastic disease[208].

In this experiment, we aim to investigate whether transplantation of normal cells in the context of an injured, neoplastic-prone microenvironment might impact on the evolution of carcinogenic process.

A rat model of chemically-induced hepatocarcinogenesis was used. Animals were given a single dose of diethylnitrosamine (DENA), followed by two injections of retrorsine (RS), a pyrrolizidine alkaloid that imposes a persistent block on hepatocyte cell cycle. At the end of this protocol, rats were either given no further treatment or injected, via the portal circulation, with 4 million normal hepatocytes isolated from a syngeneic donor. A second group of animals was similarly exposed to DENA+RS protocol followed by transplantation of normal hepatocytes.

5.3.1 The induction of hepatic nodules by sequential exposure to DENA+RS.

Experimental strategy



As predicted, sequential exposure to DENA and RS resulted in the occurrence of numerous hepatocyte nodules, both on the liver surface (up to 30/liver, mean 18±5, and on sectioning.

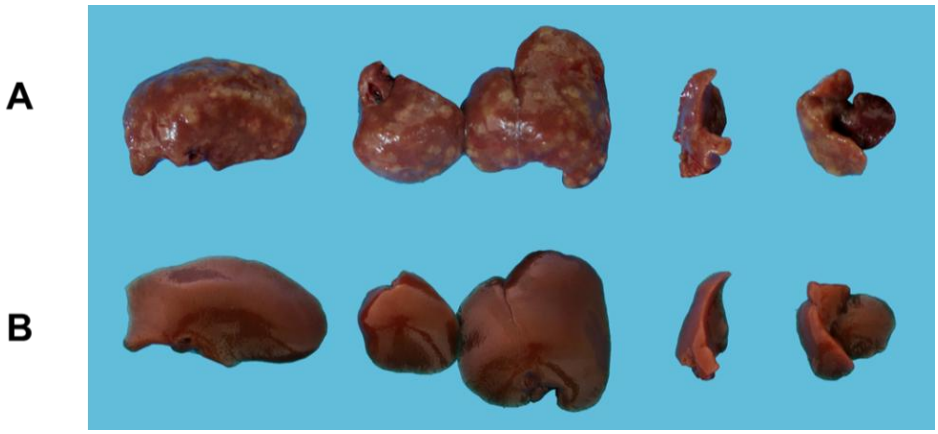


Fig.32 Liver samples of rats exposed to DENA+RS (panels A and C) or DENA+RS+Tx (panels B and D and E) and killed 4 months later. Panel A (DENA+RS) shows numerous white-greyish nodules present on liver surface, while no such lesions are present in liver lobes of panel B (DENA+RS+Tx).

Nodular lesions were whitish-grey in colour and resembled those induced by other classical protocols of chemical hepatocarcinogenesis in the rat, both on macroscopic appearance and upon histological analysis (figure 32).

They also expressed the GST 7-7 marker enzyme, further supporting their putative preneoplastic nature (figure 33).

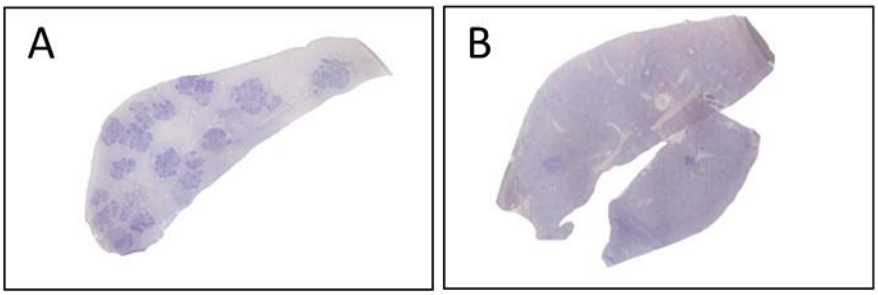


Fig.33 Whole cryostat sections of the same livers processed for immunohistochemical detection of GST 7-7. A: note the presence of several GST 7-7-positive lesions (dark blue) in the liver (DENA+RS), while few such lesions are present in panel B (DENA+RS+Tx).

The histology of transplanted and repopulated livers was also remarkably different compared to non-transplanted group (figure 34). Only residual cords of megalocytes were seen in between clusters of donor-derived cells in transplanted rats. The latter displayed normal size, normal histology and were perfectly integrated into the host liver, with no evident demarcation from resident hepatocytes. Proliferation of oval cells was also greatly reduced in livers receiving hepatocyte transplantation.

Surrounding liver was mainly occupied by enlarged (megalocytic) hepatocytes, with rare clusters of very small hepatocytes that have been referred to as regenerative nodules[177]; mild to intense proliferation of oval cells was also observed .

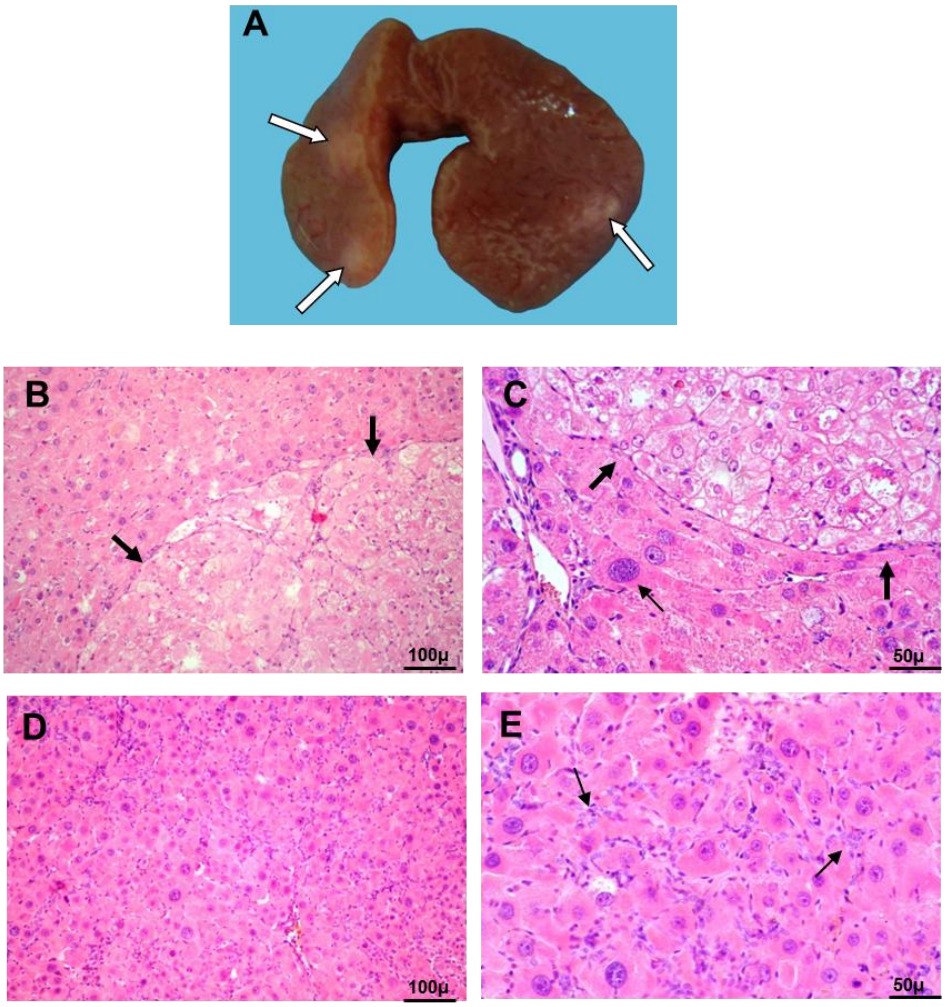


Fig.34 Liver samples of rats exposed to diethylnitrosamine + retrorsine ((DENA+RS) and killed 4 months later (see text for details). Panel A shows at least 3 white-greyish nodules (arrows) in the caudate lobe .B-E histological appearance of H&E stained liver sections is shown; panels A and B show a liver nodule (thick arrows), sharply demarcated from surrounding enlarged hepatocytes (megalocytes, thin arrow in panel C). In panels and D the typical alterations induced by RS on rat liver can be observed, with extensive megalocytosis and moderate to intense proliferation of ductular epithelial cells (panel E, thin arrows).

5.3.2 Liver size and hepatocyte proliferation.

Parameters related to liver size and liver DNA content in both transplanted and non-transplanted groups were also measured. No significant differences were seen in final body weight and liver weight. Interestingly, a slight increase in liver DNA

content was observed in the group receiving transplantation (6.42±0.12 vs. 5.64±0.34 mg DNA/liver/per 100g b.w., $p<0.05$).

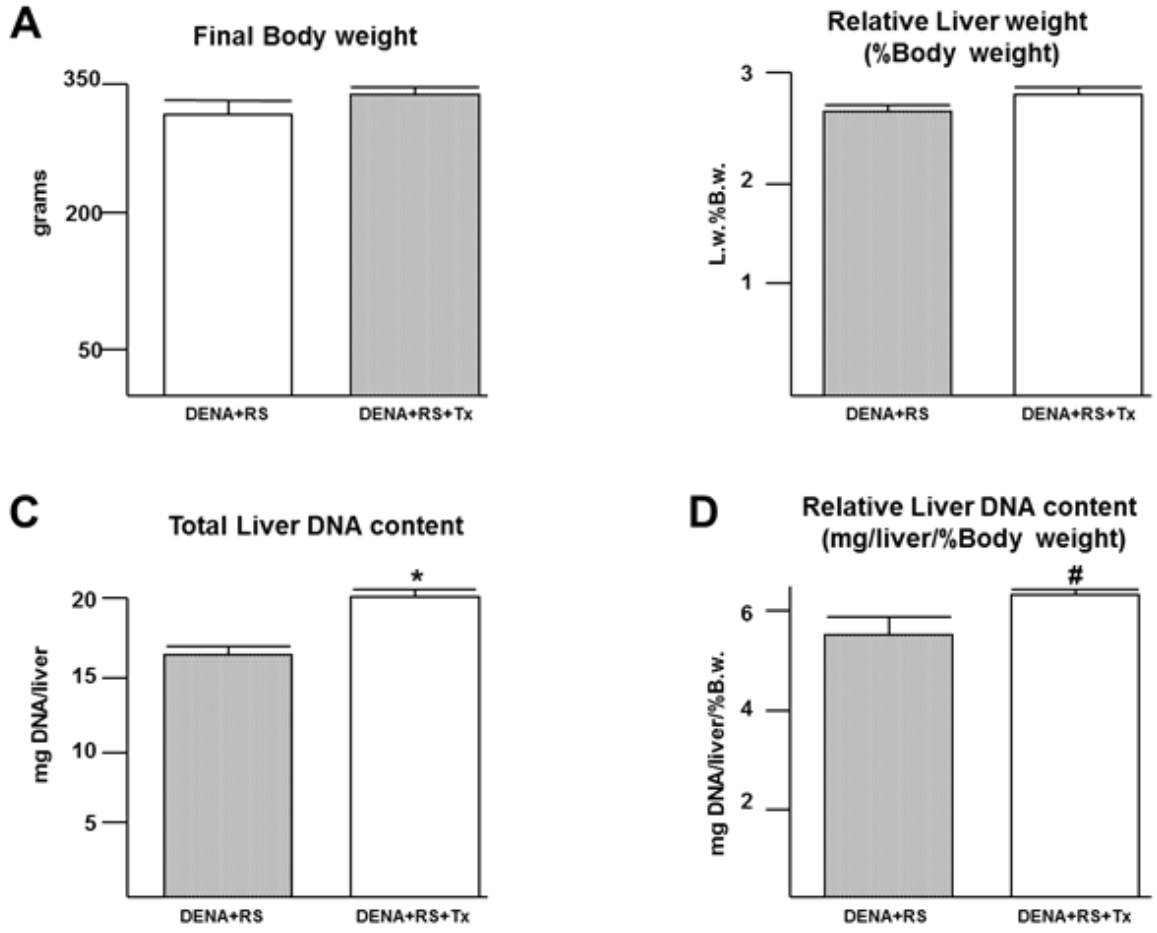


Fig.35 Slight increases in relative liver weight and liver DNA content were observed in the group receiving transplantation, although only the latter reached a level of statistical significance (panels C and D)

5.3.3 Liver repopulation by transplanted cells in DENA+RS-treated liver.

We have repeatedly shown that pre-exposure to RS sets the stage for extensive repopulation of the host liver by transplanted normal hepatocytes[207]. However, no

studies had been published so far on the effect of a combined treatment with DENA and RS on liver repopulation.

Percent of host liver replacement by donor-derived cells was therefore evaluated in these experiments using a computer assisted image analyzer. High levels of repopulation were observed in all transplanted animals, with an average of 53±7% of total liver replacement by DPP-IV-expressing hepatocytes (figure 36). The distribution of transplanted cells clusters was generally homogeneous throughout the recipient lobes, although some areas displayed lower levels of repopulation.

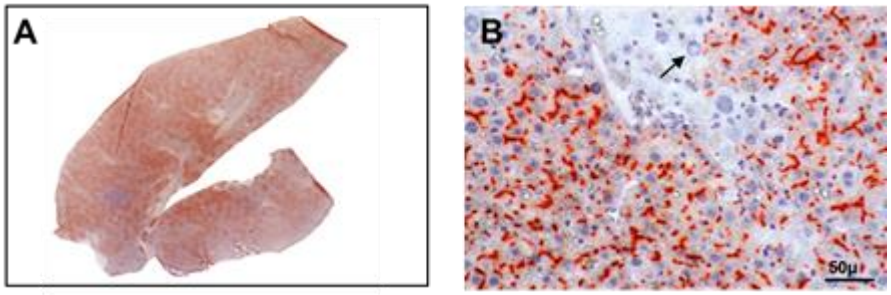


Fig.36 Panel A:whole cryostat section of a liver samples processed for dual histochemical-immunohistochemical detection of DPP-IV activity (orange-rust) and GST 7-7 expression; note the extensive repopulation of the host liver by DPP-IV positive, donor-derived hepatocytes. In panel B a photomicrograph of a transplanted liver is shown: only residual endogenous megalocytes were present (thin arrow)

5.3.4 The effect of hepatocyte transplantation on the incidence of nodules in the liver of DENA+RS-treated rats.

Another aim was to determinate whether transplantation of normal hepatocytes and the associated extensive liver repopulation had any influence on the growth of nodules induced by sequential exposure to DENA and RS. The number of GST 7-7-positive focal lesions was greatly reduced in rats receiving DENA+RS and transplantation as compared to the control group given DENA+RS only (3±2 vs. 19±8 lesions per cm^2). This was also reflected in the relative area occupied by

nodular lesions, below 2% in the group receiving DENA+RS and transplanted cells (1.8±0.3%), down from a control value of 8.5±2.8%; this represents a net decrease of almost 80% between group 1 and group 2. Furthermore, the mean size of the 3 largest GST 7-7 positive nodules found in each animal of the two groups was reduced by a factor of about 5 in transplanted vs. non-transplanted rats.

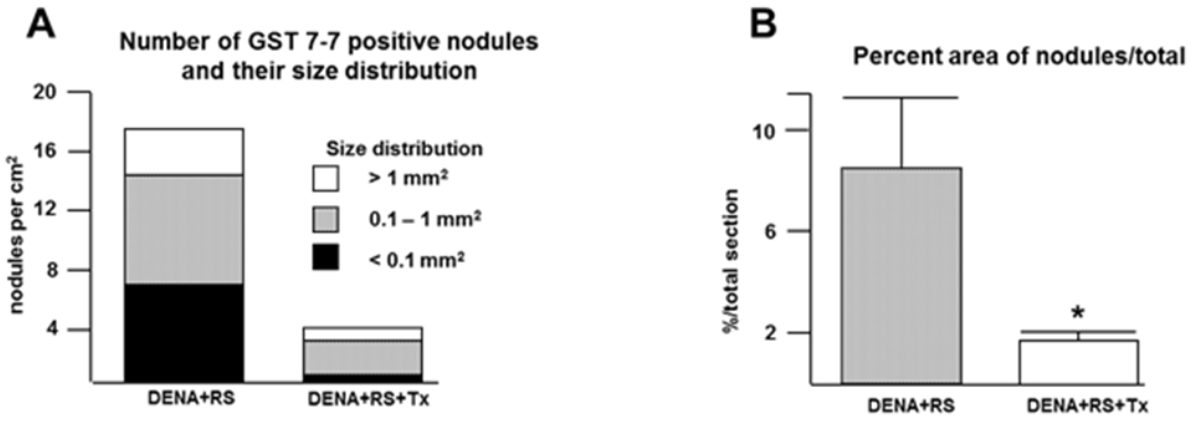


Fig. 37 number and size distribution of GST 7-7 expressing lesions is compared, while panel B shows the percent area occupied by nodular lesions in each group.

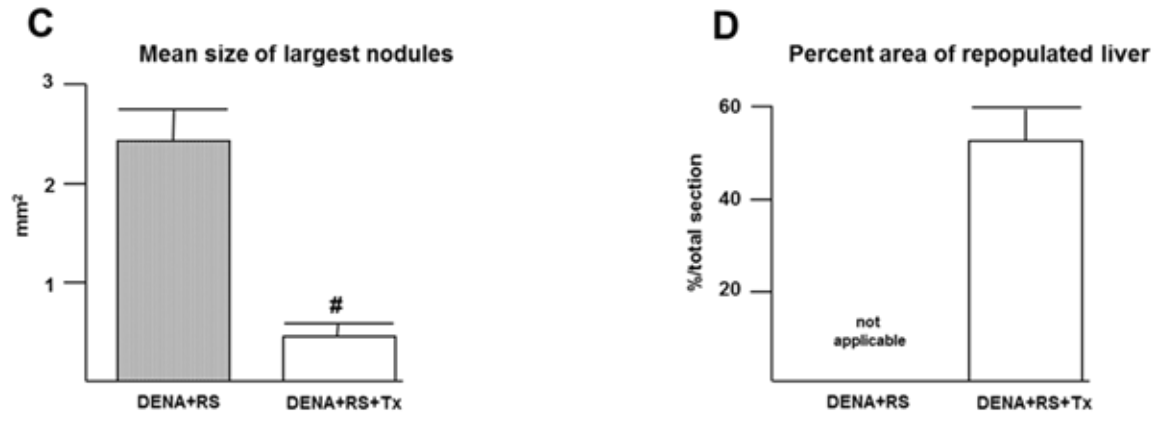


Fig. 38 In panel C, the 3 largest GST 7-7-positive nodules from each animal were considered, and their mean size was compared in the two groups. Finally, panel D reports the extent of liver repopulation by transplanted cells in rats exposed to DENA+RS+Tx. Data are mean±SE. Significantly different from control, *p<0.05; #p<0.001 (two-tailed t-test)

5.3.5 SA-β-GAL decrease in transplanted animals

The activity of SA-β GAL in the transplanted animals is reduced compared with the animals that received only DENA and RS. Two serial sections of animals transplanted after 2 months show that the liver is repopulated by normal hepatocytes and only in the area not repopulated is there some β-gal activity. (figure 39 panel A and B). DENA-RS exposed hepatocytes express senescence-associate β-Galactosidase in all the surrounding liver (panel C) but not inside the nodules (panel D).

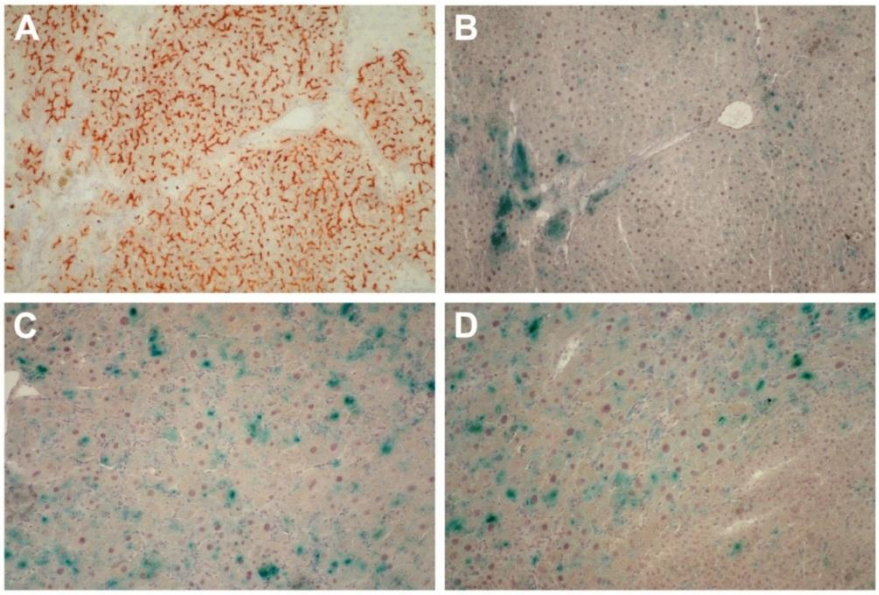


Fig.39 A: DPPIV activity in the repopulated liver. B-D SA-β-gal activity detected in an animal transplanted with normal hepatocytes (B) and in a disease liver treated with DENA+RS (C and D).

5.3.6 DNA damage foci

As reported in the preceding chapter, exposure to RS results in the appearance of persistent DNA damage foci, containing proteins involved in the chronic DDR. It became therefore important to ascertain whether transplantation of normal hepatocytes and the associated liver repopulation would alter the distribution of such foci in DENA+RS-exposed rat liver.

As reported in figures 40 and 41, the number of nuclei expressing γ-H2AX is higher in livers treated with DENA+RS without transplant compared to animals receiving DENA+RS followed by hepatocyte transplantation. Similar findings were seen for the expression of 53BP1-positive nuclear foci.

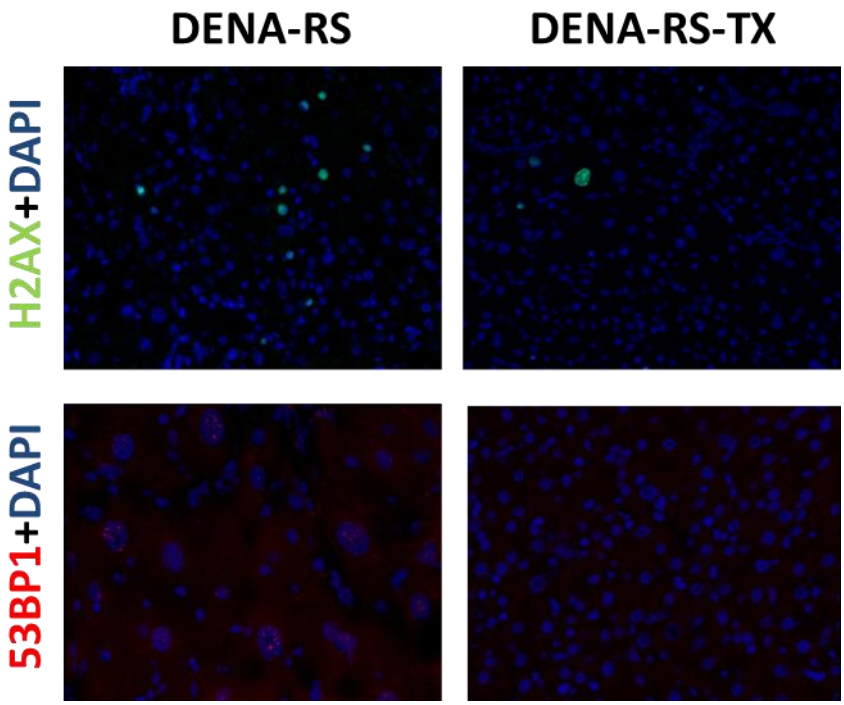


Fig.40. Immunofluorescence staining for H2AX and 53BP1 expression in DENA- RS-treated animals, with or without transplant. Nuclei are stained with DAPI.

The double staining in immunofluorescence shows that the transplanted normal hepatocytes (CD26 positive) were negative for the presence of γ-H2AX, while rare surrounding residual megalocytes expressed the senescence marker.

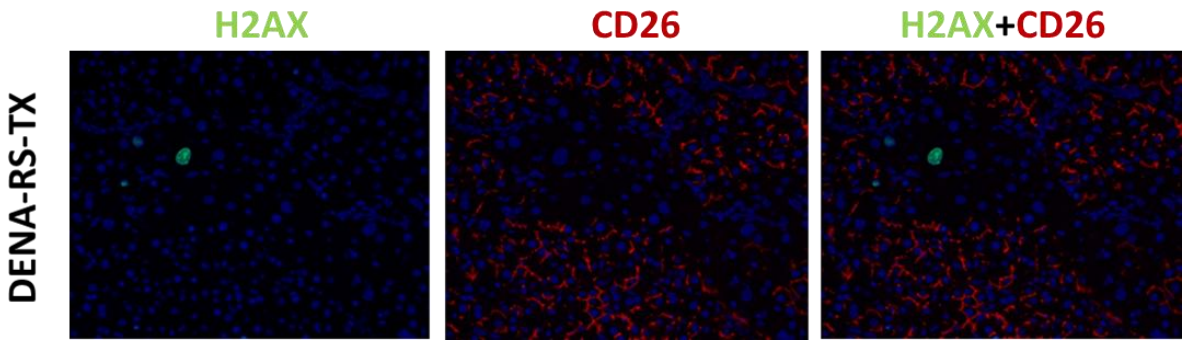


Fig.41 Immunofluorescence staining for H2AX(green), CD26(red) and merge in liver treated with DENA-RS and transplanted with normal cells. Nuclei are stained with DAPI.

6 Discussion

The first relevant observation presented in this thesis relates to the complex relationship between aging and cancer. As already mentioned in the Introduction, the basic observation that increasing age is the strongest risk factor for the neoplastic disease, has no satisfactory explanation as yet. This observations would concern both human and other animal species. We provide evidence to suggest that an altered tissue landscape induced by the aging process can foster the growth of altered cells, setting the stage for neoplastic progression and the emergence of overt cancer.

When altered hepatocytes isolated from chemically-induced rat liver nodules were orthotopically transplanted into either young (3- to 5-mos old) or aged (18- to 20-mos old) recipients, two important findings were observed:

- Firstly, very limited expansion of transplanted nodular hepatocytes was seen in the liver of young animals, indicating that pre-neoplastic cell populations isolated from hepatic nodules are not endowed with any measurable degree of *growth autonomy*, in that they were unable to form nodules when transferred in the orthotopic environment of a young host. This in turn indicates that their focal growth at the primary site is also likely to depend on stimuli originating from the local microenvironment, as opposed to intrinsic alterations in the cell cycle control. In this respect, they do in fact behave like normal hepatocytes[174].

- Secondly, the same preparations of isolated nodular cells grew significantly upon injection into aged host liver, generating large cell clusters, expanding to form new hepatic nodules and progressing, in a few cases, to HCC. These results clearly indicate that the microenvironment of the aged liver is able to provide critical component(s) to sustain the selective growth of transplanted nodular hepatocytes, implying a possible a mechanistic explanation for the link between aging and cancer.

Grisham and co-workers were among the first to suggest a direct role for the local tissue microenvironment to explain the association between cancer and aging[208]. These authors reported that tumorigenic liver epithelial cells injected directly into the hepatic parenchyma of young and old rats could grow and form tumors only in the latter group, while the liver of young recipients was able to suppress the neoplastic phenotype of the injected cells[209-210].

Recent work by DeGregori *et al*.[211] has extended a similar concept to the hematopoietic system. Following a series of elegant studies, they have proposed the hypothesis that the aged microenvironment of the bone marrow selects for the emergence of leukemogenic cell clones, thereby contributing to the increase in leukaemia in older age. The results presented in this dissertation are in line with the above propositions, providing further insights into the complex relationship between aging and cancer. In our studies, hepatocytes isolated from well characterized primary liver nodules were used and no *in vitro* passage was involved in the transplantation experiments; thus, the continuity of the carcinogenic process was essentially maintained, adding to the relevance of the results to the *in vivo* situation. Furthermore, nodular hepatocytes that were used in our studies are not fully neoplastic and they cannot be propagated *in vitro* under standard conditions,

behaving in this regard like the normal cell counterparts. In this respect, this is the first report showing that the aged liver microenvironment supports the selective growth of a pre-neoplastic cell population, extending the impact of the aging process to an early phase of the carcinogenic process. On the other hand, our findings also bear mechanistic implications towards explaining the link between aging and cancer. One of the factors that have been invoked to account for the increased frequency of cancer in the elderly is a decline in the immune-surveillance, such that altered cells on the pathway to cancer are no longer detected and deleted as age progresses[210-212]. While such proposition does fall within the broader realm of microenvironment-orientated hypotheses, our present results are not consistent with a major involvement of the immune system in this phenomenon, for at least two reasons.

- Firstly, small clusters of transplanted hepatocytes were found in the liver of young recipients as late as 8 months post-transplantation, indicating that they were not deleted by the immune system.

- Secondly, similar results were observed when normal hepatocytes were transplanted into the liver of young animals, indicating that the lack of growth of transplanted hepatocytes in liver microenvironment of the younger host is not unique to nodular/altered cells.

Taken together, such findings do not support the contention that a failing immune system can explain the increased growth of transplanted cells (including normal hepatocytes!) in the aged host liver. As expected, the liver of aged animals expressed increased levels of SA-β-gal, which is still considered as a reliable marker of cell senescence. Cell senescence has been initially interpreted as a fail-

safe mechanism to avoid the risk of neoplastic transformation in cells harbouring damaged DNA[197]. However, its biological significance has been reconsidered to incorporate the overwhelming evidence that senescent cells can in fact foster the growth of premalignant and malignant cells. These effects are at least partly mediated by a specific secretory pattern, referred to as SASP[213-214], which includes a growing list of factors secreted by senescent cells, comprising cytokines, growth factors and proteases. A major component of SASP is the inflammatory cytokine IL-6, which is also able to reinforce the senescence phenotype through an autocrine loop mechanism[122]. Interestingly, liver tissue from older animals expressed higher levels of IL-6 compared to younger controls, consistent with the activation of a SASP response in the aging liver. While being expressed by senescent cells, IL-6 is also a cytokine with an established role in liver regeneration and repair[215-216], thus making it a likely candidate contributing to the growth of transplanted nodular hepatocytes in the aged liver.

The above results obtained during transplantation studies in the aged liver are similar, in principle, to those observed using the RS-based model for liver repopulation, although the magnitude of the phenomenon is far greater in the latter case. However, it was still more surprising to find that the analogies between age-induced and RS-induced alterations in the liver microenvironment extend beyond the effect on hepatocyte transplantation. As reported in this thesis, exposure to RS was associated with widespread, persistent changes in resident hepatocytes that were consistent with the induction of a senescence phenotype. Such changes include diffuse expression of SA-β-gal, induction of markers associated with cell cycle arrest, such as p21 and p27; signs of persistent activation of a DDR with the presence of DNA-SCARS, as evidenced by ATM-, 53BP1- and γ-H2AX-positive

nuclear foci; over-expression of positive regulators of cell growth and cell cycle progression, such as mTOR, cyclin D1, CDK4 and PCNA. In addition, RS-exposed liver expressed high levels of IL-6, the pro-inflammatory cytokine that is also a major component of the SASP, as mentioned above. It is noteworthy that all alterations induced by RS were long lasting, in that they were detected as late as 6 weeks after exposure to the alkaloid, implying the seemingly irreversible nature of these changes. Thus, it is apparent that the microenvironments of the aged and RS-exposed livers share both the ability to stimulate the growth of transplanted hepatocytes and a fundamental attribute of the aging process, i.e. the emergence of senescent cells; in both cases, the effect of RS is of far greater magnitude.

According to a most comprehensive model proposed in the literature, the senescence phenotype emerges when a cell integrates two types of signals: one that reads for growth and one that imposes a block in the replicative cycle[199,217]. If these two signals persist for a sufficient length of time, one of the possible outcomes is cell senescence. For example, DNA damaging agents do not induce senescence in quiescent cells; however, they do so if the presence of persistent DNA damage and cell cycle arrest is coupled with growth promoting stimuli. Under these conditions, the cells express markers related to cell cycle block, such as p53, p16, p21 or p27, as well as markers associated with growth stimulation, including cell hypertrophy and up-regulation of cell cycle related cyclins. While this model is largely built on data derived from *in vitro* studies, our present results suggest that a similar scenario occurs in rat hepatocytes exposed to RS *in vivo*, according to a treatment protocol that is conducive to massive liver repopulation by transplanted hepatocytes[174,177]. RS and other related pyrrolizidine alkaloids have long been known for their ability to cause a persistent cell cycle block on hepatocytes[178],

possibly via induction of DNA damage[15]. Enlarged, hypertrophic hepatocyes (megalocytes) emerging in RS-treated rats were unable to complete DNA synthesis or undergo mitotic division[218]. Our present studies suggest that this is possibly mediated via induction of CDK-inhibitors p21 and p27.

Considering the other side of the coin, i.e. the growth stimulatory signals, RS-treated hepatocytes also express high levels of positive regulators of the cell cycle, including, cyclin D1, CDK4 and PCNA[200], suggestive of a state of hyper-stimulation imposed on a background of replicative arrest. In addition, a relevant finding of the present studies is that exposure to RS is also associated with a long lasting increase in the levels of mTOR protein, which plays a central role in the integration of cell growth signals[200,206]. There is evidence to indicate that mTOR pathway is critically involved in determining two possible cell fates, quiescence or senescence, depending on the conditions associated with the cell cycle block: inhibition of mTOR leads to cellular quiescence, while activated mTOR drives the cell to senescence[199,201].

It was interesting to find that regenerative nodules emerging in rat livers exposed to RS+PH did not express markers related to cell senescence; in fact, their phenotype was in sharp contrast to that observed in surrounding tissue (figure 28-29). The cell of origin of these nodules is still controversial[204-205]; however, it is well established that they slowly expand and eventually repopulate almost entirely the surrounding liver[183], replacing megalocytic hepatocytes in a process that can be likened to endogenous repopulation[219]. Such sequence of events indicates that small hepatocytes in regenerative nodules have a competitive growth advantage compared to surrounding senescent hepatocytes, possibly driven, at least in part, by IL-6[220], secreted by the same surrounding megalocytes. However, it is important

to point out that, when isolated normal hepatocytes are transplanted into the liver of RS-treated animals, they are able to overrun the emergence of endogenous regenerative nodules, leading to massive repopulation by donor-derived cells[174,177]. As already mentioned, the senescence phenotype has historically been interpreted as a means to oppose or minimize the possibility for the emergence of neoplastic cells in tissues experiencing widespread damage, including DNA damage. While this view is still largely entertained, it is now complemented, and often obscured, by a more recent propositions which regards the accumulation of senescent cells as a powerful driving force for neoplastic disease in several tissues. This hypothesis was first formulated by Judith Campisi over a decade ago[221] and has since been receiving increasing support[222]. Thus, while the essence of cell senescence continues to be interpreted as barrier against the risk of cancer[223], it is now well established that senescent cells can fuel the neoplastic process by altering the tissuemicroenvironment[70].

This effect is exerted, at least in part, via expression of a peculiar phenotype which is referred to as SASP, as described in the Introduction. A main component of SASP are cytokines, including pro-inflammatory cytokines such as IL-6. It is therefore noteworthy that both aging and exposure to RS were associated with increased IL-6 expression in the liver tissue.

However, it is easily predictable that the significance of cell senescence is still to be fully elucidated. Far from being a mere passive state of cells at the end of their replicative lifespan, the senescent phenotype is already emerging as a new state of differentiation which can exert far reaching effects on tissue function, cell turnover and repair[224]. The results presented in this thesis are in line with the widely held view that cell senescence can contribute to the emergence of neoplastic disease, by

stimulating the growth of transplanted altered/nodular hepatocytes; this effect was observed both in aged animals and in animals exposed to RS. However, it was also observed that the same liver microenvironment was also able to support the expansion of transplanted normal hepatocytes, suggesting that cell senescence and the associated SASP can indeed sustain normal tissue regeneration and repair, under appropriate conditions. This implies that factors secreted by senescent cells are not specific for altered cells on the pathway to cancer, but they can also be exploited by normal cells expressing specific receptors for those factors. In this scenario, a cell competition strategy is possibly enforced, whereby the prevailing cell type will be the one that is better equipped to integrate the stimuli generated by the surrounding microenvironment, including, as a relevant component, senescent cells and their SASP.

The last section of this thesis represents an initial attempt to test the hypothesis outlined above. It describes studies aimed at reversing the RS-induced alterations in the liver microenvironment and, in doing so, at verifying whether this would modulate the growth of preneoplastic lesions promoted by RS. Animals given the genotoxic agent diethylnitrosamine (DENA), followed by RS, developed large hepatocyte nodules within a few months. Transplantation of normal hepatocytes in the context of a neoplastic-prone liver microenvironment is able to exert a significant impact on the development of nodular pre-neoplastic lesions. Injection of $4x10^6$ hepatocytes, isolated from a normal syngeneic donor, into the liver of a recipient animal pre-exposed to a carcinogenic protocol, resulted in a sharp delay and/or reduction in the incidence of hepatocyte nodules. This effect was accompanied by an extensive repopulation of the host liver by transplanted cells, resulting in a profound modification of the neoplastic-prone tissue landscape.

These results represent a proof of principle that an altered microenvironment is indeed a powerful driving force in neoplastic progression; most importantly, they clearly indicate that strategies aimed at “normalizing” such an altered microenvironment might impact on the rate of progression of neoplastic process.

7 References

1. Hoare M., Das T., Graeme A. *Ageing, telomeres, senescence, and liver injury* Journal of Hepatology 2010 vol. 53: 950–961

2. Poynard T., Bedossa P., Opolon P. *Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups*. Lancet 1997;349:825–832.

3. Keswani R.N., Ahmed A., Keeffe E.B. *Older age and liver transplantation: a review*. Liver Transpl 2004;10:957–967.

4. Regev A., Schiff E.R. *Liver disease in the elderly*. Gastroenterol Clin North Am 2001;30:547–563.

5. Campisi J., D'Adda di Fagagna F. *Cellular senescence:when bad things happen to good cells* Nature Reviews molecular cell biology 2007 8, 729-740

6. Hayflick L. *The limited in vitro lifetime of human diploid cell strains.* Exp. Cell.Res. 1965 .37.614-636

7. Sozou P.D., Seymour R.M. *To age or not to age* Proc Biol Sci. 2004 7;271(1538):457-63

8. Di Leonardo A., Linke S. P., Clarkin K. & Wahl G. M. *DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts*. *Genes Dev.* 8, 2540–2551 (1994).

9. Herbig U., Jobling W. A., Chen B. P., Chen, D. J. & Sedivy, J. *Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)*. Mol. Cell 14, 501–513 (2004).

10. Ogryzko, V.V., Hirai T.H., Russanova V.R., Barbie D.A. & Howard B.H. *Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent.* Mol. Cell. Biol. 16, 5210–5218 (1996).

11. Serrano M., Lin A. W., McCurrach M. E., Beach D. & Lowe S. W. *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a*. Cell 88, 593–602 1997

12. Ben-Porath I., Weinberg R.A. *When cells get stressed: an integrative view of cellular senescence*. J. Clin. Invest. 2004 113, 8–13

13. Müge Ogrunc and d'Adda di Fagagna F*. Never-ageing cellular senescence*
Eur J Cancer. 2011; 47(11): 1616–1622.

14. .D'Adda di Fagagna F. *Living on a break: cellular senescence as a DNA-damage response* .Nat Rev Cancer. 2008 8 (7):512-22.

15. Rodier F.,Muñoz D.P. et al. *DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion* Journal of Cell Science 2011 124, 68-81

16. D'Adda di Fagagna F., Reaper P.M., Clay-Farrace L., Fiegler H*. et al*. *A DNA damage checkpoint response in telomere-initiated senescence*. Nature. 2003 Nov 13;426(6963):194-8.

17. Mu J.J., Wang Y., Luo H. *et al. A Proteomic Analysis of Ataxia Telangiectasia-mutated (ATM)/ ATM-Rad3-related (ATR) Substrates Identifies the Ubiquitin-Proteasome System as a Regulator for DNA Damage Checkpoints* The journal of Biological Chemistry 2007 Vol. 282, N°. 24, pp. 17330–17334

18. Rodier F., Campisi J. *When DNA damage goes invisible*.Cell Cycle. 2009 15;8(22):3632-3.

19. Brown J.P., Wei W. and Sedivy, J.M. *Bypass of senescence after disruption of p21CIPl/WAFl gene in normal diploid human fibroblasts* (1997) Science 277, 831-834

20. Beauséjour, C. M., Krtolica, A., Galimi, F., Narita, M. *et al*. *Reversal of human cellular senescence: roles of the p53 and p16 pathways*. J.(2003) Embo J 22, 4212-4222

21. Rodier, F., Coppé, J. P., Patil, C. K., Hoeijmakers, W. A. *et al.* J. *Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion.*(2009) Nat Cell Biol 11, 973-979

22. Coppé, J. P., Patil, C. K., Rodier, F., Sun, Y. *et al*. *Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic ras and the p53tumor suppressor* (2008) PLoS Biol 6, 2853-2868

23. Toussaint O., Remacle J. *et al*. *From the Hayflick mosaic to the mosaics of ageing: role of stress-induced premature senescence in human ageing*. Int J Biochem Cell Biol 2002 34:1415-1429

24. Bavik C., Coleman I., Dean J.P. *et al*. *the gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms.* 2006 Cancer Res 66: 795-802.

25. Robles S.J, Adami G.R. *Agents that cause DNA double strand breaks lead to p16(INK4A) enrichment and the premature senescence of normal fibroblasts*. Oncogene 1998 16:1113-1123

26. Parrinello S., Samper E., Krtolica A. *et al*. *Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts*. Nat Cell Biol 2003 81: 5:741-747

27. Prescott J.C. , Blackburn E.H. *telomerase: Dr Jekyll or Mr Hyde?* Curr Opin Genet Dev 1999 9:368-373

28. Harrington L., Robinson M.O. *Telomere dysfunction: Multiple paths to the same end .* Oncogene 2002 21:592-597

29. Shay J.W., Wright W.E. *Senescence and immortalization: role of telomeres and telomerase*. Carcinogenesis 2005 26:867-874.

30. Campisi J. *Senescent cells, tumor suppression, and organismal aging: Good citizens, bad neighbors.* Cell 2005 120:513-522

31. Von Zglinicki T., Saretzki G., Ladhoff J. *et al*. *Human cell senescence as a DNA damage response*. Mech Ageing Dev 2005 126: 111-117

32. Pilch D.R., Sedelnikova O.A., Redon C. *et al.: Caracteristics of gamma-H2AX foci at DNA double strand breaks sites*. Biochem Cell Biol 2003 81:123-129

33. Petrini J.H., Stracker T.H. *the cellular response to DNA double-strand breaks:defining the sensors and mediators*. Trends Cell Biol 2003 13:458-462

34. Vidanes G.M., Bonilla C.Y., Toczyski D.P. *Complicated tails: Histone modifications and the DNA damage response*. Cell 2005 121: 973-976

35. Thiriet C., Hayes J.J. *Chromatin in need of a fix:Phosphorylation of H2AX connects chromatin to DNA repair*. Mol Cell 2005 18:617-622

36. Jackson S.P., *"Sensing and repairing DNA double-strand breaks*," *Carcinogenesis*, vol. 23, no. 5, pp. 687–696, 2002

37. Sonoda E., Hochegger H., Saberi A, Taniguchi Y., and S. Takeda*, "Differential usage of non-homologous end-joining and homologous recombination in double strand break repair," DNA Repair*, vol. 5, no. 9-10, pp. 1021–1029, 2006

38. Tanaka T., Halicka H. D., Huang X, Traganos F., and Z. Darzynkiewicz, *"Constitutive histone H2AX phosphorylationand ATM activation, the reporters of DNA damage by endogenous oxidants,"* Cell Cycle, vol. 5, no. 17, pp. 1940–1945, 2006.

39. Tanaka T., Huang X., Halicka H.D. et al., *"Cytometry of ATM activation and histone H2AX phosphorylation to estimate extent of DNA damage induced by exogenous agents*," Cytometry Part A, vol. 71, no. 9, pp. 648–661, 2007

40. Takahashi A. and OhnishI T., *"Does γH2AX foci formation depend on the presence ofDNA double strand breaks?" Cancer Letters*, vol. 229, no. 2, pp. 171–179, 200

41. Vilenchik M.M. and Knudson A.G., *"Endogenous DNA double-strand breaks: production, fidelity of repair, aninduction of cancer*," Proceedings of the National Academy of Sciences of the United States of America, vol. 100, no. 22, pp.12871–12876, 2003

42. Khanna K.K. and Jackson S. P. *DNA double-strand breaks: signaling, repair and the cancer connection.* Nat.Genet. 2001 27: 247–254

43. Jackson S.P. *Sensing and repairing DNA doublestrand breaks*. Carcinogenesis 2002 23: 687–696

44. Podhorecka M., Skladanowski A. and Przemyslaw Bozko *H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy* Journal of Nucleic Acids Volume 2011doi:10.4061/2011/920161

45. Sonoda E., Hochegger H., Saberi A. *et al*. *"Differential usage of non-homologous end-joining and homologous recombination in double strand break repair," DNA Repair*, vol. 5, no. 9-10, pp. 1021–1029, 2006.

46. Rothkamm K, Löbrich M*. Evidence for a lack of dna double strand break repair in human cells exposed to very low x-ray doses* Proc Natl Acad Sci 2003 ;100(9):5057-62

47. Rogakou E.P, Pilch D.R, Orr A.H. *et al*. *"DNA double-stranded breaks induce histoneH2AX phosphorylation on serine 139,"* Journal of Biological Chemistry, vol. 273, no. 10, pp. 5858–5868, 1998.

48. Helt C.E., Cliby W.A., Keng P.C.*et al. "Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage,"* Journal of Biological Chemistry, vol. 280, no. 2, pp. 1186–1192, 2005.

49. Rogakou E.P., Pilch D.P., Orr A.H. *et al*. *"DNA double-stranded breaks induce histonEH2AX phosphorylation on serine 139,"* Journal of Biological Chemistry, vol. 273, no. 10, pp. 5858–5868, 1998.

50. Kastan M.B. and Lim S.D., *"The many substrates and functions of ATM*," Nature ReviewsMolecular Cell Biology, vol.1, no. 3, pp. 179–186, 2000.

51. Bakkenist C.J and Kastan M.B., *"DNA damage activate ATM through intermolecular autophosphorylation and dimer dissociation,"* Nature, vol. 421, no. 6922, pp. 499–506, 2003

52. Celeste A., Petersen S., Romanienko P.J.*et al., "Genomic instability in mice lacking histone H2AX*," Science, vol. 296,no. 5569, pp. 922–927, 2002

53. Celeste A., Difilippantonio S., Difilippantonio M.J.*et al*., *"H2AX haploinsufficiency modifies genomic stability and tumor susceptibility," Cell*, vol. 114, no. 3, pp. 371–383, 2003.

54. Petersen S., Casellas R., Reina-San-Martin B. *et al*., "*AID is required to initiate Nbs1/γ-H2AX focus formation and mutations at sites of class switching*," *Nature*, vol. 414, no. 6864, pp. 660–665, 2001.

55. Bassing C.H., Chua K.F., Sekiguchi J.*et al*., *"Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX,*" Proceedings of the National Academy of Sciences of the United States of America, vol. 99, no. 12, pp. 8173–8178, 2002.

56. Reina-San-Martin B., Difilippantonio S., Hanitsch L., Masilamani R.*F et al. "H2AX is required for recombination between immunoglobulin switch regions but not for intra-switch region recombination or somatic hypermutation," Journal of Experimental Medicine*, vol. 197, no. 12, pp. 1767–1778, 2003.

57. Xie A., Puget N., Shim I. et al., "*Control of sister chromatid recombination by histone H2AX," Molecular Cell*, vol. 16, no.6, pp. 1017–1025, 2004.

58. Kao J., Milano M.T., Javaheri A. et al., *"γ-H2AX as atherapeutic target for improving the efficacy of radiation therapy," Current Cancer Drug Targets*, vol. 6, no. 3, pp. 197–205, 2006.

59. Rogakou EP, Boon C, Redon C, Bonner WM. *Megabase chromatin domains involved in DNA DSBs in vivo* J Cell Biol. 1999 6;146(5):905-16

60. Sedelnikova OA, Rogakou EP, Panyutin IG, Bonner WM. *Quantitative detection of (125) IdU-induced DNA double-strand breaks with gamma-H2AX antibody*. Radiat Res 2002; 158: 486–492.

61. Bhogal N, Jalali F, Bristow RG*. Microscopic imaging of DNA repair foci in irradiated normal tissues*. Int J Radiat Biol 2009; 85: 732–746.

62. Dellaire G, Bazett-Jones D. *Beyond repair foci: subnuclear domains and the cellular response to DNA damage.* Cell Cycle 2007; 6: 1864–1872

63. Bouquet F, Muller C, Salles B. *The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage*. Cell Cycle 2006; 5: 1116–1122.

64. Liu SK, Olive PL, Bristow RG*. Biomarkers for DNA DSB inhibitors and radiotherapy clinical trials*. Cancer Metastasis Rev 2008; 27:445–458

65. Kinner A, Wu W, Staudt C, Iliakis G. *Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin.* Nucleic Acids Res 2008; 36: 5678–5694)

66. Matsuoka S., Ballif B.A., Smogorzewska A. *et al. "ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage,"* Science, vol. 316, no 5828, pp.1160–1166, 2007.

67. Srivastava N., Gochhait S., de Boer P. and R. N. K. Bamezai, *"Role of H2AX in DNA damage response and human cancers,* "Mutation Research, vol. 681, no. 2-3, pp. 180–188, 2009.

68. Sun Y., Jiang X., Chen S., *et al. "A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM,"* Proceedings of the National Academy of Sciences of the United States of America, vol. 102, no. 37, pp.13182–13187, 2005

69. Sun Y., Xu Y., Roy K and B. D. Price, *"DNA damage induced acetylation of lysine 3016 of ATM activates ATM kinase activity*," Molecular and Cellular Biology, vol. 27, no. 24, pp. 8502–8509, 2007

70. Davalos AR, Coppe JP, Campisi J, Desprez PY *Senescent cells as a source of inflammatory factors for tumor progression*. .Cancer Metastasis Rev. 2010 Jun;29(2):273-83

71. von Zglinicki, T., Saretzki, G., Docke, W., and Lotze, C. *Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence?* Exp. Cell Res. 2005 220, 186–193.

72. Chen, Q. M., Bartholomew, J. C., Campisi, J., Acosta, M., Reagan, J. D., and Ames, B. N. *Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication*. Biochem. J.1998 332(Pt 1), 43–50.

73. Blander, G., de Oliveira, R. M., Conboy, C. M., Haigis, M., and Guarente, L. *Superoxide dismutase 1 knock-down induces senescence in human fibroblasts*. J. Biol. Chem.2003 278, 38,966–38,969.

74. Ramsey MR, Sharpless NE *ROS as a tumour suppressor?* Nat Cell Biol 8; 1213-1215 2006.

75. Langen RC, Korn SH, Wouters EF *Ros in the local and systemic pathogenesis of COPD* Free Radic Biol Med. 2003 Aug 1;35(3):226-35

76. Vassilis G and Thanos D Halazonetis *Oncogene-induced senescence: the bright and dark side of the response* Current Opinion in Cell Biology 2010, 22:816–827

77. Braig M. and Schmitt C. *Tumor Development Oncogene-Induced Senescence: Putting the Brakes on Cancer Res* 2006;66:2881-2884., 2006.

78. Jay P. Reddy & Yi Li *Oncogene-Induced Senescence and its Role in Tumor Suppression* J Mammary Gland Biol Neoplasia 2011 16:247–256

79. Coppe JP, Patil CK, Rodier F, Krtolica A, Beausejour C, Parrinello S, et al *.A humanlike senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen.* PLoS One 2010;5:e9188.

80. Salminen A, Ojala J, Kaarniranta K, Haapasalo A, Hiltunen M, Soininen H. *Astrocytes in the aging brain express characteristics of senescence-associated secretory phenotype*. Eur J Neurosci 2011;34:3–11.

81. Erusalimsky JD, Kurz DJ. *Cellular senescence in vivo: its relevance in ageing and cardiovascular disease.* Exp Gerontol 2005;40:634–42.

82. Albert R. Davalos & Jean-Philippe Coppe & Judith Campisi & Pierre-Yves Desprez *Senescent cells as a source of inflammatory factors for tumor progression* Cancer Metastasis Rev 2010 29:273–283

83. Kumar S, Millis AJ, Baglioni C*. Expression of interleukin 1-inducible genes and production of interleukin 1 by aging human fibroblasts*. Proc Natl Acad Sci U S A 1992;89(10):4683–7

84. Campisi *et al. Cellular senescence:a link between cancer and age-related degenerative disease?* Seminars in Cancer Biology 2011

85. Acosta JC, et al. *Chemokine signaling via the CXCR2 receptor reinforces senescence.* Cell 2008 133:1006–1018.

86. Kuilman T, et al. *Oncogene-induced senescence relayed by an interleukin dependent inflammatory network*. Cell 2008 133:1019–1031

87. Kortlever RM, Higgins PJ, Bernards R *Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence*. Nat Cell Biol 2006 8:877–884.

88. Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR *Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7.* Cell 2008 132:363–374

89. Mantovani A., Allavena P., Sica A., Balkwill F. *Cancer-related Inflammation* Nature 2008 vol.454

90. Lin W.W., Karin M. *A cytokine-mediated link between innate immunity, inflammation,and cancer*. J Clin Invest 2007 117:1175–1183

91. Apte R.N., *The involvement of IL-1 in tumorigenesis, tumor invasiveness, metastasis and tumor-host interactions*. Cancer Metastasis Rev 2006 25:387–408.

92. Arturo V., Orjalo A., Bhaumik D, Gengler BK*, et al*. *Cell surface-bound IL-1α is upstream regulator of the senescence-associated IL-6/IL-8 cytochine network* PNAS 2009 vol 106 n°40b17031-17036.

93. Colotta F., Allavena P., Sica A., Ghirlanda C. and Mantovani A. *Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.* Carcinogenesis 2009 vol.30 n°7 1073-1081

94. Naugler W.E., Karin M. *NF-kB and cancer-identifying targets and mechanisms*. Curr Opin Genet Dev 2008 18:19–26.

95. Freund A, Orjalo A, Desprez PY, Campisi J*: Inflammatory networks during cellular senescence: causes and consequences.* Trends Mol Med 2010, 16:238-248

96. Krtolica A., Parrinello S. *et al. Senescent fibroblasts promote epitelial cell growth and tumorigenesis:a link between cancer and aging*. Proc Natl Sci 2001; 98:12072

97. Bilousova G., Marusyk A., Porter C.C., Cardiff R.D., DeGregori J*. Impaired DNA replication within progenitor cell pools promotes leukemogenesis.* PLoS Biol. 2005;3:e401.

98. Xue W., Zender L., Miething C., Dickins R.A. *et al. Senescence and tumour clearance is triggered by p53* Nature 2007 445, 656-660

99. Ju Z., Jiang H., Jaworski M., Rathinam C. *et al. Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment*. Nat Med. 2007;13:742•

100. Adams P.*D. Healing and hurting: molecular mechanisms, functions and pathologies of cellular senescence*. Mol Cell 2009;36:2–14.

101. Tchkonia T., Morbeck D.E., Von Zglinicki T., *et al*. *Fat tissue, aging, and cellular senescence*. Aging Cell 2010;9:667–84.

102. McElhaney J.E., Effros R.B*. Immunosenescence: what does it mean to health outcomes in older adults?* Curr Opin Immunol 2009;21:242–418.

103. Rosenstiel P., Derer S., Till A., Häsler R. *et al*. *Systematic expression profiling of innate immune genes defines a complex pattern of immunosenescence in peripheral and intestinal leukocytes.* Genes Immun 2008;9:103–14

104. Melov S. *et al*. *Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle*. Nucleic Acids Res 1995, 23:4122-6

105. Jeyapalan J.C., Sedivy J.M. *Cellular senescence and organismal aging* Mech Ageing Dev. 2008 129(7-8):467-74

106. Sikora E., Arendt T., Bennett M, Narita M *Impact of cellular senescence signature on ageing research* Ageing Res Rev. 201110(1):146-52

107. Dimri G.P, Lee X., Basile G., *et al*. *A biomarker that identifies senescent human cells in culture and in aging skin in vivo.* Proc Nati Acad Sci *USA.* 1995;92:9363-9367

108. Pendergrass W.R., Gray M., Wold M.S*., et al. Analysis of the capacity of extracts from normal human young and senescent fibroblasts to support DNA synthesis in vitro*. J Cell Biochem. 1999 1;73(2):176-87*.*

109. Lee B.Y., Han J.A., Im J.S., Morrone A., *et al. senescence-associated beta gal is lysosomial* . Aging Cell 2006;5(2):187-95.

110. Severino, J., Allen, R. G., Balin, S., Balin, A. & Cristofalo, V. J. *Is beta-galactosidase staining a marker of senescence in vitro and in vivo?* Exp. Cell Res.2000 257,162–171

111. Wang C., Jurk D., Maddick M., Nelson G. *et al. DNA damage response and cellular senescence in tissues of aging mice*. Aging Cell. 2009 ;8(3):311-23

112. Itahana K. *et al. Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1*. Mol. Cell. Biol.2003 23, 389–401.

113. Ruas M. & Peters G. *The p16INK4a/CDKN2A tumor suppressor and its relatives.* Biochim Biophys. Acta 1998 1378, F115–F177.

114. Sharpless N. E. & DePinho R. A. *The INK4A/ARFn locus and its two gene products*. Curr. Opin. Genet. Dev.1999 9, 22–30

115. Sherr C. J., and Roberts J. M. *CDK inhibitors: positive and negative regulators of G1-phase progression.* Genes Dev 1999 13, 1501-1512

116. Bringold, F. and Serrano, M. *Tumor suppressors and oncogenes in cellular senescence* Exp Gerontol 2000 35, 317-329

117. Ramirez, R. D., Morales, C. P., Herbert, B. S., Rohde *et al. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions.* Genes Dev 200115, 398-403

118. Coppe, J.P. et al. *The senescence-associated secretory phenotype: The dark side of tumor suppression.* Annual Review of Pathology: Mechanisms of Disease,2010 5, 99–118.

119. Sherr C.J. and DePinho R.A. *Cellular senescence: mitotic clock or culture shock?* Cell 2000 102, 407-410

120. Brookes S., Rowe J., Gutierrez Del Arroyo A. *et al*. *Contribution of p16(INK4a) to replicative senescence of human fibroblasts* Exp Cell Res 2004 298,549-559

121. Gamerdinger M., Hajieva P., Kaya A.M, Wolfrum U. *et al. Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3*. EMBO J. 2009 8;28(7):889-901

122. Young A.R., Narita M. *SASP reflects senescence*. EMBO Rep. 2009 ;10(3):228-30.

123. Collado M. & Serrano M. *The power and the promise of oncogene-induced senescence markers*. Nature Rev. Cancer 2006 6, 472–476

124. Chang, B. D. *et al. Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent*. Proc. Natl Acad. *Sci.* 99, 389–394

125. Shelton D.N., Chang E., Whittier P.S., Choi D. *et al*. *Microarray analysis of replicative senescence*. Curr. Biol.1999 9, 939–645

126. Yoon I.K., Kim H.K., Kim Y.K., Song I.H. *et al*. *Exploration of replicative senescence-associated genes in human dermal fibroblasts by cDNA microarray technology.* Exp Gerontol 2004;39(9):1369)

127. Vasto S. *et al*. *Inflammatory networks in ageing, age-related diseases and longevity*. Mech Ageing Dev 2007;128(1):83–91.

128. Ferrucci L *et al*. *A flame burning within*. Aging Clin Exp Res 2004;16(3):240–3

129. Bruunsgaard H. *The clinical impact of systemic low-level inflammation in elderly populations. With special reference to cardiovascular disease, dementia and mortality*. Dan Med Bull 2006;53(3):285– 309 6.

130. Maggio M. *et al*. *Interleukin-6 in aging and chronic disease: a magnificent pathway*. J Gerontol A Biol Sci Med Sci 2006;61(6):575–84

131. Franceschi C. *et al. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans*. Mech Ageing Dev 2007;128(1):92–105.

132. jian-Lin-Ren J.,Pan Ya-Pi-Lu *et al*. *Inflammatory Signaling and Cellular Senescence*, Cell. Sign 2009

133. Franceschi C. *Continuous remodeling as a key to aging and survival*. Biogerontology 2003; 4:329-334.

134. Franceschi C., Bonafe M., Valensin S., Olivieri F. *et al*. *Inflamm-aging. An evolutionary perspective on immunosenescence.* Ann NY Acad Sci 2000; 908:244-254

135. Makoto Goto *Inflammaging (inflammation + aging): A driving force for human aging based on an evolutionarily antagonistic pleiotropy theory?* BioScience Trends *2008; 2(6):218-230.*

136. Franceschi C., Bonafe M. *Centenarians as a model for healthy aging*. Bio- chemical Soc Trans 2003; 31:457-461

137. Franceschi C**.** *Inflammaging as a major characteristic of old people: can it be prevented or cured?* Nutr Rev. 2007 ;65:S173-6.

138. Franceschi C., Valensin S., Fagnoni F., Barbi C., Bonafe M. *Biomarkers of immunosenescence within an evolutionary perspective: the challenge of heterogeneity and the role of antigenic load.* Exp Gerontol 1999; 34:911-921.

139. Chen J. *Senescence and functional failure in hematopoietic stem cells*. Exp Hematol 2004, 32:1025-1032.

140. Zhou S., Greenberger J.S., Epperly M.W., Goff J.P. *et al*. J*: Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts.* Aging Cell 2008, 7:335-343.

141. Krishnamurthy J., Ramsey M.R., Ligon K.L., Torrice C. *et al. p16INK4a induces an agedependent decline in islet regenerative potential*. Nature 2006, 443:453-457.

142. Molofsky A.V., Slutsky S.G., Joseph N.M., He S. *et al*. *Increasing Ink4a expression decreases forebrain progenitors and neurogenesis during ageing*. Nature 2006, 443:448-452.

143. Drummond-Barbosa D. *Stem cells, their niches and the systemic environment: an aging network.* Genetics 2008, 180:1787-1797

144. Chung H.Y., Cesari M., Anton S., Marzetti E. *et al. Molecular inflammation: underpinnings of aging and age-related diseases.* Ageing Res Rev 2009, 8:18-30.

145. Franceschi C., Motta L., Motta M., Malaguarnera M *et al. The extreme longevity: the state of the art in Italy*. Exp Gerontol. 2008;43(2):45-52

146. Candore G., Balistreri C.R., Listì F., Grimaldi M.P. *et al*. *Immunogenetics, gender, and longevity*. Ann N Y Acad Sci. 2006;1089:516-37

147. Troen B.R. *The biology of aging*. Mt Sinai J Med. 2003;70(1):3-22.

148. Anisimov V.N. *Biology of Aging and Cancer* MD Cancer Control. 2007;14(1):23-31.

149. Irminger-Finger I. *science of cancer and aging*, J Clin Oncol. 2007 10;25(14):1844-51

150. Song Y.S. *et al*.: *Distinct ROS and biochemical profiles in cells undergoing DNA damage-induced senescence and apoptosis*. Mech Ageing Dev 2005 12:580-5909

151. William C. Burhans and Martin Weinberger *DNA replication stress, genome instability and aging* Nucleic Acids Research 2007 vol 35 n°22 7545-7556

152. Longo V.D. and Finch C.E*. Evolutionary medicine: from dwarf model systems to healthy centenarians?* Science 2003, 299, 1342–1346).

153. Anisimov V.N. *Relationships between cancer and aging: a multilevel approach* Biogerontology 2009 Aug;10(4):323-38.

154. Anisimov V.N. *Carcinogenesis and aging*. Adv Cancer Res. 1983;40: 365-424.

155. Dix D., Cohen P. *On the role of aging in carcinogenesis*. Anticancer Res. 1999;19:723-726.

156. Parkin D.M., Bray F.I., Devesa S. *Cancer burden in the year 2000: the global picture*. Eur J Cancer. 2001;37(suppl 8):S4-S66.

157. Balducci L., Ershler W.B. *Cancer and ageing: a nexus at several levels*. Nat Rev Cancer. 2005;5:655-662.

158. Anisimov V.N., Ukraintseva S.V., Yashin A. *Cancer in rodents: does it tell us about cancer in humans?* Nat Rev Cancer. 2005;5:807-819.

159. Peto R., Parish S.E., Gray R.G. *There is no such thing as ageing, and cancer is not related to it.* IARC Sci Publ. 1985;(58):43-53.

160. Miller R.A. *Gerontology as oncology: research on aging as the key to the understanding of cancer.* Cancer. 1991;68:2496-2501.

161. Simpson A.J*. A natural somatic mutation frequency and human carcinogenesis*. Adv Cancer Res. 1997;71:209-240.

162. Krtolica A., Campisi J. *Cancer and aging: a model for the cancer promoting effects of the aging stroma*. Int J Biochem Cell Biol. 2002 Nov;34(11):1401-14.

163. Atamna H., Killilea D.W., Killilea A.N., Ames B.N. *Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging*. Proc Natl Acad Sci 12;99(23):14807-12*.*

164. Linton P.J., Dorshkind K. *Age-related changes in lymphocyte development and function.* Nat Immunol. 2004;5(2):133-9

165. Zhang Y., Herman B. *Ageing and apoptosis* .Mech Ageing Dev. 2002;123(4):245-60

166. Ostan R., Bucci L., Capri M., Salvioli S. *et al*. *Immunosenescence and immunogenetics of human longevity.* Neuroimmunomodulation. 2008;15(4-6):224-40. Epub 2008

167. Weissman I.L. *Translating stem and progenitor cell biology to the clinic: barriers and opportunities* Science 2000 287:1442-1446

168. Kay M.A., Woo S.L.C.: *Gene therapy for metabolic disorders*. Trends Genet 1994, 10:253–257

169. Matas A.J., Sutherland D.E., Steffes M.W., Mauer S..M *et al*. *Hepatocellular transplantation for metabolic deficiencies; decrease of plasma bilirubin in Gunn rats*. Science 1976, 192:892–894

170. Groth C.G., Arborgh B., Bjorken C., Sundberg B., Lundgren G. *Correction of hyperbilirubinemia in the glucuronyl-transferase deficient rat by intrapor*tal hepatocute transplantation Transplant Proc. 1977 ;9(1):313-6.

171. Rhim A., Sandgren E.P., Degen J.L., Palmiter R.D, Brinster RL: *Replacement of diseased mouse liver by hepatic cell transplantation*. Science 1994, 263:1149–1152

172. Overturf K., Al-Dhalimy M.., Tanguay R, Brantly M*. et al*. *Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tryosinaemia type I*. Nat Genet 1996, 12:266–273

173. Laconi E., Oren R., Mukhopadhyay D.K., Hurston E., *et al. Long term, near total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine*. Am J Pathol 1998, 158:319–329

174. Sandgren E.P. Et al. *Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene*. Cell 1991 66:345-256

175. Mignon A. Guidotti J.E., Mitchell C. *et al*. *Selective repopulation of a normal mouse liver by Fas/CD95 resistant hepatocytes.* Nat.Med 1998 4: 1185-1188

176. Laconi S., Pillai S., Porcu P.P. *et al. Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine*. Am J Pathol 2001, 158:771-777

177. Peterson J.E.: *Effects of the pyrrolizidine alkaloid lasiocarpine-N-oxide on nuclear and cell division in the liver of rats*. J Pathol Bacteriol 1965, 89:153-171.

178. Thompson N.L., Hixson D.C., Callanan H., Panzica M. *et al. A Fischer rat substrain deficient in dipeptidyl peptidase IV activity makes normal steady-state RNA levels and an altered protein. Use as a liver-cell transplantation model.* Biochem J 1991;273:497-502.

179. Afzelius B.A., Schoental R.J. *The ultrastructure of the enlarged hepatocytes induced in rats with a single oral dose of retrorsine, a pyrrolizidine (Senecio) alkaloid*. Ultrastruct Res 1967, 20:328-345.

180. Jago M.V. *The development of the hepatic megalocytosis of chronic pyrrolizidine alkaloid poisoning.* Am J Pathol 1969, 56:405-422.

181. Samuel A., Jago M.V. *Localization in the cell cycle of the antimitotic action of the pyrrolizidine alkaloid, lasiocarpine, and of its metabolite, dehydroheliotridine*. Chem Biol Interact 1975, 10:185-197

182. McLean E. *The toxic actions of pyrrolizidine (senecio) alkaloids*. Pharmacol Rev 1970, 22:429-483.

183. Mattocks A.R.*Chemistry and Toxicology of Pyrrolizidine Alkaloids*. 1986. FL, Academic Press, Orlando

184. Laconi E., Sarma D.S.R, Pani P. *Transplantation of normal hepatocytes modulates the development of chronic liver lesions induced by a pyrrolizidine alkaloid, lesiocarpine Carcinogenesis* 1995 16:139-142.

185. Laconi S. Laconi E. *Principles of hepatocyte transplantation*. Semin Cell Dev Biol 2002 13:433-438.

186. Laconi E *Differential growth: from carcinogenesis to liver repopulation* . Am J Path 2000 156:389-392

187. Laconi S., Pani P., Pillai S., Pasciu D. *et al*. *A growth constrained environment drives tumor progression in vivo* Proc Natl Acad Sci 2001 3;98(14):7806-11.

188. Marongiu F., Doratiotto S., Montisci S. *et al. Liver Repopulation and Carcinogenesis: Two Sides of the Same Coin?* Am. J .of Pathol. 2008 Vol.172 n°4

189. Who *Pyrrolizidine alkaloids* Environ Health Criteria 1988;80

190. Semple-Roberts E., Hayes M.A., Armstrong D., Becker R.A.*et al*. *Alternative methods of selecting rat hepatocellular nodules resistant to 2-acetylaminofluorene*. Int J Cancer. 1987 Nov 15;40(5):643-5

191. Gossrau R. *Peptidases II. Localization of dipeptidylpeptidase IV (DPP IV). Histochemical and biochemical study*. Histochemistry. 19793;60(2):231-48

192. Smith P.K., Krohn R.I., Hermanson G.T., Mallia A.K. *et al. Measurement of protein using bicinchoninic acid*. Anal. Biochem. 1985; 150: 76-85.

193. Schmittgen T.D., Livak K.J. *Analyzing real-time PCR data by the comparative C(T) method.* Nat. Protoc. 2008; 3: 1101-1108.

194. Pasciu D., Montisci S., Greco M., Doratiotto S. *et al*. *Aging is associated with increased clonogenic potential in rat liver in vivo* Aging Cell (2006

195. Seglen P.O. *Preparation of isolated rat liver cells*. Methods Cell Biol. 1976;13:29-83.

196. Di Napoli M. and Shah I.M. *Neuroinflammation and Cerebrovascular Disease in Old Age: A Translational Medicine Perspective* J Aging Res. 2011; 2011: 857484.

197. Serra M.P., Marongiu F., Sini M. and Ezio Laconi Hepatocyte senescence in vivo following preconditioning for liver repopulation Hepatology 2012 In press

198. Rodier F., Campisi J*. Four faces of cellular senescence*. J Cell Biol 2011; 192: 547-556

199. Albrecht J.H., Poon R.Y., Ahonen C..L, Rieland B.M., Deng C, Crary GS*. Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver*. Oncogene. 1998; 16: 2141-2150

200. Demidenko Z.N., Blagosklonny M.V .*Growth stimulation leads to cellular senescence when the cell cycle is blocked.* Cell Cycle. 2008; 7: 3355-3361.

201. Pitzalis S., Doratiotto S., Greco M., Montisci S*. et al*. *Cyclin D1 is up-regulated in hepatocytes in vivo following cell-cycle block induced by retrorsine*. J. Hepatol.2005; 43: 485-490

202. Schmelzle T., Hall M.N. *TOR, a central controller of cell growth*. Cell. 2000; 103: 253-262.

203. Vasto S., Carruba G., Lio D., Colonna-Romano G. *et al*. *Inflammation, ageing and cancer* Mech Ageing Dev. 2009 ;130(1-2):40-5

204. McLean E.K. *The toxic actions of pyrrolizidine (senecio) alkaloids*. Pharmacol Rev. 1970;22: 429-483

205. Laconi S., Doratiotto S., Montisci S., Pani P., Laconi E*. Repopulation by endogenous hepatocytes does not reconstitute liver mass in rats treated with retrorsine*. Cell Transplant.2008; 17: 1415-1421.

206. Coleman W.B., Best D.H. *Cellular responses in experimental liver injury: Possible cellular origins of regenerative stem-like progenitor cells*. Hepatology 2005; 41; 1173–1176.

207. Pichard V., Ferry N. *Origin of small hepatocyte-like progenitor in retrorsine treated rats*. J. Hepatol. 2008; 48: 368–369.

208. Serra M.P.,Doratiotto S., Marongiu F. and Ezio *Laconi Normal hepatocyte transplantation delays the emergence of chemically-induced pre-neoplastic nodules in rat liver* Cell transplantation 2011 Epub ahead of print

209. Hayes M.A., Roberts E., Farber E. *Initiation and selection of resistant hepatocyte nodules in rats given the pyrrolizidine alkaloids lasiocarpine and senecionine*. Cancer Res. 45:3726-3374; 1985.

210. McCullough K.D., Coleman W.B., Smith G.J., Grishan J.W. *Age-dependent regulation of the tumorigenic potential of neoplastically transformed rat liver epithelial cells by the liver microenvironment.* Cancer Res. 1994; 54: 3668-71

211. Grisham J.W. *Migration of hepatocytes along hepatic plates and stem cell-fed hepatocyte lineages.*Am J Pathol. 1994 144(5):849-54

212. Grisham J.W. *Hepatocyte lineages: of clones, streams, patches, and nodules in the liver.* Hepatology. 1997 Jan;25(1):250-2.

213. Henry C.J, Marusyk A., Zaberezhnyy V., Adane B., DeGregori J. *Declining lymphoid progenitor fitness promotes aging-associated leukemogenesis*. Proc Natl Acad Sci U S A. 2010; 107: 21713-8)

214. Cramer D.W., Finn O.J. *Epidemiologic perspective on immune-surveillance in cancer*. Curr Opin Immunol. 2011; 23: 265-71

215. Coppé J.P., Desprez P.Y., Krtolica A., Campisi J. *The senescence-associated secretory phenotype: the dark side of tumor suppression*. Annu Rev Pathol 2010; 5: 99-118.

216. Demidenko Z.N., Zubova S.G., Bukreeva E.I., Pospelov V.A*. et al*. *Rapamycin decelerates cellular senescence*. Cell Cycle 2009; 8: 1888-1895

217. Cressman D.E., Greenbaum L.E., DeAngelis R.A., Ciliberto G *et al*. *Liver Failure and Defective Hepatocyte Regeneration in Interleukin-6-Deficient Mice*. Science 1996; 274:1379-1383;

218. Streetz K.L., Luedde T., Manns M.P., Trautwein C. *Interleukin 6 and liver regeneration*. Gut. 2000; 47: 309-12

219. Pichard V., Ferry N. *Origin of small hepatocyte-like progenitor in retrorsine treated rats*. JHepatol 2008; 48: 368–369

220. Laconi S., Curreli F., Diana S., Pasciu D. *et al. Liver regeneration in response to partial hepatectomy in rats treated with retrorsine: a kinetic study*. J Hepatol 1999; 31: 1069-1074.

221. Menthena A., Koehler C.I., Sandhu J.S., Yovchev M.I. *et al*. *Activin A,p15INK4b signaling, and cell competition promote stem/progenitor cell repopulation of livers in aging rats.* Gastroenterology 2011; 140: 1009-1020

222. Leontieva O.V., Gudkov A.V., Blagosklonny M.V. *Weak p53 permits senescence during cellcycle arrest*. Cell Cycle 2010; 9: 4323-4327

223. Campisi J. *Cancer, aging and cellular senescence*. In Vivo. 2000; 14: 183-8

224. Gorgoulis V.G., Halazonetis T.D. *Oncogene-induced senescence: the bright and dark side of the response*. Curr Opin Cell Biol. 2010; 22: 816-27

225. Kuilman T., Michaloglou C., Mooi W.J., Peeper D.S. The essence of senescence. Genes Dev. 2010; 24: 2463-79

226. Jun J.I., Lau L.F. *The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing*. Nat Cell Biol. 2010; 12: 676-85.