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**Regenerative medicine: shedding light into the link between aging and cancer**

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**Running header:** Regenerative medicine: aging and cancer

## ABSTRACT

The evidence linking aging and cancer is overwhelming. Findings emerging from the field of regenerative medicine reinforce the notion that aging and cancer are profoundly interrelated in their pathogenetic pathways. We discuss evidence to indicate that age-associated alterations in the tissue microenvironment contribute to the emergence of a neoplastic-prone tissue landscape, which is able to support the selective growth of pre-neoplastic cell populations. Interestingly, tissue contexts that are able to select for the growth of pre-neoplastic cells, including the aged liver microenvironment, are also supportive for the clonal expansion of normal, homotypic, transplanted cells. This suggests that the growth of normal and pre-neoplastic cells is possibly driven by similar mechanisms, implying that strategies based on principles of regenerative medicine might be applicable to modulate neoplastic disease.

**Key words:** Aging, cancer, regenerative medicine, hepatocyte transplantation, tissue fitness

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## **INTRODUCTION**

The evidence linking aging and cancer is overwhelming, to the point that the question pertaining to the possible basis of such strong relationship is unescapable for researchers involved in both fields. However, finding answers to this question has revealed particularly difficult, largely due to the biological intricacies of both the aging and the neoplastic processes. In this context, it is of paramount importance to consider whether aging and cancer represent two chronologically parallel, but biologically unrelated processes, or whether they result from shared etiologies and/or pathogenetic mechanisms. If in fact the latter is true, a better understanding of basic alterations associated with aging may help elucidating major biological driving forces leading to the emergence of the neoplastic phenotype; most importantly, strategies aimed at delaying the aging process may also have a beneficial impact on the morbidity and/or mortality from neoplastic disease.

### **Aging and tissue function**

A precise definition of aging remains elusive: it is commonly described as a progressive accumulation of cell and tissue damage, leading to decreased functional proficiency and increased susceptibility to disease<sup>1</sup>. At cellular level, alterations in all macromolecular components have been reported. The yellow-brown granular pigment lipofuscin was one of the first to be described: it consists of aggregates of oxidized lipids covalently linked to proteins and contributes to the typical “brown atrophy” of tissues found in aged individuals<sup>2</sup>. In fact, spontaneous non-enzymatic biological side-reactions, including glycation, have been proposed to represent a main mechanism of aging in higher animals, as part of the more general free radical theory of aging<sup>3,4</sup>.

In more recent years, a decline in the efficiency of proteostasis, i.e. the integrated systems that oversee cellular, tissue and organismal protein quality control, has gained centre stage as a candidate driver of biological aging<sup>5</sup>. Intracellular proteostasis is normally ensured by the activity of chaperones and

two complimentary proteolytic pathways: the ubiquitin-proteasome and the autophagy systems. Changes in each step of these pathways have been reported with age. Chaperons are essential for initial protein folding and during transferring of proteins across cellular organelles<sup>6</sup>, a process requiring ATP, whose availability may be limited in old age<sup>7</sup>. Furthermore, repair of damaged proteins may be compromised in aging, due to decreased activity of dedicated enzymes<sup>8</sup>. Similarly, both proteasomal function and autophagy decline with age<sup>9</sup>, increasing the risk for the piling up of aggregates both inside and outside the cell<sup>5,9</sup>.

Aging is also associated with epigenetic changes, such as altered DNA methylation patterns and histone modifications, which lead to the progressive and profound alteration of transcriptional profiles of coding and non-coding RNA<sup>10,11</sup>. Experimental evidence suggests that such large-scale alterations are linked to the inflammatory status and are in response to environmental stimuli and/or nutrient availability<sup>12</sup>. The decline of the proliferative capacity in aging cells is tightly associated with a general loss of histones, and with an imbalance between activating and repressive histone modifications<sup>13,14</sup>. In addition, DNA methylation patterns are modified with age, and methylation status of some specific regions (termed clock CpGs) can accurately predict age<sup>15,16</sup>. A recent study reported that, in senescent cells, >30% of chromatin is dramatically reorganised, including the formation of large-scale domains of H3K4me3 H3K27me3 over lamin-associated domains, as well as large losses of H3K27me3 outside these domains<sup>10</sup>, which is linked to the transcriptional downregulation of lamin B1 in senescence<sup>10,17,18</sup>. In general, age is associated with global DNA hypomethylation and local hypermethylation in some specific regions<sup>12</sup>. This, together with histone modification changes associated with inflamm-aging and oxidative stress, can affect the activation or the repression of specific transcriptional programs, including those involved in the expression of cytokines, oncogenes and tumor suppressor genes, thus predisposing the tissue to chronic inflammatory diseases associated with age and cancer<sup>11,19</sup>.

On the other hand, the hypothesis that aging might be sustained by chronic and progressive damage accrued on the molecular repository of the genetic information, i.e. DNA, has historically received special attention<sup>20-23</sup>. Both endogenous and exogenous sources of DNA damage can contribute to genotoxicity, including the recently reported uptake of circulating nucleic acids, which can act as mobile genetic elements, possibly fuelling mutagenesis and promoting cellular aging<sup>24</sup>. While most spontaneous or induced DNA alterations are short lived, a small fraction, including double-strand breaks and inter-strand crosslinks, are more difficult to repair and may persist<sup>25,26</sup> leading to changes in chromatin structure and deregulated transcription<sup>27,28</sup>.

Irrespective of the specific altered targets, be they membrane lipids, proteins or DNA, the underlying implication is that the gradual increase in macromolecular derangement translates into a decreased fitness at cellular and tissue level, which is the a main hallmark of aging<sup>29-31</sup>. As an example, regenerative capacity in vertebrates, which is an integrated complex functional response, declines with age in several organs<sup>32</sup>, including liver<sup>33</sup>. Based on these premises, any hypothesis postulating a pathogenetic link between aging and cancer should consider whether and how a decreased cell/tissue fitness, such as found in old age, could favour the emergence of a neoplastic phenotype.

### **Aging vs. neoplastic disease**

As mentioned above, two opposing paradigms have been proposed to account for the association between aging and cancer. A widely entertained view is that aging and cancer represent parallel, but unrelated biological processes. This possibility is in fact implied in a mainstream paradigm, which proposes to explain the increased risk of neoplastic disease associated with age as a direct consequence of DNA damage occurring in rare cells<sup>34,35</sup>. According to this interpretation, the main target and rate-limiting step in the origin of age-associated neoplasia is the time-dependent appearance of rare cells harboring critical genetic (oncogenic) alterations. In this view, emphasis is placed on chronological aging (more time is available for mutagenesis in rare cells), while little or no

relevance is attributed to widespread molecular changes, including genetic and epigenetic changes, occurring in the bulk of the aging tissue, which underlies functional decline (biological aging).

On the other hand, it is now widely recognized that the pathogenesis of neoplastic disease is heavily dependent on cues emanating from the tissue and/or systemic environment where the process occurs<sup>36,37</sup>. Within this conceptual framework, the role of age-related changes in the origin of cancer has often been referred to the progressive waning of effector mechanisms of immune-surveillance<sup>38</sup>, which are thought to be relevant for the clearance of putative pre-neoplastic cells<sup>39,40</sup>. However, the question regarding the precise role of immune-surveillance in controlling the growth of cancerous and/or pre-cancerous lesion is still open, given the known ability of cancer to induce tolerance<sup>41,42</sup>. An alternative, albeit not mutually exclusive hypothesis builds on the assumption that a landscape of decreased tissue fitness may provide the opportunity for the selection of mutant cells with oncogenic potential, increasing the risk of cancer through a process of “adaptive oncogenesis”<sup>43,44</sup>.

### **The tumour promoting effect of a chemically-induced low-fitness tissue environment**

Taking advantage of an experimental model developed in our laboratories, several years ago we investigated the role of a severely growth-constrained tissue environment on the expansion of pre-neoplastic and neoplastic cell populations. Experiments were conducted in rats treated with pyrrolizine alkaloids (PAs), a class of naturally-occurring compounds which are known for their ability to impose a long-lasting block in the cell cycle of hepatocytes<sup>45</sup>. Initial studies indicated that a brief exposure to retrorsine (RS), a commercially available PA, given in two doses, was able to suppress the capacity of the liver to restore liver mass after 2/3 partial hepatectomy (PH), and the effect persisted for at least several months<sup>46</sup>. Such an experimental setting was then used as a model system to test the growth behaviour of transplanted pre-neoplastic hepatocytes. The latter were isolated from chemically-induced liver nodules and injected, via portal vein, into the liver of rats pre-treated with RS, as described above. The fate of donor-derived cells in the recipient liver was

monitored over time using the dipeptidyl-peptidase type IV-deficient (DPPIV<sup>-</sup>) syngenic rat system for hepatocyte transplantation, in which donor animals express the DPPIV enzyme (DPPIV<sup>+</sup>), while host rats are DPPIV<sup>-</sup>. Results were clear-cut: nodular hepatocytes grew very rapidly, giving rise to large liver nodules and hepatocellular carcinoma within 4-6 months upon transplantation in animals treated with RS<sup>47</sup>. By contrast, the same cells were unable to expand to any extent when transferred into the liver of normal young hosts not exposed to RS<sup>47</sup>. Thus, the growth-compromised tissue environment induced by the alkaloid was able to sustain the expansion of pre-neoplastic hepatocytes; moreover, and very importantly, the liver microenvironment of healthy, untreated young recipients was not permissive for the growth of the same pre-neoplastic cell population.

Conceptually related results were reported by Marusyk et al. in mouse hematopoietic tissue, where it was shown that exposure to irradiation leads to a decline in fitness of hematopoietic stem cells and promotes selection of precursors harbouring advantageous oncogenic mutations, thereby contributing to leukemogenesis<sup>8</sup>.

Similar to radiation, PAs, including RS, are genotoxic compounds<sup>49</sup>. Moreover, exposure to RS generates persistent DNA adducts in vivo<sup>50</sup>, a possible contributing factor to the long lasting block on cell cycle exerted by this alkaloid<sup>46</sup>. In line with such interpretation, subsequent studies indicated that the phenotype induced by RS on rat hepatocytes in vivo is consistent with the chronic activation of a DNA damage response<sup>51</sup>. It also displays several markers of cell senescence, including the senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal), the phosphorylated form H2A histone family, member X ( $\gamma$ -H2AX), p53 binding protein 1 (53BP1), and the ataxia-teleangiectasia-mutated (ATM) gene product, among others<sup>51</sup>.

Cell senescence implies a stable cell cycle arrest and has long been regarded as a fail-safe mechanism to limit the risk of neoplastic transformation following genotoxic insult<sup>52</sup>. However, it is now evident from several studies that the presence of senescent cells in tissues can also fuel carcinogenesis, possibly via components of the senescence associated secretory phenotype (SASP), which includes

cytokines, growth factors and matrix-remodelling enzymes<sup>53</sup>, and/or through release of extracellular vesicles or cytoplasmic bridging, which have also been reported to occur in cell senescence<sup>54-56</sup>.

Thus, cell senescence and the concomitant SASP stand as possible mediators for the growth stimulation of transplanted nodular hepatocytes in RS-exposed liver tissue<sup>50</sup>.

### **The tumour promoting effect of the aged tissue environment**

The finding of cell senescence in a chemically-induced tissue environment that is supportive for the growth of pre-neoplastic cell populations raises an important question: are aged organs, which typically harbour senescent cells, also characterized by the emergence of a neoplastic-prone tissue landscape, i.e. conducive to selection of altered/pre-neoplastic cells? Studies reported by McCullough et al. over 20 years ago suggested such possibility<sup>57</sup>. Neoplastically transformed epithelial cell lines, generated *in vitro* and transferred into the liver of syngeneic recipients of different age, expressed their full tumorigenic potential only in aged hosts, while their growth was suppressed upon injection into young animals<sup>57</sup>. These results were highly intriguing. However, their interpretation in the context of the present discussion is rather difficult, given that cells were already neoplastic at the time of transplantation, and their responsiveness to any tissue environmental cues might therefore be different compared to pre-neoplastic counterparts. Furthermore, they were of *in vitro* origin, adding uncertainty to their potential relevance to the process as it occurs *in vivo*. Nevertheless, this type of evidence laid the grounds for a direct testing of the hypothesis that advancing age might be associated with alterations in the tissue environment, which are conducive to the selective growth of putative pre-neoplastic cells.

Taking advantage of the experimental setting described in the preceding paragraphs, we have recently explored this possibility. Cells isolated from pre-neoplastic nodules were infused in the liver of rats of different age and their fate was followed over time using the histochemical marker DPPIV (see



above and ref.<sup>47</sup>). Results were unequivocal: very limited growth of transplanted primary nodular hepatocytes was seen in the liver of young (3 to 5 month-old) hosts over a period of 8 months, as reported in previous studies<sup>47</sup>. On the other hand, the same pre-neoplastic cells formed visible nodules in the majority of recipients (17/18) following infusion into the liver of aged (18-20 month-old) syngeneic rats<sup>58</sup>. It should be mentioned that in both groups cells were injected through the portal circulation and they were therefore seeded in the liver parenchyma mainly as single hepatocytes, with ample possibility to interact with the host tissue environment.

As expected, aged liver showed increased expression of SA- $\beta$ -gal. However, other markers which are often associated with cell senescence were not found elevated in old recipients. Thus, whether senescent cells and/or their SASP components are involved in the promoting effect exerted by the aged liver microenvironment on transplanted pre-neoplastic hepatocytes remains an important question to be investigated in future studies.

Once again, some analogies with the above findings are present in studies on mouse leukemogenesis carried out in DeGregori's research laboratories<sup>59</sup>. It was reported that the reduced fitness of B-lymphopoiesis associated with aging selects for the emergence of cells with favourable oncogenic mutations, increasing the risk of leukaemia in the old animal. Thus, while the decline in the fitness of B-lymphopoiesis in aged mice coincided with altered receptor-associated kinase signalling, the fusion protein Bcr-Abl provided a much greater competitive advantage to old B-lymphoid progenitors compared with young progenitors, restoring kinase signaling pathways. Such enhanced competitive advantage translated into increased promotion of Bcr-Abl-driven leukemias. Furthermore, a chronic inflammatory milieu in the aged bone marrow appears to be involved in the genesis of the reduced-fitness phenotype of B cell progenitors<sup>60</sup>.

A decline in the ability of the aged liver to recover its mass following tissue loss has been reported by several studies<sup>61,62</sup>. Replicative capacity is an important functional attribute of the differentiated hepatocyte, given the potential exposure of the liver to dietary-born toxins or metabolic insults<sup>63</sup>. It

is noteworthy that hepatocytes isolated from old donors were found to express a cell-autonomous decrease in proliferative potential upon transplantation under strong selective conditions *in vivo*<sup>64</sup>. It is therefore reasonable to propose that an aged tissue landscape, endowed with reduced cell replicative capacity, might facilitate the selective expansion of a more competitive cell population, such as the pre-neoplastic hepatocytes. This hypothetical scenario would be in line with the interpretation suggested for mouse leukemogenesis<sup>59</sup>.

### **The aged liver microenvironment supports the growth of normal transplanted hepatocytes.**

The evidence discussed thus far suggests the existence of a pathogenetic link between a low-fitness/aged tissue landscape and the selective growth of pre-neoplastic cell populations. The microenvironment of an aged/growth-constrained liver appears able to generate stimuli that are conducive to the emergence of altered cells, increasing the risk of neoplastic disease. A crucial question at this point is whether these stimulatory signals bear any specificity towards altered/pre-neoplastic hepatocytes or, alternately, they can also sustain the growth of normal cell counterparts. Answering this question is important on two grounds. (i) It may shed light on the nature of phenotypic differences between normal and pre-neoplastic hepatocytes and/or (ii) it may help defining biological and molecular mechanisms mediating the stimulatory effect of the aged microenvironment on the growth of pre-neoplastic cells. For example, if cell senescence and/or SASP have a major role in this phenomenon, one would expect to observe some specificity of the effect towards pre-neoplastic cells. In fact, several reports associate the secretory activity of senescent cells to promotion of carcinogenesis<sup>65,66</sup>. However, no studies thus far have linked SASP components and/or other phenotypic features of senescent cells to normal tissue proliferation and/or regeneration.

Given such premises, the fate of normal hepatocytes transplanted in the liver of either young or old recipients becomes a significant issue. Results obtained by our research group provided the first evidence that the microenvironment of the aged rat liver is indeed able to foster the clonal expansion

of transplanted normal hepatocytes, while the same cells displayed very limited growth upon infusion into the liver of young hosts<sup>67</sup>. Analogous findings were reported later by Menthen et al.<sup>68</sup>. They transplanted cells isolated from fetal rat liver in syngeneic recipients of different age and observed a 4-5-fold increase in the size of donor-derived cell clusters in older hosts.

From the foregoing, it is justified to conclude that the same tissue environment of the aged liver, which is able to promote the growth of pre-neoplastic hepatocytes, exerts a seemingly comparable effect on *bona fide* normal hepatocytes (figure 1). Importantly, no signs of neoplastic transformation were seen with the latter cell type after over two years of observation<sup>67,68</sup>.

The above conclusion is further supported by the striking results obtained when normal hepatocytes are transplanted into the growth-constrained/low fitness microenvironment induced in the liver following exposure to RS. As mentioned in the preceding discussion, such treatment imposes a long lasting block on hepatocyte cell cycle and generates a powerful driving force for the rapid expansion of transplanted pre-neoplastic hepatocytes, leading to their progression to cancer. When normal hepatocytes are infused in RS-treated liver, they also proliferate extensively, setting out a most remarkable process, which culminates in the massive repopulation of the host liver by the donor-derived cell progeny<sup>69</sup>. Furthermore, the repopulated liver shows a normal histology and performs normal functions for the entire lifespan of the recipient animal<sup>70</sup>.

Taken together, these findings strongly indicate that both the age-associated and the RS-induced low fitness liver microenvironments, which represent neoplastic-prone tissue landscapes, are also supportive for the growth of phenotypically normal cells. In light of such evidence, it is reasonable to conclude that similar mechanisms are likely to be involved in the selective expansion of both normal and pre-neoplastic hepatocytes under the experimental conditions referred to above<sup>69</sup>. If cell senescence and/or SASP components do play a role in this context<sup>51</sup>, this would imply that they are also able to exert an effect on normal cell and tissue regeneration, adding a possible new facet to the biological significance of cell senescence.

On the other hand, the emergence of either normal or pre-neoplastic hepatocytes in a background of decreased tissue fitness suggests that mechanisms of cell competition could be at play. This was in fact suggested by Mentena et al. to explain the selective expansion of fetal hepatic cells in the liver of aged recipients<sup>67</sup>. Cell competition is now recognized as a pervasive biological mechanisms allowing for clearance of cells that, although viable, are less fit than their neighbours<sup>71</sup>. As such, it is thought to be involved in several physio-pathological processes<sup>72</sup>. Interestingly, it has been reported that in epithelial tissues, normal cells sense and actively eliminate the neighbouring transformed cells via cytoskeletal proteins by a process that has been referred to as epithelial defence against cancer (EDAC), which is interpreted as a form of cell competition<sup>73</sup>. However, in a tissue landscape with widespread decreased fitness in “normal” cells, rare “mutants” with favourable alterations may take the lead and set in motion a process that could result in “adaptive oncogenesis”<sup>43,44</sup>. Within this conceptual framework, it is noteworthy that normal cells, endowed with normal fitness, can also act as efficient competitors when tissue function is compromised, as documented in the preceding discussion.

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## **Normal cell transplantation delays carcinogenesis.**

As an important corollary to the above interpretation, it is possible to predict that normal cells could in fact be exploited in a cell competition strategy to counteract the emergence of altered/pre-neoplastic cells, under conditions of overall decreased tissue fitness, most notably with reference to proliferative capacity. Stated otherwise, regenerative medicine could come to the rescue by abolishing or limiting the competitive advantage of altered cells in a growth compromised tissue landscape.

Recent findings suggest such possibility<sup>74</sup>. Animals were initially exposed to a protocol for the induction of hepatocellular carcinoma (HCC) followed by transplantation of normal hepatocytes. The latter resulted in extensive repopulation of the host liver and a prominent decrease in the incidence of both pre-neoplastic and neoplastic lesions compared to control group not receiving transplantation

(figure 2)<sup>74</sup>. Further studies revealed that extensive hepatocyte senescence was induced by the carcinogenic protocol in the host liver; however, senescent cells were largely cleared and replaced following infusion of normal hepatocytes and this was associated with a decrease in the levels of the inflammatory cytokine (and main SASP component) IL6<sup>(74)</sup>.

Clearly, the above results cannot be taken to suggest that regenerative medicine, by means of normal cell transplantation, is a realistic option to be applied in the context of neoplastic disease, either in experimental models or, more to the point, in the clinical setting. However, they serve to reinforce the notion that strategies aimed at normalizing a neoplastic-prone tissue landscape, including age-associated tissue microenvironments, can modulate the evolution of neoplastic disease.

## CONCLUSION

We have discussed evidence to indicate that age-associated alterations in the tissue microenvironment contribute to the emergence of a neoplastic-prone tissue landscape, which is able to promote the selective growth of pre-neoplastic cell populations. Possible mechanisms responsible for this effect are the accumulation of senescent cells and/or their release of secretory products, including pro-inflammatory cytokines and growth factors. Alternately, or in combination, an age-associated progressive decrease in tissue fitness, notably proliferative fitness, may favor the emergence of more competitive cell variants, which might harbor pro-neoplastic genetic/epigenetic alterations. This process can be interpreted as “adaptive oncogenesis”.

Interestingly, tissue contexts that are able to select for the growth of pre-neoplastic cells, including the aged liver microenvironment, are also supportive for the clonal expansion of normal, homotypic, transplanted cells. This suggests that the growth of normal and pre-neoplastic cells is probably driven by similar mechanisms, implying that strategies based on principles of regenerative medicine might be applicable to modulate neoplastic disease.

As it has been aptly remarked, if aging is the strongest risk factor for cancer, the immediate implication is that the best protection against cancer is to be young<sup>75</sup>. Most importantly, the finding that aging and cancer are not just coincidentally associated, but are profoundly interrelated in their pathogenetic pathways, leads to the suggestion that a most effective strategy to prevent cancer is by promoting healthy chronological aging and delaying biological aging<sup>76</sup>.

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## FIGURE LEGENDS

**Figure 1.** Neither normal, nor primary pre-neoplastic hepatocytes grow to any significant extent upon transplantation into the liver of young syngeneic hosts. However, selective expansion of both cell types is seen in the liver of aged recipients (see<sup>48,57</sup> for details).

**Figure 2.** Normal hepatocyte transplantation delays chemically-induced liver carcinogenesis (See<sup>64</sup> for details).

**CELL  
TRANSPLANTATION**  
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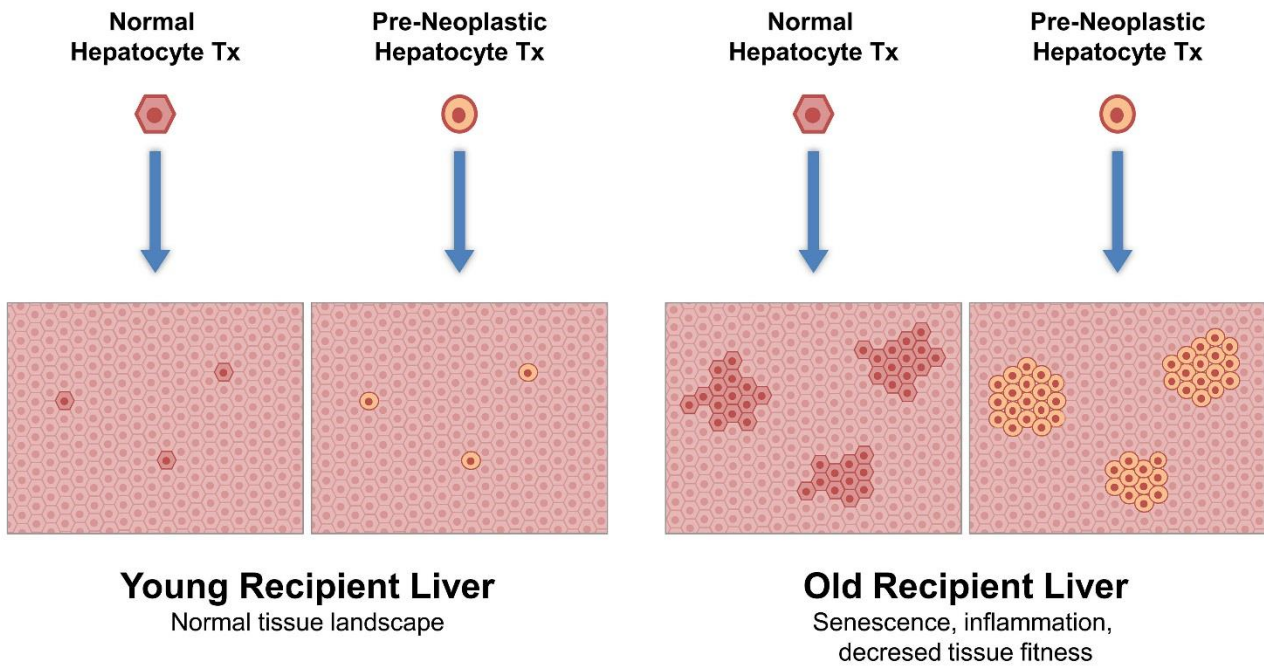
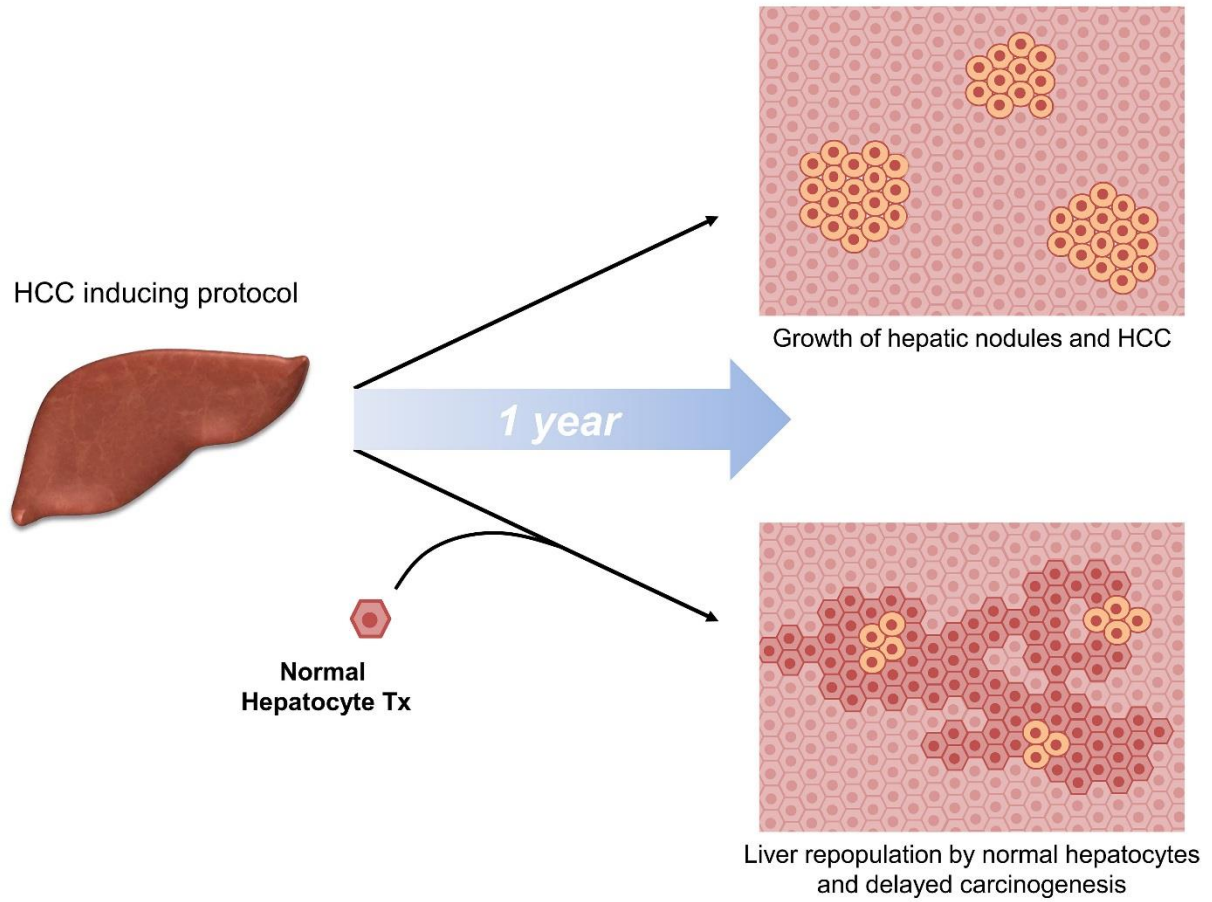


Figure 1

# CELL TRANSPLANTATION

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**Figure 2** TRANSPLANTATION  
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