CHAPTER 8

GENERAL DISCUSSION AND PERSPECTIVE

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In recent years, substantial evidence has emerged that cellular senescence acts as a potent antitumor mechanism. Replicative senescence stops the expansion of aged cells that have exhausted their proliferative potential. Similarly, premature senescence in response to harmful stresses, such as oncogenic signaling, actively halts the proliferation of cells at risk of oncogenic transformation. In fact, various precancerous lesions including pulmonary adenomas, prostate intraepithelial neoplasia, lymphomas and mammary tumors, show senescence biomarkers[1,2]. In these settings, a 'driver' mutation triggers the activation of an oncogene or the loss of a tumor suppressor gene, setting in motion a program that contributes to the formation of a benign lesion. The senescence response manifests itself after an initial phase of cell proliferation, halting further expansion. Progression towards malignancy in this case can happen only in the event of additional tumorigenic alterations. In the work described in this thesis, by studying the mechanisms crucial for maintenance of oncogene-induced senescence (OIS), we aimed to identify genes and signaling pathways involved in senescence escape and thereby find factors contributing to oncogenic transformation.

Senescence associates with a distinct metabolic profile

In Chapter 1 of this thesis, we reviewed the current knowledge on metabolic changes in malignant cells. Recent studies demonstrate that the deregulation of cellular metabolism is a key factor in driving oncogenic transformation. Cancer cells upregulate aerobic glycolysis and shift their metabolism towards biosynthesis, thereby providing the energy and building blocks that are necessary for the creation of progeny and tumor expansion (reviewed in Chapter 1). As described in Chapter 2, the molecular mechanisms underlying metabolic reprogramming are complex and require an alteration(s) in multiple growth pathways with PI3K/AKT/mTOR signaling as a prime example. Direct communication between the components of the cell cycle machinery and metabolic enzymes also plays a role in metabolic rewiring to meet proliferative needs.

In spite of the widely recognized importance of OIS and OIS escape in development of cancer, only few studies have explored the role of cellular metabolism in this setting (described in Chapter 1). Through an unbiased and comprehensive analysis of cellular metabolism we found that OIS cells show a distinct metabolic profile (described in Chapter 3). Entry into OIS associates with an increased rate of glucose oxidation in mitochondria, lower uptake of glutamine and a higher rate of fatty acid (FA) secretion. As described below, in this thesis we have revealed a pivotal role of oxidative metabolism in OIS and tumorigenesis (described in Chapter 4). However, the regulation of glutamine and lipid metabolism in senescence and escape thereof requires further studies. Notably, changes in glutamine and lipid metabolism in senescence directly oppose those in cancer. In contrast to lower glutamine utilization found in senescent cells, cancer cells increase glutamine uptake and utilization by upregulating ASCT2 and SN2 glutamine transporters and glutaminase 1 (GLS1), a first

enzyme of glutaminolysis[3,4]. In fact, cancer cells are dependent on glutamine for survival[5]. Along these lines, the silencing of GLS1 delays tumor growth[6-8] and glutamine withdrawal decreases the viability of cancer cells[9]. The observation of lower glutamine utilization in OIS raises the question as to how components of glutaminolysis are regulated in senescent cells. Interestingly, the pharmacological inhibition of GLS1 in endothelial cells leads to a growth arrest that is associated with the induction of several senescence markers including senescence-associated β-galactosidase (SA-β-Gal) activity and $p16^{INK4A}$ and $p21^{Cip1}$ protein levels[10]. This suggests that GLS1 levels and/or its activity are downregulated in OIS. Alternatively, lower levels of glutamine transporters might prevent the uptake of glutamine in senescence. Studying the regulation of the particular components of glutaminolysis in OIS should be accompanied by stable isotope labeling with C^{13} labeled glutamine tracer. While several of such studies have been performed in cancer cells, a global overview of glutamine metabolism in OIS has not been reported yet (and conceivably several mechanisms exist depending on the oncogenic insult). Finally, an important question that needs to be answered is whether glutamine metabolism has a causal role in OIS and is not merely a consequence of cell cycle arrest. This is of particular interest since lower utilization of glutamine has been linked to quiescence[11]. Although a cancer's dependence on glutamine in combination with the antitumor function of OIS suggests an instrumental role of glutamine in the regulation of malignant transformation, functional studies are yet to be performed.

By means of metabolic profiling we found that OIS cells have a higher rate of FA secretion. Similarly, others have recently reported an increase in the steady-state levels of free FA in senescence[12]. Detailed analysis of FA metabolism in that study revealed that the upregulation of FA levels was not due to increased FA synthesis, which in fact was lower in senescent cells, but rather caused by a higher rate of FA oxidation. Taking into account the antitumor function of OIS, a decrease in FA biosynthesis is in line with the observation that cancer cells commonly upregulate *de novo* FA synthesis by increasing activity and expression of several lipogenic enzymes[13]. Yet, cancer cells similarly to OIS cells have also been shown to upregulate FA oxidation. The expression of carnitine palmitoyltransferase-1 isoform C (CPT1), an enzyme activating FA oxidation, is increased in cancer cells which stimulates FA oxidation-derived ATP production and resistance to glucose deprivation[14]. This suggests that increased FA oxidation in OIS is just a consequence of the activation of an oncogene rather than an important mechanism regulating OIS. In agreement with that, the inhibition of FA oxidation upon CPT1 depletion did not prevent a senescence-associated cell cycle arrest[12]. In light of these findings, a more detailed analysis of FA metabolism is required to understand its exact role in senescence and malignant transformation.

Oxidative metabolism underlies OIS program

In Chapter 4 of this thesis, by integrating metabolic profiling with functional analyses, we provide evidence that increased oxidative metabolism is a direct mediator, rather than

only a phenomenon associated with OIS. We demonstrate that the rate of conversion of pyruvate to citrate, a combined reaction of pyruvate dehydrogenase (PDH) and citrate synthase (CS) is highly increased in OIS triggered by the mutant BRAF oncogene. This causes higher tricarboxylic acid (TCA) cycle activity and increased respiration. While PDH has been shown to be a central metabolic regulator in diabetes[15,16], heart disease[17,18] and more recently has been suggested to contribute to cancer[19-21], its regulation and function in the context of cellular senescence had yet to be characterized and understood. We show that PDH is activated in OIS due to the deregulation of PDH regulatory enzymes: PDK1 and PDP2. Conversely, the abrogation of OIS concurs with PDH inhibition. Finally, normalization of PDK1 or PDP2 levels inhibits PDH and leads to OIS escape. These data, taken together, strongly support a critical role of PDH in OIS and establish a functional link between OIS and metabolic (de)regulation.

Our study not only provides novel insights into the role and regulation of metabolic rewiring in senescence, but also points to the existence of close communication between OIS machinery and a key mitochondrial signaling axis. Yet, several aspects of this interaction remain to be resolved. For example, it is still uncertain how $BRAF^{V600E}$ deregulates *PDP2* and *PDK1* transcription. Previously, *PDK1* has been shown to be a HIF-1α-responsive gene[22,23]. Among many genes regulated by the HIF-1α protein, a large subset is associated with cellular senescence, including cell-cycle regulators[24,25] and several members of the senescence-messaging secretome (SMS)[26]. However, our previous study did not show a clear HIF-1αtranscriptome signature in cells bypassing OIS[27]. This suggests that in OIS oncogenic BRAF acts on PDK1 via another pathway. Interestingly, PDK1 can be regulated also on the level of post-translational modification. Specifically, oncogenic tyrosine kinases such as FGFR1, were reported to localize in the mitochondria, where they phosphorylate and activate PDK1, thereby promoting cancer cell metabolism and tumor progression[21]. Although changes in PDK1 phosphorylation in OIS were not analyzed in our study, it is possible that, next to being regulated at the transcriptional level, PDK1 is also controlled by phosphorylation. One of the potential mediators of PDK1 phosphorylation is MEK, a tyrosine/threonine kinase acting downstream of BRAF. Such regulation of PDK1 could explain our finding that PDH is activated in RAS-induced senescence, even in the absence of changes in PDK1 and PDP2 expression levels.

In addition to this, the precise nature and the mechanism of the connection between the higher mitochondrial respiration, redox stress and senescence program await better understanding. An increased production of reactive oxygen species (ROS) has been reported to induce and mediate RAS-induced senescence[28] and has been associated with BRAF-induced senescence[29]. Similar to this, our study demonstrated that an increase in respiration associates with a rise in redox stress in OIS cells. Although only correlative, it is consistent with the idea that ROS induction is an important mediator of senescence. Even so, functional

studies, in particular on how antioxidants affect OIS, are required to demonstrate that ROS is a cause rather than a consequence of senescence.

Our observation of increased TCA cycle activity in OIS raises the question as to whether any of the TCA cycle metabolites have a direct function in regulating senescence program. In fact, acetyl-CoA is not only an important intermediate for macrosyntheses, but it also a precursor for the acetylation of proteins. For example, acetylation of histones has been shown to be dependent on the acetyl-CoA levels[30,31] and is an essential process in the release of DNA for replication and therefore cell cycle progression[32,33]. Although in our analyses we did not directly measure acetyl-CoA, higher PDH activity predicts an increase in acetyl-CoA levels. Thus, by regulating histone acetylation, acetyl-CoA might control the cell cycle and thereby the senescence program.

Our study on oxidative metabolism and PDH regulation was focused on only one senescence type, OIS. Nevertheless, also therapy-induced senescence (TIS) has been recently associated with enhanced glucose utilization in the TCA cycle[34]. Along these lines, growth arrest upon inhibition of the melanoma-driver BRAF is accompanied by the induction of several senescence features[35] and an increase in oxidative metabolism[36,37]. These studies show that enhanced oxidative metabolism is not restricted to OIS, but in fact represents a general senescence feature. Yet, whether PDK1-PDP2-PDH axis (de)regulation plays role in different types of senescence, remains to be answered.

PDK1 as a potential therapeutic target

Increased tumorigenicity correlates with a shift from oxidative phosphorylation towards glycolysis[38]. Similarly, fully transformed cancer cells are known to shut down oxidative metabolism in favor of lactate production, a phenomenon called aerobic glycolysis or “the Warburg effect” (also described in Chapter 1)[39,40]. In this context, the increased oxidative metabolism upon PDH activation in OIS counteracts the tumor-supporting metabolic profile. Considering that for malignant transformation cells need to evade the senescence program, pathways crucial for senescence likely represent factors (de)regulated in tumors. In Chapter 4, we show that (de)regulation of PDH not only controls OIS, but also represents an important factor in malignant transformation. We find that ectopic expression of the PDH inactivating kinase PDK1 promotes melanoma growth in mice. Conversely, PDK1 depletion leads to cell death of melanoma cells, both *in vitro* and *in vivo*. Remarkably, depletion of PDK1 both prevents outgrowth of tumors and, perhaps more importantly from a clinical point of view, causes regression of established human melanomas. These results not only indicate a pro-oncogenic capacity of PDK1, but also reveal that PDK1 may serve as a potential novel therapeutic target in melanoma. The observation that PDK1 depletion induces senescence in primary cells but cell death in melanoma cells calls for more clarity about the mechanism responsible for these different sensitivities. One explanation might be a distinct level of dependence on glycolysis between cancer and non-transformed cells (reviewed in Chapter

1). PDH activation upon PDK1 depletion diverts pyruvate into the mitochondrial TCA cycle. This indirectly suppresses glycolysis and thereby selectively kills cancer cells highly addicted to glycolysis. Alternatively, the activation of mitochondrial respiration might cause much higher redox stress in cancer cells than that in non-transformed cells. In fact, melanoma cells were reported to have elevated levels of mitochondrial respiration compared to melanocytes and to be largely dependent on mitochondrial activity for energy production[41,42]. Hence, further stimulation of oxidative metabolism upon PDH reactivation likely overloads the capacity of the oxidative chain, thereby boosting ROS production and melanoma cell death. The finding that pharmacological inhibition of PDKs with dichloroacetic acid (DCA) in melanoma potentiate the antitumor effects of a pro-oxidative drug elesclomol further supports such a scenario[43].

Having demonstrated the potential therapeutic function of PDK1 inhibition, we also studied how this result can aid current melanoma therapy, especially in the context of targeted $BRAF^{V600E}$ inhibition. This is of particular interest as, although targeted inhibition of melanoma-driving BRAF pathway initially causes a substantial tumor regression, most melanomas eventually become resistant and patients surrender to recurrent disease[44-46].

Remarkably, PDK1 depletion causes synergistic toxicity with targeted BRAF inhibition, even eliminating cultured melanoma cell populations resistant to the BRAF inhibitor. This observation, together with the fact that PDK1 is an important metabolic regulator, suggests a role for metabolic reprogramming in the emerging of resistance. In fact, several studies have recently indicated that targeted inhibition of BRAF pathway acts on the metabolic level. For example, BRAF inhibition in melanoma downregulates glucose transporters GLUT1, GLUT3 and the first enzyme of glycolysis, hexokinase 2 (HK2)[47], thereby potently suppressing the uptake of glucose[48-50]. At the same time, BRAF inhibition activates the oxidative metabolism, leading to increased mitochondrial respiration and ROS production[36,37,51]. While a decrease in glucose metabolism is restored upon development of BRAF inhibitor resistance, drug-resistant melanomas show high rates of mitochondrial respiration and oxidative stress, regardless of the presence of BRAF inhibitor. Importantly, high oxidative metabolism renders resistant melanomas prone to cell death, induced by pro-oxidants including the clinical trial drug elesclomol[47,51]. All of this together indicates that PDH is activated in response to BRAF pathway inhibition in melanoma. In this regard, PDK1 depletion in presence of BRAF inhibitor would potentiate the activation of PDH, oxidative metabolism and thereby redox stress. Although this is likely to be a mechanism of synergy between PDK1 and BRAF inhibition, it requires validation, especially as information on PDK-PDP-PDH axis regulation in response to BRAF inhibition is lacking.

Our study on the role of PDK1 in response to BRAF inhibition does not go beyond cell culture. Clearly, an *in vitro* situation does not recapitulate the metabolic tumor microenvironment[52] nor the tumor cell heterogeneity that exists *in vivo*[53]. Therefore, a validation of synergy

between PDK1 and BRAF inhibition in melanoma cells killing in *in vivo* models is a critical point to be addressed. Particularly important from a therapeutic point of view, it should be recapitulated with the use of specific PDK1 inhibitors instead of shRNA-mediated PDK1 depletion. Several PDKs inhibitors that could be used for that purpose are commercially available[54]. Although these inhibitors are not fully selective for PDK1 alone but act also on other PDK isoforms, the fact that they all activate PDH justifies their use, at least in first proof-of-concept experiments.

Finally, an important question to be answered is how broad the effectiveness of the inhibition of PDK1 as a therapeutic target exactly is. Since we found that PDK1 depletion synergizes with BRAF inhibition in melanoma and because BRAF mutations are also found in thyroid, colonic and ovarian carcinoma[55-57], these tumor types would be the first choice to test the effect of PDK1 inhibition. But any therapeutic value of PDK1 regulation might go beyond BRAF-driven tumors. The fact that PDK1 inhibition has been reported to decrease viability of small cell lung cancer, breast cancer and glioblastoma cancer cells[19,58,59] implies that this approach should be explored for several cancer settings.

Other senescence mediators with a possible role in malignant transformation

In Chapter 5, Chapter 6 and Chapter 7 we describe other approaches we undertook, next to metabolic profiling, to identify novel senescence mediators. With a function-based short hairpin (sh)RNA screen we identified seven genes contributing to OIS (Chapter 5). One of them, *RASEF*, was characterized in more detail, because we found it was frequently methylated in melanomas. Although we focused on *RASEF*, other genes also represent interesting potential tumor suppressors for further characterization. While RASEF depletion abrogated OIS, causing continued cell proliferation*,* restoration of its expression in melanoma acted cytostatically (in some melanoma cell lines). This is in agreement with a potential tumor suppressive role of *RASEF.* An important limitation of our study however, is that it does not go beyond cell culture. Clearly, a more definitive characterization of *RASEF* will require *in vivo* studies. To confirm a tumor suppressive function of RASEF in an *in vivo* setting, a knock-in $BRAF^{V600E}$ mouse (Tyr::CreER; BRaf CA) would be a suitable model[60]. This mouse model was previously used to validate the importance of *PTEN* loss for development of BRAF-driven melanoma[61]. Also unclear remains the mechanism of action of *RASEF* in OIS and malignant transformation. Interestingly, RASEF is required for mutant BRAF to drive the increase of *IL6, IL8* and *C/EBPβ* transcripts, components of SMS contributing to OIS[27]. This observation implies communication between senescence-associated and *C/EBPβ*-dependent inflammatory cytokines, and signaling involving RASEF. Together, the results support a model in which RASEF controls OIS by regulating components of the senescence secretome.

Additionally, we performed mass spectrometry-based screening of the proteome and phosphoproteome in cycling, senescent, and senescence-escaping cells. By this approach

we identified many factors involved in the extracellular matrix (ECM) remodeling to be specifically regulated in senescence (Chapter 6). Furthermore, we revealed novel site-specific phosphorylation of components of PI3K/AKT/mTOR and RAS/MEK/ERK signaling network, previously undetectable by phospho-antibodies (Chapter 7). Interestingly, several studies have indicated important roles for components of ECM remodeling both in senescence and in cancer[62,63]. Therefore, functional studies on ECM organization and regulation could further increase our understanding of the mechanism underlying OIS and malignant transformation. Also, as PI3K/AKT/mTOR and RAS/MEK/ERK pathways are well-established regulators of both senescence and malignant transformation, efforts are being made to efficiently target their components for clinical use[64]. In this regard, by providing novel mechanistic insight into the signaling pathways involved, our study contributes to finding specific inhibitors for anticancer therapy.

A feedback loop comprising cytokines, metabolic regulators and transcription factors controls OIS

We and others have demonstrated that senescent cells adapt a robust inflammatory transcriptome signature (called SMS or SASP)[27,62,65-69]. The C/EBPβ transcription factor is a major player for the establishment of the resulting secretory phenotype[27]. Previously, we have shown that TSC22, a transcription factor and critical OIS mediator, acts downstream of C/EBPβ on the inflammatory secretome[70]. Here, we demonstrate that also components of PDK-PDP-PDH axis influence the SMS (Chapter 4). Inhibition of PDH activity leads to a decrease in the expression levels of *IL6*, *IL8* and *C/EBPβ* transcripts. Conversely, C/EBPβ depletion averts PDH activation by preventing $BRAF^{V600E}$-induced changes in the expression of both PDP2 and PDK1. Furthermore, we find that yet another mediator of OIS identified in this thesis, RASEF, also regulates senescence-associated and *C/EBPβ*-dependent inflammatory cytokines (Chapter 5). Taken together, these findings not only highlight the central role of specific interleukins in OIS but also suggest the existence of a complex auto-stimulatory feedback mechanism, in which cytokines, metabolic regulators and transcription factors operate to coordinate the control of OIS.

In conclusion, our study provides important insight into the mechanism underlying OIS, a vital program preventing malignant transformation. By three independent approaches: metabolic profiling, shRNA screens and mass spectrometry analysis, we identify novel factors having a pivotal role in senescence. We find several seemingly unrelated elements including the metabolic regulators PDK-PDP-PDH and the signalling molecule RASEF to act together with components of the SMS and the cell cycle machinery to regulate the senescence program in a coordinated fashion. Moreover, we provide pre-clinical evidence that these senescence mediators play a role in malignant transformation. The major challenge is to further explore the feasibility of their targeting for the clinical intervention of cancer.

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