The ambiguous role of C/EBPδ in pancreatic cancer

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Chapter 8

Discussion and Future Perspective

DISCUSSION

C/EBPδ is a versatile transcription factor with divergent, context-dependent downstream effects. It is for instance required for the differentiation of fat cells and lipid droplet formation, but also for the differentiation of myeloid cells and keratinocytes [1-4]. Furthermore, C/EBPδ is induced by inflammatory stimuli and subsequently activates the innate immune response to wards off pathogens [5]. Throughout adulthood and in terminally differentiated tissues, C/EBPδ expression is rare and tightly controlled. Consequently, prolonged increases or decreases in its expression levels can prompt unwanted cellular responses including excessive inflammation and aberrant proliferation. In cancer, C/EBPδ has been shown to act as a tumor suppressor or as a tumor promoter, depending on tumor origin and cell type. The aim of this thesis is to shed light on its role in pancreatic ductal adenocarcinoma (PDAC) tumor biology and associated clinical outcomes.

Putative mechanisms suppressing C/EBPδ in PDAC

C/EBPδ is highly expressed in the nuclei of normal pancreatic ductal cells but in PDAC cells, its expression is reduced and even lost in some cases. This pattern is reminiscent of a tumor suppressor role, urging the investigation of the underlying mechanisms of C/EBPδ inactivation. From large genomic datasets it has become apparent that the *CEBPD* gene is neither mutated nor deleted in PDAC, making it a suitable candidate for therapeutic re-expression in these cells. Instead, literature suggests different mechanisms that potentially account for C/EBPδ-suppression or inactivation in various cancers. In hepatocellular carcinoma, C/EBPδ is targeted for proteasomal degradation by the tyrosine kinase Src [6]. However, Src-inhibition in PDAC cells did not restore C/EBPδ expression (observation not included in this thesis). Furthermore, the activity of transcription factors can be regulated epigenetically. In breast cancer and acute myeloid leukemia, C/EBPδ is suppressed by promoter hypermethylation, and in cervical carcinomas histone modifications have been described to regulate C/EBPδ levels [7-9]. We thus tested whether C/EBPδ is epigenetically suppressed in PDAC cell and treated MIA PaCa-2 cells with the DNA methyltransferase inhibitor decitabine (5-aza-2'-deoxycytidine). This did not result in increased C/EBPδ expression, suggesting that C/EBPδ expression is not suppressed through hypermethylation in these cells. Treating HEK 293T cells with histone deacetylase (HDAC) inhibitors did enhance C/EBPδ-activity (**chapter 7**) although it is unclear whether this occurred due to altered histone acetylation levels of *CEBPD* or that of an upstream regulator.

Of note, C/EBPδ protein expression is also suppressed in epidermoid carcinoma cells with exogenously overexpressed mutant Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) [10]. Given that more than 90% of PDACs harbor an activating KRAS mutation, it is sensible to expect a similar association in these cells [11]. Mutations in KRAS are often followed by further amplifications of the gene and increased mRNA expression as a result [12]. KRAS mRNA levels in the tumors of patients included in the TCGA Pancreatic Adenocarcinoma (PAAD) dataset indeed negatively correlate with *CEBPD* mRNA levels. Altogether, these relations point towards a KRAS-dependent suppression of *CEBPD* in PDAC [13]. Targeting KRAS as an upstream regulator of C/EBPδ seems thus plausible but remains challenging. While inhibitors of KRASG12C [14] and KRASG12D [15] are currently making their way into the clinic, inhibitors of KRASG12V variant are still lacking.

Downregulation of C/EBPδ associates with disease progression and is affected by C/EBP-family redundancy

The low expression of C/EBPδ in PDAC nuclei as opposed to normal pancreatic duct cells implies a tumor-suppressor role in this disease and suggest that clinical outcomes may be correlated to its expression. Indeed, we describe in **chapter 2** that patients can be dichotomized by primary tumor cell C/EBPδ levels, which positively correlate with patient survival and a decreased likelihood of lymph node involvement.

Given the complexity of tumor biology, it is however unlikely that C/EBPδ acts as a sole determinant of such clinical parameters. Instead, its activity is affected by the presence of co-factors and of structurally related proteins including its family members. In **chapter 5** we consequently assess the influence of the highly homologous C/EBPβ and C/EBPγ on the correlation of C/EBPδ with patient survival and lymph node status. Although C/EBPδ is the most consistent predictor of patient survival, it turns out that in the (near) absence of C/EBPδ, both, C/EBPβ and C/EBPγ can partially compensate for low levels of C/EBPδ and associate with patient survival superior to that of patients in whom all three C/EBPs are lowly expressed by tumor cells. Lymph node involvement is a clinically and therapeutically highly relevant parameter that negatively associates with C/EBPδ expression. It describes the presence of tumor cells in regional lymph nodes which can be predictive of patient survival and therapeutic efficacy. Next to C/EBPδ, we also established in **chapter 5** that C/EBPβ acts as a powerful predictor of lymph node involvement and that C/EBPβ and C/EBPδ can compensate for the lack of each other in that respect. C/EBPγ on the other hand antagonizes C/EBPδ and rises the likelihood of lymph node involvement. C/EBPβ and C/EBPγ should thus be included when dichotomizing patients by C/EBPδ expression. Importantly,

similarly reciprocal behaviors might exist between the remaining C/EBP-family members (especially C/EBPα but also C/EBPε and C/EBPζ) and other members of the superfamily of basic leucine zipper (bZIP) transcription factors, depending on the respective biological contexts (discussed in **chapter 1**). Before we can use C/EBPδ as a prognostic marker in PDAC, it is therefore of importance to establish the reciprocal interaction with its various binding partners, as well as compensation for and by family members beyond C/EBPβ and C/EBPγ. Co-IP and ChIP-seq with the various bZIP-members would be a good starting point for such investigations.

C/EBPδ limits PDAC cell tumorigenesis through retardation of the cell cytoskeleton

The finding that low levels of C/EBPδ associate with disease progression prompted us to investigate the tumor biology specifically regulated by C/EBPδ. This is the focus of **chapter 2** and **3** where we established that in vitro, C/EBPδ re-expression limits PDAC cell proliferation and migration as well as clonogenic outgrowth in 2-dimensional and 3-dimensional anchorage-independent settings. Suppression of cell proliferation is often caused by a delay in the cell cycle, mediated through aberrant regulation of cyclin-dependent kinases or their inhibitors. Alternatively, enhanced apoptosis, necrosis or senescence can account for decreased cell numbers. During our investigations however, we found that none of these mechanisms were significantly altered by C/EBPδ.

Instead, mRNA data obtained from PDAC cells over-expressing *CEBPD* or a scrambled control implies that C/EBPδ regulates genes associated with the cytoskeleton. This is in line with the observation that re-expression of C/EBPδ limits PDAC cell motility and altogether led us to hypothesize that C/EBPδ alters the cytoskeletal dynamics in these cells. The cytoskeleton is comprised of actin filaments, intermediate filaments (keratin or vimentin) and microtubules. It is a highly dynamic structure involved with cell anchorage and morphology, cell division, polarity, intracellular organization and motility [16]. Alterations of the cytoskeleton thus likely affect all of the characteristics investigated in **chapter 2** and **3** of this thesis, i.e., migration, proliferation, and clonogenicity.

What remains obscure is the precise mechanism through which C/EBPδ regulates cytoskeletal dynamics. In mouse embryonic fibroblasts, C/EBPδ enhances cell size, spreading and adhesion through activation of SOCS3, TUBB2, KRT16/20 and RND3 [17]. In this thesis, we build on these observations and find C/EBPδ-mediated suppression of gelsolin (GSN) and epidermal growth factor receptor (EGFR; which cooperate to mediate cytoskeletal remodeling), as a potential mechanism through which C/EBPδ exerts its diverse effects.

What might further contribute to the inhibition of proliferation, migration and clonogenicity, albeit not being discussed in this thesis, is a C/EBPδ-mediated suppression of the stemness marker nestin (NES); While most such markers used in PDAC (CD24, CD44, CXCR4, EPCAM, CD133 (PROM1), ABCG2 and ALDH-1 (ALDH1A1)) remain unaffected by C/EBPδ, the mRNA data obtained in **chapter 3** show that c-Met (MET) and especially NES are strongly suppressed upon induction of C/EBPδ. In addition to governing cancer cell stemness, nestin was found to be an important mediator of cellular migration and TGF-β1-mediated epithelial-to-mesenchymal transition in PDAC. Accordingly, knock-down of nestin reduced cell motility and reverted cells towards an epithelial-like phenotype [18]. Furthermore, suppression of nestin correlated with decreased tumor take rate in vivo [18]. In **chapter 3**, we show that C/EBPδ likewise limits cell motility and outline briefly that it induces morphological changes reminiscent of EMT which we further support by showing a pronounced induction of C/EBPδ-mediated E-cadherin (CDH1) expression. Additionally, it has been shown in various cancers that transition towards an EMT phenotype oftentimes occurs with a concomitant increase of stemness markers [19]. C/EBPδ-mediated nestin might thus - next to the previously described GSN and EGFR - function as a powerful integrator of the effects of C/EBPδ on proliferation and clonogenicity (i.e. stemness), migration and EMT in PDAC cells.

C/EBPδ interferes with different steps of the metastatic cascade

In the introduction of this thesis, we briefly touched upon the metastatic cascade. This cascade describes the different steps and checkpoints that tumor cells need to pass in order to form distant lesions. These distant lesions are the major cause of PDAC-related death, rather than primary tumor growth, and thus warrant further investigation. In this thesis, we have used preclinical models to look at some of these steps, seeking to evaluate the role of C/EBPδ throughout the metastatic cascade. We found that C/EBPδ negatively regulates at least two of the metastatic steps; dissemination from the primary tumor through reduced (chemotactic) migration, and clonal outgrowth, representative of metastatic colonization.

C/EBPδ does not affect tumor cell invasiveness

In **chapter 3**, we have modeled the effect of C/EBPδ on the initiating step of the metastatic cascade – tumor cell motility – and found that overexpression of C/EBPδ significantly impairs PDAC cell migration. This is often used to claim an effect on the formation of distant metastases. Yet, while tumor cell motility can be a rate limiting step in the initial shedding of cells from the primary tumor, it is distinct from tumor cell invasiveness, another major predictor of metastasis

formation. Cell motility is largely determined by cell adhesion molecules and remodeling of the cytoskeleton while invasiveness is mediated by the secretion of matrix degrading enzymes (MMPs). According to the current literature, MMP1/2/8/9/13 are key drivers of PDAC invasiveness [20, 21]. According to our RNA-seq data, C/EBPδ indeed suppresses MMP13. Also, given the significant correlation of primary tumor C/EBPδ expression and lymph node involvement in patients, we hypothesized that C/EBPδ affects the invasive capacity of PDAC cells. Consequently, we tested the effect of C/EBPδ on PDAC cell invasion and subjected C/EBPδ-inducible and control PDAC cells to a chick chorioallantoic membrane model (CAM) invasion assay. To this end, PDAC cells are suspended in Matrigel and grafted onto the densely vascularized membrane, the CAM. This lies underneath the egg shell and can be accessed by carefully opening up the latter. Regrettably, C/EBPδ induction appears to be insufficient to noticeably change these cells' invasiveness and the lack of a C/EBPδ-mediated difference on CAM invasion implies that it has no or very weak effects at this step of the metastatic cascade (**chapter 3**).

Notably, in urothelial carcinoma, knockdown of C/EBPδ limited invasion through suppression of MMP2 [22], emphasizing once more that C/EBPδ's functions are context-dependent and that findings made in one disease cannot be extrapolated to other biological circumstances.

Extravasation is unaffected by tumor cell-intrinsic C/EBPδ but greatly impacted by stromal C/EBPδ

Extravasation, i.e. the invasion of circulating tumor cells from a blood vessel into the surrounding tissue is another indispensable step of metastasis formation. To complement our analysis of the role of C/EBPδ in the metastatic cascade, we thus employed a micro-flow chamber lined with microvasculature endothelial cells, and tested whether the extravasation potential of PDAC cells is affected by C/EBPδ expression. Regrettably, also here, wild type cells and cells with induced C/EBPδ showed similarly low attachment to the endothelial cell layer and no extravasation through this layer (**chapter 3**).

The above notwithstanding, C/EBPδ appears to play a powerful role in metastasis formation in non-tumor cells, as shown in **chapter 6**. In that study, C/EBPδ was systemically deleted in mice and the rate of metastasis formation from a tail-vein injection model was assessed. Compared to wild type animals, mice devoid of C/EBPδ showed a significantly reduced metastases count. Although we found that extravasation happened in a platelet-activating factor receptor (PAFR)-dependent manner, the stromal cell type and underlying mechanisms governing C/EBPδ-mediated extravasation were not identified in **chapter 6**. Here, we discuss

additional potential scenarios; We quantified *CEBPD* expression in vasculature-associated cells using publically available single cell-sequencing data [23] which reveals that *CEBPD* is highly expressed in pericytes and vasculature-associated fibroblasts. These cell types are thus expected to be primarily affected by the ablation of *CEBPD*. Pericytes tightly wrap around endothelial cells to maintain vascular integrity and prevent leakage. Insufficient or defective pericyte coverage has been suggested to enhanced blood vessel permeability and thereby facilitate tumor cell extravasation [24-26]. Although a precise mechanism of C/EBPδ-mediated pericyte integrity has not been described, it is possible that ablation of C/EBPδ affects pericytes in their function and thereby permits tumor cell extravasation.

Another putative explanation of the observed phenomenon lies in the inflammation-associated properties of C/EBPδ; It has been reported that in brain pericytes, C/EBPδ suppresses the IL-1β-induced expression of intracellular adhesion molecule 1 (ICAM-1) and monocyte chemoattractant protein 1 (MCP-1) to limit the shuttling of immune cells from the blood into tissues. Conversely, knocking out C/EBPδ has been shown to enhance the induction of ICAM-1 and MCP-1 [27]. This implies that C/EBPδ-/- animals harbor enhanced capabilities of immune cell-shuttling towards irritated sites which is expected to hamper the outgrowth of tumor metastases.

Whether decreased immune cell shuttling actually contributes to the lack of metastases in C/EBPδ-/- mice remains to be proven. Generally, both hypotheses fit the notion of C/EBPδ being a tumor promoter in cells participating in extravasation which will be interesting to investigate in more detail in the future.

C/EBPδ limits clonogenic outgrowth of PDAC cells in vitro and in vivo

Clonal outgrowth of extravasated tumor cells at a distant site forms the final step of the metastatic cascade. In **chapter 2**, we have shown that the clonogenicity of PDAC cells in vitro is significantly suppressed by C/EBPδ. We next tested this observation in vivo using a peritoneal metastasis model in mice. In this model, C/EBPδ-inducible and control PDAC cells are directly injected into the peritoneum of nude mice, omitting the need for invasion, intravasation and extravasation to form metastases. Half of the animals were fed doxycycline to induce C/EBPδ in the injected tumor cells. These experiments are not part of any chapter in this thesis but yielded interesting insights worth being discussed; While the average peritoneal carcinomatosis index (PCI, defined by the number and size of tumor nodules) did not differ between the treatment and control groups, the number of animals devoid of metastases was enhanced in the group bearing C/EBPδ-induced cells (4/8 mice compared to 1/8 mice in the control groups). Although this

difference lacks significance, it implies that C/EBPδ can reduce the clonogenic outgrowth of PDAC cells also in vivo. Interestingly, the recognition that the PCIs did not differ between the groups implies that proliferation may be unaffected under these physiological circumstances.

While different steps of the metastatic cascade and the respective effects of C/EBPδ have been assessed in this thesis, this cascade is affected by many more factors than those described here. The interaction of tumor and stromal cells in the primary tumor is for instance a major determinant of tumor development and aggressiveness. To study these interactions, more sophisticated models are currently being developed. They range from PDAC organoid models to patient-derived multicellular 3-dimensional structures (top-down approach) to on-chip solutions (bottom-up approach) where tumor cells are in close proximity with stromal cells [28-30]. These models will allow us to better assess the contributions of different types of fibroblasts, endothelial and immune cells on carcinogenesis, and to study the role of extracellular matrix (ECM) compounds such as integrins and hyaluronans on tumor cells. This is especially interesting in PDAC, a disease marked by an abnormally strong desmoplastic reaction. Binding of tumor cell CD44 to hyaluronan for instance increases cell survival, ECM turnover and cell invasiveness and we know from **chapter 1** that C/EBPδ induces CD44 at least in breast cancer to promote stemness [31, 32]. Experimental models considering the effects of stromal cells and the ECM are thus eagerly awaited and hold great promise for an improved understanding of PDAC development and a faster development of targeted therapies.

Oxygen tension co-determines C/EBPδ's downstream activities

In **chapter 2**, we showed that C/EBPδ limits the proliferation and clonogenicity of PDAC cells in vitro, a prime indicator of decreased tumor cell aggressiveness. In **chapter 4**, we test this finding in a subcutaneous mouse model and partially disprove the tumor-suppressor hypothesis by showing that in hypoxic tumors, C/EBPδ in fact promotes proliferation. While in line with the current literature, this observation is in disagreement with the fact that C/EBPδ-expression in primary PDAC tumors correlates with prolonged survival and decreased lymph node involvement. C/EBPδ in vivo might thus take on a dual role and promote proliferation in hypoxic regions but limit cell motility and clonal outgrowth at distant sites. What further hampers the interpretation of the data derived from subcutaneous xenografts and hypoxic monocultures is the lack of a species-specific stroma. The interaction with immune cells or fibroblasts might yet again revert or counteract the effect of C/EBPδ on tumor cell proliferation. Irrespectively, these findings urge the consideration of disease-specific

physiological factors in pre-clinical models and to establish improved models, as were described above.

Implications for C/EBPδ in PDAC not discussed in this thesis

A predicted role of C/EBPδ in drug-induced therapy resistance in PDAC

Through various RNA-seq analyses – not all of which were published in this thesis – we learned a lot about C/EBPδ and its potential contributions in addition to those that we experimentally tested and confirmed in this thesis. **Chapter 4** for instance lists the most up-and down-regulated genes upon C/EBPδ induction under hypoxia and under normoxia. The attentive reader will have recognized the gene ABCG1, an ATP-binding cassette transporter that shuttles molecules across membranes, from the introductory literature review. Here, we cite two articles concerned with the upregulation of the related membrane transporters ABCB1, ABCC2 and ABCA1. These are induced by C/EBPδ upon anti-cancer drug treatment with cisplatin, paclitaxel and temozolamide and enhance the efflux of these drugs. ABCG1 is induced by saracatinib in hepatocellular carcinoma cells through yet unresolved mechanisms, and mediates oxaliplatin-resistance [33]. Oxaliplatin is part of FOLFIRINOX, the standard chemotherapeutic regimen administered to PDAC patients. These insights imply that also in PDAC, C/EBPδ might function as a mediator of drug resistance via the activation of membrane transporters, urging careful examination of such a relation and the suitability of concomitant C/EBPδ-inhibition.

Is C/EBPδ a suitable target in PDAC treatment?

After thorough investigation of the multiple roles of C/EBPδ in diverse cancers (**Chapter 1**), the question remains whether reactivation of C/EBPδ might benefit PDAC patients. After all, C/EBPδ is a powerful regulator of cell differentiation. On the other hand, **Chapter 1** illustrates quite clearly that C/EBPδ can induce several undesirable, tumor-promoting mechanisms. Also in PDAC, it engages in a positive feedback loop with HIF-1α, which contributes to carcinogenesis (**Chapter 4**). The fact that C/EBPδ promotes the adaption of tumor cells to a hypoxic environment makes it an unfeasible target for reactivation in any solid, potentially hypoxic cancer. Interrupting the C/EBPδ/HIF-1α axis might emphasize the tumor-suppressive effects of C/EBPδ, making it a more suitable therapeutic target. Unfortunately, clinical inhibitors of HIF-1α are still lacking.

Furthermore, C/EBPδ has been described to act as a mediator of EMT which promotes cell mobility and invasiveness. Although we have shown that C/EBPδ in fact limits the motility of PDAC cells in vitro, this thesis also emphasizes that in vitro results do not necessarily represent the in vivo scenario. It is likely that

confounding physiological factors including tissue hypoxia affect the downstream effects of C/EBPδ and its binding partners in unanticipated ways. Before considering C/EBPδ as a target in PDAC treatment, much more research using near-physiological preclinical models will be needed.

Irrespective of the desirability of C/EBPδ expression, its activation is further hampered by the lack of specific activators. This thesis (**chapter 7**) did uncover cytoskeletal signaling and CDK-inhibitors as enhancers of C/EBPδ activity. CDK-inhibitors have indeed been suggested and tested for the treatment of PDAC [34-35]. Furthermore, effects on the closely related C/EBPα, C/EBPβ and C/EBPγ have been ruled out. Yet, the nature of these inhibitors implies a broad range of 'off-target'-effects; inhibition of CDKs will affect a wide range of genes, not only those directly related to cell cycle progression. As induction of endogenous *CEBPD* is not feasible yet, targeted approaches for the over-expression of exogenous C/EBPδ are currently underway. Those include the delivery of C/EBPδ-encoding peptides coupled to cell penetrating molecules and have proven effective in caspase 8 activation in prostate cancer cells [36]. In the future, targeted genomic modifications using CRISPR-based approaches might also be suitable for the modulation of C/EBPδ expression. However, this path is yet limited by insufficient spatiotemporal control, immunogenicity and ethical concerns [37, 38].

Future recommendations and clinical implications

In highly simplified cell culture models, a common theme emerges that C/EBPδ-induced differentiation limits carcinogenic properties including cell cycle progression and EMT. In more advanced preclinical models however, this picture is oftentimes reversed and C/EBPδ contributes to enhanced stemness, drug resistance, hypoxia adaption and pro-tumor inflammation. This urges scientists to not entirely isolate tumor cells from their natural environment when studying C/EBPδ but to include stromal cells, ECM compounds, and physiological parameters to create the most optimal experimental conditions. Importantly, these conditions also affect the expression of co-factors of C/EBPδ which can largely determine its down-stream effects and potentially account for the varied C/EBPδ-mediated downstream effects observed in different contexts. At the same time, it will be interesting and valuable to study the effect of C/EBPδ in stromal cells which, as we showed in **chapter 1** can contribute to disease progression. Like tumor cells, fibroblasts, macrophages, and other stromal cell types may serve as potential targets for anti-cancer therapies. The various effects of C/EBPδ in different contexts and tissues also point towards the risk of inducing C/EBPδ in the 'wrong' cell type. Targeted activation or the targeted delivery of

pharmaceutical activators will therefore be an important subject to study prior to targeting or boosting C/EBPδ safely.

The clinical reactivation of C/EBPδ in PDAC cells does not per se sound very appealing. Hypoxia and other physiological factors preclude a clear prediction of its effects in patients and observations made in other cancers do not bode well. Abrogating the C/EBPδ/HIF-1α axis might make reactivation of C/EBPδ a feasible option; without HIF-1α activation, C/EBPδ is expected to decrease tumor cell aggressiveness and to restrain the metastatic cascade at different points. As a side effect, reactivating C/EBPδ induces a partial mesenchymal-to-epithelial transition in PDAC which might make cells more susceptible to chemotherapeutics [39]. On the other hand, we have discussed above that C/EBPδ can upregulate the expression of membrane transporters to promote drug efflux.

In conclusion, the broad range of C/EBPδ's downstream activities limits the predictability of outcomes following reactivation in patients. Many factors speak for a beneficial outcome of its re-expression but as many factors argue against it. Context-specific research taking stromal and physiological components into account will be needed to determine the true effects of C/EBPδ in PDAC and whether a context-specific reactivation will genuinely benefit patients.

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