Regulation of TGF-β signaling and EMT in cancer progression

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Chapter 7

General discussion

Chapter 7

TGF-β signaling in cancer progression; switching from tumor suppressor to tumor promotor

Lung, breast and pancreatic cancer are the common cancer types worldwide [1]. Over 90% of the observed mortality of patients with solid cancers is caused by distant metastasis [2]. Many efforts have been made in early detection and better treatment to improve survival of patients with these cancer subtypes. However, no drugs have entered the clinic that would selectively interfere with the critical steps of the metastatic process [3]. Additionally, therapy resistance decreasing the efficacy and potency of anti-cancer treatment remains a major concern [4]. Further research is needed to unravel the precise mechanisms at molecular and cellular level of how cancer cells communicate with each other and with their (tumor micro) environment. This will enable innovative, more effective and less toxic approaches of cancer therapy in a personalized manner. My research has focused on elucidating the mechanisms that control the function of transforming growth factor-β (TGF-β) and its role in normal and breast, lung and pancreatic cancer progression.

TGF-β is a secreted pleiotropic cytokine, which initiates signals via specific cell surface serine/threonine kinase receptors and intracellular SMAD transcriptional effectors [5]. It plays critical roles in cancer initiation and progression; perturbation in TGF-β receptor/SMAD signaling can contribute to cancer in a biphasic manner (Figure 1) [6]. In normal or early stages of tumors, TGF-β inhibits proliferation and promotes apoptosis of normal and pre-malignant cancer cells and acts as a tumor suppressor [7]. Consistent with this notion is that the genetic inactivation of SMAD4, a critical component of canonical TGF-β intracellular signaling, frequently occurs in pancreatic ductal adenocarcinomas [8, 9]. Loss of SMAD4 counteracts the TGF-β-induced cell cycle arrest and cell death. This results in that pancreatic cells start to proliferate faster, in particular as the oncogene *K-RAS* is frequently activated in pancreatic cancer [10]. In **Chapter 5**, we observed that pancreatic cancer cells with SMAD4 deficiency still respond to TGF-β with a significant upregulation of certain *N*-glycans but not *O*-glycans and glycosphingolipids (GSLs). In addition, our data in combination with the results from others [11], showed that activated R-SMADs together

with SOX4 (but independent of SMAD4) can regulate the TGF-β-induced upregulation of *N*-glycans.

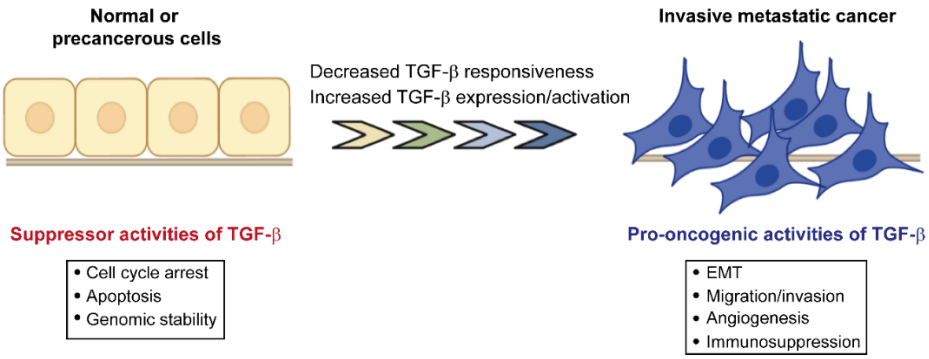

Figure 1. Biphasic role of TGF-β signaling in cancer progression. In the early stages of tumorigenesis, TGF-β signaling acts as a tumor suppressor by inducing apoptosis and inhibiting the proliferation, but promotes EMT, cell migration, invasion, angiogenesis and the formation of an immunosuppressive tumor microenvironment at later stages of disease leading to metastasis.

In advanced stages, cancer cells are resistant to the TGF-β-induced cytostatic effects but remain TGF-β sensitive. TGF-β then starts to act as a tumor promotor by inducing EMT, cell migration and invasion [12]. Compared to the frequently mutation of *SMAD4* in pancreatic cancer [13] and *TβRII*, *SMAD4* and *SMAD2* inactivation in colorectal cancer [14-16], the core elements of the TGF-β receptor/SMAD pathway in breast and lung cancer cells remain intact [17, 18]. By acting in concert with the activation of oncogenes or inactivation of tumor suppressor genes, TGF-β-induced SMAD-dependent signaling is a strong driver of EMT resulting in the tumorigenesis in these two cancer types [19, 20]. Through my investigation on TGF-β-induced EMT, important new insights were obtained in particular how TGF-β receptor function and stability is regulated by ubiquitin and glycosphingolipids in normal and cancer cells (see below). However, even after so many years of research, current knowledge still does not provide a full picture on how TGF-β switches from a tumor suppressor to a tumor promoter. Therefore, identification of new regulators of TGF-β signaling helps to move closer to unraveling this mystery at a molecular level and to design new therapeutic approaches.

Chapter 7

TGF-β-induced epithelial to mesenchymal plasticity driving cancer metastasis and therapy resistance

EMT is a fundamental process that the epithelial cells gain malignant properties to carcinoma cells, not only including migratory and invasive behaviors, but also stronger resistance to chemotherapy and immunotherapy [21]. In particular when cells adopt a epithelial/mesenchymal (E/M) phenotypic state, displaying both epithelial and mesenchymal makers, they are thought to be aggressive and metastatic. The phenomenon of cancer cells displaying an E/M hybrid state is also referred to epithelial-to-mesenchymal plasticity (EMP) [22]. Blocking EMT may effectively impair the formation of metastases as well as prevent resistance against anti-cancer treatments. Since TGF-β is the main driver of EMT in breast and lung cancer [23, 24] and that high TGF-β signaling activity is associated with poor prognosis [25, 26], it can be a prime candidate for novel anti-cancer strategies. However, besides tumor promotion, TGF-β is also crucial for healthy tissue maintenance, making it a challenging target for cancer therapy. This explains the severe toxicities that are observed when TGF-β signaling is entirely blocked by directly inhibiting ligand/receptor function [27, 28], despite indeed showing clinical anti-cancer benefit [29]. Next to my curiosity in its mechanism of action, I have been interested in identifying specific novel modulators or effectors of TGF-β-induced EMP of cancer cells, with the idea that this may specifically interfere with the pro-oncogenic effects of TGF-β while leaving its tumor suppressor functions intact.

Pre-clinical evidence has shown that therapeutic efficacy of standard chemotherapy on the primary tumor may in fact be counterbalanced by the induction of the survival and dissemination from the heterogeneous tumor of a subset of cancer cells [30]. Thus, the chemotherapy-resistant cells presenting after therapy are frequently more invasive and metastatic. Besides, as TGF-β targeting cannot induce killing of cancer cells, the combination of TGF-β-induced EMT targeting therapy and chemotherapy is expected to more effectively inhibit both primary tumor growth and metastasis formation (Figure 2). Indeed, pre-clinical cancer models with combination treatments have shown successes, with the caveat that general TGF-β inhibitors were used [31]. Combining chemo, radio or

immune therapy with more selective TGF-β targeting agents will hopefully provide more effective and less toxic therapy of cancer patients.

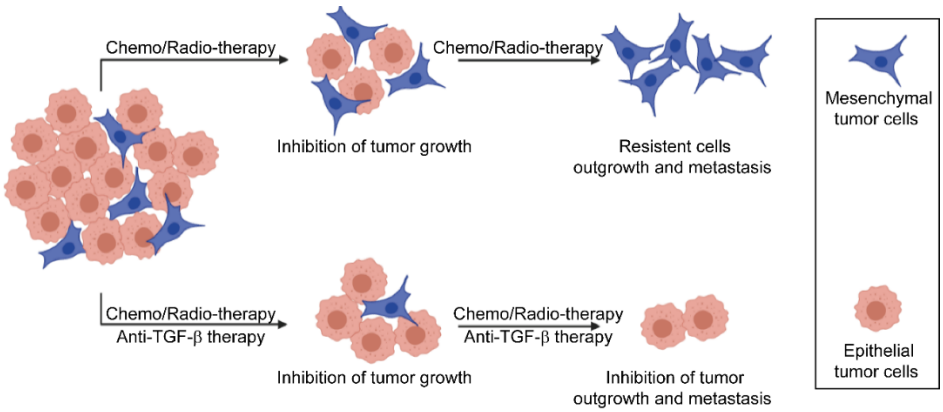

Figure 2. Chemo/radio-targeted therapy alone inhibits the growth of (epithelial-like) tumor cells in primary tumor but may result in the dissemination of a subset of (mesenchymal) cancer cells and metastasis formation. The combination of chemo-/radiotherapy with TGFβ targeting blocks the pro-invasive (mesenchymal) phenotype, therapy resistance and metastasis.

Methods to investigate TGF-β signaling and EMT and identify novel druggable targets

To overcome the limitation of current TGF-β inhibitors to target cancer metastasis and therapy resistance, we have explored the possibility to indirectly inhibit TGF-β pro-oncogenic responses in cancer cells by targeting druggable regulators or effectors on TGF-β signaling and TGF-β-induced EMT. In **Chapter 2**, we provide different methods to investigate TGF-β signaling and TGF-β-induced EMT in breast cancer cells. For gain (cDNA) or loss of function (shRNA or siRNA) or compound screens, SMAD3/SMAD4-dependent CAGA-transcriptional reporter (with or without fluorescent protein) assay has frequently been used (also by myself) to identify the positive or negative regulators of TGF-β signaling (as shown in **Chapter 3**) [32]. In addition, EMT reporter cell lines such as A549 lung adenocarcinoma-vimentin-red fluorescent protein (RFP), have been applied in high throughput genetic or compound screens for regulators of EMT using RFP-tagged vimentin expression as a read out (as shown in **Chapter 3**) [33]. TGF-β-induced SMAD2

phosphorylation, target gene expression and expression of EMT markers and migration are usually used as secondary assays to validate the findings from reporter assays. Moreover, omics screens such as the mass spectroscopy-based proteomics, metabolomics and glycomic screens with cells undergoing TGF-β-induced EMT have been utilized for the regulators and effectors of TGF-β signaling and EMT (as shown in **Chapter 6**) [34, 35]. To select most important targets for further analysis, data mining of the identified genes/proteins with online databases can be used to investigate whether these hits are mis-expressed in cancer tissues or related to the prognosis of cancer patients. Except for these *in vitro* analytical tools, the *in vivo* xenograft zebrafish model is rapidly gaining momentum as xenograft *in vivo* cancer model for intravasation and extravasation [36]. To this end, different human cancer cells from breast, lung and pancreatic labeled fluorescently, are injected into Duct of Cuvier (Doc) of the zebrafish, and the intravasation and extravasation of cancer cells can be investigated as shown in **Chapter 3**, **Chapter 5** and **Chapter 6** [37]. To interrogate the role of specific genes/proteins in the EMT process, genes are mis-expressed in different cancer cells, or if possible, pharmacological modulators of the targets are added to the egg water of the zebrafish. Thereafter, some of the targets can be further tested using genetic and pharmacological approaches and explored in mouse xenograft models, and in each case the results in zebrafish were confirmed and validated [38]. Taken together, all these platforms are effective tools for target identification and validation and drug screening, not only for developing new anti-cancer treatments by antagonizing TGF-β-induced cancer progression, but also other growth factor signaling pathways.

Opposing roles of USP19 isoforms in TGF-β signaling and EMT in breast and lung cancer

Alterations of ubiquitin enzymes and deubiquitinases (DUBs) have emerged as important mechanisms by which TGF-β signaling is dynamically regulated in cancer progression [39]. SMAD7, a negative regulator of TGF-β signaling, interacts with SMURF E3 ubiquitin ligases and brings them to the TGF-β type I receptor (TβRI), resulting in SMURF-mediated polyubiquitylation routes of the receptor for degradative

endocytosis [40]. The ubiquitination of TβRI can be reversed by the removal of ubiquitin chains by DUBs such as USP4, USP15 and USP11 [32, 41, 42], which rescue the TβRI from degradation. In **Chapter 3**, we identified the distinct roles of two ubiquitin specific protease (USP)19 splice variants in regulating TGF-β signaling in breast and lung cancer cells. USP19 is mainly expressed as two isoforms, one with a carboxy-terminal transmembrane (TM) domain that targets it to the endoplasmic reticulum (ER) with the active site facing the cytosol and herein referred as USP19-ER isoform; another USP19-CY isoform without the TM domain and localizes in cytoplasm [43]. In our study, we found that USP19-CY promotes TGF-β signaling by deubiquitinating the TβRI and increasing its stability in the plasma membrane (Figure 3). In contrast, the USP19-ER isoform inhibits TGF-β signaling by sequestering TβRI in the ER, thereby leading to lower TβRI levels in the plasma membrane and making the cells less TGF-β responsive. The inhibitory action of USP19-ER occurs in a DUB activity independent manner. Moreover, these two USP19 splice variants were found to display opposing effects on TGF-β-induced EMT and cell migration of breast and lung cancer cells. Importantly, the USP19-CY enhances the invasion of breast cancer cells in the zebrafish model, and its expression is also correlated with the poor prognosis of breast cancer patients. Indeed, USP19-CY is the major isoform of USP19 splice variants in lung and breast cancer cell with a much higher expression level than the USP19-ER isoform. Therefore, identification of specific inhibitors that target USP19-CY or specific compounds that shift splicing from USP19-CY to USP19-ER, may contribute to the development of effective therapy of breast and lung cancer.

Interplay of glycosphingolipids and TGF-β signaling in EMT

Glycosylation is a common posttranslational modification on protein in the plasma membrane or the secreted proteins that is involved in various cellular processes [44]. Aberrant glycosylation leads to pathological processes including uncontrolled cell proliferation, EMT and migration

during tumor progression [45]. In **Chapter 4**, we provide an overview of glycosylation in TGF-β signaling and epithelial-to-mesenchymal transition in cancer and discuss glycans as potential biomarkers for cancer diagnosis. In **Chapter 6,** we used two well-established EMT cell models, which are breast epithelial NMuMG cells and lung cancer A549 cells to investigate the glycosylation changes during TGF-β-induced complete EMT. We observed a strong downregulation of GSLs in particular *a*-series gangliosides in cells undergoing TGF-β-induced EMT. Furthermore, the genetic or pharmacological inhibition of the *a*-series ganglioside synthesis mediated by ST3GAL5 promotes TGF-β signaling and TGF-β-induced EMT, cell migration, and invasion (Figure 3). Mechanistically, we observed that the *a*-series gangliosides enriched in lipid rafts stimulate the ubiquitination and degradation of TβRI. Furthermore, we unraveled that high ST3GAL5 expression, a key enzyme in the synthesis of *a*-series ganglioside, is associated with a good prognosis of lung cancer patients. Consistently, the low expression of ST3GAL5 has been shown to be associated with a high grade and a poor prognosis in patients with bladder cancer [46]. Thus, ST3GAL5 may be a potential prognostic biomarker and therapeutic target that can contribute to improve lung and bladder cancer therapy in the future.

Further perspectives and clinical translation

In this thesis, we uncovered the roles and molecular mechanisms of regulators of TGF-β signaling and TGF-β-induced EMT, i.e., ubiquitination and glycosylation, in breast, lung and pancreatic cancer subtypes. A variety of drugs including neutralizing antibodies and small molecular inhibitors have been developed to inhibit TGF-β signaling [47]. Indeed, the results with TGF-β targeting agents as anti-cancer agents in (pre) clinical models have shown very promising results [48]. Therefore, there has been an immense interest from academia, biotech and pharma industries to translate the fundamental studies into clinically approved drugs. However, the TGF-β inhibitors that have been tested in the (pre)clinical stages of multiple cancer types, were abandoned (or evaluation is still ongoing) for further clinical development and no TGF-

β targeting agents are currently clinically approved. For example, AP12009, an antisense oligodeoxynucleotide complementary to TGF-β2 mRNA, has been applied in the phase IIb clinical trial (NCT00761280) to study its efficacy and safety in high-grade glioma patients. Although it did not show a significant effect in controlling tumor growth at 6 months, a delayed response and a superior 2-year survival rate were observed [49]. However, the project was terminated because of recruitment issues. In addition, the TβRI kinase inhibitor LY2157299 has been investigated in different cancer types including pancreatic cancer, breast cancer and glioma. Despite the phase II studies was initiated, the combination of LY2157299 with other treatment in patients who experienced solid malignancy has no effect on improving overall survival [50] or final data are not yet available. As illustrated earlier in this discussion, combining targeting TGF-β with other types of cancer therapy may provide additive/synergistic antitumor effects.

The USP19-ER and USP19-CY isoforms are expressed because of the alternative USP19 splicing and we unraveled the USP19-CY as a breast cancer promoter in **Chapter 3**. We identified herboxidiene, a splicing modulator that targets the core component of the spliceosome [51], as the regulator of the alternative splicing of USP19 by inducing a shift from the USP19-CY isoform to USP19-ER (Figure 3). Excitingly, herboxidiene, consistent with our expectation on its effect on USP19 splicing, was to antagonize the EMT process and migration of breast cancer cells. Given the global effects of spliceosome inhibition of splicing modulators, specifically targeting USP19 splicing events with oligonucleotide-based therapeutics can be an alternative method to more specifically manipulate the ratio of USP19 isoforms to inhibit TGF-β-induced EMT of cancer cells.

As the stimulatory effect of USP19-CY on TGF-β signaling, but not the inhibitory effect of USP19-ER, is dependent on the DUB activity, the targeting of the catalytic activity of USP19-CY can be explored as a way to target USP19-induced promotion of TGF-β signaling. We tested a selective small molecule USP19 inhibitor in breast cancer cells which mainly express USP19-CY and found that it potently suppressed TGF-β signaling, TGF-β-induced EMT, cell migration *in vitro* and invasion in the zebrafish xenograft model (Figure 3). Further studies such as the *in vivo*

mice experiments of this USP19 inhibitor are needed to validate its efficacy in the inhibition of tumor development.

In Chapter 6 we found that *a*-series gangliosides inhibit TGF-β-induced EMT in lung cancer cells. Knock down of ST3GAL5, a key enzyme that generates these gangliosides, promotes TGF-β signaling and EMT of lung cancer cells. These and other results in **Chapter 6**, suggest that it could be of therapeutic interest to screen for compounds/drugs that activate the expression or catalytic activity of ST3GAL5 in lung cancer as a potential therapeutic way for lung cancer treatment. Moreover, the specific delivery (via nanoparticles) of the *a*-series gangliosides, including GM3, GM2 and GM1a, to lung cancer cells may also offer therapeutic effects (Figure 3).

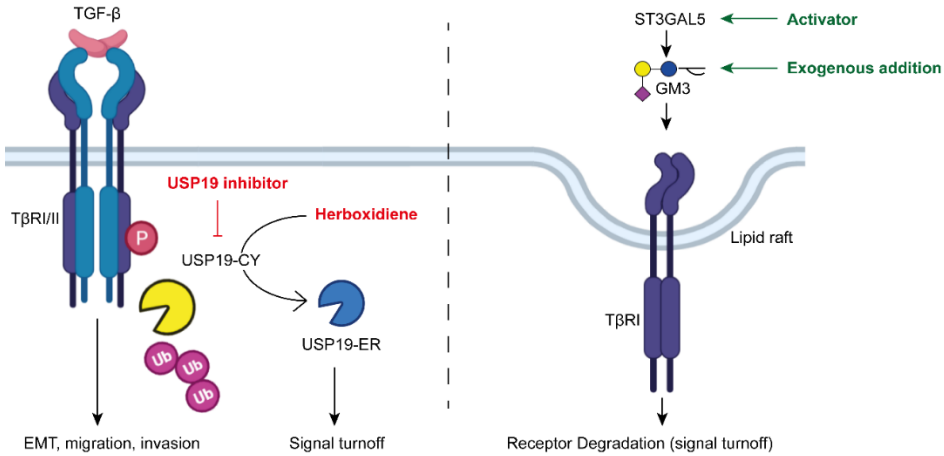

Figure 3. Potential anti-TGF-β regulator therapies. USP19 inhibitor eliminates USP19-CY-induced activation of TGF-β signaling, EMT, cell migration and invasion. Besides, the splicing modulator herboxidiene leads to a shift from USP19-CY to USP19-ER, thereby inhibiting TGF-β signaling and TGF-β-induced responses (left panel). Activation of ST3GAL5 or the exogenous addition of the *a*-series gangliosides including GM3 can enhance the gangliosides-mediated inhibition of TGF-β signaling.

In conclusion, this thesis is focused on elucidating the mechanisms that control TGF-β-induced EMT of cancer cells and manipulating this process for exploration of new therapeutic approaches. I hope that the newly generated insights will contribute to the improved survival and quality of life of cancer patients.

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