Regulation of BMP and TGFβ signaling pathway in cancer progression

Ren, J.

Summary and Perspectives

The main aim of my thesis was to reveal the anti-/pro-invasive metastatic role of BMP/TGFβ signaling in breast cancer and explore possible therapeutic interventions.

To do so, we first wanted to establish a rapid and inexpensive model in our laboratory to investigate the (potential) functional role of genes and proteins that regulate or mediate the tumor-suppressive and tumor-promoting effects of TGFβ family members in breast cancer cells. We opted for zebrafish embryo models in which we injected fluorescently labeled cancer cells (and fibroblasts). In embryos in which the immune system has not yet developed, human/mouse cells are not rejected [1, 2]. At the embryonic stage, the transplanted cells can be easily visualized, as the zebrafish are transparent, particularly casper mutant zebrafish [3]. Human/mouse cells communicate with the zebrafish host. By using genetically engineered *fli1*:*EGFP* zebrafish [4], all vessels are labeled in green, allowing us to easily track the migration and invasion of cancer cells. Two zebrafish models were established: (1) one in which cells were injected into circulation via the duct of Cuvier, which allows us to examine the level of extravasation of cancer cells into the avascular tail fin area; and (2) one in which cells were injected into the perivitelline space, which allows us to examine how cancer cells intravasate into the bloodstream. Moreover, the latter model also allows us to assess the effect of cancer cells in promoting angiogenesis surrounding the grafted tumor mass (**Chapter 2**). Moreover, we further adapted the perivitelline space model by coinjecting breast cancer cells with fibroblasts/cancer associated fibroblasts (CAFs), as discussed in **Chapter 3**. This allows us to look *in vivo* at the effect of the interplay of breast cancer cells and CAFs on the intravasation of cancer cells. Moreover, by adding drugs/small-molecule compounds to the egg water, zebrafish xenograft models are very amendable to pharmacological (and toxicity) studies.

In **Chapter 3**, by analyzing all secreted BMP antagonists in clinical breast cancer datasets, we found a strong correlation between high *GREM1* mRNA expression and poor distant metastasis-free survival of breast cancer patients. Analysis of many breast cancer cell lines surprisingly showed that nearly all cell lines had no detectable *GREM1* expression. Further analysis of different cells in the breast cancer tumor microenvironment showed that *GREM1* was exclusively and highly expressed in CAFs at the invasion front. Grem1 maintains stemness and promotes invasion of breast cancer cells in a paracrine manner. In addition, Grem1 mediates the fibrogenic activation of CAFs in an autocrine manner, suggesting that *GREM1* expression can serve as a marker for activated fibroblasts in the cancer stroma. TGFβ secreted by tumor cells

was found to be a strong promotor of *GREM1* expression in CAFs, and Grem1 appeared to be capable of inducing TGFβ and TGFβ target genes in CAFs, thereby creating a feed-forward loop. Moreover, activated CAFs strongly promoted breast cancer cell invasion. In this study, we tried but failed to produce a Grem1-neutralizing nanobody that inhibits breast cancer progression. However, our failure does not mean impossible. Further efforts to develop neutralizing antibodies or small molecular compounds that can block the function of Grem1 are still warranted. We also wondered whether Grem1 was upregulated in the serum of (breast) cancer patients and whether it could be used as a diagnostic or prognostic marker.

After showing that Grem1 is a factor in the tumor microenvironment that could restrict BMP signaling and promote CAF activation, thereby creating a favorable niche for breast cancer cells to invade and metastasize, we continued to explore the intrinsic cellular factors that could regulate BMP signaling in breast cancer cells, which are detailed in **Chapter 4**. We found that progressive loss or suppression of BMP-SMAD1/5 signaling by TGFβ-induced activation of MAPK/ERK could be an important factor for metastatic cancer development. We hypothesized that MEK activation induces the activation of a phosphatase, which triggers pSMAD1/5 dephosphorylation. Our ongoing genetic screening attempt suggests that the phosphatase PPM1A may be a candidate. A parallel study shows that FK506 potently activates BMP signaling in breast cancer cells, whereas TGFβ signaling is not affected. Next, we demonstrated that a synergistic effect arose upon restoration of BMP signaling *in vitro* and *in vivo* by combining U0126 and FK506 at suboptimal concentrations. A strong inhibition of cancer cell metastasis was thereby observed.

In **Chapter 5**, we investigated the effector function of a component of the AP1 transcription complex, *i.e.,* JUNB, the expression of which is strongly induced by TGFβ in breast cancer cells. We found that JUNB is required for the expression of many late invasion-mediating genes, and in particular, signaling components of the WNT pathway are induced. WNT7B was shown to potentiate TGFβ-induced breast cancer cell invasion, creating a feed-forward regulatory network. Intriguingly, we found enhanced MAPK/ERK activation in cells stably expressing WNT7B upon TGFβ stimulation. Thus, linking these results to those in **Chapter 4**, the question can be raised as to whether the WNT7B or WNT pathway can create a feed-forward regulatory network that is also involved in sustained MAPK/ERK activation and BMP signaling inhibition in response to TGFβ stimulation.

Cancer immunotherapy is emerging as an efficient cancer treatment that improves the prognosis of patients with a broad variety of hematological and solid malignancies [5, 6]. Specifically, the application of immune checkpoint inhibitors (ICIs) was shown to boost the immune system and eradicate cancer cells of patients [6]. However, only a few cancer patients respond to ICIs therapy (less than 10-15%). TGFβ is a potent immune suppressor within the tumor microenvironment, and recent studies have revealed roles of TGFβ in tumor immune evasion and poor responses to cancer immunotherapy. TGFβ also regulates the generation and effector functions of many immune cell types [7]. Importantly, TGFβ-activated CAFs are the main determinant for ICIs failure in colorectal and metastatic urothelial cancer [8, 9]. Thus, targeting TGFβ pathway inhibition represents an attractive strategy to enhance immune checkpoint blockade. Our key finding, detailed in **Chapter 6**, is that tumor immunogenicity is a dominant feature predicting responsiveness to dual inhibition of TGFβ signaling and PD-L1. In an immunogenic MC38 tumor model, inhibition of TGFβ signaling further improved the overall survival induced by anti-PD-L1 mAb treatment. The antitumor activity of the combination treatment is associated with higher levels of $CD8^+$ T cells infiltration in tumors than were seen in tumors treated with either agent as a monotherapy. However, an enhancement effect of combination treatment was not observed in the poorly immunogenic KPC1 tumor model. As mentioned, TGFβ-induced activation of CAFs can work as a physical stromal barrier and prevent immune cell infiltration. We found that Grem1 is a contributor of CAFs activation, as detailed in **Chapter 3**. We are therefore keen to explore whether a Grem1-neutralizing antibody can improve the treatment efficiency of ICIs in immune-excluded and immune-desert tumors.

Overall, our studies elucidated the possibility of manipulating BMP/TGFβ signaling to achieve inhibition of breast cancer metastasis, including boosting BMP signaling via blockade of the BMP antagonist Grem1 extracellularly or via stimulation of small-molecule compounds intracellularly, preventing TGFβ signaling to allow accumulation of pro-oncogenic stimuli. We also highlight the importance of selecting appropriate cancer types when adopting dual inhibition of PD-L1 and TGFβ signaling. I hope my research will aid in more efficient clinical cancer therapies.

References

1. Konantz M, Balci TB, Hartwig UF, *et al*. Zebrafish xenografts as a tool for in vivo studies on human cancer. Ann N Y Acad Sci 2012, 1266(1):124-37.

2. Mizgirev I, Revskoy S. Generation of clonal zebrafish lines and transplantable hepatic tumors. Nat Protoc 2010, 5(3): 383-94.
3. White RM, Sessa A, Burke C, B *et al*. Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2008 , 2(2):183-9.
4. Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002, 248(2):307-18.
5. Dance A. Cancer immunotherapy comes of age. Science 2017, 355(6330):1220-2.
6. Baumeister SH, Freeman GJ, Dranoff G, *et al*. Coinhibitory pathways in immunotherapy for cancer. Annu Rev Immunol 2016, 34:539-73.
7. Ganesh K, Massagué J. TGFβ inhibition and immunotherapy: Checkmate. Immunity 2018, 48(4):626-8.
8. Mariathasan S, Turley SJ, Nickles D, *et al*. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. Nature 2018, 554(7693):544-8.
9. Tauriello DVF, Palomo-Ponce S, Stork D, *et al*. TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis. Nature 2018, 554(7693):538-43.