Endoglin and the immune system: immunomodulation and therapeutic opportunities for cancer

Schoonderwoerd, M.J.A.

8

General discussion

SUMMARY OF MAIN OBSERVATIONS

Although the role of endothelial endoglin expression has been extensively been studied and related to tumor progression, the role of endoglin expression on non-endothelial cells has only recently drawn significant attention. The role of endoglin beyond the endothelium is the central theme of the studies described in this thesis. The current scientific view of endoglin beyond the endothelium is summarized in **Chapter 2.** Reviewing recent literature on the role of non-endothelial endoglin expression revealed that this is still unclear and that many contradictory results have been published. However, endoglin seems to be expressed particularly, but not exclusively, on cells sensitive to Transforming Growth Factor β (TGF-β), like fibroblasts, monocytes, regulatory T cells, and some tumor cells. **Chapter 3** shows that endoglin is expressed on Cancer Associated Fibroblasts (CAFs) at the invasive front of colorectal cancer (CRC) tissues, where it regulates invasion and stimulates tumor metastasis. Surprisingly, we found an opposite role for endoglin expression on fibroblasts in the early stages of CRC carcinogenesis as shown in **Chapter 4**. Fibroblast specific deletion of endoglin led to an increased number of colonic adenomas. Interestingly, this was accompanied by altered myeloid responses within the intestine. To further investigate the endoglin expressing CAFs in a more advanced tumor model, a pancreatic cancer mouse model was used in **Chapter 5.** These data showed that targeting of endoglin expressing cells in the tumor microenvironment does not inhibit tumor growth. To further explore the crosstalk between endoglin and immunomodulatory molecules, we investigated a novel therapeutic strategy by targeting endoglin with the endoglin neutralizing TRC105 combined with an antibody against the immune checkpoint inhibitor programmed cell death (PD)-1 in **Chapter 6**. In addition to increased therapeutic efficiency, these data also revealed an endoglin expressing subset of Tregs in the tumor microenvironment, which can be targeted by the endoglin neutralizing antibody TRC105. Targeting endoglin-expressing Tregs enhanced the effect of PD1 checkpoint inhibitor immunotherapy. Finally, in **Chapter 7**, we investigated the role of the tumor-draining lymph nodes during PD-1/PD-L1 checkpoint inhibitor therapy. These data revealed that tumor draining lymph nodes play a pivotal role during immunotherapy, strengthening the current view on the application of neoadjuvant immunotherapy in cancer treatment.

Endoglin

Endoglin was initially identified in 1985, expressed on a pre-B leukemia cell line [1]. Most later studies, however, were almost exclusively focused on the role of endoglin in angiogenesis. Endoglin is highly expressed by activated endothelial cells and plays a crucial role in (developmental) angiogenesis [2]. Loss of endoglin in mice results in an embryonic lethal phenotype around embryonic day 10.5, due to impaired

vascular and cardiac development [3, 4]. Endoglin is a homodimeric transmembrane receptor composed of disulfide bond-linked subunits of 95 kDa [5]. Endoglin has a short cytoplasmic domain, which exposes its co-receptor function [6]. Therefore, it requires additional receptors to induce signaling. Activation of the activin receptor-like kinase (ALK)1 and ALK5 pathways leads to the downstream phosphorylation of the smad1/5/8 pathway, resulting in the transcription of distinctive target genes. Interestingly endothelial endoglin expression can be regulated by TGF-β, bone morphogenetic protein (BMP)-9 [7], and hypoxia [8]. As discussed in **Chapter 2**, more recent scientific work has shown endoglin expression on a variety of cells. Although studies of endoglin on cells beyond the endothelium have increasingly been published, contradictory results have been reported, which might partially be explained by the experimental setup and culture conditions. For example, in **Chapter 3**, we show that endoglin knockout in cultured fibroblasts results in a senescent-like phenotype implying that fibroblasts need endoglin to survive in cell culture. Furthermore, the vast majority of cultured fibroblasts express endoglin, whereas in normal tissue, endoglin expression is low to non-detectable. This suggests that endoglin is hard to study in cultured cells.

Endoglin, a negative regulator of the TGF-β pathway?

The main cell types that express endoglin are endothelial cells, pro-B-Cells, a subset of monocytes, regulatory T-cells (Tregs), keratinocytes, fibroblasts including CAFs, mesenchymal stromal cells (MSCs), and some epithelial cells. Many, if not all, of these cell types, are dependent on TGF-β for their differentiation or cell maintenance. As indicated above, endoglin expression in endothelial cells can be regulated by its ligands and hypoxia, whereas for other cells this has not yet been established. In endothelial cells endoglin has been reported to act as a negative regulator of the TGF-β/ALK5 pathway. Stimulation of the endoglin dependent/ALK1 signaling pathway, indirectly inhibits the TGF-β ALK5 signaling pathway, thereby stimulating endothelial cell proliferation [9]. Next to the endoglin expressing CAFs, in **Chapter 6** we show an endoglin expressing subset of Tregs. Interestingly, in contrast to the suppressive signal mediated by the TGF-β in T-cells, cross-linking of endoglin substantially enhanced T-cell proliferation, indicating that endoglin by itself mediates signal transduction via activation of ERK 1/2 leading to T-cell proliferation [10].

In CAFs, we have shown that inhibiting endoglin signaling prevents the invasive behavior of CAFs. Furthermore, targeting endoglin signaling resulted in decreased experimental liver metastasis in a mouse model, suggesting that endoglin contributes to the invasive behavior of CAFs. In humans, endoglin expressing CAFs were found to correlate with decreased metastasis-free survival in stage II CRC. Moreover, high levels of TGF-β in patients with colorectal cancer is associated with disease progression [11, 12]. Since targeting of these endoglin expressing CAFs resulted in decreased

metastasis formation, it seems likely that endoglin does not have a negative feedback function in CAFs as shown in other cells. Another hypothesis is that since TGF-β and hypoxia regulate endoglin, the observed decrease in metastasis-free survival is dependent on TGF-β and endoglin as a bystander effect. The exact role of endoglin on CAFs is still unknown and needs to be further investigated in the future.

MSCs as CAF precursors

CAFs are a key component of the tumor microenvironment (TME) with distinctive functions, including matrix deposition and remodeling, signaling interactions with cancer cells, and crosstalk with infiltrating leukocytes [13]. Some studies describe that CAFs can be derived from Mesenchymal Stem Cells (MSCs) [14, 15], which might explain the expression of endoglin on a subset of fibroblasts since endoglin is one of the criteria for defining MSCs [16]. MSC were first identified in the bone marrow and can differentiate into mesenchymal tissue such as bone, adipose tissue and cartilage. [17] More recent research has shown a possible role during inflammation, immune response, wound healing and cancer progression [17]. In our patient samples and mouse models, we observed a subset of, potentially MSC-derived, endoglin expressing CAFs. Previous work has been shown that a significant proportion of CAFs can be derived from the bone marrow [18]. Interestingly, these bone marrow derived fibroblasts expressed higher levels of TGF-β 1 [18]. Moreover, the activation of fibroblasts by TGF-β [19] family ligands promotes the activity of the smad transcription factors, which drives the expression of alpha Smooth Muscle Actine (αSMA), an activation marker of fibroblasts [20]. in **Chapter 3**, we describe that these αSMA positive endoglin positive CAFs are responsible for the metastasis of CRC tumor cells to the liver of the mice. Targeting these CAFs using TRC105 resulted in decreased formation of experimental metastasis in mice. This same reduction in metastatic formation was observed in breast cancer once TRC105 was administrated [21]. Assessing the effects of endoglin in tumor progression using TRC105 will not discriminate between endoglin targeting of CAFS, Endothelial cells or other cells in the TME. Therefore, we explored the effects of endoglin in a fibroblast specific endoglin knockout mouse in **Chapter 4**. Interestingly we found increased tumorigenesis when we genetically deleted endoglin from the fibroblasts. In early stage lesions at the end of the experiment, we observed an increase in the macrophages and neutrophils. This indicates that there is a role for endoglin expressing fibroblasts and immune cell recruitment in the bowel. However, our data showed that depleting neutrophils had no effect on the tumorigenesis and therefore that neutrophils seem not to be responsible for the increased tumorigenesis.
Interestingly, in the first Dextran Sulfate Sodium (DSS) cycle, we found decreased myeloid cells (CD11B+) both in the blood and the intestines. Especially the Ly6C population was significantly reduced upon fibroblast specific endoglin deletion. This

indicates a potential protective role for Ly6C+ population. Others have shown that once the Ly6C high population was introduced into the blood, it restored the DSS induced damage [18], indicating that these Ly6C cells are partially responsible for the intestinal integrity during DSS induces colitis. Further research is necessary to investigate the role of myeloid cells upon fibroblast specific endoglin deletion and increased lesion formation up on Azoxymethane (AOM) DSS induced tumorigenesis. Endoglin on fibroblasts might have a dual role like TGF-β, which acts in a tumor preventative manner during early tumorigenesis and a pro-tumorigenic manner in the late stages of tumor development and metastasis. However, the dual role of TGF-β for fibroblasts is yet to be determined.

Targeting CAFs

CAFs are one of the most abundant cell types in the TME and are thought to have a prominent role in cancer pathogenesis. Mechanistically, CAFs secrete cytokines, chemokines and growth factors and are responsible for Extracellular Matrix (ECM) remodeling enabling cancer cells to invade through the TME [22]. Therefore, CAFs have been an obvious target in solid tumors and extensively studied. As described above, αSMA is a marker for a CAF subset called myofibroblast like CAFs (myCAF). Depletion of all αSMA positive myofibroblasts in a genetic Pancreatic ductal adenocarcinoma (PDAC) mouse model resulted, increased aggressiveness of the tumors, an influx of Tregs, increased epithelial–mesenchymal transition (EMT) marker expression and increased stemness of the pancreatic cancer cells. This resulted in enhanced tumor progression and subsequently reduced mouse survival [23]. Both in CRC and PDAC, high endoglin expressing CAFs were observed, targeting them in CRC reduced metastatic spread. However, in PDAC this did not seem to be the case. In vitro, CAFs isolated from both human and mouse pancreatic tumors showed high endoglin expression both *in-vitro* as *in-vivo*. Once targeted with TRC105, we could not detect any significant differences in αSMA expressing cells. Furthermore, no differences were found in tumor growth and immune influx of mouse bearing pancreatic tumors. Changes in immune cells were observed in CRC upon targeting with TRC105 (**Chapter 6)**. This striking difference between these 2 tumor types, both displaying high endoglin expressing cells might be explained the tumor mutational burden which is higher in the MC38 (CRC) tumor cells compared to the pancreatic tumor cells (KPC-3). This mutational burden leads to more immunogenic antigens that can in turn lead to immune influx and responsiveness to immunotherapy. Therefore, since TRC105 did not improved survival in mice, this needs to be further investigated. As shown in the **Chapter 6**, especially the Fc receptors play an important role in the efficacy of TRC105 in CRC, which was not determined in the pancreatic models. Next to endoglin targeting by TRC105, we used a fibroblast specific endoglin knockout mouse to investigate fibroblast specific endoglin deletion.

This fibroblast-specific endoglin deletion did not affect tumor volume and the cytokine profile in the tumor, suggesting that endoglin expressing CAFs do not contribute to the development and progression of pancreatic cancer. Interestingly others have shown that the depletion of a subset of CAFs expressing Fibroblast Activated Protein (FAP) effectively inhibits pancreatic tumor growth [24]. The depletion of the FAP-expressing cells also increased the anti-tumor effects of α-CTLA-4 and α-PD-L1, indicating that FAP positive cells can cause immune suppression. This indicates that targeting CAF subsets can considerably enhance anti-tumor responses. It remains to be elucidated why targeting of the abundantly present endoglin expressing subsets does not affect tumor growth in PDAC. In addition to targeting CAFs other possible interventions are altering CAF activation or function, CAF normalization, and ECM normalization [25]. The diverse function of CAFs and the interconvertibility of subtypes presents a challenge for CAF targeting agents. Many of the CAF targeting therapies are now undergoing clinical testing in phase I, II and III trials. However, none of the CAF targeted therapies has yet been approved for clinical use [26, 27].

Immune modulation

Immune modulation in cancer refers to a range of therapies aimed to eradicate cancer by using the immune system. There are multiple types of immune modulation, of which the immune checkpoint inhibitors are the most successful and widely accepted in the clinic. they belong to one of the most promising cancer therapies of the 21st century. Reactivating the immune system is an essential tool to target tumors that are not responding to conventional treatment. Currently, a hand full of antibodies have been approved for clinical use. Some of them target the PD-1/PD-L1 interactions and are currently approved to treat melanoma and lung cancer patients. **Chapter 7** describes the essential contribution of the tumor-draining lymph nodes (TDLNs) to therapy efficiency of immunotherapy in mouse models. Once the TDLNs are removed, mice fail to respond to therapy. Interestingly, others have shown that these TDLNs were responsible for enhanced anti-tumor T cell immunity by seeding the tumor with progenitor T cells resulting in improved tumor control [28]. In **Chapter 6** we have shown that TRC105, combined with anti-PD-1, increases the number and activation of T-cells, significantly enhancing the survival of mice induced with colorectal cancer. Interestingly, we observed a significant decrease in the percentage of Tregs accompanied by an influx of CD8+ T-cells. When we investigated endoglin expression on Tregs, we found a subset of endoglin expressing Tregs, signifying that TRC105 can directly bind Tregs and could induce antibody-dependent cellular cytotoxicity (ADCC) in mouse models for CRC. Decreased Tregs were also observed in the blood of patients treated with TRC105 [29].

Taken together, this suggests that TRC105 is not only an anti-angiogenic antibody but possibly also acts as an immunoregulatory antibody by targeting the Tregs within the TME of CRC tumors. With increasing knowledge of endoglin expression beyond the endothelium, it might be that endoglin targeting directly targets other cell types. In cancer, TRC105 has been clinically evaluated. Although encouraging results have been published [30], a recent phase III trial in angiosarcomas (TAPPAS trial) showed no differences in the progression-free survival between the standard of care Votrient and a combination of Votrient and TRC105 in advanced angiosarcoma. Further research is needed to gain more knowledge of responders and non-responders to therapy. Liu and Paauwe et al. [30] give an exciting overview of pre-clinical and clinical targeting of endoglin.
Remarkably, some tumor cells might be directly targeted with TRC105 since many reports have shown endoglin expressing tumor cells. Although endoglin's role on tumor cells is under debate and might be tumor type specific, targeting with TRC105 might induce ADCC in tumor cells. Supporting data has been found in urothelial sarcoma patients treated with TRC105 in which a decrease in circulating tumor cells (CTCs) was observed [29]. Several reports describe the loss of epithelial endoglin to be pro tumorigenic [31, 32]. In contrast, other reports have described a pro-tumorigenic role for endoglin expression on epithelial cancer cells [33][34][35]. As demonstrated, the role of endoglin is not fully understood and needs to be further investigated.

In conclusion, the role of endoglin on CAFs might depend on the stage of the tumor, acting in an anti-tumor manner in the developmental stage of cancer and pro-tumor manner during the metastatic process. Targeting endoglin has shown promising results hampering angiogenesis, metastatic spread, and acting as an immunoregulatory antibody in pre-clinical models. Although TRC105 failed to prove efficacious in a phase III study, the novel approach of targeting endoglin has the potential to become a valuable cancer treatment strategy by targeting multiple cell types that contribute to the TME.

REFERENCES

1. Quackenbush, E.J. and M. Letarte, *Identification of several cell surface proteins of non-T, non-B acute lymphoblastic leukemia by using monoclonal antibodies.* J Immunol, 1985. **134**(2): p. 1276-85.

2. Wikstrom, P., et al., *Endoglin (CD105) is expressed on immature blood vessels and is a marker for survival in prostate cancer.* Prostate, 2002. **51**(4): p. 268-75.

3. Arthur, H.M., et al., *Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development.* Dev Biol, 2000. **217**(1): p. 42-53.

4. Goumans, M.J. and P. Ten Dijke, *TGF-beta Signaling in Control of Cardiovascular Function.* Cold Spring Harb Perspect Biol, 2018. **10**(2).

5. Gougos, A. and M. Letarte, *Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line.* J Immunol, 1988. **141**(6): p. 1925-33.

6. Gougos, A. and M. Letarte, *Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells.* J Biol Chem, 1990. **265**(15): p. 8361-4.

7. Scharpfenecker, M., et al., *BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis.* J Cell Sci, 2007. **120**(Pt 6): p. 964-72.

8. Sanchez-Elsner, T., et al., *Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways.* J Biol Chem, 2002. **277**(46): p. 43799-808.

9. Lebrin, F., et al., *Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction.* EMBO J, 2004. **23**(20): p. 4018-28.

10. Schmidt-Weber, C.B., et al., *TGF-{beta} signaling of human T cells is modulated by the ancillary TGF-{beta} receptor endoglin.* Int Immunol, 2005. **17**(7): p. 921-30.

11. Friedman, E., et al., *High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer.* Cancer Epidemiol Biomarkers Prev, 1995. **4**(5): p. 549-54.

12. Tsushima, H., et al., *High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression.* Gastroenterology, 1996. **110**(2): p. 375-82.

13. An, Y., et al., *Crosstalk between cancer-associated fibroblasts and immune cells in cancer.* J Cell Mol Med, 2020. **24**(1): p. 13-24.

14. Karnoub, A.E., et al., *Mesenchymal stem cells within tumour stroma promote breast cancer metastasis.* Nature, 2007. **449**(7162): p. 557-63.

15. Raz, Y., et al., *Bone marrow-derived fibroblasts are a functionally distinct stromal cell population in breast cancer.* J Exp Med, 2018. **215**(12): p. 3075-3093.

16. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.

17. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-7.

18. Quante, M., et al., *Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth.* Cancer Cell, 2011. **19**(2): p. 257-72.

19. Foster, C.T., F. Gualdrini, and R. Treisman, *Mutual dependence of the MRTF-SRF and YAP-TEAD pathways in cancer-associated fibroblasts is indirect and mediated by cytoskeletal dynamics.* Genes Dev, 2017. **31**(23-24): p. 2361-2375.

20. Tomasek, J.J., et al., *Myofibroblasts and mechano-regulation of connective tissue remodelling.* Nat Rev Mol Cell Biol, 2002. **3**(5): p. 349-63.

21. Paauwe, M., et al., *Endoglin targeting inhibits tumor angiogenesis and metastatic spread in breast cancer.* Oncogene, 2016. **35**(31): p. 4069-79.

22. Gaggioli, C., et al., *Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells.* Nat Cell Biol, 2007. **9**(12): p. 1392-400.

23. Ozdemir, B.C., et al., *Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival.* Cancer Cell, 2014. **25**(6): p. 719-34.

24. Feig, C., et al., *Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer.* Proc Natl Acad Sci U S A, 2013. **110**(50): p. 20212-7.

25. Sahai, E., et al., *A framework for advancing our understanding of cancer-associated fibroblasts.* Nat Rev Cancer, 2020. **20**(3): p. 174-186.

26. Chen, X. and E. Song, *Turning foes to friends: targeting cancer-associated fibroblasts.* Nat Rev Drug Discov, 2019. **18**(2): p. 99-115.

27. Dang, H., T.J. Harryvan, and L. Hawinkels, *Fibroblast Subsets in Intestinal Homeostasis, Carcinogenesis, Tumor Progression, and Metastasis.* Cancers (Basel), 2021. **13**(2).

28. Dammeijer, F., et al., *The PD-1/PD-L1-Checkpoint Restrains T cell Immunity in Tumor-Draining Lymph Nodes.* Cancer Cell, 2020. **38**(5): p. 685-700 e8.

29. Apolo, A.B., et al., *A Phase II Clinical Trial of TRC105 (Anti-Endoglin Antibody) in Adults With Advanced/Metastatic Urothelial Carcinoma.* Clin Genitourin Cancer, 2017. **15**(1): p. 77-85.

30. Liu, Y., et al., *Endoglin Targeting: Lessons Learned and Questions That Remain.* Int J Mol Sci, 2020. **22**(1).

31. Liu, Y., et al., *Over expression of endoglin in human prostate cancer suppresses cell detachment, migration and invasion.* Oncogene, 2002. **21**(54): p. 8272-81.

32. Lakshman, M., et al., *Endoglin suppresses human prostate cancer metastasis.* Clin Exp Metastasis, 2011. **28**(1): p. 39-53.

33. Li, Y., et al., *CD105 promotes hepatocarcinoma cell invasion and metastasis through VEGF.* Tumour Biol, 2015. **36**(2): p. 737-45.

34. Zhang, J., et al., *Human epithelial ovarian cancer cells expressing CD105, CD44 and CD106 surface markers exhibit increased invasive capacity and drug resistance.* Oncol Lett, 2019. **17**(6): p. 5351-5360.

35. Hu, J., et al., *Cancer Stem Cell Marker Endoglin (CD105) Induces Epithelial Mesenchymal Transition (EMT) but Not Metastasis in Clear Cell Renal Cell Carcinoma.* Stem Cells Int, 2019. **2019**: p. 9060152.