Cell-cell interactions in the gastrointestinal tumour-microenvironment

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Chapter 11

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1 Summary main observations

The general purpose of the studies described in this thesis was to evaluate how cell-cell interactions within the gastrointestinal tumour-microenvironment contribute to the progression of tumours. Based on clinical data from gastrointestinal cancer patients, we developed different cell-culture models to resemble, as closely as possible, the human situation. Using these models we examined the interaction between tumour cells, tumour-associated myofibroblasts, endothelial cells, inflammatory cells and the extracellular matrix (ECM), together creating the tumour-microenvironment. We showed that these interactions are crucial for two major processes associated with cancer progression, the accumulation of myofibroblasts and the regulation of angiogenesis. The major interactions studied in this thesis are summarised in figure 1.

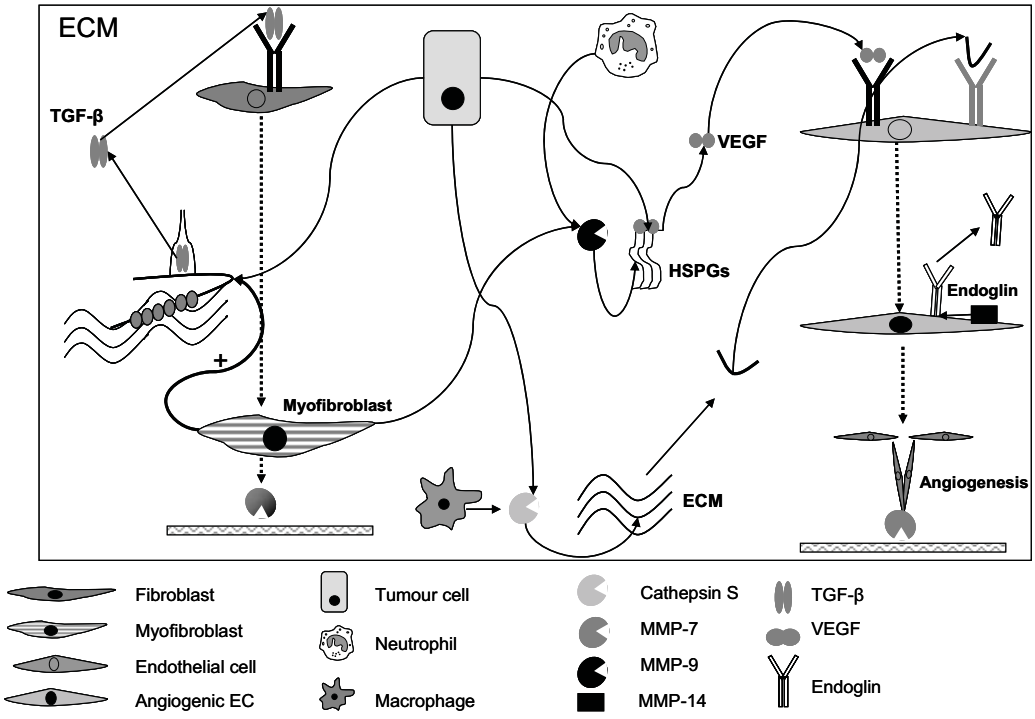

Figure 1. Cell-cell-ECM interaction within the gastrointestinal tumour-microenvironment. Myofibroblasts are generated by interaction between tumour cells and fibroblasts by activation of TGF-β1. In turn myofibroblasts secrete high amounts of TGF-β and invasion related proteinases, including MMP-9. MMP-9 is capable of cleaving ECM localised HSPGs releasing VEGF-165. However, the majority of MMP-9 required for initiating the angiogenic switch is retrieved from tumour-infiltrating neutrophils. VEGF and cathepsin S-released pro-angiogenic ECM molecules bind to quiescent endothelial cells turning them into angiogenic endothelial cells expressing Endoglin. MMP-7 is upregulated in angiogenic endothelial cells facilitating matrix degradation and subsequent invasion and sprout formation. Membrane-bound endothelial MMP-14 is the most appropriate candidate for shedding endothelial Endoglin into the circulation, thereby regulating angiogenesis.

2 Tumour-cell – fibroblast interactions; TGF-β activation and generation of myofibroblasts

Already in 1986 Dvorak *et al.*[1] described that tumours resemble "wounds that do not heal" indicating that the interaction of cells within the tumour-microenvironment has strong similarities to a chronic wound healing process. One of the cell types involved in both processes is the myofibroblast[2], a hyper-activated fibroblast displaying both vimentin and smooth muscle actin (SMA) expression[3]. The numbers of myofibroblasts are low in normal tissue except during wound healing and fibrosis, where myofibroblasts play a major role in the deposition of ECM components like collagen type-I[4]. When we evaluated gastric and colorectal cancers we observed strongly increased numbers of myofibroblasts being present in tumours compared to normal tissue (**chapter 3 and 4**). This increase is not yet present in pre-malignant colorectal adenomas, indicating the importance of cancer-associated myofibroblast in the malignant progression of tumours. The origin of myofibroblasts in colorectal cancer is still a subject of many investigations[3,5-9]. Cancer-associated myofibroblasts probably arise from the interaction between (pre-)malignant epithelial cells and surrounding tissue fibroblasts via direct cell-cell contact through membrane bound proteins like EMMPRIN [10], but also by secretion and activation of soluble growth factors like TGF-β. When we analysed TGF-β1 levels in tissue homogenates we observed that endogenously active TGF-β1 levels correlated with the SMA content in these homogenates, indicating a prominent role for TGF-β1 in the generation of cancer-associated myofibroblasts.

TGF-β has a dual role in cancer because it is tumour-suppressive in the pre-malignant stage and tumour-promoting in later stages[11,12]. This is likely attributable to the switch of TGF-β signalling from epithelial cells in normal tissue to increased mesenchymal signalling in cancers as we have shown in **chapter 3 and 5**. In contrast to normal tissue myofibroblasts, cancer-associated fibroblasts display increased TGF-β signalling. This process involves the activation of the latent TGF-β1 complex, which is the crucial regulation mechanism for TGF-β1 activity[13]. Other studies on TGF-β have mostly focussed on total TGF-β levels in clinical samples, including both latent ECM-bound TGF-β and the endogenously active TGF-β[14, 15]. In contrast, we have shown that primarily active TGF-β1 levels are related to survival of both gastric and colorectal cancer patients (**chapter 3 and 4**). Upregulation of synthesis of the latent TGF-β1 complex is already seen early in the normal-adenoma-carcinoma sequence, however, increased activation is solely present in carcinomas, indicating that *over-activation* rather than *over-expression* of TGF-β1 enables transition from benign to malignant tumours.

TGF-β activation is mediated by the interaction between tumour cells and fibroblasts (**chapter 5**). In contrast to previous observations in the literature[13,16] and our expectations, the activation of tumour cell-derived large latent TGF-β1 by tumour cells interacting with cancer-associated fibroblasts, does not seem to involve a proteolytic activation cascade in our model. Rather the minor amounts active TGF-β, secreted and possibly proteolytically activated at the tumour cell surface, mediate the first trans-differentiation of fibroblasts into myofibroblasts. Hereafter myofibroblasts probably use tumour cell-derived TGF-β, which is bound via LAP to ECM localised αvβ5 integrin. Binding of LAP to αvβ5 integrin has been described[17] and recently it was shown that myofibroblasts can use their contractile properties to non-proteolytically activate large latent TGF-β via binding to this integrin[18]. This could result in increased trans-differentiation of more myofibroblasts. Based on our data the TGF-β mediated trans-differentiation of resident fibroblasts accounts for the majority of the cancer-associated myofibroblasts and the interaction between tumour cells and fibroblasts is crucial in initiation of this process. In turn, myofibroblasts show increased secretion of TGF-β, MMPs, TIMPs and plasminogen activation system components. The strong upregulation of TGF-β in myofibroblasts both on the RNA as well as the protein level, together with upregulation of invasion and growth factor releasing related proteinases, creates a cancer enhancing feedback loop.

Chapter 5 further revealed that next to myofibroblasts also HT29 and HCT116 tumour cells are responsive to TGF-β1, but show no growth inhibition by TGF-β1. Moreover, the interaction between fibroblasts and tumour cells enhances TGF-β signalling in tumour cells, which increases the synthesis and secretion of proteolytic enzymes. Normal and pre-malignant epithelial cells are growth inhibited by TGF-β treatment, but in cancers these cells are often refractory to growth inhibiting properties by TGF-β. This might partly be due to epithelial TGF-β RI or RII mutations as observed in colorectal cancer[19], but also result from mutations in downstream TGF-β signalling molecules like Smad-4[20]. These mutations might only effect the epithelial cells and not the stromal cells which particularly show increased TGF-β signalling in colorectal cancer specimens. However, staining for nuclear accumulation of p-Smad-2 illustrated that in the majority of the tumours epithelial Smad-dependent TGF-β signalling was reduced. The tumour-promoting effects of TGF-β on epithelial cells could also involve Smad-independent pathways[19,21]. Many colon cancer cells like HT29 do not express the Smad-4 protein, but nevertheless are still responsive to stimulation with high doses TGF-β1. Stimulation of HT29 cells resulted in upregulation of many proteolytic enzymes which consequently resulted in increased invasive growth and the formation of distant metastasis-

like cell clusters, when embedded in a stroma-like ECM environment. Taken together these data imply that the interaction between tumour cells and fibroblasts generates active TGF-β1 that not induces the trans-differentiation of fibroblasts into myofibroblasts, but also upregulates MMP secretion by tumour-cells and fibroblasts, illustrating a double paracrine interaction mechanism. Other studies revealed that myofibroblasts are indeed capable of inducing invasiveness of cancer cells via upregulation of MMPs and uPA[22] or through hepatocyte growth factor/scatter factor dependent mechanisms[9,23].

Enhanced secretion of MMPs is associated with increased invasiveness of cancer cells, but more importantly also with the regulation of growth factor bioavailability **(Chapter 1)**. TGF-β enhances MMP-2 and MMP-9 secretion by myofibroblasts **(Chapter 5 and 6)**, which have often been associated with tumour angiogenesis through regulation of VEGF bioavailability. This results in another interaction within the tumour-microenvironment, i.e. with endothelial cells and thereby affecting angiogenesis.

3 Tumour- mesenchymal- endothelial cell interaction in angiogenesis

Tumour angiogenesis is of major importance for the outgrowth of tumours to provide them with nutrients and oxygen[24] and enabling tumour cells to enter the circulation and form distant metastasis[25]. When the tumour reaches the critical size of 1-2 mm the angiogenic switch occurs, resulting in the formation of its own vasculature system. The microvessel density (the number of blood vessels in tumours) is a strong prognostic parameter for the survival of cancer patients[26,27] and is analysed by specific markers on angiogenic endothelial cells, like CD34 and Endoglin[27,28]. We have shown that Endoglin levels in pre-malignant adenomas are comparable to normal mucosa, whereas in carcinomas strongly increased Endoglin levels were present (**chapter 10**). Interactions within the early malignant tumour-microenvironment are instrumental in the initiation of the angiogenic switch. Initiating factors include a hypoxic environment[24], infiltrating neutrophils[29] and the secretion of angiogenic growth factors by tumour cells[30]. For example TGF-β1, besides contributing directly to angiogenesis via Endoglin, has been shown to enhance secretion of VEGF[25,31] and in gastric cancer patients TGF-β1 levels in gastric cancers are correlated to VEGF expression[32]. In **chapter 6** we show that tumour cells produce high amounts of VEGF, the major angiogenic factor in vivo[24]. Secreted VEGF is bound to heparan sulphate proteoglycans (HSPGs) in the ECM *in vivo*[33]. Spheroids of HT29 cells represent a good model for the human *in vivo* situation as they produce ECM components, including HSPGs, which are able to bind growth factors like

TGF-β and VEGF[34]. In this way tumour cells are the first players in the network of interacting cell types. VEGF bound to HSPGs has no biological activity and processing of the HSPGs or VEGF protein is required. Therefore, the second player in the initiation of the angiogenic switch are the cells capable of secreting the proteolytic enzyme required for increasing VEGF bioavailability under given conditions; i.e., a non-vascularised hypoxic tumour environment. We have shown that MMP-9 is a major contributor of regulating VEGF bioavailability. Both neutrophils and myofibroblast show strong secretion of MMP-9. However, in our model, representing the initiation of the angiogenic switch, it seems that neutrophils are the major source of MMP-9 *in vivo*. This is probably caused by the fact that neutrophils contain MMP-9 in granules and active MMP-9 can be directly secreted upon a activation signal like a hypoxic environment. Besides MMP-9 neutrophils also contain MMP-8, which is, although to a lesser extent, also capable of releasing VEGF. In later stages, MMP-9 producing macrophages which are present at the periphery of the tumours could also contribute to VEGF release[29]. Furthermore myofibroblasts might release VEGF through secretion of MMP-2 and MMP-9 both capable of releasing VEGF if the appropriate proteolytic activators of MMP-2 and -9 are present. In addition myofibroblasts have been shown to secrete VEGF and TGF-β, which is capable of inducing angiogenesis[35]. Finally, the third player in the complex interaction between cell types regulating tumour angiogenesis is the effector cell (endothelium). To study the angiogenic switch we used 3-dimensional cultured HUVEC endothelial cells embedded in a collagen type 1 matrix. Under these conditions HUVECs represent the non-activated stage. However, in the presence of angiogenic stimuli cells enter the initiation phase of angiogenesis, followed by the resolution phase and the formation of a lumen[36]. Sprout formation requires proteolytic activity[37], and as we have shown in **chapter 7**, MMP-7 is expressed by sprouting endothelial cells. MMP-7 has previously been described to be an "epithelial" MMP, contributing to cancer progression for example by its capacity to cleave VE-cadherin from endothelial cells enhancing their proliferation[38]. In addition, MMP-7 can also cleave E-cadherin from epithelial cells, which is one of the hallmarks of epithelial cells undergoing epithelial to mesenchymal transition (EMT). EMT is a process in which epithelial cells lose epithelial markers en gain mesenchymal markers, like vimentin and smooth muscle actin. This process is thought to contribute to both metastasis of cancer and the generation of part of the myofibroblast population. Furthermore, it was shown that expression of MMP-7 regulates VEGF release from fibroblast ECM[39] and might enhance myofibroblast differentiation[40]. Our observation that endothelial MMP-7 also plays a role in angiogenic processes might render it as a new therapeutic target.

Using the bio-activity assay as described in **chapter 8,** a pilot study on colorectal (pre-) malignancies revealed that pro-MMP-7 is already upregulated in pre-malignant colorectal adenomas, whereas upregulation of MMP-7 activity is merely seen in carcinomas. This is the same phenomenon as we observed for TGF-β1 activity and further emphasizes that upregulation of protein expression can occur early in the development of carcinomas, but increased activation is merely seen in carcinomas, and therefore the activating proteins might be attractive therapeutic candidates.

To study possible therapeutic targets within the tumour-microenvironment it is important to take the human microenvironment into account. In **chapter 6** we have shown that MMP-9 mediated VEGF release involves cleavage of HSPGs, releasing VEGF-165, the most potent isoform capable of inducing angiogenesis[41]. This is in contrast to what has previously been shown for mouse VEGF-164 which is cleaved by human MMP-9 to a smaller isoform instead of being released[42]. This clearly illustrates the importance of studying cell-cell interactions within the human tumour-microenvironment. Recent advancements in the development of model systems, using human 3-dimensional endothelial spheroids and grafting them into mice[43] to create the complexity of a living organism, is a valuable contribution to study interactions in the tumour-microenvironment.

Tumour angiogenesis is not solely dependent on cell-cell interactions involving VEGF release. We have shown in **chapter 9** that cathepsins S releases pro-angiogenic molecules from the ECM. Cathepsin S in tumours is mainly produced by tumour cells and cancer-associated macrophages[44]. Interestingly, we observed increased cathepsin S secretion when colon cancer cells are cultured as spheroids, compared to monolayer conditions (Figure 2A).

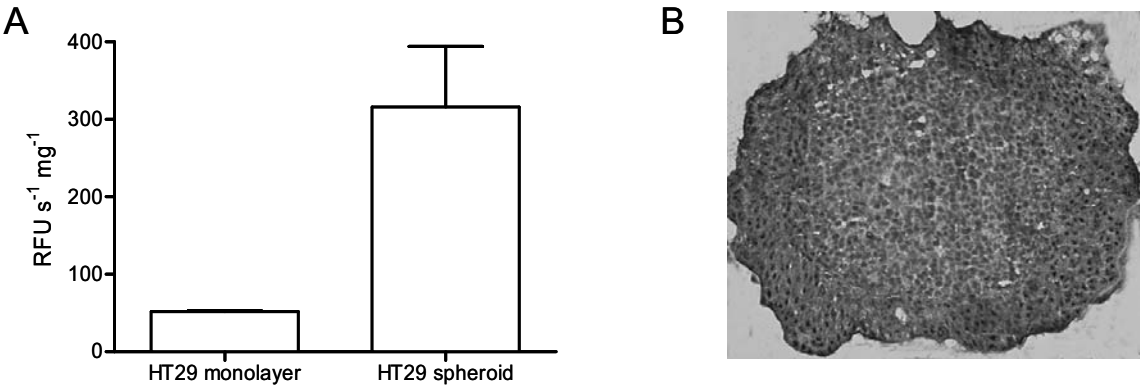

Figure 2. Cathepsin S activity in HT29 homogenates cultured under monolayer or spheroid conditions (A). Staining for laminin on HT29 spheroids (B). Full colour illustration at page 201.

It has been described that cathepsins S is capable of cleaving laminin-5, one of the major constituents of the ECM and also produced by HT29 cells[45] and unpublished data (Figure 2B). One of the proteolytically generated laminin-5 subchains, the γ-2 fragment has been shown to have angiogenic properties[44]. This implies that in the tumour-microenvironment macrophages and/or tumour cells secrete cathepsins S which could generate laminin-5 γ2 fragments from the ECM, which could in turn induce endothelial sprouting.

Equally important to regulation of angiogenesis by the presence of angiogenic factors equally important is the presence of appropriate receptors on the target cells. TGF-β is capable of inducing angiogenesis via binding to the TGF-β co-receptor Endoglin[35]. Besides membrane bound also a soluble form of Endoglin (sEndoglin) is detected in the circulation. Soluble receptors, like Endoglin and the soluble VEGF receptor, can still bind their ligands and can therefore act anti-angiogenic by scavaging pro-angiogenic factors. Several studies addressed the levels of soluble Endoglin in pathologic conditions, but are not conclusive[46-51]. We observed lower circulating sEndoglin in colorectal cancer patients levels pre-operatively, (**chapter 10**) which increased to reach levels observed in healthy controls after operation. This phenomenon might correspond to an anti-angiogenic role of sEndoglin in colorectal cancer. Furthermore, we evaluated the potential role of MMPs in the generation of this sEndoglin and show that membrane type-1 MMP (MMP-14) is the most likely candidate to mediate the cleavage of Endoglin to generate the soluble form. This indicates a possible tumour-suppressive role for MMP-14 besides its described pro-tumourogenic effects like activation of MMP-2[52] and a role vascular tubulogenesis[53]. In contrast, shedding of Endoglin from non-endothelial cells might result in a highly invasive phenotype as shown for mouse keratinocytes[54]. In human tumours MMP-14 is also highly expressed by epithelial cells and fibroblasts in malignant transformed tumours[55,56]. We also observed Endoglin expression in the normal to tumour transition zone (**chapter 10**). Further studies are required to examine the role of Endoglin and the role of epithelial or fibroblast-associated MMP-14 in shedding of non-endothelial Endoglin and elucidating another network of cell-cell interactions in the gastrointestinal tumour-microenvironment.

4 Clinical implications and perspectives of targeting the tumour-microenvironment

In this thesis studies are described on the interactions of cell-types within the tumour-microenvironment, focussing on four key players: MMPs, cathepsin S, VEGF and TGF-β in

two major processes: the generation of myofibroblasts and tumour angiogenesis. We have shown that several MMPs and cathepsins S contribute to invasive and angiogenic processes in the initiation and progression of carcinoma. Therefore, the development of therapeutic applicable inhibitors for cathepsins S, MMP-9 or MMP-7 might pose new opportunities to inhibit tumour angiogenesis. However, further studies are required to exactly evaluate the role of these proteinases in tumour-suppressive processes like the degradation of angiogenic molecules[57] or the generation of anti-angiogenic molecules[58]. Previous clinical trails with broad spectrum MMP inhibitors did not show therapeutic effects on cancer progression[59]. This might partly be explained by the fact that these inhibitors were broad spectrum MMP inhibitors inhibiting both the tumour-promoting effect of a subset of MMPs, but also impairing the tumour-suppressing effects generated by several MMPs. Furthermore, studies showing effectiveness of MMP inhibitors were mainly based on *in vitro* studies and animal models. Mouse models do not have to represent the human situation[60], as we have shown for the mechanism of VEGF release. This illustrates that both simple and complex *in vitro* systems together with animal models should be used to evaluate biological processes and therapeutic application of inhibitors[61]. Clinical MMP inhibition should therefore be examined in a human setting, taken the tumour-microenvironment, including activators, docking molecules and inhibitors, into account[59]. Next to that, the selection of specific inhibitors for certain proteinases might be a challenge. Most MMPs and cathepsins have similar catalytic clefts. Using the phage display technique as described in **chapter 9** it might be easier to bias selection of specific proteinases inhibitors towards specific and functionally active inhibitors.

Besides inhibition of MMPs or other cancer-related proteinases another therapeutic approach could be to use the proteolytic activity to deliver pro-drug constructs which are locally activated by the increased proteolytic activity in the tumour, more specifically targeting tumour cells and create high intra-tumour concentrations of anti-cancer drugs. As we have shown, for example, MMP-7 activity is low in normal tissue and adenomas, whereas activation is strongly increased in carcinomas, also in tumour-associated endothelial cells. MMP-7 sensitive pro-drug constructs might therefore provide possible advantages above systemic delivery of anti-cancer drugs.

Finally targeting the tumour-microenvironment via direct inhibition of growth factors could offer therapeutical benefits. Clinical inhibition of angiogenesis by neutralizing the effect of VEGF is used for the treatment of CRC and still many new inhibiting antibodies and receptor kinase inhibitors are being developed[62-64]. Clinical trails have been initiated to inhibit TGF-β activity in pathological conditions, including fibrosis and cancer. Strategies include

neutralizing TGF-β activity by monoclonal antibodies, soluble TGF-β Receptors, or ALK-5 inhibitors[31]. Based on our data targeting specifically active TGF-β, rather than the latent TGF-β reservoir, or targeting activators of latent TGF-β might have therapeutic potential. However, TGF-β plays an important role in normal tissue homeostasis and acts as a tumour suppressor in early stages of cancer development. Therefore, the therapeutic inhibition of TGF-β should be carefully considered[65], for example, by stringent selection criteria for patients applicable to receive anti-TGF-β treatment.

5 Concluding remarks

The interplay between tumour cells, inflammatory cells and fibroblasts plays a major role in cancer progression by influencing myofibroblast trans-differentiation and inducing angiogenesis in gastrointestinal cancer. For the elucidation of these processes and the development of therapeutic agents, research should be focussed on the role of the tumour-microenvironment including cells, ECM, proteolytic enzymes and their activation cascades, using 3-dimensional cell culture models to study gastrointestinal carcinogenesis. The studies described in this thesis illustrated the importance of proteolytic regulation of VEGF and TGF-β bioavailability by specific cell-cell interactions in the tumour-microenvironment.

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