Cell-cell interactions in the gastrointestinal tumour-microenvironment

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Chapter 1

General introduction

1 Gastrointestinal cancer

Cancer is the second cause of death worldwide and shows increasing incidence during the last decades[1,2]. Among the different types of neoplasia, cancer of the colon and rectum (CRC) is the second most diagnosed cancer with according numbers of deaths in western Europe[2]. Hereditary CRC accounts for 5-10% of the CRC and includes HNPCC (Lynch syndrome) and familiar adenomatous polyposis (FAP)[3, 4]. Apart from familiar predisposition, the majority of the CRC arise from environmental factors (sporadic CRC). Risk factors include age, physical inactivity, consumption of red meat and smoking. The development of colorectal tumours consists of different steps (Figure 1). Adenomas (polyps) arise from normal colonic mucosa by (environmentally induced) genetic alternations in the mucosal epithelial cells, like mutation in the APC gene and K-ras[5]. Additional mutations lead to the development of *in situ* and eventually invasive cancers[6]. Tumours cannot exceed the size of 1-2 mm without acquiring its own vasculature system, a phenomenon known as the angiogenic switch. The vascular system provides the tumour with nutrients and oxygen, preventing tumour cell necrosis, leading to further outgrowth, invasion and eventually formation of distant metastasis (Figure 1). The primary treatment of CRC consists of surgical resection of the tumour combined with adjuvant chemotherapy or radiation[7, 8].

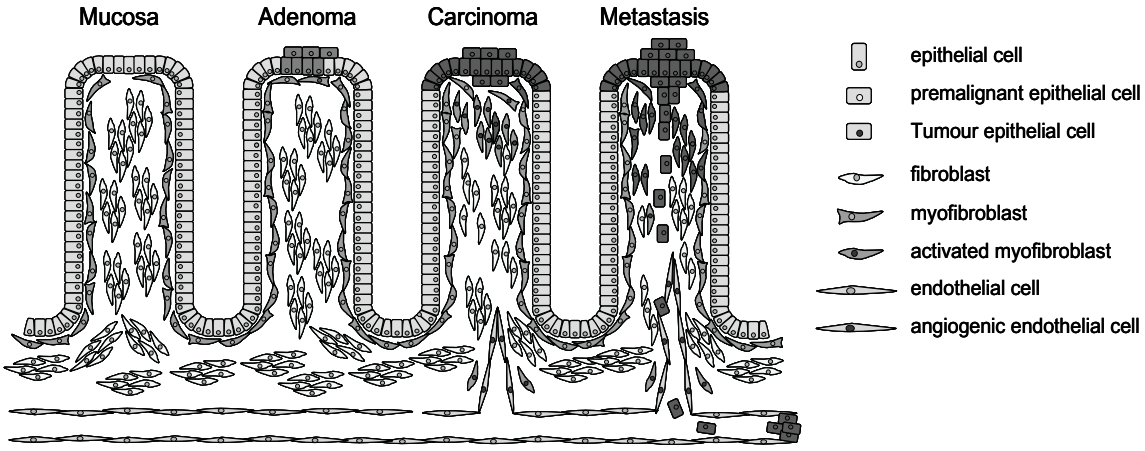

Figure 1. Schematic presentation of colorectal crypts showing different stages in the development of CRC. Hyper-proliferation of epithelial cells leads to the formation of pre-malignant colorectal adenomas, progressing into early carcinomas by additional mutations. When tumours reach a critical size, they acquire their own vasculature system by recruitment of endothelial cells, eventually leading to the formation of distant metastasis.

Gastric cancers show large geographic differences and about 5% is due to hereditary etiology[9]. Two major histopathological types are described by Laurén et al[10], an intestinal and a diffuse-type gastric cancer. Risk factors for developing gastric cancer include diet, cigarette smoking, alcohol consumption and *Helicobacter pylori* infection. Tumours arise from precursor lesions that include chronic atrophic gastritis, which can proceed into intestinal metaplasia[9]. Primary treatment for gastric carcinomas is resection of the tumour with or without adjuvant chemo- or radiation therapy.

For both CRC and gastric cancer the prognosis strongly depends on the stage of tumour at the time of diagnosis and decreases with increasing stage and the presence of lymph node or distant metastasis[2,7].

2 Cell-cell interactions in the tumour-microenvironment

Tumours consist of different cell types together creating the tumour-microenvironment. These cell types include tumour cells and the tumour stroma, consisting of surrounding fibroblasts and myofibroblast, tumour-associated endothelial cells creating the tumour's vasculature system and infiltrating immune cells, including macrophages, T-lymphocytes and neutrophils[11,12]. For a long time tumour cells have been the only focus of research, whereas the role of the tumour stroma was neglected. During the last decade more attention has been given to the role of the tissue stroma in the initiation, progression and metastasis of cancers[13-17]. All the distinct hallmarks in cancer, self-sufficiency in growth factors, insensitivity to anti-growth signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis[18], involve interactions between different cell types and the extracellular matrix (ECM) within the tumour-microenvironment. Not surprisingly, it was shown that the carcinoma-stromal ratio in CRC is an independent prognostic factor for survival[19]. This thesis focusses on interactions between tumour cells, fibroblasts, endothelial and inflammatory cells. Cross-talk between cells can occur via direct cell-cell contact or via soluble factors[12, 13, 16, 20, 21].

2.1 Cell-cell interactions via direct contact

Under normal condition cells interact via the formation of cell-cell and cell-matrix adhesions. Cadherins are Ca^{2+} dependent cell adhesion molecules, mediating cell-cell contact via their extracellular domains. Through their intracellular domain cadherins influence rearrangement of the cytoskeleton via β-catenin[22]. Besides interaction with catenins, cadherins also interact

with integrins, like αvβ3 integrin, and growth factor receptors like the TGF-β receptor II (TGF-βRII)[23,24]. Interactions between cells are required to maintain normal tissue morphology and function. In cancer these cell-cell adhesions change by rearrangement or excessive cleavage of adhesion molecules. Besides interaction of cells from similar origin (like epithelial interactions), cell-cell contact between tumour epithelial and stromal cells can influence many processes. One of the most well described molecules capable of doing so is extracellular matrix metalloproteinase inducer (EMMPRIN). EMMPRIN is a trans-membrane protein consisting of two immunoglobulin chains and a short cytoplasmic domain[25]. It is mainly expressed by epithelial cells and was shown to have an important role in cancer[26,27]. EMMPRIN expressed on malignant epithelial cells evokes the hyper-activation of neighbouring fibroblasts leading to enhanced MMP-2 expression[28,29]. Furthermore in gastric cancers a correlation was observed between EMMPRIN and the expression of VEGF, MMP-2 and MMP-9, indicating a clear relation with angiogenesis[30]. Recently a soluble form of EMMPRIN has been indentified, which is also capable of inducing MMP expression[31].

2.2 Cell-cell interactions involving soluble factors

Cells communicate via soluble factors like cytokines, chemokines and matrix-derived peptides. The extracellular matrix (ECM) of cancers is next to its supportive and nutritious functions also an excessive pool of growth factors, stimuli and chemoattractants that enable the tumour cells to grow, invade and metastasise. For instance, ECM-derived molecules like tenascin-C play a role in the recruitment of myofibroblasts[32], whereas tumour cell-derived monocyte chemoattractant protein (MCP)-1 mediates macrophage infiltration. In turn these cells secrete interleukin (IL)-8, capable of inducing angiogenesis in endothelial cells[33]. The expression of growth factors like vascular endothelial growth factor (VEGF) can be regulated via epithelial-stromal interactions[34,35]. The majority of the growth factors in the ECM are present in an inactive conformation and need processing or release. Within the tumour-microenvironment proteolytic enzymes, especially MMPs, play an important role in regulating growth factor bio-availability[36-40](Figure 2). For example, IL-8 can be activated through proteolytic processing via MMP-9[41], which results in a cascade of tumour cell-fibroblast-endothelial-inflammatory cell interactions. Another example is the initiation of the fibroblast angiogenic switch, regulated by tumour cell-derived MMP-7[42]. Next to proteolytic enzymes also integrins play a major role in activation of growth factors. Integrins consist of a group of 24 heterodimers and are involved in nearly all stages of cancer development.

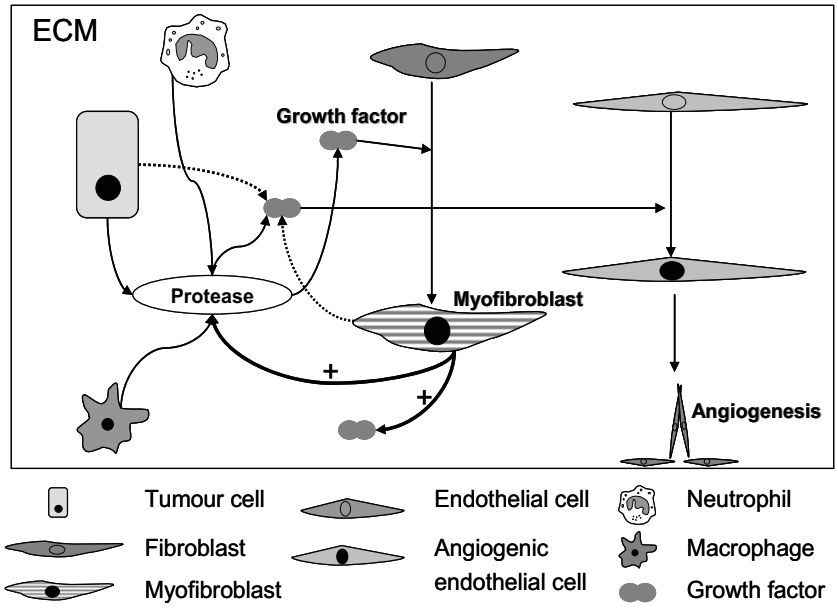

Figure 2. The tumour-microenvironment

Integrin αvβ3 and αvβ4 have been shown to be involved in regulating VEGF expression[43], and αvβ5, αvβ6 and αvβ8 integrin have been show to be involved in TGF-β binding and activation[44,45]. Besides activation of growth factors some integrins bind to MMPs[46] and interact with growth factor receptors like VEGF-Receptor-2[43,45].

Because of these important regulation mechanisms, targeting cell-cell interactions in the tumour-microenvironment is now considered as a promising candidate for therapeutic intervention[13,16,21,47]. This thesis focuses primarily on two major cell-cell interactions in the tumour-microenvironment: Tumour angiogenesis and the generation of myofibroblasts.

3 Angiogenesis

Angiogenic activity of endothelial cells is in general low, except during embryonic development, tissue repair after injury or pathological processes. The initiation phase of angiogenesis consists of endothelial cell activation, degradation of the basement membrane, subsequently followed by migration of endothelial cells. Then, in response to angiogenic stimuli, endothelial cells proliferate and differentiate to re-establish cell-cell contacts and form a lumen. Next is the so-called resolution phase in which the basement membrane is restored and the endothelial cells return to a non-activated state by inhibition of proliferation and vessel wall assembly[48,49]. Tumours depend on neo-vascularisation processes to provide oxygen and nutrients, allowing them to exceed a size of 1-2 mm[50,51].

In hypoxic environments tumour cells start secreting pro-angiogenic factors which results in endothelial branching (sprouting) towards the tumour[51]. Molecules capable of inducing endothelial sprouting are VEGF[48,50,52-54], bFGF, TGF-β[55,56], Laminin-5 γ2 chain[57], IL-8 and several others[58]. Various cell types within the tumour-microenvironment contribute to the angiogenic processes. Neutrophils have been indicated to mediate the initial angiogenic switch[59], whereas macrophages and fibroblasts contribute to angiogenesis via the secretion of various soluble factors and proteinases leading to the release of angiogenic molecules from the ECM (e.g. VEGF release from connective tissue growth factor (CTGF)[60].
In contrast, various types of cells can also mediate anti-angiogenic properties via proteinases-mediated degradation of pro-angiogenic molecules[61] or the release of anti-angiogenic ECM molecules[62]. Especially the role of MMPs seems dualistic and complex as they might act pro- and anti-angiogenic[41]. Together these data indicate that the response of endothelial cells to angiogenic stimuli is a delicate interaction between tumour cells, fibroblasts and inflammatory cells.

4 Origin and function of myofibroblasts

Already in the 1980s Dvorak *et al*[63] described tumours as "wounds that do not heal", because there are great similarities between tumours and wound healing processes. One of the cell types crucial in both conditions are the myofibroblasts. Myofibroblasts are a heterogeneous population of hyperactivated fibroblasts which simultaneously express the fibroblast marker vimentin and α-Smooth Muscle Actin (SMA)[64-66]. Myofibroblasts are characterized by increased matrix deposition together with enhanced synthesis of proteolytic enzymes and growth factors[67]. In normal colon the presence of myofibroblasts is restricted to a thin single layer along the crypt axis[68,69]. In adenomas and carcinomas a strong increase in number of myofibroblast has been shown[70]. Although their origin has not been clarified yet, several theories have been postulated[71]. Firstly, normal colonic myofibroblasts could strongly proliferate. Secondly, pericytes, vascular smooth muscle cells, or smooth muscle cells from the muscularis mucosa could differentiate and contribute to the myofibroblast population[71-73]. Thirdly, circulating precursor cells (fibrocytes) have been shown to migrate to tumours where they trans-differentiate by TGF-β1 into myofibroblasts[74-77]. Fourthly, epithelial-mesenchymal transition (EMT) of tumour cells[67,78,79] or differentiation of tumour infiltrating macrophages[80], could contribute to the carcinoma myofibroblast population. Finally, the most commonly accepted hypothesis is that myofibroblasts arise from TGF-β1 mediated trans-differentiation of resident fibroblasts[73, 81-87]. Interestingly, in most of these proposed models TGF-β plays a

major role. TGF-β can differentiate fibrocytes into myofibroblasts, initiate EMT and trans-differentiate macrophages or fibroblasts into myofibroblasts *in vitro*. Within the tumour-microenvironment tumour cells are the major TGF-β producers. However, the bio-activity of the secreted ECM-bound latent TGF-β strongly depends on the presence of activation cascades, finally leading to the fibroblast trans-differentiation. In turn, myofibroblasts interact with surrounding epithelial cells, via secreted growth factors and proteinases. In colon cancer, for example, it was shown that myofibroblasts up-regulate MMP expression[88,89], increase growth of epithelial cells[15,78,90] and stimulate the invasion of colon cancer cells[87,91-93]. Furthermore, myofibroblasts can contribute to angiogenic processes by enhanced secretion pro-angiogenic molecules[94,95], providing matrix growth factor binding proteins (like CTGF), morphogens or releasing inactive angiogenic factors from the ECM via secreted proteinases[96].

5 Growth factors in gastrointestinal cancer

Angiogenic processes as well as the generation of myofibroblasts depend on the presence of growth factors in the tumour-microenvironment. In this thesis focus is on two major growth factors involved in these processes leading to cancer progression and metastasis: Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor (TGF)-β1.

5.1 Vascular Endothelial Growth Factor

In 1971 Folkman *et al.* described a factor secreted by tumour cells which is capable of inducing angiogenesis[51], called Vascular Endothelial Growth factor (VEGF). VEGF is a key mediator of angiogenesis, both in physiological as well as pathological conditions[48,50]. Besides angiogenesis recent reports also show a role for VEGF in the suppression of epithelial apoptosis induced by chemotherapeutic agents[97]. The expression of VEGF is regulated by different cytokines including TGF-β, bFGF and a hypoxic environment via Hypoxia Inducible Factor (HIF)-1α[48,50,52]. At least four different subtypes of VEGF are known, VEGF-121, -165, -189 and -206, referring to the number of amino acids[53,98]. Except VEGF-121 all isoforms contain a heparin-binding domain which enables binding to the ECM via interactions with Heparan Sulphate Proteoglycans (HSPGs)[99,100]. VEGF-165 is the most abundant form present in the human body. This 45 kDa, disulfide bond-linked homodimer can bind to VEGF receptor type 1 and 2 with high affinity. Receptor binding results in downstream signal transduction via different signalling pathways and endothelial sprouting by stimulating proliferation and migration (Figure 3). In tumours these vessels are irregular, dilated and leaky and do not differentiate into arteries and venes[50].

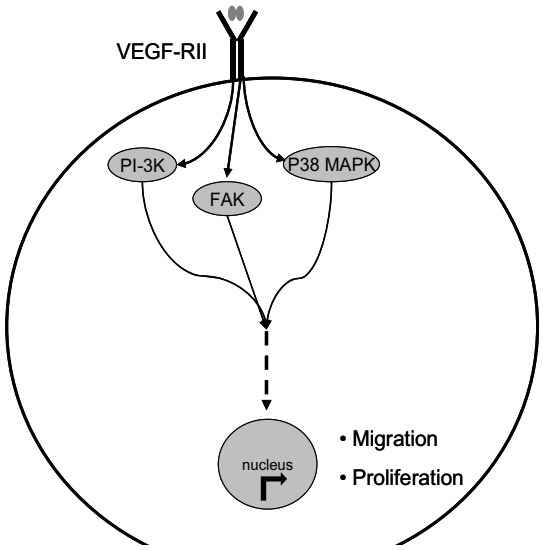

Figure 3. VEGF signalling in endothelial cells

This provides an effective route for tumour cells to enter the bloodstream and form distant metastasis. Furthermore, because these vessels are inefficient, a hypoxic environment remains present resulting in the release of VEGF and subsequent more angiogenesis. The ability of VEGF to induce angiogenesis depends on the presence of soluble VEGF[99]. Therefore, the release of ECM-bound VEGF is a critical step in the induction of angiogenesis. There are indications that proteinases play a role in the release of VEGF and in the degradation of ECM components enabling endothelial cells to form new microvessels. Proteinases which are involved in both the release and/or cleavage of VEGF include plasmin[99,101,102] and MMPs[58, 103-105]. The clinical relevance of tumour angiogenesis and especially VEGF is clearly shown by the clinical use of the VEGF inhibiting antibody bevacizumab (Avastin®), in the treatment of metastatic CRC[106,107]. Several other therapeutic agents targeting angiogenesis via blockade of the VEGF receptors or downstream receptor kinases are currently tested in clinical trails or already therapeutically applied[101,108,109].

5.2 Transforming Growth Factor-β

The TGF-β superfamily consists of 33 proteins which all have distinct functions in cell homeostasis. This family of pleiotropic cytokines contains the TGF-βs, the Bone Morphogenic Proteins (BMPs), the actividins, and several others[110,111]. In humans the TGF-β subfamily consist of three members, TGF-β1, -2 and -3, which are encoded by different genes, but show high homology[112,113]. TGF-β1, a 25 kDa disulphide-bond linked homodimer, is the most abundantly and universally expressed TGF-β member[114-118]. It is produced as a latent complex consisting of TGF-β1, non-covalently bound to the latency-associated protein (LAP) dimer, and covalently bound to Latent TGF-β Binding Protein (LTBP) for binding to the ECM[56,95,119-121]. Four subtypes of LTBPs are known in humans of which in the colon LTBP-4 is most important for the localisation[122]. Before TGF-β1 can exert its effects removal of LTBP and LAP is required[123], involving a two step process (Figure 4).

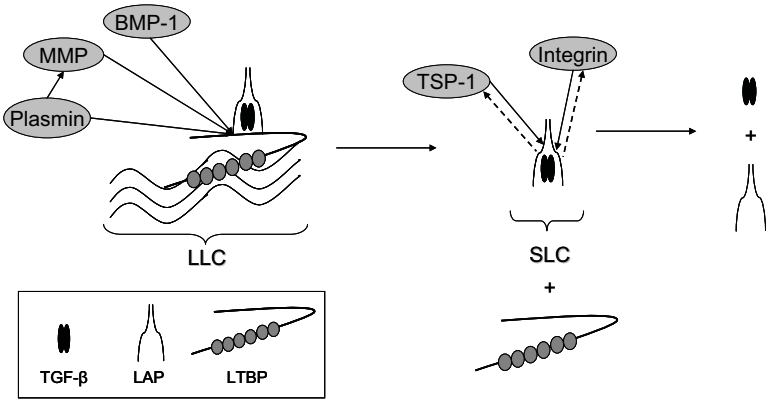

Figure 4. Latent TGF-β1 activation

First the small latent complex (SLC) consisting of the TGF-β dimer and LAP is released from the LTBP. This occurs generally via proteolytic cleavage of the LTBP by MMPs, serine proteases like plasmin, or BMP-1[56,124-127]. Secondly LAP is removed, which is mainly mediated via a conformational change involving thrombospondin-1 (TSP-1)[128,129], integrins[56, 130-132] or the neuropilin-1 receptor[133]. Furthermore, *in vivo* TGF-β1 can be activated via irradiation[134] or mechanical stress[135] and *in vitro* by extremes in pH or heat[136,137]. After active TGF-β1 is released it can bind to TGF-β Receptor type II (TGF-βRII). In epithelial cells and fibroblasts this receptor recruits TGF-βRI, generally the activin receptor-like kinase type-5 (ALK-5), which is transphosphorylated by TGF-βRII. This results in phosphorylation of the Smad-2/Smad-3 complex. Subsequent complexation with Smad-4 leads to trans-localisation to the nucleus, where the complex binds to specific promoter regions and transcription is initiated (Figure 5). Typical targets genes of TGF-β include plasminogen activator inhibitor-1 (PAI-1), fibronectin and, in fibroblasts, collagen type-I[138-141]. The Smad complex is dephosphorylated by phosphatases and the Smads are released back into the cytosol[110,111].

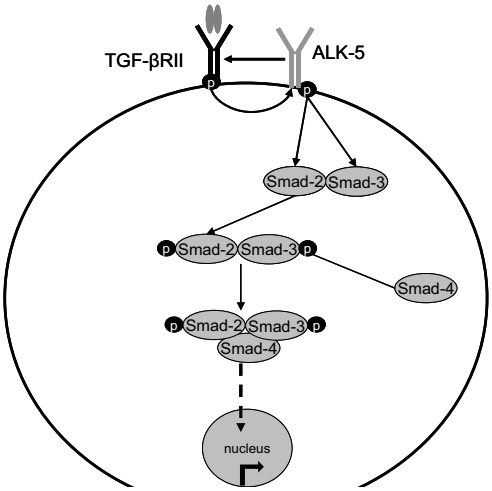

Figure 5. Smad dependent TGF-β signalling pathway

Depending on the specific cell type, TGF-β can signal via other pathways including Mitogen-Activated Protein Kinases (MAPK), Phospho-Inositide-3 (PI-3) kinases and signalling via other ALKs and Smads[117,142,143]. Besides TGF-βRI and TGF-βRII several co-receptors for TGF-β have been identified including betaglycan (TGF-βRIII)[144-146] and endoglin[147]. Endoglin (CD105) is mainly expressed on angiogenic endothelial cells and seems to be important for the effects of TGF-β on (tumour)-angiogenesis[148-153].

In normal tissue homeostasis effects of TGF-β1 on cells include inhibition of proliferation, induction of apoptosis, suppression of the immune system and inhibition of endothelial cell proliferation and migration. In pre-malignant and early stages of cancer, TGF-β1 inhibits the proliferation of epithelial cells, thereby acting as a tumour-suppressor. In later stages the cells become refractory to the inhibition of proliferation by TGF-β1 and can even be growth stimulated by TGF-β1. Furthermore, TGF-β1 affects stromal cells by mediating trans-differentiation of fibroblasts into hyper-activated myofibroblasts, thereby increasing synthesis of growth factors, ECM components and proteolytic enzymes. The effects of TGF-β on endothelial cells include induction of angiogenesis via stimulation of proliferation and migration[149]. Finally, TGF-β represses the immune response, for example, via prevention of activation and proliferation of cytotoxic T-lymphocytes, directly inhibiting the cytolytic activity of NK-cells[95,136], but also converting CD8+ T-cells into IL-17 producing cells enhancing growth by pro-survival signals[154]. All these processes and the differences in cellular responses to TGF-β contribute to the dual role of TGF-β1 in cancer progression[95,114-118,155-160]. Also in gastrointestinal cancers the TGF-β pathway functions as a key mediator in different stages of cancer. Several mutations in the TGF-β signalling pathway contribute to CRC carcinogenesis. In mice it was shown that in colon adenomas, initiated by a mutation in the APC gene, additional epithelial TGF-βRII mutations induces malignant transformation of these adenomas[158], and that Smad-3 knock-out mice develop metastatic CRC already at four months of age[161], indicating the involvement of the TGF-β pathway in the development of CRC. In patients, TGF-βRII is mutated in 90% of the microsatellite instable colorectal tumours[162] and mutation in the TGF-βRI mutations seem to be causally involved in a subset of HNPCC occurrences[163]. In the downstream signalling cascade several mutations in the Smad pathway, especially Smad-4, have been described[5,164-167]. Disruption of the gene encoding LTBP-4, important for latent TGF-β localisation in the colon, causes CRC[122]. Besides mutations in the receptors, associated proteins or downstream signalling cascade components, mutations in the TGF-β1 gene itself enhances TGF-β production by four to six fold[168], increasing the CRC risk[169]. Increased tissue and plasma TGF-β1 levels have indeed been shown in CRC, which correlate with different clinico-pathological parameters including survival[166,170-174]. In gastric cancer TGF-β expression is observed in malignant epithelial and stromal cells and it was shown that both serum and tissue TGF-β1 levels are up-regulated, correlating with lymph node metastasis and poor prognosis[112,175-183].

These data suggest a crucial role for TGF-β1 in the progression of gastrointestinal cancers. However, only a few studies focussed on the interplay between the different cells in the

tumour-microenvironment influencing the production, effects, and, of crucial importance[123], activation of latent TGF-β1.

6 Proteinases implicated in growth factor activation and receptor shedding

Proteolytic enzymes or proteinases cleave targets proteins which include basement membrane components, ECM molecules, membrane bound receptors, and growth factors (Figure 6)[37,39,184]. The role of proteinases in the progression and metastasis of cancer has extensively been studied. Among the many groups of proteases much focus has been on components of the urokinase-type plasminogen activator system[185], the cathepsins[186] and the matrix metalloproteinases[36-39,187,188].

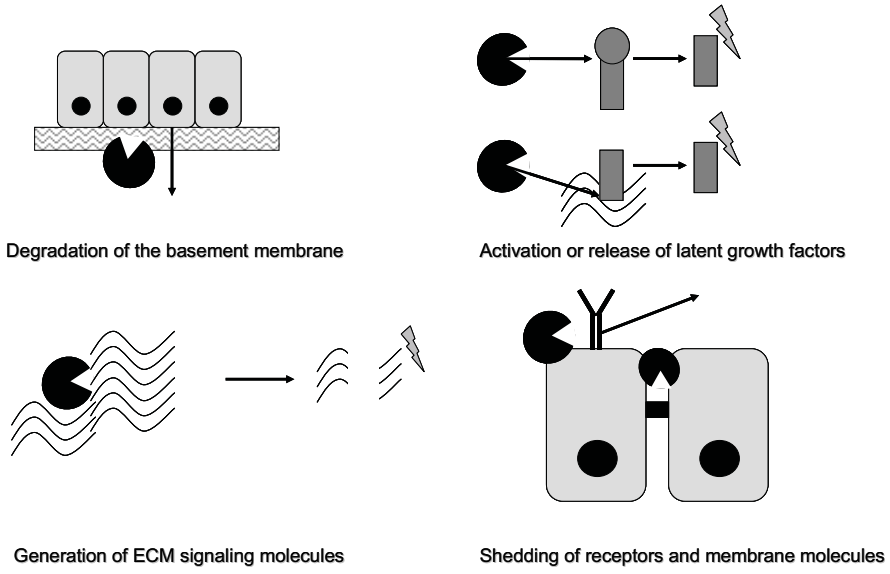

Figure 6. Contribution of proteinases to specific cleavage events in cancer

6.1 The urokinase plasminogen activation system

Plasmin is a serine protease, which is synthesised as the pro-enzyme plasminogen. Activation occurs through removal of the pro-peptide and is mediated via tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). The role of uPA in cancer has extensively been studied revealing a clear role for uPA, its receptor uPAR and plasminogen activator inhibitor-1 (PAI-1) in cancer progression via the generation of plasmin, regulation of cell/ECM interactions, cell recruitment, and in signal transduction[185,189,190]. Furthermore, clinically the plasminogen activator system has been shown to be related to tumour-parameters and to patient survival[190,191]. uPA can induce chemotaxis after binding to uPAR, but also via direct activation of pro-HGF. Furthermore, uPA activates plasmin which in turn can increase bio-availability of growth factors like bFGF, VEGF, and TGF-β[185].

6.2 The cathepsins

Cathepsins belong to the group of lysosomal cystein proteases and 11 members of this family have been identified[192]. Activation of the pro-enzyme occurs via proteolytic removal of the N-terminal propeptide sequence[193]. They have a role in the lysososmal processing of incorporated molecules in the cell[194], but can also be secreted into the pericellular space. Especially the role of cathepsin B and cathepsin S has been studied in cancer progression. Cathepsin B is up-regulated in CRC[195,196], displaying involvement in angiogenesis[197] and tumour invasion, for example directly via the activation of HB-EGF[186] or via the activation of indirectly via processing of proteins like uPA[186,198,199]. Cathepsin S is one of the most potent lysosomal cystein proteinases. It has a low pH optimum, around pH 5, and is mainly produced by macrophages and tumour cells. Activation of the pro-molecule occurs via removal of the N-terminal propeptide[186,194]. It has been shown to be involved in MHC class II antigen presentation[194,200] and is therefore involved in many immune disorders. Cathepsin S is also up-regulated in prostate cancer[201] and glioblastomas[201-205], and has a role in tumour angiogenesis[206], probably by its ability to generate pro-angiogenic matrix-derived peptides like laminin-5 γ2 chains[57, 206].

6.3 The matrix metalloproteinases

The first matrix metalloproteinase, MMP-1, was discovered in 1962 in tadpole tails[207]. Since then the MMP family has extended to a group of at least 23 zinc-bound proteinases, which are involved in remodelling of the ECM[208] and implicated in many physiological processes including embryonic development, organogenesis, wound healing processes and bone remodelling[188]. Furthermore, MMPs are involved in many pathological conditions like inflammation and the invasion of cancer cells by ECM remodelling, including degradation of the basement membrane[36,38,55,187,209]. Regulation of MMP activity is mediated via activation of the inactive zymogens and further by the expression of their natural inhibitors, α-2 macroglobulin, the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) and the membrane anchored inhibitor RECK[105,210]. Biological processes involving MMPs are therefore always strongly dependent on the balance between activation of the zymogen, and the presence of the inhibitors[38]. Recently, the MMP family was expanded with a special group of metalloproteinases, the ADAM(T)s [a Disintegrin And Metalloproteinase (with Thrombospondin motive)], which have a similar catalytic structure to MMPs and are also inhibited by TIMPs[38,104,105]. Besides their role in ECM degradation MMPs also have a prominent role in the activation of latent growth factors and hence in the communication

between cells within the tumour-microenvironment[40,41,188,209,211,212]. The role of MMPs in different stages of tumour development has been studied, showing up-regulation of various MMPs in CRC[39, 190, 213, 214]. After it was shown that MMPs contribute significantly to tumour progression clinical trials started using broad spectrum MMP inhibitors. However, these trials did not show the results expected from pre-clinical tests *in vitro* and in mice[215]. Severe side-effects combined with only minor therapeutical effects on tumour-progression led to a rapid withdrawal of the initial clinical trials[215,216]. Afterwards, more studies have focused on the specific role of MMPs within the human tumour-microenvironment to reveal the more sophisticated role of MMPs in releasing or activating growth factors and the generation of signalling molecules[38,188]. Currently more specific MMP inhibitors are developed and tested in clinical trials for various pathologic conditions[36,38,217,218].

The following MMP classification into collagenases, gelatinases, stromelysins or membrane type MMPs is based on their structural domains, natural substrates and localisation[38, 213, 219].

6.3.1 The collagenases

The collagenases consist of MMP-1, MMP-8, MMP-13 and MMP-14. Their ECM substrates include different collagens and HSPGs and the collagenases are activated by several other MMPs and plasmin. MMP-13, which can be activated by MMP-3[219], seems to have a central role in the MMP activation cascade[220], activating both MMP-2 and MMP-9 (Figure 7). Furthermore, MMP-13 expression is low in normal epithelial cells but strongly up-regulated in tumour cells, inflammatory cells and occasionally fibroblasts[221,222]. MMP-13 has a role in angiogenesis[223], and is associated with poor survival in CRC patients[220] and several other cancers[221,224-226]. In addition, MMP-1 and MMP-13 have both been implicated in LTBP cleavage leading to TGF-β activation[227, 228].

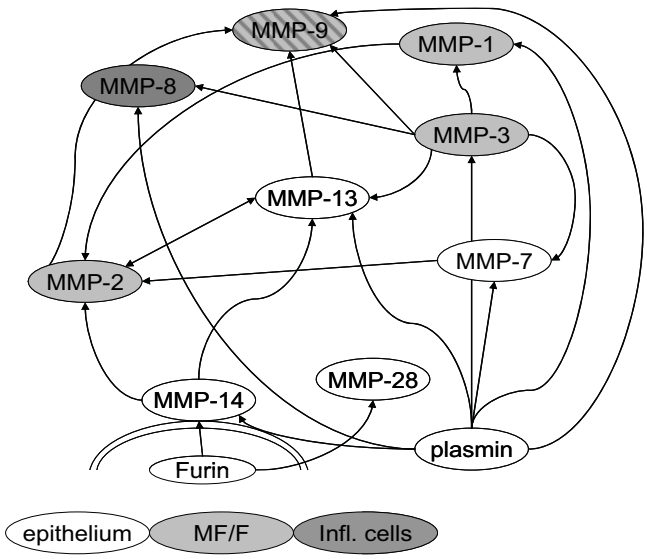

Figure 7. MMP activation cascades and cell types expressing MMPs in CRC. (M)F; (myo)-fibroblast

6.3.2 The gelatinases

MMP-2 and MMP-9 belong to the gelatinases, because of their ability to degrade gelatine (denatured collagen). Physiological substrates include collagen type IV, the major constituent of the basement membrane. Therefore, the gelatinases have been often associated with invasive processes and metastasis in CRC[229-234]. Expression of MMP-2 is mainly observed in fibroblasts, but also tumour cells are capable of producing MMP-2[39]. MMP-9 expression is found in fibroblasts and infiltrating immune cells, including macrophages and neutrophils, where MMP-9 is stored in granules[33,39]. Both MMP-2 and MMP-9 are up-regulated when cells are stimulated with TGF-β1[235-238]. Furthermore, via direct cell-cell interaction, epithelial EMMPRIN upregulates stromal MMP-2 expression[29,239]. The major activator of MMP-2 is MMP-14 in a complex also containing TIMP-2[240]. In turn MMP-2 can activate MMP-9 and MMP-13[219]. Besides by MMP-2, MMP-9 can be activated by several other MMPs and by plasmin (Figure 7). Both MMP-2 and MMP-9 have a role in angiogenic processes[241,242]. Especially MMP-9 might have a role in regulating VEGF bioavailability during the angiogenic switch[103], but the exact mechanism of this process is not known. The gelatinases are also involved in regulating TGF-β bio-activity. MMP-9, localised to the cell surface by binding to CD44, and MMP-2 have both been implicated in the proteolytic processing activation of the large latent TGF-β complex[126, 243-245].

6.3.3 The stromelysins

MMP-3, -10, and -11 belong to the stromelysins. Besides degradation of different ECM components and various cell surface molecules[104], MMP-3, which can be activated by plasmin[190], seems to be very important because of its ability to activate a large number of other MMPs including MMP-1, -7, -8, -9 and -13[219] (Figure 7). Expression of MMP-3 is mainly observed in fibroblasts and is strongly upregulated in CRC[39,246]. MMP-3 is capable of generating active insulin like growth factor (IGF)[41] and cleaving mouse VEGF-164 to smaller isoforms[247]. Interestingly, a short form of MMP-3 has also been detected in the nucleus of myofibroblasts, where it has a role in apoptosis[248]. Finally, MMP-3 has also been implicated in TGF-β activation[104].

6.3.4 The membrane type MMPs

Until now six membrane type MMPs (MT-MMPs) have been identified, which all contain a transmembrane domain. MT-1, -2 and -3 MMP are the only MMPs which have been show to be directly capable of dissolving the basement membrane, facilitating invasion[41]. The most

studied MT-MMP is MMP-14 (MT-1 MMP), which is strongly expressed by epithelial cells, endothelial cells and myofibroblasts[39,249,250]. Intracellularly MMP-14 is processed to the active form by furin[251,252] and displayed on the membrane MMP-14 is capable of activating MMP-2[253]. Besides promoting invasion by degradation of ECM components[254,255] and HSPGs[251], MMP-14 has been shown to be involved in vascular tubulogenesis[256], receptor shedding[144] and TGF-β activation[257]. MT4-MMP seems to have a role in angiogenesis, by the normalisation of tumour blood vessels[258].

6.3.5 Other MMPs

The smallest member of the MMP family is MMP-7 (matrilysin), which is mainly expressed by epithelial cells. This MMP is activated by MMP-3, -10 and plasmin and is in turn capable of activating MMP-2[219] (Figure 7). Besides degradation of ECM components, MMP-7 increases fibroblast proliferation[259], cleaves E-cadherin[104,260,261] and HB-EGF[209] and is capable of generating soluble Fas-ligand[262]. This increases apoptosis of normal epithelial cells, but not affecting tumour cells as they have decreased sensitivity to Fas-ligand induced apotosis. In addition, MMP-7 is capable of releasing active TGF-β1 bound to decorin, an ECM molecule[263].

MMP-28 (epilysin) is one of the most recently discovered MMPs[264]. It is widely expressed in the gastrointestinal epithelial cells and in several tumours[264]. Interestingly, one study described MMP-28 to be down-regulated in CRC[265]. Furthermore, MMP-28 is up-regulated during wound healing processes and is capable of activating TGF-β via cleavage of the LTBP-1[266].

6.3.6 The ADAM(T)s

The ADAMs are membrane anchored proteins, whereas the ADAM(T)s are secreted. At least 25 ADAMs have been described in humans, which are intracellularly activated by furin-like convertases. Several ADAMs have been linked to cancer, like ADAM-17, which is capable of shedding HB-EGF, IL-1 receptor, tumour necrosis factor receptor I and II and pro-TNF-α processing, and ADAM-10 which also contributes to pro-HB-EGF, pro-TNF-α, notch and E-cadherin shedding[267]. In contrast, ADAM-1 and -8 have been shown to be anti-angiogenic factors, possibly via binding of VEGF[268].

7 MMPs activating TGF-β and VEGF

The role of MMPs in the release or activation of growth factors like TGF-β and VEGF has been subject of several studies[104]. It has been shown that, *in vitro*, latent TGF-β can be activated by MMP-1[227], MMP-2[243, 245], MMP-3[119, 138], MMP-9[126, 245], MMP-13[228], MMP-14[132, 269] and MMP-28[266]. Which MMP is crucial for the activation of latent TGF-β and whether the MMPs are involved in a proteolytic cascade and therefore inhibiting one of the MMPs would block the proteolytic activation cascade, is not known yet. Not only MMPs, but also plasmin has been described to be able to activate latent TGF-β[125, 270] directly *in vitro*, but it is possible that this activation occurs indirectly via activation of MMP-3. Besides direct activation of the latent TGF-β complex the MMPs also play a role in releasing active TGF-β when it is bound to other molecules. For example, active TGF-β binds to the ECM molecule decorin, from which it can be proteolytically released by MMP-7[263]. In turn, activated TGF-β up-regulates various invasion-associated MMPs and other proteinases[235].

Several studies examined the role of MMPs in the release or activation of VEGF, but none of them has been able to elucidate the mechanism how VEGF bioavailability is regulated. Upregulation of availability can occur via cleavage of the VEGF molecule itself, generating a smaller, more soluble isoform[247], whereas VEGF release is mediated via cleavage of ECM components which bind VEGF[100].

8 Shedding of membrane molecules and receptors by MMPs

Besides the activation of growth factors MMPs and other proteinases are also involved in shedding of other membrane-bound molecules (Figure 6)[271] like integrins and receptors including Fibroblast Growth Factor Receptor-1 (FGFRI), uPAR and Her-2 and various others (reviewed in[104]). For example one of the hallmarks of Epithelial Mesenchymal Transition (EMT), a process in which epithelial cells lose expression of epithelial markers and morphology and synthesize de novo mesenchymal markers (e.g. vimentin)[272], is the loss of E-cadherin expression on epithelial cells. It has been shown that MMP-7 and MMP-3 can mediate this cleavage process[260,271]. Furthermore betaglycan (TGF-βRIII), can be shed from the membrane by MMP-14, releasing a soluble form of betaglycan[144]. Endoglin, a TGF-β co-receptor, which also can bind BMP-9[273], has a crucial role in angiogenesis and exists also a soluble form[56,148,274]. However, the release mechanism and the possible involvement of proteinases have not been elucidated yet.

9 3-dimensional cell culture models

In order to investigate complex pathological processes and cell regulatory mechanisms often model systems are used. Models can be simple by growing human primary cells or cell-lines in monolayer conditions or complex by the use of animals. Although these models are valuable and gain insight into aforementioned processes, they do not have to be representative for the human *in vivo* situation[59,275-277], especially when more than one cell type is involved. In this thesis, which focusses on the interaction between cells in the human tumour-microenvironment, we used various 3-dimensional model systems.

9.1 Multi-cellular tumour spheroids

Spheroids are aggregates of cells in a 3-dimensional conformation. The first paper describing the often applied agarose liquid overlay technique already dates from 30 years ago[278]. Wells are coated with agarose, which prevents attachment of the cells. In this way after 2-7 days multi-cellular aggregates (100-500 μm) are formed[275]. In contrast to cells growing in monolayers, culturing them as spheroids restores the morphological and functional features of cells as observed *in vivo*[279, 280]. Cells in the hypoxic centre of the spheroid die, whereas cells in the outer layers are viable cells, like cells *in vivo* surrounding blood vessels. Therefore, spheroids closely represent small avascular solid tumours or micro-metastasis[279, 281-283]. The genes upregulated in 3-dimensionally cultured cells represent the genes which are also upregulated in tumours, whereas cells grown as monolayers show different or sometimes even contradictory responses[275,284-287]. The fact that results in spheroids are in general more closely resembling the *in vivo* situation[288] is caused by a difference in the expression of growth factor receptors (like EGF of TGF-β receptors), but also by the deposition of ECM proteins, a phenomenon far less observed by cells cultures under monolayer conditions, and differences in cell adhesion molecules[288].

To study chemotaxis between tumour cells and immune cells often the Boyden chamber is used, first described by *Boyden* in 1961[289]. Spheroids form a good alternative to study tumour-cell immune cell interaction because spheroids are capable of modulating infiltrating immune cells as observed *in vivo*[290]. In addition, co-culture of spheroids with host cells, like fibroblast, can represent good models to study the influence of tumour cells on the resident fibroblasts in advanced tumours[279,291]. In conclusion, multi-cellular tumour spheroids form a very valuable tool to study tumour biology[275,279,280,288,290,292-294]. A special application of spheroids is to study angiogenesis of endothelial cells.

9.2 Endothelial spheroids

3-dimensional *in vitro* angiogenesis models are used to study all phases of angiogenesis (matrix degradation, migration, proliferation, survival and morphogenesis[295]). In contrast to monolayers, spheroids of endothelial cells do not proliferate once embedded in a collagen matrix and only form sprouts after stimulation with an angiogenic stimulus like VEGF[296, 297], therefore better representing the human setting. There are critical advantages of studying (tumour)-angiogenesis using *in vitro* models compared to animal models. As was already mentioned by Rangarajan *et al*[276] "mice are not small people" and therefore do not have to reflect the human tumour-microenvironment. For example the VEGF neutralizing antibody Bevacizumab does not bind to mouse VEGF-164, whereas it strongly inhibits human VEGF-165 induced angiogenesis[298]. Although mouse models will still be indispensable to study the pharmakinetics and toxicologic aspect of new drugs, recent advancements in the development of model systems include 3-D lymphangiogenesis models[299], and also grafting human endothelial spheroids into mice to study tumour angiogenesis[300]. This combination gives the opportunity to study the angiogenesis in a human setting, but with the advantages of studying it in the complexity of a whole organism.

10 References

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