

Keywords: cancer-associated fibroblast; cancer stem cell; metastasis; immune suppression; transforming growth factor β ; tumour invasiveness

Transforming growth factor β as regulator of cancer stemness and metastasis

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Key elements of cancer progression towards metastasis are the biological actions of cancer stem cells and stromal cells in the tumour microenvironment. Cross-communication between tumour and stromal cells is mediated by secreted cytokines, one of which, the transforming growth factor β (TGF β), regulates essentially every cell within the malignant tissue. In this article, we focus on the actions of TGF β on cancer stem cells, cancer-associated fibroblasts and immune cells that assist the overall process of metastatic dissemination. We aim at illustrating intricate connections made by various cells in the tumour tissue and which depend on the action of TGF β .

TGF β IN CANCER

Transforming growth factor β (TGF β) is a secreted polypeptide discovered as a biological activity produced by tumour cells and capable of inducing oncogenic transformation of non-cancerous cells in culture (Moses *et al*, 2016). Today we appreciate in great detail the mechanisms by which TGF β and its diverse family members regulate embryonic developmental processes and why and how they are implicated essentially in many human diseases (Akhurst and Hata, 2012). The main reason for the overwhelming implication of TGF β in human disease, including cancer, is the prominent role that TGF β has on tissue homeostasis and the fact that all chronic inflammatory and wounding processes activate this cytokine from the extracellular matrix (ECM) where it is deposited at abundant quantities and resides in an inactive form (Pickup *et al*, 2013). At the cellular level, TGF β patrols several biological events either under physiological or pathological conditions such as the cell cycle and apoptosis, epithelial to mesenchymal transition (EMT) and ECM regulation (Akhurst and Hata, 2012). At the tissue and organ level, TGF β regulates the differentiation and immunological response of B and T lymphocytes participating in the inflammatory cascade associated with cancer progression, and also regulates tissue interactions important during both embryonic organogenesis and cancer progression (Pickup *et al*, 2013).

Abnormalities in the TGF β pathway relate to cancer development characteristic examples of which are certain hereditary cancer

syndromes and many sporadic malignancies such as brain, breast, colon, liver, lung, prostate and haematopoietic malignancies. Abnormal TGF β signalling additionally encompasses diverse developmental disorders, as for example, the craniofacial cleft palate syndrome, and the autosomal dominant abnormality of the Rendu–Osler–Weber syndrome; cardiovascular pathologies including atherosclerosis, hypertension and rare abnormalities of the vasculature such as aneurysms; connective tissue and bone diseases like the Marfan syndrome and osteoporosis; muscular and reproductive disorders (Gordon and Blobel, 2008). In cancer, the homeostatic action of TGF β explains why this cytokine acts as a tumour suppressor, by directing diverse cell types towards cell cycle arrest and apoptosis, whereas some of the genes encoding for TGF β family ligands, receptors and Smads (downstream signalling proteins) become mutated in specific cancer types (Pickup *et al*, 2013). On the other hand, excessive amounts of TGF β are expressed in the extracellular milieu of many tumours, and upon activation, induce sustained signalling in most types of malignancy analysed including brain, breast, liver, prostate, haematopoietic and other malignancies (Gordon and Blobel, 2008). In particular, TGF β disrupts homeostasis and enhances tumour progression via its ability to dedifferentiate many cell types, suppress the development of immune cells and indirectly allow vascular growth (Padua and Massagué, 2009).

Transforming growth factor β signals via the same key signalling molecules under pro-tumourigenic and physiological homeostatic conditions. However, the signalling outcome of these pathways may be very different in normal vs malignant cells. The

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Received 19 February 2015; revised 14 July 2016; accepted 19 July 2016; published online 18 August 2016

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main difference between normal and tumour cell signalling relies in the prevalence of oncogenic molecules in the tumour cells, which might lead to disrupted cellular behaviour and pathogenic phenotypic outcome. The central mediators of TGF β signalling activity involve receptors on the cell surface named type II (T β RII) and type I (T β RI), which bind the ligand as a heterotetrameric complex, leading to activation of their intrinsic protein kinase activity (Akhurst and Hata, 2012). T β RII is the primary ligand-binding receptor that trans-phosphorylates T β RI, whose kinase activity becomes released from negative inhibition by chaperones. This causes the phosphorylation of Smad proteins – namely, Smad2 and Smad3 – which then assemble with Smad4 into a heteromeric complex. The latter accumulates on chromatin, resulting in an integrated action of chromatin-bound Smad complexes with the other signalling (non-Smad) molecules summarised below. Immediate early target genes of TGF β -Smad signalling include the inhibitory Smads, such as Smad7, which accumulate upon signalling and negatively regulate the pathway at the level of T β RI degradation, Smad2/Smad3 phosphorylation by T β RI and at the level of chromatin-bound Smad complexes, which are transcriptionally blocked by Smad7 (Akhurst and Hata, 2012).

Alternatively, the T β RII–T β RI receptor complex can recruit other signalling proteins, such as ubiquitin ligases, adaptors to protein and lipid kinases or small GTPases, which mediate diverse molecular activities collectively referred to as non-Smad signalling (Akhurst and Hata, 2012). The Smad and non-Smad signalling effectors most often coordinately regulate different sets of genes in a tissue-dependent and pathogenesis stage-dependent manner.

In the following sections, we will focus mostly on the biological actions of TGF β but also make reference to signalling and mechanistic details wherever possible, pointing out how TGF β can contribute to the biology of cancer stem cells (CSCs) and various stromal cell types in order to facilitate cancer metastasis. Due to limitations in the length of this article, we deliberately cover few instrumental cases from the older literature and base most of our examples on more recent but also few scientific reports.

TGF β SIGNALLING IN CANCER STEM CELLS

Similar to its complex role in cancer progression, TGF β can have a dual function concerning the biology of CSCs, inhibiting or sustaining their function. As an example, TGF β has been reported to suppress breast cancer tumourigenesis via two independent mechanisms: by reducing the CSC/early progenitor pools or by promoting the differentiation of a committed but highly proliferative progenitor subset to a less proliferative and more differentiated one (Tang *et al*, 2007). In the presence of TGF β , the breast CSC population was less abundant and lost its self-renewal capacity, moreover this cytokine induced the expression of mucin-1 and cytokeratin-18, luminal markers identifying slowly proliferating cells and negatively affected the expression of ‘basal genes’, such as cytokeratin-14 or frizzled-7, normally expressed by more proliferative cells (Tang *et al*, 2007). In a study of diffuse-type gastric carcinoma, TGF β has been described to decrease the cancer-initiating cell population (side population), leading to a decrease in tumour formation and tumour size *in vivo*; this study pointed out how TGF β acted via the negative regulation of ABCG2, a transmembrane transporter responsible for the active efflux of chemotherapeutics, probably conferring a metabolic or survival impairment to the CSCs, which were then eradicated (Ehata *et al*, 2011). The negative effect of TGF β on the side population of gastric carcinoma can also be ascribed to the negative regulation on aldehyde dehydrogenase 1 (ALDH1) and REG4 (regenerating islet-derived family, member 4), which leads to a decrease in the ALDH1⁺ population, correlating to poor

prognosis in different tumours (Katsuno *et al*, 2012). The ALDH1⁺ population exhibits self-renewal capacity and displays tumour initiating and tumour progression potential *in vivo* and these CSC features are significantly suppressed by TGF β (Katsuno *et al*, 2012).

On the other side, several studies underline how TGF β has a positive role on the CSC population promoting or sustaining stemness of the pool of CSCs in diverse types of malignancy including breast cancer (Bruna *et al*, 2012; Lo *et al*, 2012; Bhola *et al*, 2013), liver cancer (You *et al*, 2010; Mima *et al*, 2012), gastric cancer (Hasegawa *et al*, 2014), skin cancer (Oshimori *et al*, 2015), glioblastoma (Ikushima *et al*, 2009; Peñuelas *et al*, 2009) and leukaemia (Naka *et al*, 2010). In hepatocellular carcinoma (HCC), TGF β upregulates the expression of the stem cell marker CD133, via a Smad-dependent transcriptional mechanism and by promoting CD133 promoter demethylation based on a negative effect on the DNA methyltransferases DNMT1 and DNMT3 β , thus enhancing the tumourigenic potential of the CD133⁺ population *in vivo* (You *et al*, 2010). Another marker of HCC stem cells, the adhesion molecule CD44, potentiates TGF β signalling and mesenchymal differentiation (Mima *et al*, 2012). Genetic ablation of T β RII in mice, followed by chemically induced carcinogenesis of the bladder also attests to the positive role TGF β signalling has in the generation of a bladder CSC population that promotes tumour invasiveness and aggressive behaviour (Liang *et al*, 2016).

In the brain tumour glioblastoma, TGF β selectively induces self-renewal of the glioma-initiating cells but not of normal glial progenitors, via the Smad-dependent induction of leukaemia inhibitory factor (LIF) and the sequential activation of the LIF–Janus kinase–STAT pathway (Peñuelas *et al*, 2009). This pathway leads to the increase in self-renewal potential (neurosphere formation), prevention of neurosphere differentiation *in vitro* and to higher tumour incidence and tumour size *in vivo* (Peñuelas *et al*, 2009). In this pathological scenario, an autocrine TGF β loop maintains the self-renewal of glioma-initiating cells, acting on its direct target Sox4, which, in turn, binds to the stem cell transcription factor Oct4, and together induce expression of another stemness gene, Sox2 (Ikushima *et al*, 2009; Ikushima *et al*, 2011). In particular, the downregulation of Sox4 and Sox2 expression by TGF β inhibitors diminishes stemness features and induces glioma CSC differentiation *in vitro*, whereas genetic attenuation of Sox4 leads to improved survival after intracranial injection of these glioma CSCs *in vivo* (Ikushima *et al*, 2009).

Leukaemia initiating cells (LICs) support the growth and relapse of chronic myeloid leukaemia; LICs with nuclear localisation of the transcription factor Foxo3a show decreased Akt kinase phosphorylation and exhibit characteristic enrichment in CSCs in this type of malignancy, because LIC apoptosis is suppressed and thus sustained progression of the malignancy is promoted (Naka *et al*, 2010). These findings propose that TGF β regulates Akt phosphorylation and Foxo3a nuclear localisation, which is required for the stemness properties of LICs (Naka *et al*, 2010). Accordingly, a new therapeutic perspective can be based on the adjuvant administration of TGF β receptor kinase inhibitor to the established imatinib treatment used against chronic myeloid leukaemia, in order to eradicate more effectively the LIC pool (Naka *et al*, 2010).

Crosstalk between TGF β and other developmental pathways is also relevant. The cooperation between TGF β and tumour necrosis factor α (TNF α) positively affects the acquisition of a CSC phenotype in breast cancer due to the induction of EMT. In particular, the TGF β -induced EMT-generated breast CSCs have a claudin-low phenotype – which is normally associated with mesenchymal features and more aggressive cancer behaviour – and possess self-renewal potential, increased tumourigenicity *in vivo* and resistance to the chemotherapeutic drug oxaliplatin (Asiedu *et al*, 2011). Moreover, TGF β cooperates with the WNT pathway in breast cancer to induce EMT and maintain the

resulting mesenchymal and stem cell state in an autocrine fashion (Scheel *et al*, 2011). Blocking the TGF β -induced WNT pathway in this model of breast cancer, results in decreased cell migration and stem cell self-renewal *in vitro*, lowers tumourigenic potential and lowers macro and micrometastatic incidence *in vivo*. A screen for stemness genes enriched in non-small-cell lung carcinomas that exhibit high metastatic potential pointed to the importance of musashi-2, a regulator of protein synthesis (Kudinov *et al*, 2016). Attempting to genetically silence musashi-2 in these lung CSCs depleted the stem cell pool in part by causing induction of epithelial proteins, such as the junctional claudins, and by suppressing the translation of mRNAs for T β RI and Smad3 and of Snail1 and Snail2, transcriptional mediators of EMT (Kudinov *et al*, 2016). This model suggests that TGF β signalling promotes lung adenocarcinoma CSCs and their metastatic potential and that the TGF β pathway is under the translational control of musashi-2.

TGF β IN CANCER METASTASIS

Cancer metastasis is a multistep process that engages several cell types in addition to the primary tumour cell. Here we summarise evidence presented according to the major cell lineages that mediate metastatic dissemination. In recent years, different studies have demonstrated how TGF β is implicated in metastasis (Padua and Massagué, 2009; Hansen *et al*, 2014). It has been reported that TGF β is able to create a tissue microenvironment permissive to the metastatic dissemination (Pickup *et al*, 2013), and that TGF β can contribute to the local invasion, blood-borne metastatic dissemination and colonisation of distant organs (Calon *et al*, 2012; Calon *et al*, 2014).

TGF β and fibroblasts in the tumour stroma. The origin of cancer-associated fibroblasts (CAFs) still remains an open question, due to the possible multiple origin of these cells and the differences described in CAF populations within specific tumour subtypes or even within distinct areas of the same tumour (Cirri and Chiarugi, 2011). Resident CAFs can originate from the differentiation of resident fibroblasts via the action of TGF β , and these CAFs then sustain the proliferative, migratory and invasive behaviour of cancer cells (Calon *et al*, 2014). Cancer-associated fibroblasts can also originate from the trans-differentiation of pericytes or inflammatory cells via the so-called mesenchymal to mesenchymal transition, which can be mediated by TGF β , among other cytokines (Buess *et al*, 2007; Cirri and Chiarugi, 2011). In addition, CAFs can be generated from bone marrow-derived mesenchymal stem cells (BM-MSC), which can be recruited at the tumour or inflammatory site and be committed to fibroblast differentiation by the locally released cytokines and growth factors (Karnoub *et al*, 2007; Mishra *et al*, 2008; Quante *et al*, 2011). At last, CAFs can derive from endothelial cells via the TGF β 1-induced endothelial mesenchymal transition process, or endEMT; in this event, the endothelial markers CD31/PECAM are downregulated, whereas the mesenchymal marker fibroblast-specific protein-1 is induced and the resulting CAFs localise at the invasive front of the tumour (Zeisberg *et al*, 2007).

A hallmark of activated fibroblasts or myofibroblasts is their ability to synthesise many ECM proteins and build a specialised cytoskeleton that incorporates the α -smooth muscle actin (α SMA). Transforming growth factor β once again has a major role in mediating this terminal differentiation process. The TGF β 1 effect on pulmonary fibroblast to myofibroblast differentiation is illustrative of this process. Transforming growth factor β drives pulmonary fibroblasts to acquire an irreversible post-mitotic phenotype associated with the induction of type I, II, III and IV collagen expression and secretion, reorganisation of the actin

cytoskeleton, increase in α SMA expression and incorporation of α SMA into stress fibres, one of the clear hallmarks of the myofibroblast phenotype. Interestingly enough, the Smad proteins differentially regulated the TGF β 1-induced morphological and functional changes, in particular Smad2 but not Smad3 affected α SMA, whereas both Smad2 and Smad3 affected collagen regulation (Evans *et al*, 2003).

In the context of cancer, early experiments using fibroblast-specific ablation of T β RRI in mice demonstrated that fibroblasts have homeostatic roles within a tissue such as prostate or stomach, as loss of T β RRI led to excessive secretion of hepatocyte growth factor (HGF) and resulting in hyperproliferation of the epithelial cells in these tissues, supporting tumourigenic progression (Bhowmick *et al*, 2004). This experiment was one of the first that established the key role of stromal cells in controlling epithelial carcinogenesis via paracrine mechanisms. In the tumour microenvironment, TGF β is produced by terminally differentiated CAFs, acting in an autocrine fashion on the same CAFs that have produced it (stromal TGF β -activation) while the epithelial cancer cells can also produce TGF β , which acts in a paracrine way on the fibroblasts (epithelial TGF β -activation) (Calon *et al*, 2014; Hawinkels *et al*, 2014). More specifically, CAFs can produce TGF β 1 via a Smad4-dependent autocrine signalling loop, which promotes their differentiation to myofibroblasts and supports a sustained acquired myofibroblast phenotype (Kojima *et al*, 2010). In particular, TGF β 1 promotes and crosstalks with the autocrine SDF-1 (stromal cell-derived factor 1)/CXCR-4 chemokine receptor pathway, and the latter helps to maintain high expression of TGF β 1, thus resulting in a synergistic positive effect on the myofibroblast functions and on their persistence in invasive breast cancer specimen (Kojima *et al*, 2010). Moreover, colorectal cancer cells secrete TGF β 1 and this leads to the hyperactivation of the TGF β signalling pathway in the CAFs, causing enhanced expression of target genes such as the ECM modulators plasminogen activator inhibitor 1, matrix metalloproteases 2 and 9 (MMP-2, MMP-9) and α SMA (Hawinkels *et al*, 2014). Further evidence of the positive effect CAFs have on tumour cells has been described in colon cancer, where, CAFs activate extracellular TGF β , which causes secretion of interleukin-11 (IL11) by the CAFs, that in turn triggers GP130/STAT3 signalling in the colorectal cancer cells (Calon *et al*, 2012). This mechanism confers a survival advantage to specific colorectal cancer clones with metastatic potential, whereas pharmacological blockade of the TGF β response in the stromal CAFs significantly impairs the tumour initiation (Calon *et al*, 2012). Furthermore, TGF β enhances the attachment and co-migration of colon cancer cells and CAFs (Figure 1), positively affecting the metastatic burden of these colon carcinomas to the liver. Beyond this effect, TGF β increases cancer cell proliferation in the primary tumour and in the metastasis, and TGF β -treated colon cancer cells more efficiently bind to endothelial cells, whereas CAFs exposed to TGF β upregulate proteins involved in cell-cell attachment and cytokines that can sustain cancer cell survival during dissemination (Gonzalez-Zubeldia *et al*, 2015). In a parallel scenario studied in bladder carcinoma, the urinary bladder CAFs oversecrete TGF β 1, which then causes EMT on the bladder epithelial cells via transcriptional induction of the long non-coding RNA (lncRNA) *ZEB2NAT* (Zhuang *et al*, 2015). *ZEB2NAT* positively regulates the expression of the pro-EMT transcriptional repressor ZEB2, which establishes the bladder carcinoma EMT and promotes the invasive behaviour of the tumour cells (Zhuang *et al*, 2015).

Furthermore, the TGF β effect on CAFs appears to be associated to their metabolic reprogramming. Transforming growth factor β activation by epithelial breast cancer cells MDA-MB-231 can induce differentiation of surrounding fibroblasts into CAFs, which causes their metabolic shift towards catabolic and glycolytic pathways – related with the processes of mitophagy and autophagy

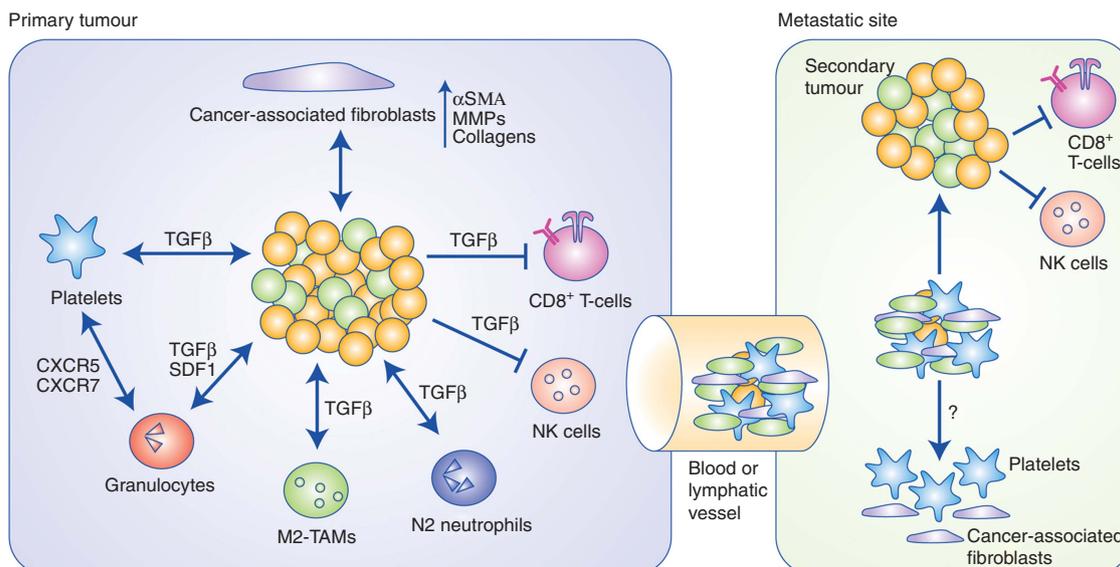


Figure 1. Pleiotropic effects of TGF β in the tumour microenvironment. Representation of a primary tumour with cancer stem cells (CSCs) or metastasis-initiating cells (light green) and the bulk of the tumour cells (orange). Different cell types surrounding the tumour mass can positively contribute to tumour progression, invasiveness and metastatic dissemination, acting on the tumour cells but also receiving input from the tumour cells (bi-directional arrows). Cells that can inhibit tumour progression are linked to the tumour mass with a negative arrow. The contributions of TGF β and additional cytokines and chemokines are highlighted next to each arrow. As graphic simplification, haematogenous or lymphatic metastatic dissemination shows solely CSCs co-migrating with stromal cells and platelets. At the secondary site, metastatic colonisation and growth is shown; whether stromal cells dissociate from the metastasising clonal population or whether they are replaced by new local stromal cells is currently unknown (?).

– as well as a decrease in mitochondrial activity and consequent oxidative stress (Guido *et al*, 2012). As a result, CAFs generate metabolites (L-lactate, ketone bodies and glutamine) that sustain the mitochondrial metabolism and the anabolic growth of the adjacent cancer cells at the expense of their own metabolic efficacy. In a parallel study of co-culture between NIH3T3 fibroblasts and 4T1 mouse breast cancer cells, the TGF β -induced autophagy on the fibroblasts (which resemble CAFs in this *in vitro* model) provided survival signals that enhanced the tumourigenic potential of the cancer cells in xenograft assays (Liu *et al*, 2016). A screen for chemical agents that could block the activation of CAFs by TGF β revealed that cardiac glycosides, such as digoxin, which naturally regulate contractility of the heart muscle, effectively blocked CAF activation and cell contractility induced by TGF β (Coleman *et al*, 2016). According to these views, CAFs serve as metabolic engines for the benefit of tumour growth and expansion, and inhibition of CAF function is beneficial for the host organism carrying the tumour.

Transforming growth factor β not only acts on fibroblasts but can also have a role in increasing the myofibroblast population via recruiting and promoting differentiation of mesenchymal stem cells, thus sustaining the trophic role of myofibroblasts on tumour growth and supporting cancer cell invasiveness and subsequent metastatic dissemination. A recent study has pointed out how TGF β released by prostate cancer cells via exosomes, but not soluble, secreted TGF β , can trigger the differentiation of BM-MSC into myofibroblasts (Chowdhury *et al*, 2015). In particular, exosomal TGF β shifts the physiological adipogenic differentiation of BM-MSC towards the more pathological myofibroblast phenotype. The resulting myofibroblasts exhibit high expression of α SMA, MMPs, HGF and vascular endothelial growth factor-A (VEGF-A). The latter provides pro-angiogenic signals by acting on endothelial cells and experimentally exhibited a tumour promoting role based on a 3D co-culture model. Furthermore, osteopontin (OPN), which is normally present in the ECM, can influence TGF β signalling. In particular, OPN triggers $\alpha_v\beta_3$ integrin to induce the

myeloid zinc finger 1 transcription factor causing an increase in TGF β 1 expression, and this allows the differentiation of mesenchymal stem cells into myofibroblasts (Weber *et al*, 2014). In this scenario, the obtained CAFs enhance the tumour occurrence *in vivo* when co-injected with breast cancer MDA-MB-231 cells, supporting the general concept of a trophic role of CAFs towards carcinoma cells.

TGF β effect on immune cells in the tumour stroma. One of the main reasons behind tumour progression is the ineffective immune response against cancer (immune suppression), or the development of immune tolerance towards cancer-associated and cancer-specific antigens (Yang *et al*, 2010). It is well established that TGF β is essential for the regulation of the innate and adaptive immune system under physiological or pathological conditions (Flavell *et al*, 2010). In cancer, in particular, it seems that TGF β antagonises the effective innate and adaptive immunity responses, in order to promote cancer growth and metastatic dissemination, as depicted in Figure 1. A most illustrative example has been the complete loss of cancer growth in mice where T β RII was knocked out in T lymphocytes, causing resistance to TGF β and generation of regulatory T cells, which elicit a potent anti-tumoural response (Gorelik and Flavell, 2001). A recent example that expands on the previous study and underlines the significance of TGF β effect on adaptive immunity provides more mechanistic insight. CD8 $^+$ T cells become unresponsive against tumour antigens due to the upregulation of the transcription factor FOXP1 (Stephen *et al*, 2014). FOXP1 blocks CD8 $^+$ activity due to a direct inhibitory effect on c-Jun, and upon TGF β stimulation, FOXP1–Smad2/3 complexes accumulate in pre-activated CD8 $^+$ T cells, which results in an inhibitory effect on c-Myc expression and on cell proliferation. As a result, the tumour suppressive effect of CD8 $^+$ T cells is completely abrogated (Stephen *et al*, 2014). In addition to differentiation, TGF β regulates the residence of CD8 $^+$ T cells in epithelial tissues. In the skin, CD8 $^+$ T-cell residence depends on the proper expression of integrin receptor complexes on the surface

of the epithelial cells, and on the regulated activation of latent TGF β in the T-cell microenvironment (Mohammed *et al*, 2016). This event provides clues about the importance of a regulated degree of activation of TGF β that can have homeostatic functions, whereas upon deregulation of the balance, TGF β can exhibit its pro-tumourigenic activities. Interestingly, it has been demonstrated how restricted loss of Smad4-dependent signalling in the T-cell population alters the physiological communication between adaptive immunity cells and tissue parenchyma (the epithelial cells in the latter retaining the normal function of Smad4), allowing the arousal of spontaneous epithelial cancers in the gastrointestinal tract, thus further underlining that a productive adaptive immune response is essential for effective tumour suppression (Kim *et al*, 2006).

Beyond T cells, macrophages have important roles in the tumour stroma. Macrophages can differentiate in two different lineages: M1 or classically activated, and M2 or alternatively activated, in response to different microenvironmental cues. The so-called tumour-associated macrophages (TAM) express a comparable phenotype to M2 macrophages and TGF β 2 can induce M2 macrophage polarisation, as described in Figure 1, whereas knock out of T β R11 results in less functional M2 macrophages (Gong *et al*, 2012). Interestingly, it has recently been described how M2-TAM can actively produce TGF β , which induces EMT and acquisition of cancer stem cell features in an *in vitro* model of HCC, leading to a worse prognosis in patients (Fan *et al*, 2014a). Moreover, TGF β has a similar effect on pro-tumourigenic N2 neutrophils and blocking TGF β signalling switches the cellular phenotype to the anti-tumourigenic N1 neutrophil population (Pickup *et al*, 2013). In addition to this, TGF β has an antagonistic effect on the activation of antigen-presenting dendritic cells and impairs the maturation of natural killer cells (Figure 1), thus inhibiting an effective innate response towards cancer (Pickup *et al*, 2013).

Breast cancer cells carrying knockout for T β R11 present more aggressive and highly metastatic behaviour to the lungs of mice compared with sibling carcinoma cells having wild-type T β R11 (Yang *et al*, 2008). The invasive periphery of such tumours is highly enriched in myeloid cells that express the Gr-1 and CD11b antigens on their surface, and their immunological ablation decreases the rate of metastatic dissemination. The T β R11 knockout cancer cells oversecrete chemokines, SDF-1 and CXCL5, which then attract the myeloid cells to the primary tumour; as a paracrine response, the myeloid cells oversecrete TGF β 1 and activate MMPs in the microenvironment, which facilitates invasiveness and dissemination (Yang *et al*, 2008). In particular, the lung tissue becomes remodelled in response to the extracellular activity of the MMPs secreted by the myeloid cells, which appear as groups intercalated with tumour colonies (Yan *et al*, 2010). Interestingly, genetic ablation of T β R11 in the myeloid cells suppresses the metastatic homing of breast cancer cells, emphasising the importance of this stromal cell type (Meng *et al*, 2016). Basic fibroblast growth factor (bFGF) could rescue the bone metastasis when injected intravenously in the mice with the T β R11 knockout myeloid cells, demonstrating that TGF β signalling in the myeloid cells controls bFGF secretion, which then acts on the metastatic carcinoma cells to assist their homing (Meng *et al*, 2016). Similar studies with T β R11 ablation in the myeloid cells revealed a role for the chemokine CCL9, which is secreted by the TGF β -responsive myeloid cells and acts on the carcinoma cells promoting their survival and facilitating their metastatic homing (Yan *et al*, 2015). In agreement with the mouse model, the orthologous to CCL9 human chemokine CCL23 is highly expressed in the peripheral blood myeloid cells from patients with aggressive cancer (Yan *et al*, 2015). The metastatic process is also linked to the action of platelets. Invading and intravasating breast cancer cells associate with platelets, which provide a rich source of TGF β , initiating

breast epithelial EMT (Labelle *et al*, 2011). The interaction between platelets and breast cancer cells initiates upon unknown signalling pathways that mediate activation of the nuclear factor κ B, which synergises with TGF β signalling to elicit robust EMT, tumour cell migration and intravasation leading to lung metastasis. In addition, the tumour-associated platelets release chemokines CXCL5 and CXCL7, which recruit granulocytes (Labelle *et al*, 2011). The granulocytes sustain the metastatic colonisation via their enhanced MMP activity, and they co-migrate with the cancer cells through the lung capillaries.

Unexpectedly, the concept of TGF β -mediated immune suppression can also be exploited therapeutically, as demonstrated in therapy trials of the brain tumour glioblastoma using oncolytic Herpes simplex viral particles (Han *et al*, 2015). Pre-treatment of the tumour-bearing animal with a single dose of TGF β prior to the administration of the oncolytic virus was able to effectively suppress resident natural killer and microglial cells so that the virus could elicit more robust cytotoxicity and limit tumour growth by prolonging life expectancy (Han *et al*, 2015).

TGF β in metastatic dissemination. Transforming growth factor β generally has a positive role in cancer dissemination and metastasis due to the shift of the TGF β response from growth arrest to invasion and metastatic dissemination in the primary tumour (Roberts and Wakefield, 2003; Pickup *et al*, 2013). In addition, TGF β can enhance metastasis by positively affecting neoangiogenesis and lymphangiogenesis, by promoting aggregation of cancer cells and CAFs, transendothelial migration of metastatic cell clones, by inducing microRNAs and lncRNAs with pro-metastatic effects and by acting on the tumour microenvironment in order to allow a permissive milieu to dissemination (Padua and Massagué, 2009; Peñuelas *et al*, 2009), as described in Figure 1.

Seminal studies aiming at identifying genes that control the metastatic behaviour of breast cancer cells that selectively colonise the bone identified a cohort of secreted factors that collectively contribute to bone destruction and generation of a permissive microenvironment for the establishment of metastatic cancer (Kang *et al*, 2003). The metastatic cells secrete polypeptides such as IL11, which mobilises osteoclasts to start damaging the bone, and connective tissue growth factor, which promotes local angiogenesis. The abundance of these two cytokines in the metastatic microenvironment is further boosted by the local release of TGF β from rich bone depots, leading to an enhanced loop of cytokine–bone destruction–TGF β and back to cytokine release (Kang *et al*, 2003). Unexpectedly, the release of TGF β from its storage in normal bone, during osteolysis promoted by the metastatic breast cancer cells, is also responsible for the deterioration of the associated muscle that eventually leads to cachexia (Waning *et al*, 2015). Transforming growth factor β acting on muscle cells induces expression of the NADPH oxidase Nox4, which oxidises signalling proteins in the muscle, including the ryanodine receptor and the calcium release channel, both playing critical roles in maintaining physiological calcium levels for the function of the muscle. The resulting deterioration in calcium availability weakens the force-generating capacity of the muscle and initiates cachexia (Waning *et al*, 2015).

More intriguing is the mechanism by which TGF β acting in the primary breast tumour induces angiopoietin-like 4, which is retained by the metastatic cancer cells and facilitates their extravasation specifically to the lung (Padua *et al*, 2008). Angiopoietin-like 4 released from metastatic cells disrupts endothelial cell–cell adhesions in lung capillaries, promoting delivery of the tumour cells to the distant tissue. This is an example whereby the action of TGF β in the primary tumour, conditions the cancer cells with capacities for manipulation of the microenvironment at the target metastatic site. Transforming growth factor β can also promote the formation of a pro-metastatic

extracellular environment and can actively influence the interaction between cancer cells with ECM and stromal cells. It has been recently reported how TGF β can induce the expression of the metalloprotease ADAM17, which promotes the release of the adhesion protein ALCAM from metastatic prostate cells *in vivo* and *in vitro* (Hansen *et al*, 2014). ALCAM shedding appears to be required for the effective prostate cancer metastasis to the bone and positively correlates with the tumour burden in subcutaneous and orthotopic *in vivo* models. Moreover, the tumour-shed ALCAM mediates TGF β -induced migration and bone metastasis without affecting primary tumour growth; persistent ALCAM shedding in the metastatic bone microenvironment also promotes the survival and proliferation of metastatic cells (Hansen *et al*, 2014). An alternative pro-metastatic mechanism induced by TGF β signalling in prostate carcinomas involves one of the most highly sensitive target genes of the TGF β pathway, one encoding for the plasma membrane protein PMEPA1 (Fournier *et al*, 2015). The regulatory function of PMEPA1 involves interaction with Smad proteins and ubiquitin ligases, causing a relative decrease in TGF β signalling. Interestingly, upon prostate cancer metastasis, PMEPA1 expression is downregulated by as yet uncharacterised mechanisms, causing a release of TGF β /Smad signalling from the negative control of PMEPA1 and promoting the homing of metastatic cells to bone (Fournier *et al*, 2015).

Moreover, TGF β and the TGF β co-receptor family member Endoglin can promote tumour angiogenesis, thus positively affecting metastatic dissemination (Pardali and ten Dijke, 2009). Transforming growth factor β can directly activate endothelial cell proliferation and migration, promotes the capillary formation *in vitro* and neoangiogenesis *in vivo* (Pardali and ten Dijke, 2009). Moreover, this cytokine can induce the expression of VEGF, the major growth factor promoting vascularisation, and this can be promoted by hypoxia, a frequent condition in the microenvironment of growing tumours (Pardali and ten Dijke, 2009). Furthermore, TGF β can recruit inflammatory cells that secrete pro-angiogenic factors and it can promote the synthesis of MMP, integrins and plasminogen activators, which have an important role in the initiation and progression of angiogenesis (Pardali and ten Dijke, 2009).

The importance of TGF β for the sprouting of lymphatic vessels and of the lymphatic network in the skin has also been reported, and furthermore, how inhibition of the TGF β pathway allows the lymphatic microvascular endothelial cells to migrate, proliferate and form functional lymphatic vessels in response to the growth factor VEGF-C, an important promoter of lymphangiogenesis (Oka *et al*, 2008; James *et al*, 2013). These findings suggest an inhibitory role of TGF β on lymphangiogenesis in this particular context. Intriguingly, the transcription factor SIX1 has been described to enhance the Smad-dependent TGF β signalling and this results in the transcriptional induction of the growth factor VEGF-C, and promotion of lymphatic metastatic dissemination, thus pointing out the mechanism by which TGF β impacts on this physiological process (James *et al*, 2013; Liu *et al*, 2014). In addition, metastasis of breast cancer cells that undergo EMT through the lymphatic system also involves upregulation of the chemokine CCL21, which is secreted by lymphatic endothelial cells responding to TGF β (Pang *et al*, 2016). CCL21 signals by binding to its receptor CCR7, whose expression is also upregulated by TGF β on the surface of the breast carcinoma cells that exhibit EMT (Pang *et al*, 2016). This mechanism attracts the metastatic cells to the lymphatic vessels for intravasation and subsequent dissemination to local lymph nodes.

Modern techniques, such as intravital microscopy, allow detection of a switch in TGF β signalling during metastasis. It has been elegantly described how TGF β differentially regulates cell migration, affecting the type of systemic dissemination of tumour cells: cancer cells exhibiting high levels of TGF β activity are highly

motile and can intravasate as single cells, whereas cells with lower TGF β activity are characterised by cohesive and collective movement, which allows their lymphatic dissemination (Giampieri *et al*, 2010). Moreover, it has been underlined that only cells with high TGF β levels are poorly differentiated and are actually able to migrate, whereas TGF β signalling cannot cause the same effect in highly differentiated cells. Reasonably, it is important for TGF β signalling to be switched off at the secondary tumour site in order to enable proliferation of the metastatic cells (Giampieri *et al*, 2010). Three dimensional co-culture studies also demonstrated that mesenchymal stem cells that secrete TGF β promote a characteristic directional migration of breast carcinoma cells, which exhibit elongated morphology and develop strong traction between them, factors that facilitate tumour cell invasiveness (McAndrews *et al*, 2015).

Transforming growth factor β is classically associated with the induction of EMT and with migratory and invasive cell properties, which allows the local and systemic dissemination of selected clones of primary tumour cells in order to establish metastasis at a distant organ. As an example, in HCC, the tyrosine kinase receptor Axl induces and modulates the autocrine TGF β pathway and this positively correlates with HCC invasion, transendothelial invasion and metastatic dissemination *in vivo* (Reichl *et al*, 2015). Such a role of Axl downstream of TGF β has also been observed in breast carcinoma (Li *et al*, 2015) and supports the double-positive loop whereby tumour progression may be promoted by the Axl-TGF β -Axl double axis, a prominent target for new therapeutic intervention. Intriguingly, TGF β signalling also induces expression of Axl in the pancreas and the skin under non-cancerous, homeostatic conditions (Bauer *et al*, 2012).

In breast cancer, the nuclear factor NR4A1 activates TGF β /Smad signalling by increasing the half-life of T β RI on the plasma membrane, and by inducing Smad7 degradation via an AXIN2-RNF12/ARKADIA ubiquitylation machinery in cancer cells and in CAFs (Zhou *et al*, 2014). This results in the acquisition of TGF β -induced EMT features and in the increase of cell migration, invasion and metastasis *in vitro* and *in vivo*. Interestingly, NR4A1 appears to integrate signals from different pathways as follows: it mediates the IL1 β and TNF α actions on EMT and cancer cell migration *in vivo*; and it promotes TGF β signalling, emphasising the cooperative role of these cytokines in malignancy.

Non-coding RNAs in metastatic dissemination. TGF β can also have a positive role on metastatic dissemination via the induction of lncRNAs, as it has been described for *lncRNA-ATB* (Yuan *et al*, 2014). *lncRNA-ATB* upregulates the homeobox and zinc finger transcription factors ZEB1 and ZEB2 due to a competitive effect on the microRNA (*miR*)-200 family, which base pairs with the *lncRNA-ATB*, thus permitting ZEB1/ZEB2 synthesis and resulting in EMT and invasion of HCC cells. In this respect, *lncRNA-ATB* functionally mimics the action of *ZEB2NAT*, which operates in CAFs (Zhuang *et al*, 2015), as described earlier. *lncRNA-ATB* also binds the *IL11* mRNA promoting an IL11-STAT3 signalling loop responsible for dissemination of HCC cells. This effect is independent from the *miR-200* inhibition and contributes to an increased colonisation and metastasis occurrence in the lungs. Bladder cancer metastasis is as well promoted in part due to the TGF β -induced upregulation of the lncRNA *Malat1* (Fan *et al*, 2014b). Upon TGF β stimulation *Malat1* associates with the Polycomb corepressor subunit Suz12, and this induces an EMT phenotype in bladder cancer, concerning in particular the repression of E-cadherin, and promoting migration and invasion *in vivo* (Fan *et al*, 2014b). *Malat1* and its protein partner Suz12 appear to be crucial for this process, as their silencing abrogates the migratory and invasive properties promoted by TGF β .

Similar mechanisms involve microRNAs, such as the oncogenic *miR-155*, which negatively regulates the expression of the

transcription factor C/EBP β , a differentiation factor for the mammary epithelium (Johansson *et al*, 2013). As a consequence, breast cancer cells become insensitive to the TGF β growth inhibitory effects and acquire EMT features, promoting their invasion and dissemination to the lungs of tumour-bearing mice (Johansson *et al*, 2013). In particular, when *miR-155* silences C/EBP β in the cancer cells, autocrine production of TGF β 1 or downstream phosphorylation of Smad3 is not perturbed, confirming that the *in vivo* effects are due to a switch in TGF β response, and not due to ineffective TGF β signalling. In a distinct mechanism, TGF β signalling is enhanced in pancreatic adenocarcinomas in part because of the downregulation of the *miR-323-3p* (Wang *et al*, 2016). The *miR-323-3p* downregulates expression of Smad2 and Smad3 and relatively high expression of this microRNA maintains a controlled, low activity of TGF β signalling that serves homeostatic functions. An epigenetic mechanism affecting Smad2 and Smad3 expression operates in lung adenocarcinomas (Tang *et al*, 2015). High TGF β signalling promoting EMT and invasive, metastatic growth is achieved by high expression of Smad2 and Smad3, which is coordinately regulated by the cytoplasmic protein profilin-2. Tumour cells overexpress profilin-2, which sequesters the histone deacetylase HDAC1 in the cytoplasm, thus releasing the transcriptional control from Smad2 and Smad3, which also become overexpressed (Tang *et al*, 2015). Whether this mechanism in lung carcinomas involves non-coding RNAs remains to be examined.

Conversely, microRNAs can be positive regulators of TGF β signalling by targeting the negative feedback mechanism of this pathway that is mediated by Smad7. Such an example is the miR-1269, which downregulates Smad7 and the transcription factor HoxD10 in colorectal cancers (Bu *et al*, 2015). By negatively affecting Smad7 expression, miR-1269 promotes TGF β signalling and colorectal carcinoma metastasis to the liver, whereas TGF β itself transcriptionally induces expression of the miR-1269, forming a feed-forward signalling loop that counteracts the classical negative feedback loop of Smad7 in the context of tumour metastasis (Bu *et al*, 2015).

CONCLUSION

TGF β signalling is dysregulated in different types of cancers, thus affecting the overall progression to malignancy. For this reason TGF β has been considered a valuable target in oncology. Current approaches with TGF β inhibitors, such as the low molecular weight T β RI kinase inhibitor LY2109761, revealed an acquired chemoresistance in cancer patients (Akhurst and Hata, 2012). However, a careful clinical study where the dose of the improved T β RI inhibitor LY2157299 was tested in glioma patients revealed strong beneficial effects and complete lack of cardiotoxic side effects revealing the promise of anti-TGF β therapy (Rodon *et al*, 2015). Despite this recent progress, another way to obtain effective therapeutic treatment could be to apply personalised medicine based on the genetic background of the individual, which can define the TGF β response; this approach is viewed as particularly challenging considering the intricacy of this pathway (Akhurst and Hata, 2012), but it may soon gain applicability in the oncology clinic. Another effective approach could consist in finding biomarkers related to TGF β signalling – as for example USP15 in glioblastoma – which can be used to discriminate patient populations into those that are responsive and non-responsive to TGF β , in order to apply targeted therapeutic approaches only to the responsive cohort (Eichhorn *et al*, 2012). Similarly, a strategy that can be potentially useful to stratify colorectal cancer patients according to their prognosis has been recently suggested (Calon *et al*, 2015). This relies in a gene signature that is induced by TGF β

specifically in the colorectal tumour stroma (i.e. mainly the CAFs), rather than in the epithelial tumour, and such an approach might prove invaluable for the future use of TGF β signalling inhibitors, so that the inhibitors selectively interfere with the crosstalk between cancer cells and the tumour stroma. All such cases convincingly raise the TGF β pathway as a promising therapeutic target against all types of tumours, although deeper insight into the means for achieving personalised therapeutic benefits has to be gained.

ACKNOWLEDGEMENTS

We acknowledge funding by the Ludwig Institute for Cancer Research, the Swedish Cancer Society, the Swedish Research Council and the Marie Curie Initial Training Network 'IT-Liver' under the European Union FP7 program. We thank all past and present members of our group for their contributions to the scientific work emanating from our laboratory. Due to space limitations, we have been unable to include all relevant publications in our discussion. We apologise to those authors whose relevant work has not been included in this review article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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