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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 (TGFβ), regulates essentially every cell within the malignant tissue. In this article, we focus on the actions of TGFβ 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 TGFβ.

TGFβ IN CANCER

Transforming growth factor β (TGFβ) 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 TGFβ 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 TGFβ in human disease, including cancer, is the prominent role that TGFβ has on tissue homoeostasis 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, TGFβ 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, TGFβ 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 TGFβ 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 haematopoetic malignancies. Abnormal TGFβ 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 Blobe, 2008). In cancer, the homoeostatic action of TGFβ 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 TGFβ 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 TGFβ 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, haematopoetic and other malignancies (Gordon and Blobe, 2008). In particular, TGFβ disrupts homoeostasis 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 homoeostatic conditions. However, the signalling outcome of these pathways may be very different in normal *vs* malignant cells. The

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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 TGFβ signalling activity involve receptors on the cell surface named type II (TβRII) and type I (TβRI), which bind the ligand as a heterotetrameric complex, leading to activation of their intrinsic protein kinase activity (Akhurst and Hata, 2012). TβRII is the primary ligand-binding receptor that trans-phosphorylates TβRI, 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 TGFβ-Smad signalling include the inhibitory Smads, such as Smad7, which accumulate upon signalling and negatively regulate the pathway at the level of TβRI degradation, Smad2/Smad3 phosphorylation by TβRI and at the level of chromatin-bound Smad complexes, which are transcriptionally blocked by Smad7 (Akhurst and Hata, 2012).

Alternatively, the TβRII–TβRI 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 TGFβ but also make reference to signalling and mechanistic details wherever possible, pointing out how TGFβ 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.

TGFβ SIGNALLING IN CANCER STEM CELLS

Similar to its complex role in cancer progression, TGFβ can have a dual function concerning the biology of CSCs, inhibiting or sustaining their function. As an example, TGFβ 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 TGFβ, 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, TGFβ 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 TGFβ 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 TGFβ 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 TGFβ (Katsuno *et al*, 2012).

On the other side, several studies underline how TGFβ 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), TGFβ 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 DNMT3β, 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 TGFβ signalling and mesenchymal differentiation (Mima *et al*, 2012). Genetic ablation of TβRII in mice, followed by chemically induced carcinogenesis of the bladder also attests to the positive role TGFβ 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, TGFβ 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 TGFβ 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 TGFβ 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 TGFβ regulates Akt phosphorylation and Foxo3a nuclear localisation, which is required for the stemness properties of LICs (Naka *et al*, 2010). Accordingly, a new therapeutic prospective can be based on the adjuvant administration of TGFβ 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 TGFβ and other developmental pathways is also relevant. The cooperation between TGFβ and tumour necrosis factor α (TNFα) positively affects the acquisition of a CSC phenotype in breast cancer due to the induction of EMT. In particular, the TGFβ-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, TGFβ 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 TGFβ-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 TβRI and Smad3 and of Snail1 and Snail2, transcriptional mediators of EMT (Kudinov *et al*, 2016). This model suggests that TGFβ signalling promotes lung adenocarcinoma CSCs and their metastatic potential and that the TGFβ pathway is under the translational control of musashi-2.

TGFβ 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 TGFβ is implicated in metastasis (Padua and Massagué, 2009; Hansen *et al*, 2014). It has been reported that TGFβ is able to create a tissue microenvironment permissive to the metastatic dissemination (Pickup *et al*, 2013), and that TGFβ can contribute to the local invasion, blood-borne metastatic dissemination and colonisation of distant organs (Calon *et al*, 2012; Calon *et al*, 2014).

TGFβ 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 TGFβ, 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 TGFβ, 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 TGFβ1-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 (αSMA). Transforming growth factor β once again has a major role in mediating this terminal differentiation process. The TGFβ1 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 αSMA expression and incorporation of αSMA into stress fibres, one of the clear hallmarks of the myofibroblast phenotype. Interestingly enough, the Smad proteins differentially regulated the TGFβ1-induced morphological and functional changes, in particular Smad2 but not Smad3 affected αSMA, whereas both Smad2 and Smad3 affected collagen regulation (Evans *et al*, 2003).

In the context of cancer, early experiments using fibroblast-specific ablation of TβRII in mice demonstrated that fibroblasts have homoeostatic roles within a tissue such as prostate or stomach, as loss of TβRII 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, TGFβ is produced by terminally differentiated CAFs, acting in an autocrine fashion on the same CAFs that have produced it (stromal TGFβ-activation) while the epithelial cancer cells can also produce TGFβ, which acts in a paracrine way on the fibroblasts (epithelial TGFβ-activation) (Calon *et al*, 2014; Hawinkels *et al*, 2014). More specifically, CAFs can produce TGFβ1 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, TGFβ1 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 TGFβ1, 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 TGFβ1 and this leads to the hyperactivation of the TGFβ 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 αSMA (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 TGFβ, 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 TGFβ response in the stromal CAFs significantly impairs the tumour initiation (Calon *et al*, 2012). Furthermore, TGFβ 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, TGFβ increases cancer cell proliferation in the primary tumour and in the metastasis, and TGFβ-treated colon cancer cells more efficiently bind to endothelial cells, whereas CAFs exposed to TGFβ 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 TGFβ1, 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 TGFβ 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

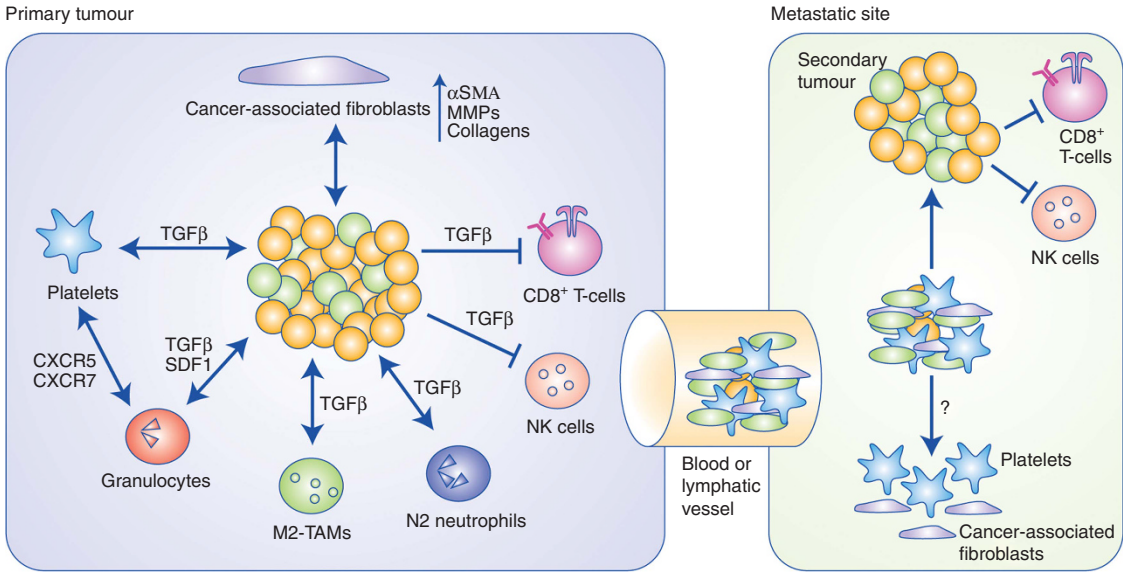

Figure 1. Pleiotropic effects of TGFβ in the tumour microenvironment. Representation of a primary tumour with cancer stem cells (CSCs) or metastasis-initiating cells (light green) and the bulk of the tumour cells (orange). Different cell types surrounding the tumour mass can positively contribute to 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 TGFβ 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 TGFβ-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 TGFβ revealed that cardiac glycosides, such as digoxin, which naturally regulate contractility of the heart muscle, effectively blocked CAF activation and cell contractility induced by TGFβ (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 TGFβ released by prostate cancer cells via exosomes, but not soluble, secreted TGFβ, can trigger the differentiation of BM-MSC into myofibroblasts (Chowdhury *et al*, 2015). In particular, exosomal TGFβ shifts the physiological adipogenic differentiation of BM-MSC towards the more pathological myofibroblast phenotype. The resulting myofibroblasts exhibit high expression of αSMA, 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 TGFβ signalling. In particular, OPN triggers $\alpha_v\beta_3$ integrin to induce the myeloid zinc finger 1 transcription factor causing an increase in TGFβ1 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.

TGFβ 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 TGFβ 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 TGFβ 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 TβRII was knocked out in T lymphocytes, causing resistance to TGFβ 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 TGFβ 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 TGFβ 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, TGFβ 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 TGFβ in the T-cell microenvironment (Mohammed *et al*, 2016). This event provides clues about the importance of a regulated degree of activation of TGFβ that can have homoeostatic functions, whereas upon deregulation of the balance, TGFβ 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 TGFβ2 can induce M2 macrophage polarisation, as described in Figure 1, whereas knock out of TβRII results in less functional M2 macrophages (Gong *et al*, 2012). Interestingly, it has recently been described how M2-TAM can actively produce TGFβ, 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, TGFβ has a similar effect on pro-tumourigenic N2 neutrophils and blocking TGFβ signalling switches the cellular phenotype to the anti-tumourigenic N1 neutrophil population (Pickup *et al*, 2013). In addition to this, TGFβ 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 TβRII present more aggressive and highly metastatic behaviour to the lungs of mice compared with sibling carcinoma cells having wild-type TβRII (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 TβRII 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 TGFβ1 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 TβRII 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 TβRII knockout myeloid cells, demonstrating that TGFβ 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 TβRII ablation in the myeloid cells revealed a role for the chemokine CCL9, which is secreted by the TGFβ-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 orthologuous 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 TGFβ, 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 κB, which synergises with TGFβ 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 TGFβ-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 TGFβ 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).

TGFβ in metastatic dissemination. Transforming growth factor β generally has a positive role in cancer dissemination and metastasis due to the shift of the TGFβ response from growth arrest to invasion and metastatic dissemination in the primary tumour (Roberts and Wakefield, 2003; Pickup *et al*, 2013). In addition, TGFβ 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 TGFβ from rich bone depots, leading to an enhanced loop of cytokine–bone destruction–TGFβ and back to cytokine release (Kang *et al*, 2003). Unexpectedly, the release of TGFβ 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 TGFβ 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 TGFβ 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 TGFβ 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 TGFβ-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 TGFβ signalling in prostate carcinomas involves one of the most highly sensitive target genes of the TGFβ 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 TGFβ signalling. Interestingly, upon prostate cancer metastasis, PMEPA1 expression is downregulated by as yet uncharacterised mechanisms, causing a release of TGFβ/Smad signalling from the negative control of PMEPA1 and promoting the homing of metastatic cells to bone (Fournier *et al*, 2015).

Moreover, TGFβ and the TGFβ 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, TGFβ 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 TGFβ for the sprouting of lymphatic vessels and of the lymphatic network in the skin has also been reported, and furthermore, how inhibition of the TGFβ 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 TGFβ on lymphangiogenesis in this particular context. Intriguingly, the transcription factor SIX1 has been described to enhance the Smad-dependent TGFβ 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 TGFβ 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 TGFβ (Pang *et al*, 2016). CCL21 signals by binding to its receptor CCR7, whose expression is also upregulated by TGFβ 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 TGFβ signalling during metastasis. It has been elegantly described how TGFβ differentially regulates cell migration, affecting the type of systemic dissemination of tumour cells: cancer cells exhibiting high levels of TGFβ activity are highly motile and can intravasate as single cells, whereas cells with lower TGFβ 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 TGFβ levels are poorly differentiated and are actually able to migrate, whereas TGFβ signalling cannot cause the same effect in highly differentiated cells. Reasonably, it is important for TGFβ 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 TGFβ 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 TGFβ 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 TGFβ 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-TGFβ-Axl double axis, a prominent target for new therapeutic intervention. Intriguingly, TGFβ signalling also induces expression of Axl in the pancreas and the skin under non-cancerous, homoeostatic conditions (Bauer *et al*, 2012).

In breast cancer, the nuclear factor NR4A1 activates TGFβ/Smad signalling by increasing the half-life of TβRI 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 TGFβ-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 IL1β and TNFα actions on EMT and cancer cell migration *in vivo*; and it promotes TGFβ signalling, emphasising the cooperative role of these cytokines in malignancy.

Non-coding RNAs in metastatic dissemination. TGFβ 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 TGFβ-induced upregulation of the lncRNA *Malat1* (Fan *et al*, 2014b). Upon TGFβ 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 TGFβ.

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

transcription factor C/EBPβ, a differentiation factor for the mammary epithelium (Johansson *et al*, 2013). As a consequence, breast cancer cells become insensitive to the TGFβ 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/EBPβ in the cancer cells, autocrine production of TGFβ1 or downstream phosphorylation of Smad3 is not perturbed, confirming that the *in vivo* effects are due to a switch in TGFβ response, and not due to ineffective TGFβ signalling. In a distinct mechanism, TGFβ 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 TGFβ signalling that serves homoeostatic functions. An epigenetic mechanism affecting Smad2 and Smad3 expression operates in lung adenocarcinomas (Tang *et al*, 2015). High TGFβ 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 TGFβ 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 TGFβ signalling and colorectal carcinoma metastasis to the liver, whereas TGFβ 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

TGFβ signalling is disregulated in different types of cancers, thus affecting the overall progression to malignancy. For this reason TGFβ has been considered a valuable target in oncology. Current approaches with TGFβ inhibitors, such as the low molecular weight TβRI 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 TβRI inhibitor LY2157299 was tested in glioma patients revealed strong beneficial effects and complete lack of cardiotoxic side effects revealing the promise of anti-TGFβ 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 TGFβ 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 TGFβ signalling – as for example USP15 in glioblastoma – which can be used to discriminate patient populations into those that are responsive and non-responsive to TGFβ, 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 TGFβ 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 TGFβ signalling inhibitors, so that the inhibitors selectively interfere with the crosstalk between cancer cells and the tumour stroma. All such cases convincingly raise the TGFβ 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.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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