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Transforming growth factor-β, MAPK and Wnt signaling interactions in colorectal cancer

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ABSTRACT

In non-cancerous cells, transforming growth factor-β (TGFβ) regulates cellular responses primarily through Smad signaling. However, during cancer progression (including colorectal) TGFβ promotes tumoral growth via Smad-independent mechanisms and is involved in crosstalk with various pathways like the mitogen-activated protein kinases (MAPK) and Wnt. Crosstalk between these pathways following activation by TGFβ and subsequent downstream signaling activity can be referred to as a crosstalk signaling signature. This review highlights the progress in understanding TGFβ signaling crosstalk involving various MAPK pathway members (e.g., extracellular signal-regulated kinase (Erk) 1/2, Ras, c-Jun N-terminal kinases (JNK) and p38) and the Wnt signaling pathway.

1. Introduction

Globally, colorectal cancer (CRC) was the third most commonly diagnosed cancer in 2012 with over 1.36 million new cases (9.7% of all cancers). Then, it led to almost 694,000 deaths (i.e., 8.5% of all cancer deaths) [1]. Australia and New Zealand have one of the highest incidence rates globally (44.8 and 32.2 per 100,000 in men and women respectively), whilst the lowest rates are found in Middle Africa (4.5 and 3.5 respectively per 100,000 in men and women respectively) [1].

Diagnostically, various staging systems have been developed to describe progression of the severity of the disease (e.g., TNM Classification of Malignant Tumours, Australian Clinico-pathological Staging (ACPS) System [2] and Dukes' staging system [3]). These staging tools, usually obtained from patho-histological analyses of CRC biopsies, help clinical oncologists to assess size, location and the spread of the cancerous lesion to other parts of the body and aid in patient treatment and management. Many studies have shown that CRC survival rates primarily dependent on how advanced the cancer is at initial diagnosis. Despite the availability of numerous screening strategies (Table 1) aggressive surgical therapies and extensive research on the genomic, molecular and cellular basis of CRC, detection at the earliest stages remains elusive.

If detected early, CRC is associated with excellent 5-year survival (>90%) following simple (often curative) surgical resection, while patients diagnosed with later stage cancers (ACPS or Dukes' C or D) experience recurrence and distant metastases leading to particularly poor 5-year survival rates of less than 10% [4]. This progressive decrease in survival rates between early to late stage CRCs (90–10%) has been shown to be associated with the disruption of a number of well-established signaling pathways (Supplementary Table 1). These include, but are not limited to, transforming growth factor-beta (TGFβ)-Smad signaling, mitogen-activated protein kinase (MAPK) signaling pathways and Wnt signaling. This review will briefly discuss TGFβ ligands, their receptors, TGFβ canonical signaling through Smads and will highlight recent findings concerning its role/s in CRC and extensively focusing on the signaling crosstalk of TGFβ with the above-mentioned pathways. TGFβ, or proteins in associated pathways, could be used as early detection biomarkers that may, in the long term, improve survival and management of the global CRC health burden. A list of potential early detection biomarkers for CRC is provided in Table 2.

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Table 1
Currently available/emerging CRC screening strategies, ns: not specified.

Test	Sensitivity (%)	Specificity (%)	Frequency	Year developed	Comments	Ref.
Traditional assays						
Colonoscopy	>95	95–99	Every 10 years from age 50	1969	The current gold standard, but invasive, expensive and requires bowel preparation	[5]
Sigmoidoscopy	98–100	35–70	5 years	1976	Only screens the distal colon and rectum	[6]
Double contrast barium enema (DCBE)	45	90	5 years	1920s–1930s	Detects only 30–50% of tumors detected by colonoscopy. Recommended only if endoscopic screening options are not available.	[7]
Computed tomography (CT) colonography	90	86	5 years	1994	Becoming accepted as an alternative to colonoscopy.	[8]
Guaiac fecal occult blood test (GFOBT)	16–38	98–99	Yearly	1967	Detects traces of blood released from bowel cancers or their precursors (polyps or adenomas) into the stool. Results may be affected by consumption of red meat and vitamin C. All positive FOBT tests are often followed up with colonoscopy.	[9]
Fecal immunochemical test (FIT)	56–89	91–98	Yearly	1978	Specific antibodies are used against the globin component of hemoglobin. Unaffected by dietary intake, but the epitope may be destroyed by bacterial enzymes in the stool giving false negatives	[9]
Fecal DNA	52–91	93–97	3 years	2003	Identifies genetic alterations involved in adenoma-carcinoma progression. ColoSure™ test, for example, detects methylation of the vimentin gene, an epigenetic marker.	[10]
Carcinoembryonic antigen (CEA)	43	90	ns	1969	Not suitable for routine detection, but useful for monitoring recurrence.	[11]
Emerging assays						
Colon capsule endoscopy (CCE)	>80	64–95	ns	2006	A non-invasive technique in which a capsule containing a wireless camera is swallowed and transmits images of the inside of the digestive tract to an extracorporeal monitor. Second generation colon capsule endoscopy has a diagnostic sensitivity of 89% or higher to identify polyps >5 mm.	[12,13]
MicroRNA	50–90	>70	ns	2009	miR92 reported as elevated in the plasma of CRC patients compared with controls	[14,15]
Blood RNA (ColonSentry)	72	70	Anytime	2008	Blood-based test which measures the RNA of seven-gene biomarker panel (ANXA3, CLEC4D, LMNB1, PRRG4, TNFAIP6, VNN1 and IL2RB) extracted from peripheral blood cells	[16]
SEPT9	67–96	81–99	ns	2008	Blood-based test which measures the methylated SEPT9 DNA in plasma	[16]

2. TGFβ superfamily ligands

The TGFβ superfamily consists of a large family of secreted cytokines that regulate a multitude of cellular functions and disease pathogenesis. The superfamily is divided into three major subfamilies; TGFβ, activin/inhibin/nodal branches and BMP/GDF (bone morphogenetic proteins/growth differentiation factors), all of whom possess diverse and complementary physiological effects. The TGFβ subfamily members, named for their cell transforming activities (i.e., cell growth and differentiation) from *in vitro* assays are now unequivocally known to be involved in both tumor suppression and tumor progression (i.e., proliferation, invasion and metastases). Activin and inhibins are well known positive and negative regulators of follicle-stimulating hormone respectively [17]. Nodal along with LEFTY-1 and LEFTY-2 is required for formation of mesoderm and axial patterning during embryonic development [18,19]. The GDF and BMP subfamily proteins have major roles in skeletal development [20], neurogenesis [21], and regulation of ovarian folliculogenesis [22].

The bona fide TGFβ subfamily consists of three TGFβ isoforms, TGFβ1, TGFβ2 and TGFβ3 encoded by three different genes located on different chromosomes (19q13.1, 1q41 and 14q24 respectively) but which are thought to function through the same receptor signaling systems. All TGFβ ligands are produced and secreted *in vivo* as 'latent' inactive zymogen complexes containing a mature TGFβ dimer in a non-covalent complex with latency associated peptides (LAP) that are bound to their respective latent TGFβ binding proteins [23]. The LAP domain ensures that 'inadvertent' release of TGFβ does not occur in normal cells under normal physiological conditions. Latent TGFβ can be activated *in vivo* through a variety of mechanisms. These include activation either by proteases (e.g., plasmin) [24] and/or various matrix metalloproteinases (MMP-2 and MMP-9) [25] by cleavage of the LAP. Alternatively, conformational changes in the LAP mediated by integrins αvβ6 [26], αvβ8 [27], and thrombospondin-1 [28] allow the release of active TGFβ1 from its associated LAP. The activation of TGFβ1 by integrin αvβ6 is restricted to epithelial tissues as αvβ6 is only expressed in those cells. Equally, the expression of TSP-1 in some epithelial tissues suggests the possibility that αvβ6 and TSP-1 may operate in tandem to activate latent TGFβ1. A recent study has shown that methylation of the TSP-1 gene results in suppression of TGFβ1 activation in CRC [29]. Integrin αvβ8-mediated activation, however, depends on the presence of MT1-MMP (MMP-14) [27]. It is therefore clear that TGFβ1 can be activated via a number of different mechanisms and in various cellular contexts. These allow it to play an important role in different cellular contexts and functions. As such, it is not surprising that alterations in plasmin or plasminogen binding [30] and alterations in expression of MMP-2 and MMP-9 [31], integrin αvβ6 [32] and active TGFβ1 [33] have been found collectively to be associated with poor CRC prognosis and subsequently poor survival.

3. TGFβ receptors

The TGFβ receptors were identified by methods involving affinity labeling of cells with radio-iodinated TGFβ (^{125}I-TGFβ) ligand and subsequent mapping of receptors to which this bound. Three different receptors, namely type I (53 kDa), type II (73–95 kDa) and type III (110 kDa) were identified depending on their molecular weights [58]. The type I and type II receptors were found to contain serine/threonine kinase domains and activity,

Table 2
Potential early detection biomarkers for CRC. nd: not determined.[a]

Candidate biomarker	Sample type	Mechanism of identification	Can discriminate	Sensitivity (%)	Specificity (%)	Ref.
Individual biomarkers						
Alpha 1-antitrypsin	Serum	Protein expression levels		87	73	[34]
Amphiregulin	Blood/serum	Protein expression levels	Controls from Dukes' A CRC	nd	nd	[35]
CEA	Blood/serum	Protein expression levels	Controls from Dukes' A–D stage CRCs	53	93	[34–37]
CXCL11	Blood/serum	Protein expression levels	Controls from Dukes' A CRC	nd	nd	[35]
CXCL5	Blood/serum	Protein expression levels	Controls from Dukes' A CRC	nd	nd	[35,38]
IL6	Blood/serum	Protein expression levels		27	95	[35]
IL8	Blood/serum	Protein, mRNA expression levels	Controls from Dukes' A CRC	30	95	[35,39]
Methylated Septin 9 (SEPT9)	Blood	DNA methylation		67–96	81–99	[40–44]
MMP7	Serum	mRNA expression levels		58	100	[34]
Suppressor of cytokine signalling (SOCS) 2 and SOCS6	Tumors	Protein expression levels		nd	nd	[45]
uPAR	Serum	mRNA expression levels		nd	nd	[34]
Collagen type X alpha1 (CPL10A1)	Serum	Protein expression levels	Controls from Adenoma and colon cancer	63	85	[46]
Metastasis associated in colon cancer 1 (MACC1)	Tumor samples	Protein expression levels		nd	nd	[47]
Biomarker panels						
Tumor associated monocyte genetic finger print	Blood monocyte samples	Gene expression		92.6	92.3	[48]
IGFBP2, DKK3 and PKM2	Blood	Protein expression levels		73	95	[49]
BMP3, NDRG4, VIM, TFPI2 and a mutant KRAS	Stool	DNA methylation	Cancer from controls	68–86	77–92	[50–53]
			Adenoma (size >1 cm) to controls	52–73	85–92	
			Adenoma (size ≥1 cm) to controls	45–62	85–92	
miR-19a-3p, miR-223-3p, miR-92a-3p and miR-422a	Serum	mRNA expression levels		84.3	91.6	[54]
miR-601 and miR-760	Plasma	mRNA expression levels	CRC to normal controls	83.3	69.1	[55]
			Adenomas to normal controls	72.1	62.1	[55]
miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, and miR-652	Plasma	mRNA expression levels	Polyps from controls	88	64	[56]

[a] The data presented in this table only summarises biomarkers from research published in the last 5–6 years. For a more detailed review on this topic please see "Biomarkers for Early detection of Colorectal Cancer and Polyps: Systematic Review" by Shah et al. [57].

whilst the type III receptors lacked any similar domain [59]. The detailed structure of these three receptors is illustrated in Fig. 1.

3.1. TGFβ type III receptors

The transforming growth factor type III receptor (TGFβR3) betaglycan is the most ubiquitously expressed type III receptor. Betaglycan acts as an accessory receptor by presenting TGFβ ligands to the type II receptors and promoting signaling [60]. The short cytoplasmic tail of betaglycan consists of a class I PDZ binding motif that binds to GAIP-interacting protein C-terminus (GIPC). GIPC interaction with betaglycan increases the stability of betaglycan at the cell surface and promotes TGFβ1 and TGFβ2 mediated gene expression in Mv1Lu mink lung epithelial and L6 myoblast cells [61]. More recently the GIPC-betaglycan interaction has been shown to inhibit TGFβ-mediated Smad signaling and migration in breast cancer cells. However, the exact mechanism/s by which this occurs has yet to be characterized [62].

3.2. TGFβ type II receptor

Transforming growth factor type II receptor (TGFβR2) is a transmembrane serine/threonine kinase receptor with a signal peptide, a cysteine-rich *N*-glycosylated extracellular domain, a transmembrane domain, and a cytoplasmic serine/threonine kinase domain flanked by a short juxtamembrane domain and C-terminal tail [63]. TGFβR2 can bind to all three TGFβ ligands, but cannot participate in downstream signaling in the absence of TGFβR1. The presence of betaglycan is also essential to facilitate high affinity TGFβ binding to TGFβR2 which then participates in downstream signaling. As yet, only TGFβs are known to bind to TGFβR2 in any extracellular context.

3.3. TGFβ type I receptor

Transforming growth factor type I receptor (TGFβR1) is also a transmembrane serine/threonine kinase receptor and closely resembles TGFβR2 in structure. TGFβR1 contains a signal peptide,

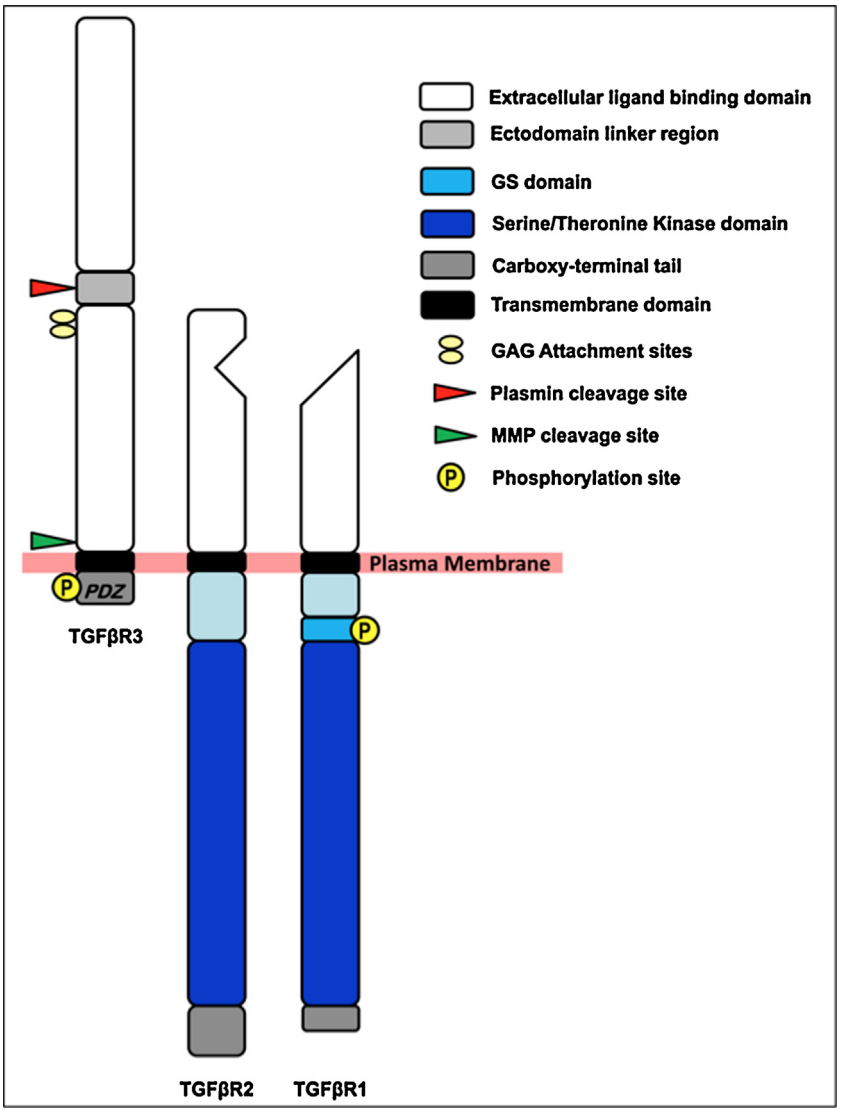

Fig. 1. Structure of TGFβ receptors type I (TGFβR1), type II (TGFβR 2) and type III (TGFβR3).

a cysteine-rich *N*-glycosylated extracellular domain, a cytoplasmic kinase region with 41% sequence homology to TGFβR2 and a very short C-terminal tail [64]. A unique feature of TGFβR1 is its highly conserved 30 amino acid region preceding the cytoplasmic kinase region that is called the GS domain because of the characteristic SGSGSG sequence it contains. Ligand-induced phosphorylation of serine and/or threonine residues in the GS region is required for signaling. TGFβR1 forms a heterodimer with TGFβR2 and this complex collectively takes part in TGFβ-mediated downstream signaling [64].

4. Canonical signaling of TGFβ receptors through Smads

Intracellular TGFβ signaling is complex and affects various cellular functions, both directly and indirectly. A well-characterized signaling pathway that is initiated by active heterodimeric TGFβ receptors is through Smads, although Smad-independent TGFβ signaling pathways are also known to exist [65]. TGFβ signaling via Smads is facilitated by TGFβR1 and TGFβR2, which form both homodimeric and heterodimeric complexes required for signaling. Dysfunction in one or more components of the functional TGFβ complex has been associated with cancers (including CRC) and these are briefly discussed later in this review.

Canonical Smad signaling (Fig. 2) is initiated by preferential binding of active TGFβ1 to TGFβR2 that then recruits, binds and transphosphorylates TGFβR1 in the GS region, inducing protein kinase activity. Active TGFβR1 then phosphorylates Smad2 and Smad3 which form a complex with Smad4 and translocate to the nucleus, where in combination with various DNA-binding co-activators, co-repressors and transcription factors, they regulate expression of TGFβ responsive genes [66]. The Smad2/3/4 complex induces expression of the cyclin-dependent kinase inhibitor p21, which then leads to cell growth arrest. Puzzlingly, Smad4 can only translocate into the nucleus when bound to receptor Smads (Smad1/2/3/5/8) whilst Smad2 and Smad3 can translocate into the nucleus in a Smad4-independent manner [67] implying a regulatory role for Smad4 rather than a simple signal transmission from cytoplasm to nucleus. Studies on various tumor cells suggest that TGFβ-mediated cell migration is not always dependent on Smad signaling, but also the activation of various mitogen-activated protein kinases (MAPK) and Rho GTPases that can be activated by non-canonical Wnt signaling pathways.

5. Non-canonical signaling of TGFβ receptors

Increasing evidence over the past few years has revealed that the complexity of TGFβ signaling responses is influenced not only

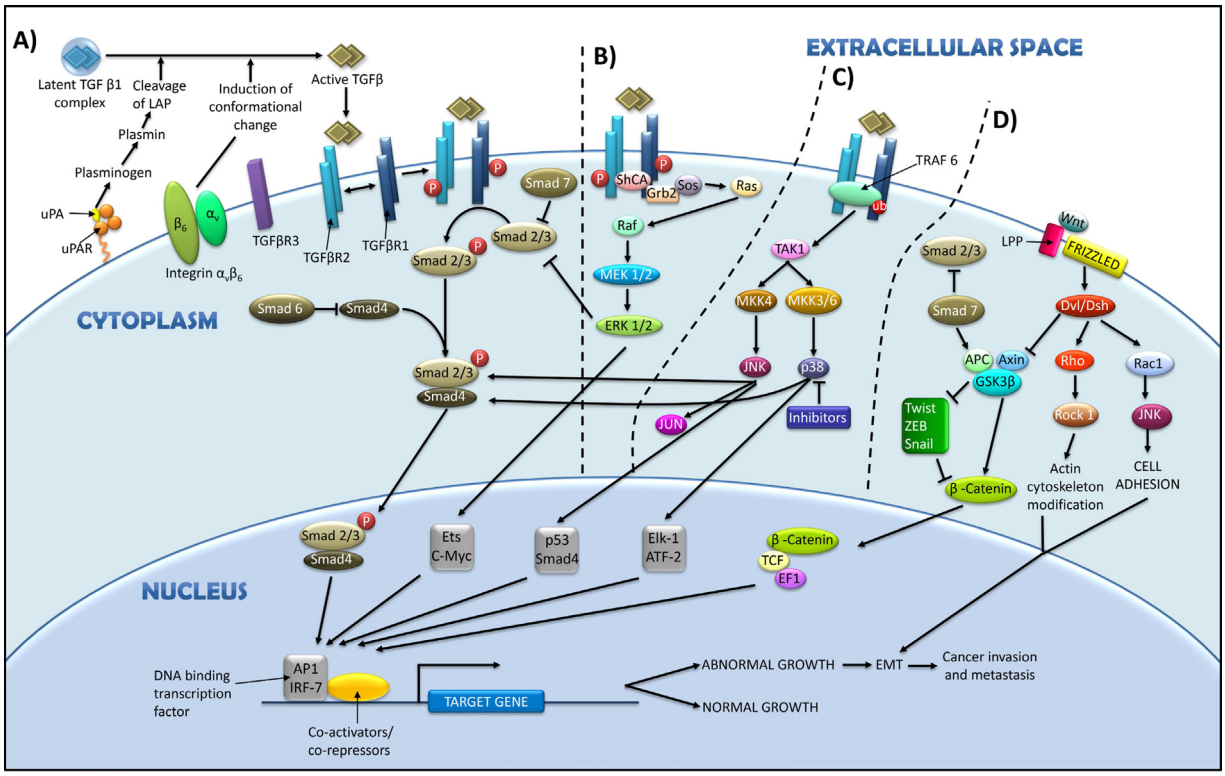

Fig. 2. Outlines the (A) Smad dependent TGFβ pathway. Latent TGFβ is activated through cleavage of LAP by plasmin or by conformational change induced by integrin αvβ6. TGFβR3 presents the active TGFβ to phosphorylated TGFβR2 which recruits, binds and phosphorylates TGFβR1. The active TGFβR1 phosphorylates Smad2 and Smad3. Active Smad2 and Smad3 form a complex with Smad 4 (Smad2/3/4) and translocate into the nucleus, where in combination with various DNA-binding co-activators, co-repressors and transcription factors regulate the expression of TGFβ target genes. Smad6 can inhibit the formation of Smad2/3/4 complex and Smad7 can inhibit the Smad2/3 interaction with TGFβR1 thereby preventing TGFβ associated Smad signaling. (B) Ras/Erk non-Smad pathway. The phosphorylated TGFβR1 and TGFβR2 recruit and phosphorylate ShcA which associated with Grb2/Sos to activate Erk through Ras, Raf and MEK1/2. Erk then regulates downstream transcription factors to control the EMT. (C) JNK/p38 non-Smad pathway. TGFβ receptors interact with polyubiquitinated TRAF6 which recruit TAK1 to activate JNK and p38 pathways via MKK4 and MKK3/6 respectively. Active JNK/p38 then regulate the EMT by controlling the downstream transcription factors. (D) Wnt pathway interactions. Smad7 and APC-Axin-GSKb3 complex interact which other to either enhance or suppress the activity of Smads. The APC-Axin-GSKβ3 complex can activate β-catenin and blocks its inhibitors Twist, Zeb and Snail. Dvl/Dsh can control two important criteria for the EMT—actin cytoskeletal modifications and cell adhesion through activation of Rho and Rac1 pathways. Rac1 then activates JNK pathway to further contribute to the EMT.

by core pathway components including ligands, receptors, Smads and Smad-dependent transcription factors, but also by the ability of TGFβ receptors to activate other Smad-independent (i.e., non-canonical) pathways through crosstalk resulting in downstream cellular responses. The mechanisms of crosstalk include, but are not restricted to, regulation of co-activators and co-repressors recruited during the process of transcription, regulation of receptor Smads activity through the mitogen-activated protein kinase (MAPK) pathway, regulation of inhibitory (I)-Smads (Smad6,7) expression and other interactions that could activate or inhibit certain molecules in the pathways [68]. A few pathways associated with TGFβ signaling crosstalk in CRC and other cancers include, but are not limited to, MAPK pathways [69] like extracellular signal-regulated kinase (Erk) 1/2 [70], Ras [70], p38 MAPK and c-Jun N-terminal kinases (JNKs), phosphoinositol-3-kinase (PI3K), protein phosphatase 2A (PP2A), Wnt [71] and RhoA [72]. This review specifically focuses on MAPK and Wnt signaling pathways.

5.1. Mitogen-activated protein kinase (MAPK) cascades

The MAPK cascades are key membrane-to-nucleus signaling modules that respond to various stimuli resulting in the phosphorylation and activation of transcription factors required for gene expression [69]. Downstream activation of distinct MAPK pathways of Erk1/2, stress-activated protein kinases (SAPK)/JNK, Ras and p38 MAPK can be regulated by TGFβ1 in either a slow or a rapid manner. Slow activation (~15 min) of these pathways is mediated by Smad-dependent responses whilst the rapid activation is thought to be mediated by Smad-independent responses [73]. It has been shown that TGFβ has the potential to rapidly (within 3–6 min) activate Erk and Ras MAPK pathways [74]. Rapid activation of Ras by TGFβ in epithelial cells resulted in increased activity of TGFβ-induced Erk MAPK signaling leading to increased invasion and metastasis [74]. The aberrant activation of MAPK pathways by TGFβ may therefore play a key role in diverting the TGFβ response towards pro-oncogenic outcomes by promoting invasion and metastasis in CRC.

5.1.1. Erk MAPK pathway

The Erk 1/2 pathway is traditionally known to promote cell growth and survival [75], but under certain conditions it can have a pro-apoptotic effects. The Erk pathway is dysregulated in one-third of all human cancers [76] and is involved in pathogenesis, disease progression, and oncogenic behavior [77,78]. During late tumourigenesis, the activation of both Erk and Ras pathways is required for TGFβ-induced epithelial mesenchymal transition (EMT) leading to cancer progression [79,80].

The Erk/MAPK signaling cascade can be activated by a wide range of effectors including peptide growth factors involved in cell growth and differentiation and integrins [81,82]. Rapid activation of Erk mediated by TGFβ has been observed in epithelial cells, breast cancer cells and fibroblasts [83]. Smad-dependent delayed activation of Erk by TGFβ is partly accounted for, but does not completely explain the rapid activation of Erk (within 3–6 min) by

TGFβ [65]. There is evidence that TGFβR1 directly participates in the activation of Erk by recruiting and phosphorylating Src Homology 2 Domain-Containing) Transforming Protein 1 (ShcA) on its serine and tyrosine residues. The phosphorylated ShcA then associates with TGFβR1 via its phosphotyrosine-binding domain and recruits growth factor receptor binding protein 2 (Grb2) and Sos proteins, leading to activation of Erk and Ras MAPK pathways [84,85] (Fig. 2). Erk and Ras then regulate target gene transcription through their downstream transcription factors and Smads to control the EMT [84].

Treatment of TGFβ-sensitive (Hs578T) and TGFβ-responsive (MDA-MB-231) breast cancer cells with TGFβ resulted in different levels of phosphorylation of Erk 2 downstream to Erk 1. TGFβ-sensitive cells showed a significant increase in phosphorylation within 5 min of treatment as compared to the TGFβ-responsive cells, suggesting that the kinetics of Erk phosphorylation induced by TGFβ may vary with cell type and/or physiological state of the cell [83]. Interestingly, a recent study by Hough et al. [86] demonstrated that TGFβR-mediated Erk phosphorylation can be cell type specific, occurring in phenotypically normal mesenchymal cells but not in the epithelial cell phenotype. This could help to explain the dysregulated activation of Erk by TGFβ observed in epithelial cancers as they are at various stages of the epithelial–mesenchymal transition. The TGFβ-mediated phosphorylation of Erk, however, was inhibited when a specific PI3K-inhibitor, LY294002, was added. Similar inhibition was observed with the use of the MEK1/2 inhibitor U0126, suggesting that both MEK1/2 and PI3K are required for TGFβ-mediated Erk activation [86]. Hough et al. then applied small molecule inhibitors to observe their effect on the activation of the downstream PI3K-activated pathways, Akt and Erk. They found that both pathways were activated through TGFβ by PI3K, though only Erk phosphorylation was sensitive (understandably) to inhibition by the MEK1/2 inhibitor U0126 [86]. Hough et al. also proposed that TGFβ-mediated Erk phosphorylation primarily follows the PI3K/Pak2/c-Raf/MEK/Erk pathway, supported by a secondary contribution from Ras, although at a greatly reduced level. Furthermore, Erk is known to phosphorylate serine or threonine residues in the PX(S/T)P or (S/T)P motif of the linker regions in receptor Smads (Smad1,2,3,5,8) which cannot migrate into the nucleus, thus inhibiting TGFβ-Smad signaling [87]. Phosphorylation of the Smad2 linker region was found to be dependent on MEK activation, which could be increased with the rapid activation of Erk by epidermal growth factor (EGF), highlighting a direct functional connection between Erk and the Smad pathway [86]. Erk induced phosphorylation of the linker region of nuclear Smads and increased the duration of Smad-targeted gene transcription by extending the half-life of C-terminal pSmad2/3 (Ser465/467). A thymidine incorporation assay examining the biological consequences of TGFβ-mediated activation of Erk, showed a 6-fold increase in DNA synthesis with TGFβ treatment that was attenuated with MEK1/2 inhibition [86].

The TGFβRs also play an important role in the Erk-TGFβ crosstalk. Primarily, the expression levels and the ratio of TGFβR2/TGFβR1 hetero-oligomers contribute to different downstream signaling modules [88]. Bandyopadhyay et al. have established that dermal cells with high TGFβR2 expression selectively activate Erk1/2 [89]. In contrast, epidermal cells with high TGFβR1 expression favor canonical TGFβR1-Smad signaling and do not activate Erk. These two findings highlight the influence of TGFβR expression on TGFβ-mediated Erk signaling. In the context of cancer, the crosstalk between Erk, TGFβRs and Smads has been shown to directly and indirectly promote cancer growth in the early stages of cancer resulting in metastasis [90–92]. It is also important to note the tyrosine kinase activity of TGFβR1 as well as its serine/threonine kinase activity could be a key to understand the broad spectrum of TGFβR associated signaling in cancer progression.

5.1.2. Ras MAPK signaling

The Ras proteins play a key role in regulating several aspects of both normal cell growth and malignant transformation in cancer signaling. The Ras pathway is deregulated in up to about 30% of tumors [93]. Chaiyapan et al. reported that mutation of K-Ras oncogene occurred in 25–35% of CRCs at early stages of progression [94]. Abnormal activation of Ras leads to increased proliferation and reduced apoptosis, promoting progression. Similar to Erk, TGFβ-mediated activation of Ras occurs through the ShcA/Grb2/Sos complex as described earlier [84] (Fig. 2). The rapid activation of Ras, within (3–6 min) by TGFβ1 and TGFβ2 during CRC tumourigenesis causes an imbalance between Erk and JNK [74]. Ras family proteins are also known to contribute to this imbalance by suppressing JNK activation through active K-Ras or by enhancing Erk activation through H-Ras [95]. Hartsough et al. have shown that Ras activation is required for TGFβ-mediated Erk1 activation and partially required for growth inhibitory effects [96]. TGFβ growth inhibitory responses in prostate cancer and CRC cells are transmuted to Smad-independent mitogenic responses in the presence of active Ha-Ras and Ki-Ras [97,98], whereby active Ras can induce and enhance the expression of TGFβ1, which explains the frequently observed high levels of active TGFβ1 during cancer [99].

It is known that most TGFβ responses are dependent on cellular context partly due to Smad interactions with cell type specific transcription factors. For instance, active Smad3 co-occupies the genome with Oct4 in human embryonic stem cells, Myod1 in myotubes and PU.1 in pro-B cells [100]. The association between Smad2/3 and transcriptional cofactors can be regulated by the Ras MAPK pathway. Smad2/3 and tumor suppressor protein p53 can directly interact and together regulate several TGFβ target genes. Overexpression of p53 in *Xenopus* animal cap cells showed increased cooperation between endogenous Smads to induce mesoderm markers [101]. This cooperation was lost when the animal cap cells were treated with fibroblast growth factor (FGF)-receptor inhibitor SU5402, indicating a relationship between p53 and FGF. Treatment with FGFefficiently promoted association of p53 and TGFβ-activated Smad2 [101]. In this mechanism, FGF signals through Ras to regulate phosphorylation of p53 at its N-terminus, which then interacts with activated Smad2/3 to regulate TGFβ-mediated tumor suppression [101]. SW480.7 colon cancer cells deficient in Smad4 having hyperactive Ras signaling do not show TGFβ-mediated antiproliferative responses, as hyperactive Ras inhibits the function of Smad2/3 by phosphorylating them on their linker regions [102]

During their study of mammary epithelial cells, Oft et al. showed that Ras and TGFβ1 are required to work in collaboration to transform benign epithelial cells to induce invasive and metastatic phenotypes [103]. Results from a recent study by Kim et al., clearly support this outcome, demonstrating that Ras expression promoted mesenchymal morphology. Employing normal MCF-10A cells and MCF-10A/Hras cells which express pro-oncogenic H-Ras, they showed increased invasive potential with TGFβ treatment that was exacerbated when H-Ras was expressed [104]. RT-PCR analysis showed that leukotriene B_4 receptor-2 (BLT2) expression was increased by H-Ras, and the treatment with BLT2 inhibitor LY255283 or depletion of BLT2 using a BLT2-specific small interfering RNA (siBLT2) greatly reduced the morphological alterations and invasiveness of MCF-10A/Hras cells in response to TGFβ treatment. The induction of BLT2 expression in MCF-10A cells showed a marked increase in invasiveness upon TGFβ treatment. This study clearly shows that Ras controls the

expression of BLT2, which responds to TGFβ treatment to promote the adoption of the mesenchymal phenotype and invasion [104].

Various studies have shown that TGFβ and Ras cooperate to induce invasion. In the intestinal epithelium, the loss/inactivation of TGFβR2 or expression of Kras alone did not result in neoplasia. However, the combination of both lead to colorectal neoplasms and eventual metastasis which were mediated through EGF [105]. Loss of Smad4 and the presence of oncogenic K-Ras can also induce expression of MMP9 and urokinase plasminogen activator (uPA), through the EGFR/NF-κB pathway, which contributes to the invasive phenotype of cancer cells through activedegradation of the extracellular matrix (ECM), liberating the cells from cell–cell interactions and enabling extravasation from the primary site [106].

In summary, there is growing evidence of crosstalk between Ras and TGFβ pathways at various levels in cell signaling cascades leading to varying outcomes that can manipulate the EMT and promote metastatic phenotypes.

5.1.3. JNK MAPK pathway

The c-Jun N-terminal kinase (JNK) cascade regulates various transcription and non-transcription factors in response to external stimuli and has been implicated in several biological processes including cell proliferation, apoptosis and tumor development. The TGFβ system has the ability to autoregulate its own expression via the JNK pathway making it an important pathway in cancer development. TGFβ treatment rapidly increased JNK activity (within 5–10 min) and induced up-regulation of urokinase plasminogen activator receptor (uPAR) by increasing the protein–DNA complex formation at the distal Activator Protein-1 (AP-1) site in the uPAR promoter region [107]. TGFβ, however, did not affect JNK protein expression [107]. As TGFβ can activate Ras within 3–6 min of TGFβ treatment, it is conceivable Ras may be required for TGFβ-mediated JNK activation.

JNKs, like Erk, are a third layer of MAPK cascade activated by upstream MKKs—MKK4 and MKK7. The rapid Smad-independent activation of JNK through TGFβ is achieved specifically through MKK4–TGFβ-activated kinase 1 (TAK1) axis [108,109]. Further upstream, tumor necrosis factor-receptor-associated factor 6 (TRAF6) associates with TGFβR2 and TGFβR1 through its c-terminal TRAF domain to activate TAK1 in a receptor kinase-independent manner. Yue et al. also reported that TGFβR2 is required for TGFβ-mediated activation of JNK which is required for up-regulation of uPAR, suggesting a complex crosstalk between these pathways [107]. Initially, it was thought that TRAF6 can only directly interact with TGFβR2. However, the activation of TGFβR2 occurs upon homodimer formation and TGFβR1 is activated by TGFβR2. This suggests that TRAF6 binds to either the active homodimer of TGFβR2 or the hetero-complex of TGFβR2 and TGFβR1 [108]. Furthermore, TGFβR1 has a TRAF6 binding motif (basic residue-X-P-X-E-X-X-aromatic/acidic residue) and the TGFβR1–TRAF6 interaction is required for TRAF6 autoubiquitylation and subsequent activation of JNK/p38 pathways via TAK1 [109]. TRAF6 with the help of TGFβ induces Lys63-linked polyubiquitination of TGFβR1, which promotes cleavage of the intracellular domain (ICD) of TGFβR1 by TNF-alpha converting enzyme, in a PKCζ-dependent manner [110]. The ICD of TGFβR1 can then translocate into the nucleus, where in association with transcriptional regulator p300 it promotes invasion by inducing the expression of *Snail*,*MMP2* and *p300* genes [111].

The TRAF6–TAK1–JNK cascade, in conjunction with the Smads, is known to regulate TGFβ-mediated apoptosis and EMT [109,112] suggesting a close link between these cellular responses. Yamashita et al. showed that TRAF6 and TGFβ-mediated apoptosis and EMT were abrogated when TRAF6 expression was knocked down [108]. A similar effect was observed with knock down of Smad3 expression. Interestingly, TAK1 also mediates TGFβ-induced signaling by phosphorylating the Smad3 linker region (pSmad3L), a feature that is also observed in CRC [113]. pSmad3L can translocate into the nucleus and regulate gene expression to mediate the development of an invasive phenotype of cancer. TGFβ can also activate JNK as part of an accessory pathway, as shown by Ventura et al., who demonstrated that JNK-deficient fibroblasts caused a significant increase in expression of TGFβ1 and TGFβR1 and decreased the expression of TGFβR2 and I-Smads [114], suggesting that JNK deficiency may cause autocrine signaling of TGFβ through a positive feedback loop. Freudlsperger et al. [115] have shown that Smad7 and TAK1 mediate TGFβ and nuclear factor-κB (NF-κB) crosstalk in head and neck cancers. TAK1 further enhances the activation of NF-κB through TGFβ. Treatment of head and neck squamous cell carcinoma (HNSCC) lines with TGFβ1 induced the phosphorylation of TAK1 along with NF-κB family member RELA (p65). RELA and TGFβ activation induced Smad7 expression that preferentially suppressed TGFβ-induced Smad and NF-κB reporters leading to malignant phenotype in HNSCC [115]. Additionally, the ability of Smad7 to interact with TGFβR1 using two modes—a three-finger-like structure in the MH2 domain and a basic groove in the MH2 domain, in contrast to only one mode for Smad6, the other I-Smad [116], suggests a dual role for Smad7: inhibition of TGFβ-Smad signaling and promotion of TGFβ-induced activation of JNK and p38 MAPK pathways.

The cooperation of Erk and JNK has been shown to jointly increase the expression of a key late stage molecule, fascin1 in gastric cancer, which promoted TGFβ-mediated invasion and metastasis [91]. Fascin1 expression was ablated by $\geq$75% when treated with the JNK and Erk specific inhibitors, SP6001125 or PD98059 respectively [91]. In addition to gastric cancer, a recent study by Herbest et al. reported increased fascin1 expression in late stage CRC was induced by β-catenin, an integral member of the Wnt signaling pathway, that has been associated with TGFβ-mediated crosstalk during cancer [117].

5.1.4. p38 MAPK pathway

The p38 MAPK pathway is often activated by various stress responses such as heat shock, osmotic shock and hypoxia leading to diverse roles in cell proliferation, differentiation, survival and migration in different cell types. It is unsurprising, therefore, that p38 MAPKs have been implicated in cancer development [78]. p38 signaling is required for cell migration and metastasis in both CRC and breast cancer [118,119]. As for JNKs, p38 MAPKs are activated by MKKs through autophosphorylation: specifically by MKK3 and MKK6 and sometimes by MKK4 [108,109]. Rapid Smad-independent activation of p38 MAPKs is achieved through a TAK1 and TRAF6 module [108,109] (Fig. 2). Knockdown of TRAF6 inhibited TGFβ-mediated EMT [108]. TGFβ-induced activation of TRAF6-TAK1-JNK/p38 pathways has been implicated in cell death, cell proliferation and EMT [110]. In breast cancer, ubiquitin-conjugating enzyme Ubc13 was shown to control metastasis through the TAK1-p38 MAPK pathway by activation of MEKK1 and TAK1 [119]. Silencing of Ubc13 resulted in decreased TAK1 phosphorylation, and the silencing of TAK1 or p38α resulted in a dramatic decrease of lung metastasis in a mouse model [119]. Wu et al. also showed that the using the p38 inhibitor, SB203580, resulted in decreased metastasis indicating that p38 inhibitors can be used as potential treatment for established breast cancers [119]. Safina et al. using MDA-MD-231 breast cancer cells, have showed that TGFβ-mediated TAK1 regulates MMP9 expression which involves NF-κB signaling, similar to K-Ras. The TAK1-NF-κB-MMP9 pathway as a whole, contributes to TGFβ-mediated metastasis [120]. p38 is also known to regulate cell invasion

through up regulation of MMP2 in prostate cancer [110,121]. The blockade of p38 MAPK activity using specific inhibitors, or by genetic alterations or cancer therapies like 5-fluorouracil, leads to cell cycle recovery and induction of autophagic cell death [118,122].

Activation of Smads is an important cellular response for TGFβRs. Cells with mutated TGFβR1 that are defective in Smad activation showed an increase in p38 MAPK signaling response to TGFβ1, but did not induce EMT [123]. However, cells lacking the cytoplasmic domain of TGFβR2 did not block TGFβ-mediated p38 MAPK activation, resulting in integrin αvβ1 mediated EMT [124]. This confirms that TGFβR2 is important for TGFβ-mediated EMT through the p38 MAPK cascade. The phosphorylation of TGFβR2 tyrosine (Tyr248) in the cytoplasmic domain by Src recruits Grb2 and Shc to TGFβR2, which associates these adapter proteins with p38 MAPK activation [125]. Galliher-Beckley and Schiemann also showed that Grb2 binding to Tyr248 of TGFβR2 is required for TGFβ-mediated mammary tumor growth and metastasis [126]. Northey et al. showed that ShcA expression and phosphotyrosine-dependent signaling are essential for TGFβ-mediated cell motility and invasion [127]. Galliher-Beckley and Schiemann, and Northey et al. also showed that loss, or reduced expression, of ShcA and/or Grb2, or mutations in their phosphorylation sites, no longer promoted TGFβ-mediated migration, invasion, or EMT [126,127]. Rather than the "standard" TGFβ-mediated activation of p38 through MKK3 and MKK6, the possible phosphorylation of TGFβR2 at Tyr248 has the potential to drive Shc and Grb2 through an alternative pathway that is required for TGFβ-mediated tumor growth and metastasis. This secondary activation of p38 through a pathway that would normally activate Erk/JNK compounds the complexity of TGFβ crosstalk with MAPK pathways in cancer.

5.2. Wnt signaling cascade

Along with numerous other transcriptional regulators such as the fibroblast growth factors (FGF) and Forkhead transcription factor families, the interplay between Wnt and TGFβ signaling is a feature of gut development and endoderm formation [128]. More recently, genome-wide association studies have found that both the Wnt and TGFβ pathways are active in lung cancer [129] and breast cancer cells [130]. It has previously been proposed that crosstalk between the Wnt and TGFβ pathways may be more extensive than suggested, especially in the context of malignancy and/or the EMT [128]. This crosstalk may be occurring at several points along the network, notably in the migration of cells as witnessed in cancer and also fibrosis [131,132].

Several studies have demonstrated the role of Smads, with initial studies on homeobox gene promoters showing that TGFβ mimics the effects of Wnt signaling on β-catenin, leading to cell cycle arrest through interactions with Smad7 [133]. Axin, a negative regulator of Wnt signaling has also been shown to interact with Smad3 as a putative adaptor, enhancing the efficiency of TGFβ signaling [134]. Wnt and FGF regulate the phosphorylation of the Smad4 linker region through glycogen synthase kinase-3 (GSK3) in the canonical MAPK/Erk site (PxTP) [128]. This phosphorylation event did not occur when HaCaT immortalized, human keratinocyte cells were treated with the MEK-specific inhibitor U0126, demonstrating the requirement of MAPK activity for GSK3-induced Smad4 phosphorylation [128]. MAPK/FGF and Wnt/GSK3 mediated phosphorylation is required for the polyubiquitination and degradation of Smad4 through E3 ligase β-TrCP [128,135,136]. As stated by Demagny et al., the MAPK/Erk and GSK3 trigger the formation of a phosphodegron bound by the E3 ligase β-TrCP, resulting in the polyubiquitination of Smad4. Demangy et al. also showed that treatment of cells expressing the TGFβ-specific reporter CAGA12-luciferase with Wnt3a or FGF2 alone did not affect TGFβ signaling activity. However, the addition of both increased TGFβ signaling activity, indicating the involvement of GSK3 [128]. This demonstrates that FGF is also required for TGFβ and Wnt crosstalk which is enhanced by activation of MAPK signaling. It is important to note that another study reported that TGFβ suppresses β-catenin/Wnt signaling and enhances cell adhesion in CRC in a Smad4-independent manner [132]. A similar study to that of Demangy et al., reported the ability of TGFβ to promote the EMT and invasion in a p38 MAPK/β-catenin/peroxisome proliferator-activated receptor γ-dependent manner in non-small cell lung cancers [137].

Numerous canonical and non-canonical Wnt signaling proteins have also been shown to act as co-factors of TGFβ signaling, including, but not limited to, Snail, Twist, β-catenin and AP-1 by either activating or suppressing the activity of various Smads (for a comprehensive review refer to [138]). These interactions have not been directly observed in CRC, though this crucial link may bridge the gap between these two signaling pathways (i.e., that of Notch, Wnt, and TGFβ/Activin signaling) which is in part mediated by the interactions of Dll1 with Smad2/3 and Tcf4 at the promoter sites [139]. A further point of interaction between the Wnt and TGFβ signaling pathways involves the regulation of the same genes independently or cooperatively. Both regulate Lef1/Tcf, which are canonical proteins involved in the EMT [140]; gastrin, a promoter of gastrointestinal cancers [141]; BAMBI, the pseudoreceptor involved in TGFβ signaling regulation in CRC [142]; and importantly Snail1 and Snail2, both of which are acknowledged as key switches that initiate the EMT in cells, and have been implicated in CRC [143–146]. Other canonical Wnt signaling molecules such as Twist and KLF8 have also been shown to be regulated by TGFβ [138].

Several other proteins involved in Wnt and TGFβ signaling have been shown to be perturbed in CRC cell lines and tissues, including Pitx2 [147], a homeodomain transcription factor, and ECM transition remodeling proteins such as heparin-degrading endosulfatases, sulfatase 1 (SULF1) and sulfatase 2 (SULF2). Recent studies have shown that FOXQ1, a member of the forkhead transcription factor family, can promote TGFβ expression and the EMT through crosstalk between the Wnt and TGFβ signaling pathways [71,148]. Fan et al. [148] showed that silencing FOXQ1 decreased cell migration and invasion which was supported by Peng et al. [71]. Interestingly, treating the cells with TGFβ1 increased FOXQ1 gene expression resulting in TGFβ-mediated the EMT within 4-days, that was suppressed upon silencing FOXQ1 expression [148]. A similar outcome was reported by Peng et al. wherein treatment of CRC cells with TGFβ1 increased FOXQ1 expression and promoted migration and invasive potential. They also demonstrated that FOXQ1 suppression by siRNA decreased the invasive and angiogenic potential and resistance to chemotherapy drugs. Peng et al. further showed that FOXQ1 is overexpressed in CRC tissues and correlates with CRC stage [71]. Indeed, other recent studies further support the fact that overexpression of FOXQ1 induces the EMT in various cancers [149–151] and has been shown to be a direct Wnt target in CRC [152].

The overall picture regarding Wnt and TGFβ signaling is that of a highly interconnected system of activators and repressors that serve to maintain cell proliferation and migration. The details of the Wnt and TGFβ pathways continue to be elucidated, with novel players such as FOXQ1 continually changing the models of potential crosstalk between these two pathways. Suffice it to say that in cancers, particularly CRC, the involvement of both these pathways is crucial not only for ECM degradation but also for metastasis as evidenced by the involvement of APC in over 60% of loss of heterozygosity (LOH) positive CRC cases [153,154]. A detailed analysis of this crosstalk system is beyond the scope of this

current review. However, there is extensive evidence indicating that such crosstalk strongly influences the EMT and metastasis.

6. Genetic alterations in TGFβ signaling components

Various intracellular signaling pathways, including the ones described above, are frequently dysregulated in CRC. Almost 75% of CRC cell lines are resistant to TGFβ-mediated growth inhibition due to the loss or mutation of one or more components of the TGFβ signaling pathway [155,156]. A detailed review of the genetic alterations of TGFβ signaling components specifically in CRC has been published by Wu et al. [158].

Genetic alterations of TGFβR2 are the most common mechanism leading to the loss of TGF-β signaling in CRC. Inactivation of TGFβR2 frequently occurs due to microsatellite instability (MSI), resulting from DNA mismatch repair defects, causing nucleotide additions or deletions in simple repeated sequences, or microsatellites in the genome [156,159]. Additionally, impairment of TGFβ-mediated anti-proliferative responses due to mutation of TGFβR1 has also been observed [160]. However, the presence of a common polymorphic variant TGFβR1*6A has been shown to increase the risk of CRC and several other cancers [161,162].

Genetic polymorphisms of TGFβ1 have also been associated with colorectal neoplasia, although meta-analyses of particular alleles demonstrated inconclusive correlation with a single mutation [163,164]. The mostly widely studied TGFβ1 genetic alterations are TGFβ1 −509 C>T, +869 T>C, +915 G>C, and −800 G>A [164]. Meta-analysis by Liu et al., has shown that the TGFβ1 −509 C>T, +869 T>C, +915 G>C, and −800 G>A polymorphisms are not associated with colorectal adenoma, but, C allele of −509 C>T and A allele of −800 G>A are associated with increased CRC risk [164]. In addition, the −509 C>T has been reported to be associated with increased risk of developing CRC by Wang et al. [165] and decreased risk of CRC by Liu et al. [163].

Mutation or deletions in Smad genes can also be an important factor during tumor development. Most commonly mutations are seen on Smad4 and Smad2, due to allelic loss or LOH that has been demonstrated in up to 60% of CRCs. Mutations in Smad4 gene (16–25%) and Smad2 gene (6%) have been associated with CRC. Smad4 and Smad2 genes along with tumor suppressor gene DCC (deleted in colorectal cancer) are localized at chromosome 18q21 [166]. Smad4 mutations are found in about 11% of familial adenomatous polyposis and 11% of hereditary non-polyposis colorectal cancer [157,167] syndromes. Smad2 mutations occur in the MH1 or MH2 domains of the molecule affecting the phosphorylation, nuclear translocation, and/or decreasing protein stability ultimately disturbing TGFβ signaling. Similar mutations or LOH of Smad3 gene (located on 15q21–q22) were reported in a human CRC cell line (SNU-769A) [160]. A later study using 36CRC cell lines and 744 primary CRC patient tumor biopsy samples concluded that approximately 4% of them carried mutations in the Smad3 gene [168]. Concurrently, the loss of Smad3 expression in gastric cancer tumors/cells has been associated with high susceptibility to cancer [169]. This multitude of genetic mutations in TGFβ signaling components, and the signaling crosstalk with various pathways during the development of cancer, enhance its ability to invade and metastasize to various organs, resulting in decreased 5-year survival.

7. Conclusion

TGFβ signaling plays major roles in regulating normal cell growth, although various cancer studies have suggested that canonical TGFβ signaling is unfaithful. It is promiscuously involved in intracellular signaling crosstalk with various pathways, including, but not limited to, Erk, JNK, Ras, p38 and Wnt. TGFβRs play a crucial role in non-canonical signaling which collectively result in changes that drive cancer progression and metastasis. The poorly understood Janus-like nature of TGFβ in cancer is likely the product of these interrelations and correlations that do not have simply one single signature. This may explain why understanding it remains elusive. This is potentially how a widely accepted tumor suppressor in benign cells "switches" to promote cancer progression. Understanding this switch to a tumor-promoting outcome remains an important question that is likely to be answered in the minutiae of less established interactions.

This review has explored many possible avenues of TGFβ crosstalk and their consequences in cancer. It is crucial to note that almost no TGFβ signaling component has a single function. For instance the dual kinase activity of TGFβR1 and the two modes of Smad7 interaction with TGFβR1 further add to complexity of TGFβ-crosstalk that is already poorly understood. This complex crosstalk in CRC, we propose, can be investigated by implementing a combination of sophisticated informatics, -omics technologies and *in vivo* studies in a spatio-temporal manner, coupled with larger protein tracking and interaction studies. Emerging multiplexed technologies such as SOMAmer® [170], proximity extension assays [35,171] and/or SureFire® assays [172] will be crucial in the coming years to perform more elaborate experiments in order to elucidate complex cell signaling behaviors within a matrix of different pathways and the crosstalk between them. It is crucial to remember that in cancer and various diseases, we cannot study these pathways in isolation but instead must transition into a matrix-oriented systems approach that more comprehensively models the spatio-temporal ramifications of signaling activities within the complexity of living cells and tissues. A better understanding of four-dimensional biology is essential to identify *in vivo* signaling signatures that are of clinical relevance, facilitating the development of more effective, targeted therapeutics to combat a global health burden.

Conflict of interest

All authors have no conflict of interest to declare in this manuscript.

Acknowledgments

The Australian proteomics community enjoyed a marvelous collaborative relationship with our friend and colleague Juan Pablo Albar. We dedicate this review to his memory.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.euprot.2015.06.004.

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