厚生労働省科学研究費補助金
第3次対がん総合戦略研究事業

脳腫瘍における幹細胞性維持機構の遮断とその臨床応用

平成22～24年度　総合研究報告書

研究代表者
宮園　浩平

平成25（2013）年5月

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厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
総合研究報告書
「脳腫瘍における幹細胞性維持機構の遮断とその臨床応用」

研究代表者：　宮園　浩平　東京大学大学院・医学系研究科・教授

研究要旨：我々はこれまで TGF-β が脳腫瘍幹細胞に作用して転写因子 Sox4、その下流の Sox2 を介して脳腫瘍幹細胞の未分化性を維持していることを明らかにした。本研究では脳腫瘍幹細胞において、TGF-β ファミリーシグナル経路がどのような標的遺伝子を活性化するか、それらの遺伝子は脳腫瘍幹細胞の未分化性維持にどのように関わっているか、さらにこれらの遺伝子群はヒト脳腫瘍においてどのように発現しているかを中心に研究を進めた。

我々はまず転写因子 Oct4 に注目し、ヒト脳腫瘍患者から得た脳腫瘍幹細胞を Oct4 に対する siRNA で処理したところ、sphere 形成能が著しく抑制された。また脳腫瘍幹細胞を Oct4 siRNA で処理すると、抗がん剤投与によりコントロールに比べて有意に細胞数の減少が見られ、抗がん剤に対する感受性が亢進したと考えられた。さらに我々は Luciferase 発現脳腫瘍幹細胞をマウス頭蓋内に移植することにより、マウスを屠殺することなく、腫瘍の形成・増大をリアルタイムで検出することを可能にした。未分化性を維持していると考えられる脳腫瘍細胞は血管周辺に局在する傾向があることを免疫組織染色で確認した。

我々はさらに TGF-β ファミリーの脳腫瘍幹細胞に対する作用の研究を進め、BMP-4 は脳腫瘍幹細胞に作用し、分化促進作用を持つことを確認した。BMP-4 は CD133 や Olig2、Sox2 の発現を低下させた。BMP-4 の標的遺伝子を探索したところ、BMP-4 の機能を阻害する細胞外タンパク質 Noggin や細胞内タンパク質 Smad6 の発現が BMP-4 投与により上昇することが明らかとなった。このため、Noggin や Smad6 の発現を siRNA でノックダウンしたところ BMP-4 の作用の増強が見られ、in vivo での腫瘍形成能も有意に抑えられた。さらに RNA-seq や DNA microarray で BMP の新たな標的遺伝子の同定に成功した。

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A. 研究目的

脳腫瘍の中でも最も悪性度の高い膠芽腫では、TGF-βががんの進行に密接な役割を果たしていることが近年示唆されている。実際に、TGF-β受容体の阻害剤の臨床試験などが国外では開始されており、TGF-βシグナルの抑制が膠芽腫の治療に有効であることが報告されている。一方、TGF-βファミリーの因子であるBMPについては、BMPが脳腫瘍幹細胞の分化を促進することはPiccirilloらによって2006年に報告されたが、その後は実験の困難さもあってほとんど研究が進められていないのが現状であった(Piccirillo et al. Nature 2006)。

我々はこれまで TGF-β が脳腫瘍幹細胞に作用して脳腫瘍幹細胞の未分化性を維持していることを明らかにした(Ikushima et al. Cell Stem Cell 2009)。また TGF-β 受容体阻害剤が脳腫瘍幹細胞の分化を促進することを見出した。しかし、脳腫瘍幹細胞に対する TGF-β 阻害剤の効果は症例によって差があることから、TGF-β が脳腫瘍幹細胞においてどのような下流シグナル分子を活性化するかを明らかにすることは個々の脳腫瘍症例に最適な治療法を決定する上で極めて重要と考えられた。

本研究では、TGF-β ファミリーの因子がどのような経路を介し、他の転写因子とともにどのような標的遺伝子を活性化するか、それらの遺伝子は脳腫瘍幹細胞の未分化性維持にどのように関わっているかを in vitro、in vivo の両面から明らかにすることを目的として研究を行った。さらにこれらの遺伝子群の脳腫瘍における発現、とくに血管組織などのニッチェとの関わりを含めて免疫組織染色などで確認することを中心に研究を進めた。

B. 研究方法

ヒト脳腫瘍幹細胞は東京大学医学部倫理委員会の承認を得て採取したものを用いた。培養は無血清で EGF と bFGF の存在下で行った。

幹細胞マーカーの発現、sphere 形成能の測定、RNAi による遺伝子ノックダウン、イムノブロッテイング、定量的 RT-PCR、ルシフェラーゼア

ッセイ、ヌードマウス頭蓋骨内への同所移植、免疫組織染色はすでに報告した手法 (Ikushima et al. 2009; Katsuno et al., 2012)により行った。Lentiviral vector による細胞の遺伝子発現は既報の通り行った (Nagano et al. 2010)。

クロマチン免疫沈降法はすでに報告されている方法を用いて行った (Horiguchi et al, 2012)。RNA-seq は東京大学医学部ヒトゲノム・遺伝子解析研究倫理審査委員会の承認を得て行った。RNA は PolyA selection の後、Epicentre 社の Scriptseq V2 およびタカラバイオ社の SMARTer Ultra Low RNA kit for Illumina Sequencing でのサンプル調製を行った。データ取得は Illumina 社の Genome Analyzer IIx または HiSeq system を用いて行った。

（倫理面への配慮）

1) この研究で行なう予定の遺伝子組み換え実験は平成 20 年 10 月 21 日の東京大学医学部組換 DNA 実験安全委員会において承認を受けており、適切な拡散防止措置がとられている。

2) 動物を用いた実験は、動物実験の講習を修了し、充分な知識と経験を有するものだけに従事させ、東京大学医学部の定める規則に従って行った。

3) 本研究で用いる臨床検体は東京大学医学部倫理委員会の承認を得て、被験者に対するインフォームド・コンセントを書面で行っている。

4) 平成 24 年度はヒト脳腫瘍細胞の RNA-seq による遺伝子解析を行うことから、倫理委員会に審査を申請し、平成 24 年 5 月 7 日に承認を得た（審査番号 G3532）。

5) ヒト ES 細胞を用いた研究は本研究計画には予定されていない。

C. 研究結果

1) Oct4 は脳腫瘍幹細胞の未分化性の維持に必須である

我々はまず Oct4 の脳腫瘍幹細胞における役割を明らかにするために Oct4 の発現を siRNA を使ってノックダウンし、その効果を検討した。ヒト脳腫瘍患者から得た 2 種類の脳腫瘍幹細胞 TGS-01 と TGS-04 は、無血清で EGF と bFGF の存在下では sphere を形成し、sphere を形成する細胞は Nestin などの幹細胞マーカーを発現している。TGS-01 と TGS-04 細胞を Oct4 に対する siRNA で処理すると sphere 形成能が著しく抑制された。同様の結果は他の 3 人の脳腫瘍患者から得た脳腫瘍幹細胞でも確認することができた。

これらの細胞における幹細胞マーカーの発現を調べたところ、コントロールの siRNA で処理した細胞は Nestin や Musashi を発現していたのに対し、Oct4 siRNA で処理した細胞ではこれらのマーカーを発現する細胞の数が有意に低下していた。一方 Tuj1（神経細胞のマーカー）、GFAP（星細胞のマーカー）を発現する細胞の数は Oct4 siRNA で処理した場合に有意に上昇していたことから、Oct4 の発現の低下により脳腫瘍幹細胞が神経細胞様細胞や星細胞などに分化する傾向を示すことが明らかとなった。

さらに TGS-01 細胞をヌードマウスの頭蓋内に同所移植したところ、コントロールの siRNA で処理した細胞を移植したさいには腫瘍の形成が見られ，マウスは 6 週以内にすべて死亡したのに対し、Oct4 siRNA で処理した細胞を移植した場合には半数以上のマウスで腫瘍の形成が見られず、これらのマウスは 80 日以上、生存した。このことからも Oct4 が脳腫瘍幹細胞の未分化性の維持に必須であることが示唆された。

我々はさらにこれらの細胞の抗がん剤に対する感受性について検討を行った。脳腫瘍の治療に使われる temozolomide (TMZ)を用いて TGS-01、TGS-04 の抗がん剤感受性を検討したところ、コントロールの siRNA で処理した細胞は TMZ を加えても細胞数の減少はわずかしか見られなかった。これに対し、これらの細胞を Oct4 siRNA で処理すると TMZ 投与により増殖抑制が見られ、コントロール siRNA に比して有意に細胞数の減少が見られた。このことは Oct4 のノックダウンにより脳腫瘍幹細胞が分化した結果、抗がん剤に対する感受性が亢進したことによると考えられた。

2) Oct4-Sox 複合体が Sox2 の発現を制御する

我々は脳腫瘍幹細胞では Oct4 をノックダウンすると Sox2 の発現が減少することを確認した。また分化した脳腫瘍幹細胞では Oct4 の発現が低下し、Sox2 の発現も低下することを確認した。クロマチン免疫沈降（ChIP）アッセイでは Sox2 のエンハンサー領域に Oct4 が直接結合して，Sox2 の発現を制御していることを明らかにした。さらに我々は脳腫瘍幹細胞では Sox2 のエンハンサー領域には Sox4 と Oct4 の両者が結合しており、また両者は複合体を形成していることを明らかにした。

続いて実際に Sox4 と Oct4 が Sox2 の発現に重要であることをルシフェラーゼアッセイを用いて確認した。このさい、Sox2 のエンハンサー領域に存在する Sox や Oct4 の結合配列に変異を加えると、Oct4-Sox4 による Sox2 の発現上昇が認められなくなることを明らかにした。

Sox2 エンハンサーには正常の神経幹細胞では Sox2 が結合して Sox2 の発現を上昇させることが知られている。しかし我々の今回の実験では、脳腫瘍幹細胞では Sox2 ではなく Sox4 が重要であることが明らかとなった。すなわち、今回の実験結果で正常の神経幹細胞と脳腫瘍幹細胞の

相違を明らかにすることができた点で極めて興味深いと思われた。これらの成果を平成23年度にJournal of Biological Chemistry誌に原著論文として発表した。

我々はOct4の抗体で腫瘍組織の染色を行ったが適切な抗体がなく、組織染色でOct4陽性の脳腫瘍細胞をin vivoで確認することはできず、他のTGF-β-Sox4-Sox2経路の標的遺伝子の検索が必要と考えられた。

3) がん細胞における Smad2/Smad3 結合部位の網羅的同定解析

TGF-β のシグナル伝達研究領域においてChIP-chip法を用いて検討を行った。その結果いかなる制御因子がTGF-βシグナル下流の転写調節にかかわっているのかを同定することができ、また直接のSmad2/Smad3の標的遺伝子を同定することが可能となった。

本研究ではがん細胞株を用いて同様の手法を用い、すでにChIPに有用な抗体が存在することからSmad2/Smad3に対する抗体を用いて、網羅的にSmad2/Smad3結合部位を同定することができた。その結果正常上皮細胞と比較して8割に及ぶ結合部位が、解析を行ったがん細胞特異的に存在することが明らかとなった。このことはこれまで知られていた細胞特異的なTGF-βの作用発現が、一部は選択的なSmad2/Smad3の結合によりもたらされていることを示唆するものである。またこうしたがん細胞特異的な結合部位に特徴的なDNA配列を解析することで、その選択的結合のメカニズムを説明できる可能性のある転写因子が同定された。

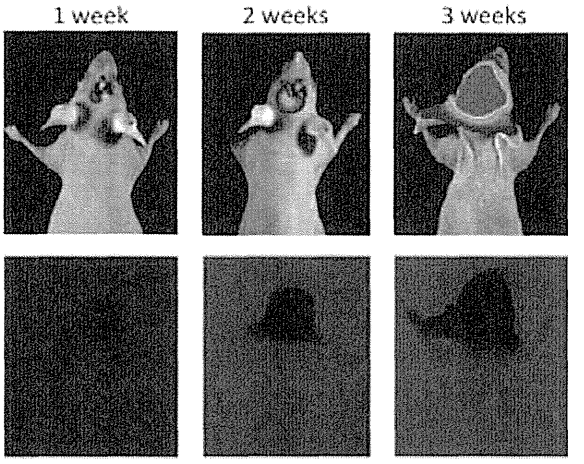
興味深いことに同様の細胞種特異的な結合部位の相違は、脳腫瘍幹細胞に対する抑制的役割が知られるBMPシグナル下流の転写因子であるSmad1/Smad5についても観察された。

4) 脳腫瘍幹細胞を用いた in vivo イメージングシステムの確立

本研究ではTGF-β-Sox4-Sox2経路の新規標的遺伝子の探索のためにChIP-seqを行う予定で研究を進めた。しかしChIP-seqを行うためには比較的大量の脳腫瘍幹細胞を準備することが必要であるが、脳腫瘍幹細胞は培養中にspontaneousに分化してしまうことが実験を行う過程で明らかとなった。我々はEOS lentiviral vectorを用いてOct4/Sox2 enhancerの活性化を指標に培養脳腫瘍幹細胞の未分化性を検出する系を立ち上げることを試みた。その結果、TGS-01細胞においてOct4/Sox2を発現する細胞を検出することが可能となった。

さらに我々はLuciferase発現脳腫瘍幹細胞を用いて、in vivoで脳腫瘍幹細胞を免疫不全マウスの頭蓋内に同所移植したさいにマウスを屠殺することなく、腫瘍の形成・増大をリアルタイムで検出する実験系を確立した。我々はさらにマウスを屠殺して脳腫瘍組織の免疫染色を行った。

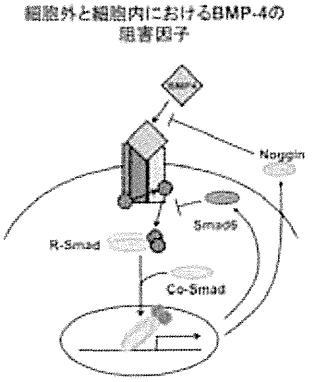
平成23年度までに未分化性を維持していると考えられる脳腫瘍細胞は血管の周辺（perivascular niche）に局在する傾向があることを免疫組織染色で確認した。一方で、最近国外の他のグループ（Ricci-Vitianiet al. 2010; Wang et al. 2010）から報告されている脳腫瘍幹細胞が血管前駆細胞へ分化するという所見を積極的に支持する結果は、この実験系では確認できなかった。さらに未分化マーカーであるMusashiや分化マーカーであるGFAPを用いて免疫組織染色を行ったが、Musashi陽性細胞は主に血管周囲のほか、脳室及び脳周囲組織に見られた。脳内の大きな病変部位には明らかな陽性部位はまれであった。一方でGFAPはMusashiとは逆に脳内の大きな病変部位における染色性が強く、脳室及び脳周囲の病変は陰性であった。



5) BMP-4 の脳腫瘍幹細胞に対する作用

これまで我々はTGF-βの脳腫瘍幹細胞を中心に研究を進めて来たが、TGF-βファミリーの他の因子の作用についても検討を行った。その結果、既報（Piccirillo et al. 2006）のとおり、TGF-βとは対象的にBMPは脳腫瘍幹細胞の分化を促進した。BMP-4は、BMP-6やBMP-9に比べてより強力に脳腫瘍幹細胞に作用し、CD133やOlig2、Sox2の発現を低下させた。BMP-4単独の投与ではin vivoでの腫瘍形成能には大きな影響を与えなかった。このため、BMP-4の標的遺伝子を探索したところ、BMP-4の機能を阻害する細胞外タンパク質Nogginや細胞内タンパク質Smad6の発現がBMP-4投与により上昇することが明らかとなった。このため、NogginやSmad6の発現をsiRNAでノックダウンしたところBMP-4の作用の増強が見られ、CD133やSox2の発現が顕著に減少した。またin vivoでの腫瘍

形成能も BMP-4 の単独処理に比較して、有意に抑えられることが明らかとなった。

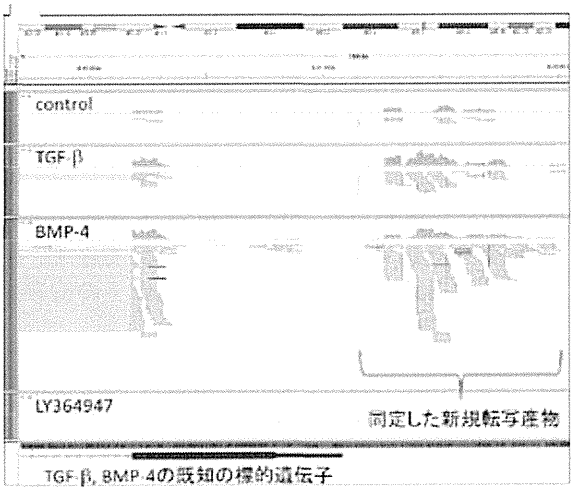


6）脳腫瘍幹細胞における TGF-β ファミリー応答性遺伝子発現変化の網羅的解析

本研究では ChIP-seq による Smad をはじめとした直接の標的遺伝子を同様に同定することを当初目指したが、幹細胞という限られたサンプルからのデータ取得が結果として困難であったため、急速に手法が確立しつつあった RNA-seq を活用することとした。平成 23 年度から開始した微量のサンプルからの RNA-seq についての複数のライブラリー調製試薬を用いた検討の結果、100 pg 以上のサンプルからのデータ取得に成功し、1 ng からある程度の定量性が確保されることが確認された。平成 24 年度は実際に脳腫瘍幹細胞を用いて比較検討のためのデータ取得を行った。

脳腫瘍幹細胞の刺激には TGF-β のほか、TGF-β 受容体阻害剤 LY364947 を用いたサンプルを用意した。さらに TGF-β ファミリーの一つである Bone morphogenetic protein 4 (BMP-4)が TGF-β とは反対に強力に脳腫瘍幹細胞分化を誘導することから、BMP-4 刺激のサンプルも合わせてデータ取得を行った。その結果ダイナミックレンジの大きい発現データセットを取得することに成功した。また、期待されたように得られたデータから機能未知の多くの転写産物が各種刺激によって発現変動することが見出された（下図）。

さらに DNA マイクロアレイを行い、BMP-4 の標的遺伝子を中心に検討を行った。その結果、BMP シグナル関連遺伝子、Transcription 関連遺伝子、Differentiation 関連遺伝子、Proliferation 関連遺伝子などが得られた。さらに脳腫瘍の予後との関連から有望な遺伝子の絞り込みを行い、約 100 個の BMP-4 標的遺伝子の絞り込みに成功した。



D&E. 考察及び結論

本研究は、近い将来、我が国で開始されることが期待される TGF-β 阻害剤の膠芽腫に対する効果との関連性を明らかにするための基礎的知見を得ることを目的としており、極めて必要性の高い研究である。また BMP の脳腫瘍に対する有効性は、基礎研究成果は報告されているもののその後の研究の進展がほとんどなく、今後の臨床応用に向けてその分子機構の研究が極めて注目されている。

TGF-β シグナルの下流で Sox4-Sox2 経路が重要であることは我々が世界に先駆けて明らかにしたものであり、さらに我々は Oct4 の重要性をも独自に明らかにしたことから、独創性の高い研究である。本研究の成果がさらに明らかとなれば将来、TGF-β 阻害剤を臨床応用するさいに治療が有効な症例を予測する上で重要となることが期待される。また in vivo イメージングを導入することで、これまで屠殺しないと腫瘍の進展を明らかにできなかったのが、マウスが生きたままで腫瘍の増大を追跡することが可能となった。さらに腫瘍組織の解析においては Luciferase 抗体などを用いて未分化な脳腫瘍細胞の局在を明らかにすることが可能となり、今後は脳腫瘍細胞の分化と腫瘍組織内における局在との関連についても研究を進めることができると期待される。

BMP-4 はすでに欧米では整形外科領域などで骨や軟骨の誘導因子として臨床応用されているタンパク質で、脳腫瘍への応用が可能となればその臨床的有用性は極めて高いと考えられる。BMP-4 の前処理のみでは脳腫瘍の in vivo での腫瘍形成や増大には有意な効果が得られなかったが、Smad6 や Noggin をノックダウンすることで BMP-4 の効果を増強し、in vivo での腫瘍形成能を有意に抑制することが可能となった。我々は BMP-4 の標的遺伝子の同定に成功しており、今後、BMP のシグナル経路や標的遺伝子がさらに

詳細に明らかになれば新たな治療法の開発につながるものと期待された。

膠芽腫は極めて予後の悪いがんの一つで、1年以内に50%の患者が死亡し、5年生存率は約3%である。膠芽腫に対しては手術以外にも抗がん剤やγナイフなどの放射線療法が行われているが、治療効果の劇的な改善は望めないのが現状である。TGF-β の阻害剤は国外で臨床試験が開始され、その効果が期待されている。一方で本研究により TGF-β や BMP を標的とした治療と脳腫瘍幹細胞との関連が明らかになり、有効な症例を予測することができれば、厚生労働行政の面でも極めて重要であると期待される。

F. 健康危険情報

無し

G. 研究発表

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H. 知的財産権の出願・登録状況

無し

研究成果の刊行に関する一覧表

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TGFβ signalling: a complex web in cancer progression

Hiroaki Ikushima and Kohei Miyazono

Abstract | The distortion of growth factor signalling is the most important prerequisite in tumour progression. Transforming growth factor-β (TGFβ) signalling regulates tumour progression by a tumour cell-autonomous mechanism or through tumour–stroma interaction, and has either a tumour-suppressing or tumour-promoting function depending on cellular context. Such inherent complexity of TGFβ signalling results in arduous, but promising, assignments for developing therapeutic strategies against malignant tumours. As numerous cellular context-dependent factors tightly maintain the balance of TGFβ signalling and contribute to the regulation of TGFβ-induced cell responses, in this Review we discuss how they maintain the balance of TGFβ signalling and how their collapse leads to tumour progression.

Perturbations of transforming growth factor-β (TGFβ) signalling are central to tumorigenesis and tumour progression through their effects on cellular process, including cell proliferation and cell invasion[1]. TGFβ receptor 2 (TGFBR2) and SMAD4 are commonly inactivated through mutation and loss of heterozygosity (LOH) in several types of carcinoma[2]. TGFBR2-inactivating mutations are frequently found in colon cancers that are associated with microsatellite instability (MSI)[3]. Absence or decreased SMAD4 expression has been found in various cancers, including pancreatic cancer, colorectal cancer, and head and neck cancer[4]. These results provide evidence that the TGFβ signalling pathway functions as a tumour suppressor that cancers must bypass for their progression. However, TGFβ signalling is also known to function as a tumour promoter. Analyses of clinical tumour samples have revealed that TGFβ signalling is strongly implicated in tumour progression. Increased TGFβ1 expression by tumour cells correlates with colorectal and prostate cancer progression[5,6]. Positive TGFβ immunostaining also correlates with metastases in breast, prostate and colorectal cancers[6–8]. Moreover, TGFβ staining is stronger in invading local lymph node metastases than in the primary tumour sites in breast and colorectal cancers[9,10]. These findings indicate that excessive TGFβ stimulation is an indispensable prerequisite for tumour progression. How do these paradoxical outcomes occur?

In addition to the tumour cell-autonomous effects of TGFβ signalling, TGFβ also has important roles in host–tumour interactions. During tumorigenesis and tumour progression, surrounding host environments, known as tumour microenvironments, affect the characteristics of tumour cells through diverse mechanisms[11]. TGFβ signalling can suppress inflammation, which can drive tumorigenesis, and can also affect the recognition and destruction of tumour cells through the regulation of immune cell function. In addition, this cytokine has multiple roles in the interaction between stromal fibroblasts and tumour cells. Recent studies have added new aspects to the role of TGFβ signalling in tumour–microenvironment interactions: cancer stem cells and their niches.

One of the complex themes in recent years has been the regulation of TGFβ signalling in cancer cells. TGFβ signalling simultaneously triggers several responses in cancer cells in a cellular context-dependent manner. Meanwhile, hundreds of factors form a complex web that regulates TGFβ signalling, and the collapse of such networks leads to a crash of the signalling pathway, resulting in the development and progression of malignant tumours. In this Review, we focus on recent insights into the regulation of TGFβ signalling in cancer cells and address how impairment of this pathway in cells and the microenvironment causes tumorigenesis and tumour progression. TGFβ also regulates cytokine and chemokine secretion and the resulting effects on the inflammatory tumour microenvironment. However, as the roles of TGFβ in the inflammatory tumour microenvironment have been discussed in other reviews[11,12], they are not covered here.

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At a glance

- Transforming growth factor-β (TGFβ) signalling is mediated by TGFβ ligands, type 1 and type 2 receptors, and Smad proteins. TGFβ also regulates non-Smad pathways.
- TGFβ stimulation inhibits cancer cell proliferation in some cellular contexts and promotes it in others. Numerous factors are involved in TGFβ-regulated cell proliferation and keep its signalling pathways balanced.
- In addition to perturbation of TGFβ signalling, disruption or mutation of regulators of TGFβ signalling can lead to a loss of balanced TGFβ signalling, resulting in the generation and progression of tumours.
- TGFβ signalling in cancer cells has dual roles in the regulation of cell death and proliferation.
- TGFβ signalling has crucial roles in the maintenance of self-renewal and tumorigenic activity of glioma-initiating cells and leukaemia-initiating cells, whereas the function of TGFβ signalling in breast cancer-initiating cells is controversial.
- TGFβ signalling is involved in several cell responses during cancer cell metastasis, and cell type-dependent and context-dependent factors contribute to the regulation of tumour metastasis.
- The TGFβ pathway has been targeted for cancer therapy using multiple strategies. Some of them are currently in clinical trials.

Regulation of cell proliferation by TGFβ

The effects of TGFβ are mediated by three TGFβ ligands — TGFβ1, TGFβ2 and TGFβ3 — through TGFβ type 1 and type 2 receptors[13–15]. TGFBR2 is the specific receptor for TGFβ ligands. TGFβ ligands, which are produced as latent high molecular weight complexes, can bind to TGFBR2 with high affinity once activated by proteolytic cleavage or structural modification of the latent TGFβ complexes[16,17]. Both type 1 and type 2 receptors contain serine/threonine kinase domains in their intracellular portions[13]. Binding of the ligand causes the formation of heterotetrameric active receptor complexes that result in the phosphorylation of the type 1 receptor by the type 2 receptor (FIG. 1). Although activin receptor-like kinase 5 (ALK5; also known as TGFBR1) transduces TGFβ signalling in most cell types, ALK1 and other type 1 receptors are also activated in response to TGFβ stimulation in certain cells[18,19].

The functional receptor complex regulates the activation of downstream Smad and non-Smad pathways[20]. The phosphorylated type 1 receptor recruits and phosphorylates receptor-regulated Smads (R-Smads). Of the five R-Smads in mammals, the TGFBR2–ALK5 complex activates SMAD2 and SMAD3, whereas the TGFBR2–ALK1 complex activates SMAD1, SMAD5 and SMAD8 (REF. 21). Activated R-Smads form heteromeric complexes with the common partner Smad (co-Smad; SMAD4 in mammals) and translocate into the nucleus[14]. As the affinity of the activated Smad complex for the Smad-binding element is insufficient to support association with endogenous promoters of target genes, Smad complexes are associated with other DNA-binding transcription factors to regulate expression. Various families of transcription factors, such as the forkhead, homeobox, zinc-finger, AP1, Ets and basic helix–loop–helix (bHLH) families, are Smad partners[22–24]. The DNA-binding Smads and their specific DNA-binding cofactors achieve high affinity and selectivity for target promoters with the appropriate binding elements[25].

Several transcriptome analyses have shown that TGFβ stimulation leads to the immediate activation or repression of expression of several hundred genes in a given cell type, and different subsets of gene responses underlie the various cellular responses to TGFβ signalling in a cell type-dependent and cellular context-dependent fashion[26,27]. To achieve specific cell responses depending on cellular context, the activated Smad pool is shared among many competing partners, each of which is used for a subset of TGFβ-responsive genes only. Moreover, the Smad complex recruits co-activators such as p300 and CREB binding protein (CBP)[28,29] or co-repressors such as retinoblastoma-like 1 (RBL1) (REF. 30) depending on which partner is selected, and this can determine whether the target gene is activated or repressed. A group of genes that are simultaneously regulated by a common Smad cofactor complex is known as a 'synexpression group' (REFS 25,27). Cells of different types or those exposed to different conditions express distinct repertoires of transcriptional partners for Smads, and link their responses to TGFβ to their cellular context. Such gene responses orchestrate and maintain cellular homeostasis, and aberrant regulation of such responses can result in various clinical disorders, including cancer. Recent studies have shown that the human homologue of maternal Id-like molecule inhibits TGFβ signalling in a synexpression group-selective manner through the abrogation of physical interaction between SMAD2 and SMAD3 and certain bHLH transcription factors[25].

In addition to Smads, which are pivotal signal transducers in TGFβ signalling, TGFβ is also known to regulate non-Smad pathways, including Erk, p38 MAPK, JUN N-terminal kinase (JNK), PI3K–Akt and small GTPases[31,32]. Non-Smad pathways also control TGFβ-mediated tumour cell-autonomous and host–tumour interactions in cancer progression.

Suppression of TGFβ-mediated growth inhibition. In carcinoma cells, TGFβ stimulation inhibits cell cycle progression in the G1 phase through the induction of cyclin-dependent kinase inhibitors (CDKIs), INK4B and p21 (REFS 33,34). TGFβ also represses the expression of *MYC*, a transcription factor that promotes cell proliferation[35]. The induction of translation-inhibitory protein 4EBP1 by TGFβ stimulation also mediates the anti-proliferative effect of this cytokine[36]. To date, several genes that antagonize the inhibitory effect of TGFβ have been identified.

Among various TGFβ antagonists, SKI (also known as c-Ski) and SKIL (also known as SnoN), which are members of the SKI family of nuclear proto-oncogenes[37], have been well characterized. SKI was first identified as the transforming protein of avian Sloan-Kettering retrovirus (v-Ski) and the human cellular SKI homologue and closely related SKIL were later cloned on the basis of sequence similarity to v-Ski. Both SKI and SKIL physically interact with SMAD3 and SMAD4, which leads to the displacement of p300 and CBP from Smad complexes and the recruitment of nuclear hormone receptor co-repressor NCOR1 and histone deacetylases (HDACs). SKI also stabilizes inactive Smad complexes

on DNA, which results in the repression of target gene transcription[38] (FIG. 2). Suppression of Smad complexes and TGFβ-mediated anti-proliferative effects explains the pro-oncogenic roles of SKI and SKIL. In addition to the antagonistic effects on Smads, SKI and SKIL have Smad-independent functions. SKIL triggers premature senescence by binding to the promyelocytic leukaemia (PML) protein[39].

The evidence supporting the pro-oncogenic function of SKI and SKIL in mammalian tumorigenesis comes from studies showing that suppression of SKIL expression in human lung or breast cancer cells inhibited tumour growth both *in vitro* and *in vivo*[40], and that downregulation of SKI in pancreatic cancer cells also reduces tumour growth[41]. Moreover, expression of SKI and/or SKIL is increased in many cancer cells and tissues, including those derived from oesophageal squamous cell carcinoma, melanoma, oestrogen receptor$^+$ (ER$^+$) breast carcinoma, colorectal carcinoma and leukaemia[37], suggesting pro-oncogenic properties of SKI and SKIL in various cancer types.

The human ecotropic viral integration site 1 (EVI1) protein contains a zinc finger domain and is transcriptionally activated by several recurrent chromosomal aberrations in acute myeloid leukaemia (AML)[42].

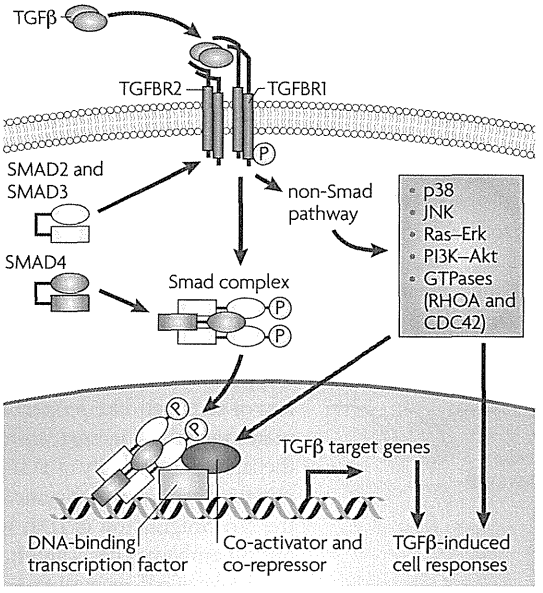

Figure 1 | **Intracellular signal transduction of TGFβ signalling.** Transforming growth factor-β (TGFβ) signalling is transduced through Smad and non-Smad pathways. TGFβ ligand binds to TGFBR2 and TGFBR1. TGFBR2 phosphorylates (P) TGFBR1, which subsequently phosphorylates and activates SMAD2 and SMAD3. Activated SMAD2 and SMAD3 form a Smad complex with SMAD4 and translocate into the nucleus. In the nucleus, the Smad complex interacts with other DNA-binding transcription factors, and co-activators and co-repressors, binds to the promoter regions of TGFβ target genes and regulates the transcription of target genes. TGFβ stimulation also activates other signalling cascades in addition to the Smad pathway. TGFβ receptors activate p38, JNK, Ras–Erk, PI3K–Akt, and small GTPases such as RHOA and CDC42.

EVI1 interacts with SMAD3 and antagonizes the growth inhibitory effects of TGFβ[43]. MEL1 (also known as MDS1) was originally identified as a member of the EVI1 gene family[44]. MEL1 and SKI were reported to be aberrantly expressed in gastric cancer cells by chromosomal co-amplification. MEL1 interacts with SKI and inhibits TGFβ signalling by stabilizing the inactive SMAD3–SKI complex on the promoter of TGFβ target genes[45]. The tumour-promoting effects of MEL1 through the inhibition of tumour-suppressive TGFβ signalling in gastric cancer cells were also demonstrated in studies *in vitro* and *in vivo*[45].

Chromosomal translocations that result in abnormally regulated *BCL6* expression are frequently observed in diffuse large B cell lymphomas and follicular lymphomas, the two most common types of non-Hodgkin lymphoma[46,47]. Recent studies have shown that BCL6 interacts with SMAD4 to suppress complex formation between SMAD4 and co-activators, which in turn represses SMAD4-mediated transcription activation and TGFβ signalling[48]. In an *in vitro* study, knock down of BCL6 expression restored TGFβ-mediated cell cycle arrest in B lymphoma cells.

Some viral gene products regulate Smad signalling and attenuate growth inhibitory activity of TGFβ. Human T cell leukaemia virus type I (HTLV-I) Tax, which is implicated in various clinical manifestations in adult T cell leukaemia, disrupts the interaction of Smad complexes with the transcriptional co-activator p300 and contributes to resistance to growth inhibition by TGFβ[49,50].

As discussed here, several proto-oncogenes exhibit their tumorigenic activity through the suppression of TGFβ signal transduction, indicating pivotal roles of TGFβ signalling in tumour progression.

In addition to proto-oncogenes, certain tumour suppressor genes cooperate with TGFβ–Smad signalling for growth inhibition, and the loss of such genes can lead to tumour progression. A Runt domain transcription factor, RUNX3, is an important tumour suppressor in gastric cancer[51]. RUNX3 is required for TGFβ-dependent induction of p21 expression as it binds to the *Cdkn1a* promoter, and along with a Smad complex synergistically activates expression of this cell cycle inhibitor[52]. RUNX3 is also involved in TGFβ-induced apoptosis in gastric epithelial cells through the induction of the pro-apoptotic protein BIM[53].

Promotion of cancer cell proliferation by TGFβ. Although TGFβ has an anti-proliferative effect on most epithelial cells and haematopoietic cells, it promotes proliferation of certain mesenchymal cells, including smooth muscle cells, through the induction of platelet-derived growth factor (PDGF)[54]. Similarly, TGFβ induces the proliferation of certain types of cancer cells, including glioma and osteosarcoma cells, through the induction of PDGFA or PDGFB[55,56]. In addition, hypomethylation of CpG islands in the *PDGFB* promoter results in a stronger induction of PDGFB expression by TGFβ and is associated with a poor prognosis in patients with glioma[55]. A bHLH transcription factor, OLIG1, is associated with SMAD2 and SMAD3 in a TGFβ-dependent

manner and synergistically promotes the expression of *PDGFB* in glioma cells[25]. *In vitro* and *in vivo* growth of glioma cells was greatly attenuated by the suppression of OLIG1 expression compared with control cells. Among glioma samples, OLIG1 is highly expressed in glioblastoma (WHO grade IV), anaplastic oligodendroglioma (WHO grade III) and oligodendroglioma (WHO grade II)[57], suggesting a pro-oncogenic role of OLIG1 through the synergistic induction of PDGFB with TGFβ signalling.

Regulation of apoptosis and autophagy by TGFβ

In addition to the regulation of the cell cycle, TGFβ also limits cancer formation through the activation of the apoptotic pathway. Downstream targets for pro-apoptotic functions of TGFβ include death-associated protein kinase (DAPK), growth arrest and DNA damage-inducible 45β (GADD45β) and BIM[58–60]. For example, BIM deficiency was shown to induce follicular lymphoma and accelerate MYC-induced generation of lymphoma in a mouse model[61].

By contrast, TGFβ also exhibits anti-apoptotic effects through the induction of differentially expressed in chondrocytes 1 (DEC1) under certain conditions[62]. DEC1 is a bHLH transcription factor that is frequently overexpressed in certain cancers, including breast carcinomas[63]; a correlation between the expression of DEC1 and tumour grade in breast cancer has been reported[64]. TGFβ-induced *DEC1* expression prevents the apoptosis of mouse mammary carcinoma cells, and a dominant-negative mutant of DEC1 prevents lung and liver metastasis of breast cancer cells *in vivo*[62].

TGFβ can also induce autophagy. During autophagy, cells digest their proteins and organelles using the lysosomal degradation pathway, leading to the maintenance of macromolecular synthesis and ATP production. Recent studies have shown that TGFβ induces autophagy and growth inhibition in certain hepatocellular carcinoma and mammary carcinoma cell lines through the transcriptional activation of autophagy (ATG) genes[65]. Autophagy has been described as a cytoprotective mechanism that is induced under conditions of nutrient deprivation[66]. The involvement of autophagy induction in the context of the tumour-suppressing and tumour-promoting effects of TGFβ needs to be further studied.

These studies indicate that TGFβ signalling in cancer cells has dual roles in the regulation of apoptosis, as well as that of proliferation.

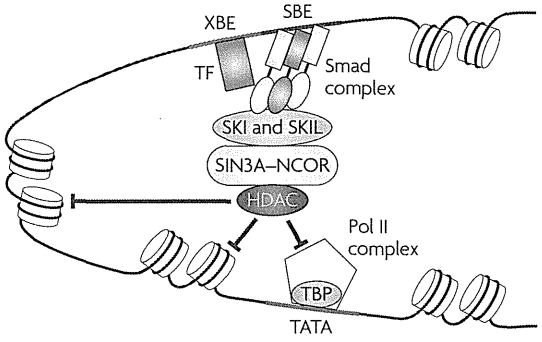

Figure 2 | **The function of SKI and SKIL.** Smad co-repressors SKI and SKIL bind to the Smad complex and recruit NCOR1–SIN3A and histone deacetylase (HDAC) activity to the target gene promoter. Smad co-repressors also repress Smad signalling through the disruption of the formation of Smads and Smad co-activator complexes. XBEs are binding elements of Smad-binding cofactors. Pol II, RNA polymerase II; SBE, Smad binding element; TBP, TATA binding protein; TF, transcription factor; XBE, X protein binding element.

TGFβ signalling in tumour-initiating cells

Recently, specific populations of cells with increased tumour-initiating capacity have been identified in many cancer types and are referred to as cancer stem cells (CSCs) or tumour-initiating cells (TICs)[67,68]. These highly tumorigenic cells often exhibit stem cell properties such as self-renewal, multipotency and the expression of stem cell markers. It has been suggested that TICs make use of a microenvironment similar to that found in normal stem cell niches for the maintenance of their stem cell-like properties. TGFβ signalling was recently identified as a niche signal in the control of haematopoietic stem cells[69], and so a broader role for TGFβ signalling in the maintenance of TICs has been proposed. Recent studies have revealed crucial roles of TGFβ signalling in TIC–niche interaction, as well as TIC-autonomous signalling pathways (TABLE 1).

Breast TIC. Tang *et al.*[70] showed that the suppression of the TGFβ pathway increased the size of the putative breast cancer-initiating cell compartment and promoted tumorigenesis by a mechanism that was independent of direct effects on proliferation. They used an immortalized and transformed human breast epithelial cell line, Ca1h, and demonstrated that the introduction of a dominant-negative TGFBR2 enhanced the proliferation of these cells, although the expression level of p21 was unchanged. They also showed that TGFβ stimulation resulted in the loss of stem cell-like properties and the ability to form mammospheres, using transformed human breast epithelial cells. The ability of TGFβ to deprive breast cancer-initiating cells of tumorigenic activity was dependent on the downregulation of ID1, which is highly expressed during embryogenesis and has been implicated in the regulation of self-renewal and differentiation. These findings suggest that TICs benefit from similar mechanisms that regulate the function of normal stem cells.

By contrast, Mani *et al.*[71] found that TGFβ signalling has an important role in the maintenance of stem cell-like properties and tumorigenic activity through the induction of epithelial–mesenchymal transition (EMT). A $CD44^{high}$ $CD24^{low}$ subpopulation that was isolated from normal and cancerous mammary glands exhibited mesenchymal properties, with decreased expression of E-cadherin and increased expression of mesenchymal markers, including N-cadherin and vimentin. Furthermore, normal and transformed mammary epithelial cells, in which EMT was induced by TGFβ stimulation, acquired stem cell-like properties, including

Table 1 | **Roles of TGFβ signalling in cancer stem cells**

Cancer type	Cells	Function of TGFβ	Refs
Breast	Immortalized human mammary epithelial cells and human tumour samples	TGFβ treatment reduces the size of the side population (SP) fraction and the ability to form tumours	70
Breast	Immortalized human mammary epithelial cells and human tumour samples	Tumorigenicity of breast cancer stem cells is maintained by TGFβ-induced EMT. A $CD44^{high}$ and $CD24^{low}$ population expresses genes associated with cells that have undergone EMT	71
Glioblastoma stage IV	Human samples	TGFβ-induced LIF expression maintains tumorigenicity of glioma stem cells. LIF expression correlates with TGFβ2, Nestin or Musashi expression in glioma tissues	74
Glioblastoma stage IV	Human samples	A TGFβ-induced SOX4–SOX2 axis maintains tumorigenicity of glioma stem cells. *SOX4* and *SOX2*, genes that are upregulated by TGFβ, are highly expressed in glioma stem cells	75
Chronic myeloid leukaemia (CML)	Mouse CML model and human tumour samples	The TGFβ–FOXO pathway maintains stem cell-like properties of leukaemia-initiating cells	85
Prostate	Cell lines from mouse xenografts	Inhibition of TGFβ signalling promotes differentiation of SP clones of prostate cancer cells	128
Pancreatic	Cell lines	TGFβ responsiveness is greater in SP cells than in main population cells, resulting in enhanced induction of EMT and invasiveness	129

EMT, epithelial–mesenchymal transition; LIF, leukaemia inhibitory factor; TGFβ, transforming growth factor-β.

mammosphere-forming ability, the expression of CD44 and low levels of CD24 expression. Transformed mammary epithelial cells with TGFβ-induced EMT also showed higher tumorigenic activity *in vivo* and fewer cells were required to initiate tumour formation. These results connect EMT and tumour-initiating properties in cancers of epithelial origin, and suggest that regulating EMT through targeted drugs might be a promising strategy to target TICs. Further studies are needed to clarify the contradictory results of the role of TGFβ signalling in the regulation of TICs in breast cancer. In addition, the involvement of the tumour microenvironment in breast cancer TICs should be further investigated.

Glioma-initiating cells. TGFβ signalling and bone morphogenetic protein (BMP) signalling have important roles in the regulation of the stem cell properties of neural stem cells[72]. Moreover, these signalling pathways are also involved in the development and progression of brain tumours. These facts have shed some light on the role of TGFβ and BMP signalling in the maintenance of brain TICs.

The overexpression of TGFβ that is commonly seen in malignant glioma has been variously implicated in glioma cell proliferation, migration, decreased apoptosis and tumour-specific immunosuppression[73]. Recent reports have unveiled pivotal roles of TGFβ signalling in the maintenance of stem cell-like properties and tumorigenic activity of glioma-initiating cells (GICs)[74,75]. TGFβ inhibitors markedly deprived GICs of glioma sphere-forming activity and self-renewal *in vitro* and tumorigenic activity *in vivo*. Inhibition of TGFβ signalling also decreased the size of $CD133^+$ and $Nestin^+$ subpopulations, markers that are associated with cell populations that have stem cell-like properties. These results indicate that microenvironmental niche-derived or GIC-autonomous TGFβ signalling maintains the glioma-initiating abilities of GICs. TGFβ mediates this activity through the activation and subsequent direct binding of a Smad complex to the promoter region of the leukaemia inhibitory factor (*LIF*) gene[74] (FIG. 3). LIF activates the JAK–STAT pathway in GICs, leading to increased GIC tumorigenesis that is secondary to their increased self-renewal and decreased differentiation. Independently of this mechanism, TGFβ induces the expression of *SOX2*, a self-renewal gene that helps to maintain stem cell-like properties in embryonic stem cells and neural stem cells[76–78]. TGFβ induces the expression of *SOX4*, and this subsequently induces the expression of *SOX2* (REF. 75).

TGFβ signalling thus maintains the stemness property of GICs through at least two independent pathways: TGFβ–LIF and TGFβ–SOX4–SOX2. GICs, as well as other TICs, are known to be more resistant to chemotherapy and radiotherapy[79,80]. These recent studies raise the possibility that a TGFβ inhibitor could be used in a combination with conventional pharmacological therapies and radiation to make malignant glioma less aggressive[81].

BMP signalling is known to induce the differentiation of embryonic neural progenitor cells into astrocytes[82]. In an analogous fashion, BMP4 inhibits the proliferation of GICs, deprives them of self-renewal capacity and induces differentiation predominantly into cells with the characteristics of normal mature astrocytes[83]. Furthermore, BMP4 reduces glioma growth and associated mortality after intracerebral engraftment of human GICs in mice. These findings suggest that BMP signalling might be a promising therapeutic agent to target GICs and prevent recurrence of malignant glioma through the induction of terminal differentiation of GICs. However, another study demonstrated that BMP-induced differentiation is

impaired in a subpopulation of GICs owing to epigenetic silencing of BMP type IB receptor (*ALK6*), resulting in a differentiation block that contributes to the pathogenesis of malignant glioma[84]. This study demonstrates not only that BMPs function in GICs, but also the importance of tumour-to-tumour variation in GICs.

Leukaemia-initiating cells. TGFβ signalling also has crucial roles in the maintenance of leukaemia-initiating cells (LICs) in chronic myeloid leukaemia (CML). TGFβ regulates AKT activation and FOXO3a localization in LICs. Furthermore, this TGFβ–FOXO pathway maintains the stem cell-like properties of LICs[85]. This study also showed that a combination of TGFβ inhibition, FOXO3a deficiency and imatinib treatment led to the efficient depletion of CML cells *in vivo*. These studies indicate that TGFβ maintains the tumorigenic activity of LICs in a different manner from that of GICs, and suggest that TGFβ maintains the tumorigenic activity of TICs in several types of cancers in a tissue-specific manner.

Targeting the pathways that maintain TICs might ultimately prove to be an effective therapeutic strategy against malignant tumours. However, such pathways could have divergent roles in TIC populations from different patients. This diversity among TICs could reflect both the differences between the oncogenic mutations expressed by the cells and their progeny, and the differences in their origin. These differences will need to be taken into account when developing treatments based on TGFβ and/or BMP signalling for any individual patient.

TGFβ signalling in tumour angiogenesis

The ability of tumour cells to induce new blood vessel formation is essential for progressive tumour growth and blood-borne metastasis. TGFβ can induce a pro-angiogenic environment and stimulates tumour angiogenesis, and increased TGFβ expression has been linked to increased microvessel density in certain tumour types, which also correlates with a poor prognosis[86]. In a xenograft model of prostate cancer, treatment with a TGFβ inhibitor reduced blood vessel formation in the tumour stroma, resulting in the inhibition of tumour angiogenesis and tumour growth[87].

The overexpression of TGFβ1 in Chinese hamster ovary cells and human prostate cancer cells significantly stimulates tumour growth and angiogenesis when these cells are injected into mice[88,89]. The mechanism of angiogenesis stimulation by TGFβ signalling includes the induction of key angiogenic factors such as connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) in both epithelial cells and fibroblasts[90,91]. In addition, TGFβ can induce the expression, secretion and activation of matrix metalloproteinase 2 (MMP2) and MMP9, and downregulate the expression of tissue inhibitor of metalloproteinase (TIMP) in tumour and endothelial cells[92]. These metalloproteinase activities result in the enhancement of migratory and invasive properties of endothelial cells, which are required for tumour angiogenesis.

Conversely, TGFβ regulates the expression of angiogenic factors and angiogenic inhibitors in some cancer cells, and inhibits angiogenesis under certain conditions.

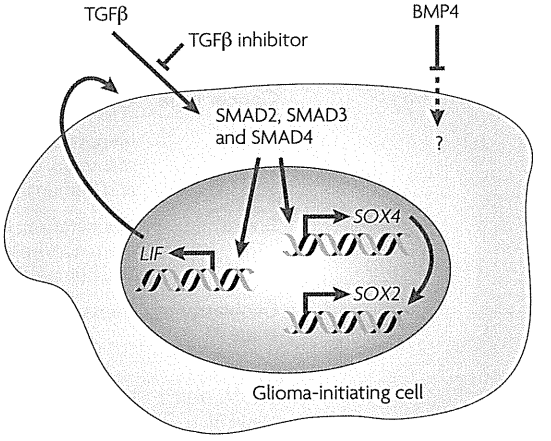

Figure 3 | **TGFβ and glioma-initiating cells.** Transforming growth factor-β (TGFβ) signalling maintains the tumorigenicity and stem cell-like properties of glioma-initiating cells through many independent pathways, two of which are the activation of the leukaemia inhibitory factor (LIF) pathway and the induction of the SOX4–SOX2 cascade. Bone morphogenetic protein 4 (BMP4) stimulation inhibits the proliferation of glioma-initiating cells and deprives them of tumorigenic activity. The mechanism of how BMP4 induces differentiation of glioma-initiating cells has not been fully determined.

In pancreatic cancer and diffuse-type gastric cancer, TGFβ induces the production of thrombospondin 1 (TSP1), a potent angiogenic inhibitor, and perturbations of TGFβ signalling result in accelerated angiogenesis and growth of tumours[93–95].

It is therefore dependent on the cellular context of tumour cells and endothelial cells whether TGFβ is a pro-angiogenic factor or an anti-angiogenic factor. Key determinants could include not only the status of the cells themselves but also the tumour–microenvironment interactions.

TGFβ signalling in metastasis

During the metastatic process, tumour cells undergo a sequence of migrations to different anatomical compartments: local invasion through the epithelial basement membrane from the primary tumour into the surrounding tissue; transport through the circulation; extravasation from the circulation at the putative metastatic site; adaptation to the new host microenvironment; and growth at the metastatic focus. Several studies in model systems have described a broad range of potential TGFβ effects on distant metastasis.

TGFβ signalling in EMT and mesenchymal–epithelial transition (MET). To invade normal tissues and metastasize to distant organs, carcinoma cells need to lose polarity and cell–cell contacts and acquire fibroblastic characteristics. This process of EMT is a crucial step for carcinoma cells to metastasize[96,97]. TGFβ was first described as an inducer of EMT in normal mammary epithelial cells, and several subsequent studies established crucial roles of TGFβ-induced EMT in tumour

progression[98]. A hallmark of EMT is the disintegration and disassembly of cell–cell junctions, including tight junctions and adherens junctions that maintain the integrity of epithelial units.

Tight junctions are mediated by transmembrane claudins, occludins and scaffold proteins such as ZO1. During TGFβ-induced EMT, these molecules are downregulated, leading to the degradation of tight junctions. TGFβ also alters cell surface protein complex structure directly through its receptor complex independently of nuclear gene regulation. PAR6 is a key component of epithelial polarity complexes that regulate the assembly of tight junctions[99]. Binding of TGFβ ligand to its receptors enables TGFBR2 to phosphorylate PAR6 and degrade RHOA, which mediates the maintenance of junctional stability[100].

Adherens junctions are mediated by homotypic interaction of the extracellular domains of E-cadherin. Several studies have focused on the mechanisms of disintegration of adherens junctions mediated by TGFβ — numerous factors, including SNAI1, SNAI2, HMGA2, ZEB1 and ZEB2, repress the expression of E-cadherin[101].

One of the key factors in tumour progression, Ras, is involved in the induction of EMT synergistically with TGFβ signalling. Mammary epithelial Eph4 cells with hyperactivation of Ras signalling undergo EMT by TGFβ stimulation and acquire an invasive phenotype[102,103]. Ras and PI3K seem to activate Src family tyrosine kinases, resulting in the destabilization of E-cadherin–β-catenin complexes and the disruption of the adherens junctions[104]. In addition, the induction of SNAI1 by TGFβ is strongly dependent on cooperation with active Ras signals, and silencing of Ras abolishes SNAI1 induction by TGFβ in some types of cells, including Panc-1 pancreatic cancer cells[105].

Another key pathway in cancer cells, MDM2–p53, also has a crucial role in TGFβ-induced EMT. In mouse mammary epithelial cells, TGFβ induces expression of *Mdm2*, and increased levels of MDM2 lead to the destabilization of p53, which is a key component of EMT[106]. Furthermore, histological analyses of human breast cancer samples demonstrated a strong correlation between TGFβ1-mediated induction of MDM2 and late-stage tumour progression[106].

It is now generally accepted that TGFβ functions as a tumour suppressor in the early phase of tumorigenesis, but can be converted to a tumour promoter during cancer progression[11]. Recent studies have shown that mutation of p53 is involved in this switching of TGFβ from a tumour suppressor to a tumour promoter[107]. In the early stages of tumorigenesis, TGFβ, working as a tumour suppressor, inhibits the proliferation of tumour cells in cooperation with wild-type p53. By contrast, after p53 is mutated, an activated Smad complex and mutant p53 cooperatively abrogate the ability of p63 to downregulate sharp-1 and cyclin G2 expression and to suppress metastasis. In addition, mutation of p53 in non-invasive tumour cells enhances the pro-invasive and migratory effects of TGFβ, whereas loss of mutant p53 expression in aggressive tumours impairs their metastatic potential.

In addition to these genetic events, epigenetic silencing is also involved in TGFβ-induced EMT. During EMT, the promoter regions of some epithelial marker genes, including that of *CDH1* (encoding E-cadherin), are hypermethylated after TGFβ stimulation[108]. Although the mechanism through which TGFβ induces methylation of these genes has not been fully determined, one of the proposed mechanisms is that TGF-β modulates the binding of maintenance DNA methyltransferase, DNMT1. If proven, this would mean that the TGFβ–Smad pathway has a crucial role in the maintenance of epigenetic silencing of genes that regulate EMT.

Although the cytokines and transcription factors involved in EMT have been well characterized, the mechanisms of its reverse reaction, MET, have received little attention. BMP7 was reported to reverse TGFβ-induced EMT in a mouse model of chronic renal injury[109]. In bone metastasis models, BMP signalling in breast and prostate cancer cells inhibited their metastatic capability by conteracting EMT[110,111]. Recent studies showed that thyroid transcription factor 1 (TTF1; the product of *NKX2.1*) inhibits EMT in response to TGFβ and restores epithelial phenotypes in lung adenocarcinoma cells, leading to the suppression of cell migration and invasion[112]. TTF1 attenuates autocrine TGFβ signalling through the downregulation of TGFβ2 expression and abrogates TGFβ-mediated induction of SNAI1 and SNAI2. TTF1 might also suppress Smad-mediated transcription of EMT-inducing molecules, as is suggested by the finding that SMAD3 physically interacts with TTF1 and regulates its transcriptional activity[113,114]. In a syngenic mouse model, expression of TTF1 in Lewis lung carcinoma cells resulted in tumour growth retardation and an increased survival rate[112]. Furthermore, TTF1 was reported to be a good prognostic marker in patients with non-small-cell lung cancer[115]. These results suggest that the modulation of EMT and MET in carcinoma cells could control the invasive properties of carcinoma cells and might be the basis of a new therapeutic strategy for the inhibition of tumour metastasis.

Priming for distant metastasis. A recent study demonstrated that TGFβ in the breast cancer microenvironment primes cancer cells for pulmonary metastasis[116]. Inhibition of TGFβ signalling in an ER^- human breast cancer cell line decreased the ability of these cells to generate lung metastases when implanted in mice. Central to this process was the vascular remodelling gene, angiopoietin-like 4 (*ANGPTL4*), which was identified as a target of TGFβ signalling in multiple breast cancer samples. Tumour cell-derived ANGPTL4 disrupted vascular endothelial cell–cell junctions, increased the permeability of lung capillaries and facilitated the transendothelial passage of cancer cells. This study also showed that a TGFβ gene response signature that included *ANGPTL4* upregulation was associated with lung metastases but not bone metastases. The reason why the signature did not provide an advantage for seeding to bone could be explained by the function of ANGPTL4. The capillary walls in the bone marrow are already fenestrated to facilitate the passage of haematopoietic cells. Therefore, tumour cells with an

enhanced ability to breach tight vascular barriers would gain little advantage in colonizing bone. This new model suggests that TGFβ can function at a distance: the induction of cytokine ANGPTL4 by TGFβ enables the actions of TGFβ to project throughout the body, enhancing the affect of TGFβ signalling on distant metastasis.

Metastatic colonization. Once distant metastases have developed, local production of TGFβ can profoundly affect the growth of these lesions. Recent studies have uncovered a prominent role for TGFβ in bone metastases, a common site of dissemination for breast and prostate cancers. The bone microenvironment consists of a rich store of multiple growth factors, including TGFβ. Metastatic cells that reach the bone activate osteoclasts that degrade the bone matrix and release the stored TGFβ. TGFβ then stimulates the cancer cells to release several osteolytic cytokines, one of which is parathyroid hormone-related protein (PTHrP)[117,118]. TGFβ induces PTHrP secretion, which in turn stimulates the production of RANK ligand (RANKL) in osteoblasts to promote the differentiation of osteoclast precursors and bone resorption[119]. Additional mediators that influence TGFβ-mediated bone metastases include a set of genes that modulate bone metastasis in a mouse model in which mice were inoculated with human ER$^-$ breast cancer cells[120]. Within this gene signature are osteolytic genes *CTGF* and interleukin-11 (*IL11*) that are induced by TGFβ–Smad signalling. CTGF mediates both angiogenesis and invasion, whereas IL-11 stimulates the expression of osteoclastogenic factors RANKL and granulocyte-macrophage colony stimulating factor in osteoblasts[121]. In mouse models of human breast cancer cell metastasis, oral administration of a TGFBR1 kinase inhibitor significantly reduced both the incidence and the extent of bone metastasis through the downregulation of PTHrP and IL-11 secreted by breast cancer cells[122–124].

The metastasis-promoting effects of TGFβ discussed here could, at least partially, explain the tumour-promoting roles of TGFβ in later stages of cancer progression[11]. On this basis, TGFβ inhibitors should be promising therapeutic agents for the suppression of metastases seeded by aggressive cancers, although their effects on other cell responses induced by TGFβ should also be carefully considered.

Future directions

TGFβ can function as a tumour-suppressing or tumour-promoting factor in cancer progression[11,125]. It is clear that a large number of cellular context-dependent factors contribute to the dynamic regulatory roles of TGFβ signalling. Under physiological conditions, TGFβ signalling is tightly regulated by numerous factors. Distortion of this balance could alter the characteristics of certain cells and induce the transformation from normal cells to cancer cells. The 'normalization' of TGFβ signalling is one of the key strategies for the development of new anticancer drugs.

The TGFβ pathway has been targeted using multiple strategies, including small-molecule inhibitors of the TGFBR1 kinase domain, TGFβ-specific neutralizing antibodies and antisense compounds[126]. Among them, a soluble antisense oligonucleotide that is specific for human *TGFβ2* mRNA, AP12009, has been used to target the TGFβ pathway *in vivo* and is currently in clinical trials for human cancers[81,127]. Other methods for targeting TGFβ signalling should enter pre-clinical and clinical trials in the future. To use modulators of TGFβ signalling in clinical practice, we will need to consider the tumour microenvironment, as it is one of the key determinants of cellular context for tumour cells[11]. Furthermore, recent studies have added new aspects to the role of TGFβ signalling in the tumour–microenvironment interaction: cancer stem cells and their niches. As discussed above, TGFβ can induce both cancer stem cell self-renewal and differentiation, depending on tumour type and other factors. Because of such complexity, TGFβ-based therapeutic strategies must be carefully considered in each case. In addition, potentially deleterious effects of these strategies in normal tissues need to be considered.

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