Differentiation plasticity regulated by TGF-β family proteins in development and disease

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During development, stem and progenitor cells gradually commit to differentiation pathways. Cell fate decisions are regulated by differentiation factors, which activate transcription programmes that specify lineage and differentiation status. Among these factors, the transforming growth factor (TGF)-β family is important in both lineage selection and progression of differentiation of most, if not all, cell and tissue types. There is now increasing evidence that TGF-β family proteins have the ability to redirect the differentiation of cells that either have fully differentiated or have engaged in differentiation along a particular lineage, and can thereby elicit 'transdifferentiation'. This capacity for cellular plasticity is critical for normal embryonic development, but when recapitulated in the adult it can give rise to, or contribute to, a variety of diseases. This is illustrated by the ability of TGF-β family members to redirect epithelial cells into mesenchymal differentiation and to cause switching of mesenchymal cells from one lineage to another. Hence, various pathologies in adults may be considered diseases of abnormal development and differentiation.

Embryonic development is marked by successive well-orchestrated differentiation events, whereby cells make decisions to differentiate along defined lineages during the formation of specific tissues or to remain uncommitted or partly differentiated, thus permitting the maintenance of stem cell and progenitor cell populations within that tissue. These cell fate decisions are driven by combinations of cell-extrinsic growth and differentiation factors that regulate stem cell pluripotency, selection of the differentiation lineage and progression of differentiation. These differentiation factors exert such control by activating or inhibiting transcription factor cascades that define the differentiation status and cell lineage pathway.

A question that repeatedly emerges from studies of cell and tissue differentiation is how far the decision to enter a differentiation pathway terminally restricts the differentiation potential of the cell, either as a result of cell-intrinsic genetic and epigenetic programming or because of the effect of the tissue environment. One may wonder how far cells that have differentiated along a certain lineage still retain the ability to reverse this differentiation, or to differentiate along an alternative pathway, in response to altered cell-extrinsic conditions. Questions regarding the plasticity in differentiation have led to a debate about the potential of cells to 'transdifferentiate' or to 'reprogramme' themselves. These issues can be addressed not only through studying the molecular and cellular aspects of normal development but also by characterizing diseases that derive from, or involve, the deregulation of differentiation.

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Effects of the TGF-β family on differentiation

Several classes of extracellular proteins act as key mediators of differentiation that regulate or define the selection and the progression of the differentiation pathways during development. Among these, TGF-β family members have essential roles in self-renewal, the differentiation potential of embryonic and somatic stem cells, selection of the differentiation lineage and progression along that lineage. The characterization of their activities during development has provided insights into the plasticity of cell differentiation. Indeed, TGF-β family proteins can, through paracrine or autocrine signalling mechanisms, induce cells that have acquired cell differentiation characteristics of one particular lineage to switch to differentiation along another lineage.

Extensive studies have led to an understanding of the mechanisms that lead to changes in gene expression in response to TGF-β family proteins. TGF-β ligands bind to their cognate receptor complexes at the cell surface; these consist of two type I and two type II serine/threonine kinase receptors, resulting in type I receptor activation and the consequent direct carboxy-terminal phosphorylation of Smads by the type I receptors. The receptor-activated Smads then associate with Smad4 and translocate into the nucleus to modulate the transcription of target genes. Together with non-Smad signalling mechanisms that emanate from TGF-β family receptors (and other receptors)[1], the Smad signalling pathways regulate the transcription programmes that define the differentiation potential and characteristics of various cell types[2,3].

The abilities of TGF-β family proteins and Smad signalling to direct and redirect differentiation have been demonstrated in various vertebrate tissue systems, including in the immune and haematopoietic compartments[4,5] and during neuronal differentiation[6]. Here we discuss observations that TGF-β family proteins actively participate in redirecting the differentiation of ectodermal and epithelial cells

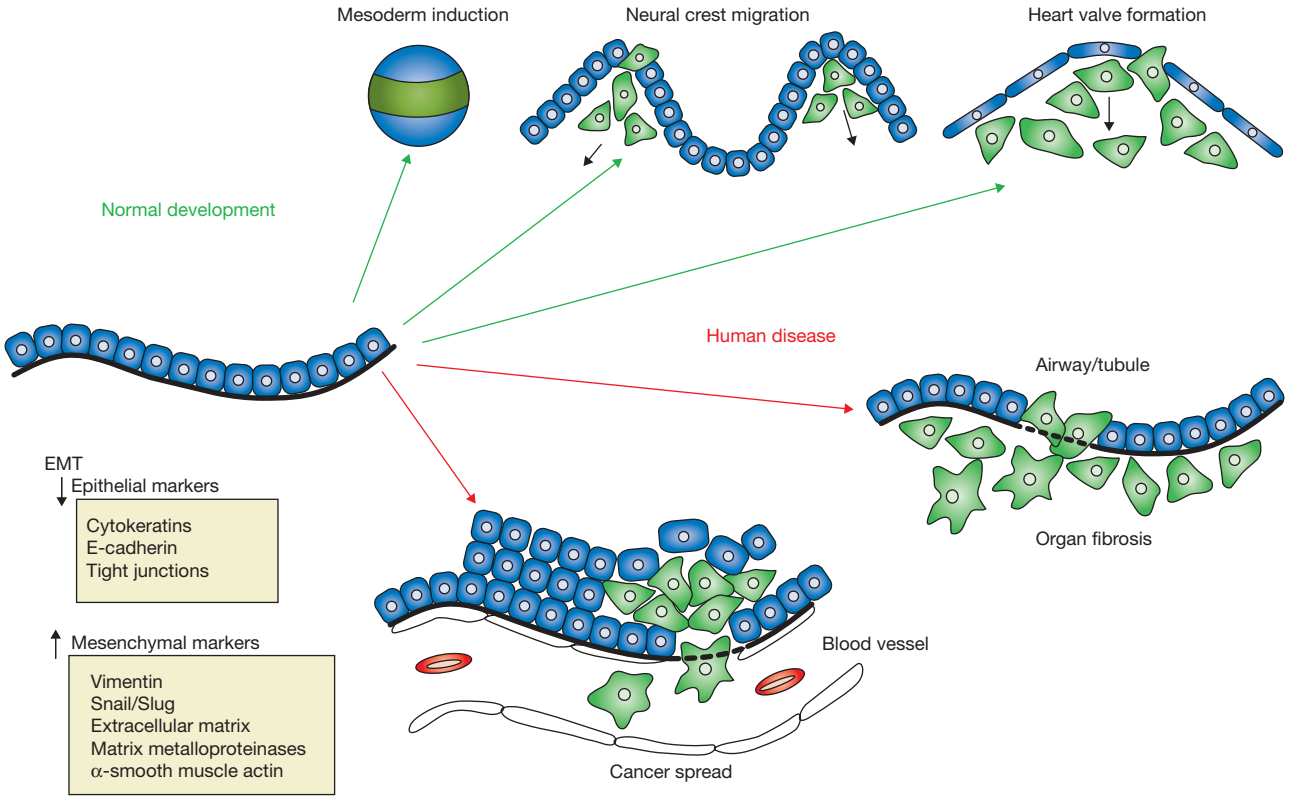

Figure 1 Epithelial–mesenchymal transition (EMT) in development and disease. EMT is integral to normal development, from gastrulation to later organogenesis. Reinstatement of this biological programme occurs in fibrosis and cancer and has a major effect on disease progression. EMT results in changes in markers as indicated by the vertical arrows. Snail and slug are transcription factors driving a mesenchymal cell transcriptional program.

towards mesodermal and mesenchymal differentiation. These activities are essential during normal differentiation but are also at the basis of various human diseases, including cancer, fibrosis and developmental pathologies.

The cellular basis for epithelial–mesenchymal transition

At several stages during normal development, epithelial cells acquire the phenotypic characteristics and behaviour of mesenchymal cells, a process named epithelial–mesenchymal transition or transformation (EMT), and in this way they contribute to the generation of different tissues and organs. Simply put, the EMT process results in a 'transdifferentiation' of epithelial cells forming a highly organized, tightly connected sheet of cells into a disorganized motile population of mesenchymal cells that phenotypically resemble fibroblasts. Although EMT is integral to normal development and occurs in a well-orchestrated fashion numerous times from gastrulation to later organogenesis, this biological programme can be reinstated in the adult under pathological conditions, particularly in fibrosis and epithelial neoplasia, in which it has a major effect on disease progression. Fibrosis involves an overgrowth of fibroblasts within an organ, together with uncontrolled elaboration of extracellular matrix protein and deposition of proteases, accompanied by the production of an inflammatory infiltrate that exacerbates this process. Neoplasia has some parallels to fibrosis but is driven by the appearance of mutation-bearing malignant epithelial cells with unlimited growth potential.

EMT commences with the dissolution of tight junctions connecting epithelial cells at apicolateral surfaces. Subsequently, basolateral adherens junction complexes are disrupted, simultaneously with reorganization of the actin cytoskeleton from a cortical adherens-associated location into actin stress fibres anchored to the focal adhesion complexes that are essential for traction during cell migration. The delocalization and downregulation of E-cadherin, an essential junction component, is a pivotal event in EMT because this process — or its converse, mesenchymal to epithelial transition (MET) — can be induced by manipulating the expression of E-cadherin. The downregulation of E-cadherin during EMT induces the release of soluble β-catenin, which activates the expression of c-Myc, cyclin D1 and matrix metalloproteinase 7, all contributing to the invasive behaviour of the resultant mesenchymal cells. These cells also display increased secretion of extracellular proteases and altered expression of extracellular matrix proteins and their receptors, resulting in a more migratory cell phenotype (Fig. 1). The final phase of EMT is a complete switch in the transcriptional profile of the cell as the expression of epithelial genes is lost and there is elevated or *de novo* expression of mesenchymal markers such as vimentin, N-cadherin and fibronectin[7–9]. In addition, in certain pathological situations, the cells differentiate further into the specialized contractile myofibroblast cell type that expresses α-smooth muscle actin and secretes a host of proteases and cytokines. These cells are particularly aggressive in both fibrotic and neoplastic conditions[10,11].

EMT in development

EMT occurs very early during vertebrate development, during gastrulation. Signalling by the TGF-β family members nodal and activin through Smad2 and Smad3 (using Smad4 as coactivator) acts in concert with other signalling pathways[12–14] to induce the formation

of the mesendoderm lineage from primitive epiblast (epithelial) cells, which subsequently gives rise to both mesoderm and endoderm, thus establishing the three primary germ layers from which the embryo develops. Successful gastrulation establishes the identity of the individual embryo, and mesoderm formation may be considered the earliest example of EMT in the embryo.

As embryonic development continues, EMT occurs as part of normal organogenesis in a variety of contexts. Delamination of migratory neural crest cells from the neuroepithelial tube gives rise to mesenchymal and other cell types and is ultimately responsible for the accurate development of head structures, as well as that of many organs and tissues of the body[15]. During cardiac development, EMT contributes to the formation of heart valves within the atrio-ventricular canal. In this system, inducing factors produced by the atrio-ventricular myocardium act on overlying endocardial cells to stimulate this change in the differentiation phenotype. *Ex vivo* studies on chick heart explants, and *in vivo* studies in transgenic and knockout mice have implicated several TGF-β family ligands in this process, particularly TGF-β2 and bone morphogenetic proteins (BMPs), as well as TGF-β family receptors and Smads[16,17]. During craniofacial development, TGF-β3 acts in an autocrine fashion within the midline epithelial seam of the secondary palate to transform these cells into palatal mesenchyme, an essential process for palatal development, as shown by complete penetrance of cleft palate in *Tgfb3*$^{-/-}$ mice[18].

EMT in fibrosis

Although mesenchymal cells are essential to formation of the body plan and normal tissue remodelling during organogenesis, the excessive accumulation of aggressive fibroblasts in adult organs can have serious consequences for morbidity and mortality, as seen in idiopathic pulmonary fibrosis, renal tubulo-interstitial nephritis, scleroderma and hepatic fibrosis. Many fibrotic conditions, some of which were previously thought to be caused by the infiltration and/or proliferation of pre-existing fibroblasts, are now known to include a significant contribution from mesenchymal cells arising through EMT. Cell lineage analysis in a tubulo-interstitial fibrosis mouse model demonstrated that 36% of renal fibroblasts are derived from renal tubular epithelium by EMT[19]. More strikingly, in a mouse model of TGFβ1-induced lung fibrosis, genetically labelled alveolar epithelial cells were shown to be the major source of myofibroblasts[20], which is consistent with the observation that lungs of patients with idiopathic pulmonary fibrosis contain numerous cells that co-stain with epithelial and myofibroblast markers[21]. Fibrotic cardiac valve disease may also involve a TGF-β-induced EMT, in this case from endothelial or endothelial progenitor cells[22]. In fibrotic liver, kidney and eye diseases, TGF-β-induced EMT has been shown to require Smad3 signalling[23,24], although MAP (mitogen-activated protein) kinase pathways have also been implicated in EMT in various systems[9].

TGF-β, EMT and metastasis

EMT also has a central role in malignant metastatic tumour spread of carcinomas, which are derived from epithelia. For certain types of tumour, cancer patients can live for many years with their disease under control, provided that the tumour does not disseminate to distant sites. However, it is the metastatic spread of tumours to vital organs, and the secretion of toxic products from these metastases, that bring about the ultimate demise of the patient. In mammalian epithelial cell cultures, TGF-β initiates cell scattering and cytoskeletal reorganization, transforming a tightly organized epithelial sheet into a motile population of cells. However, in mouse tumours, synergy between the Ras and TGF-β signalling pathways, both of which are frequently elevated during tumour progression, can result in an overt EMT, with the formation of fibroblastoid, spindle tumours that are highly invasive and contribute to metastatic spread[10,25]. In extreme cases, TGF-β induces not only EMT but also the further differentiation of fibroblasts into myofibroblasts that are contractile and have an elevated expression of pro-metastatic factors, such as matrix metalloproteinases, interleukin-8, vascular endothelial growth factor and the chemokine receptor CXCR4. This altered gene expression profile of the carcinoma cell leads to enhanced migration, invasion, and intravasation or extravasation in or out of the circulatory system[10]. Additionally, elevated expression of genes encoding chemokine ligands and receptors, such as SCF-1 (stem cell factor 1) and CXCR4, may facilitate homing and survival of carcinoma cells at the metastatic site in mouse models.

In general, human tumour metastases are not fibroblastoid in appearance, although there are increasing numbers of reports of spindle-like elements in human carcinomas[26–29]. However, it is now fairly well accepted that, during cancer spread in humans, EMT is most often transient, not necessarily complete, and reversible[30]. Indeed, the cell-extrinsic control and dynamism of EMT would be an advantage to the metastatic cell, which must not only migrate from its site of origin but also root and re-establish at a favourable secondary site.

The myofibroblast not only stimulates metastatic spread but also modulates the basic biology of the tumour by increasing the elaboration of extracellular matrix and eliciting a tissue contraction process, which results in elevated interstitial fluid pressure, similar to that seen during wound repair[31]. This has consequences for the efficiency of drug delivery to the tumour. Unlike normal tissue, which is under negative interstitial fluid pressure and thus acts like a sponge for drugs, the tumour resists fluid uptake, and consequently drug uptake, by virtue of this elevated pressure[31]. Hence, inhibition of TGF-β signalling in cancer patients, which is aimed at inhibiting EMT, may have two positive effects: a decrease in metastatic spread and an enhancement of uptake of simultaneously administered drugs delivered to the tumour cells[32]. Intra-tumoral drug delivery may also be enhanced through effects of TGF-β inhibition on the vascular system, particularly in inhibiting cellular differentiation of tumour vessel walls including their association with pericytes and smooth muscle cells[33]. Recently, with a mouse tumour allograft model, it was shown that the uptake and efficacy of nanoparticle-encapsulated cancer drugs were markedly enhanced in response to simultaneous administration of TGF-β inhibitors[33].

TGF-β-directed mesenchymal differentiation

TGF-β ligands are also important in determining the direction and extent of mesenchymal differentiation. Mesenchymal stem cells have the ability to differentiate into a variety of cell types including fibroblasts and highly specialized cell types such as skeletal muscle cells (myocytes), bone-matrix-depositing cells (osteoblasts), cartilage cells (chondrocytes) and fat cells (adipocytes). Mouse models and cell culture systems have shown that autocrine and paracrine stimulation by TGF-β is important in the maintenance and expansion of the mesenchymal stem cell progenitor populations. TGF-β inhibits differentiation along the osteoblast, skeletal myoblast and adipocyte lineages and stimulates the proliferation of these cells before they reach full

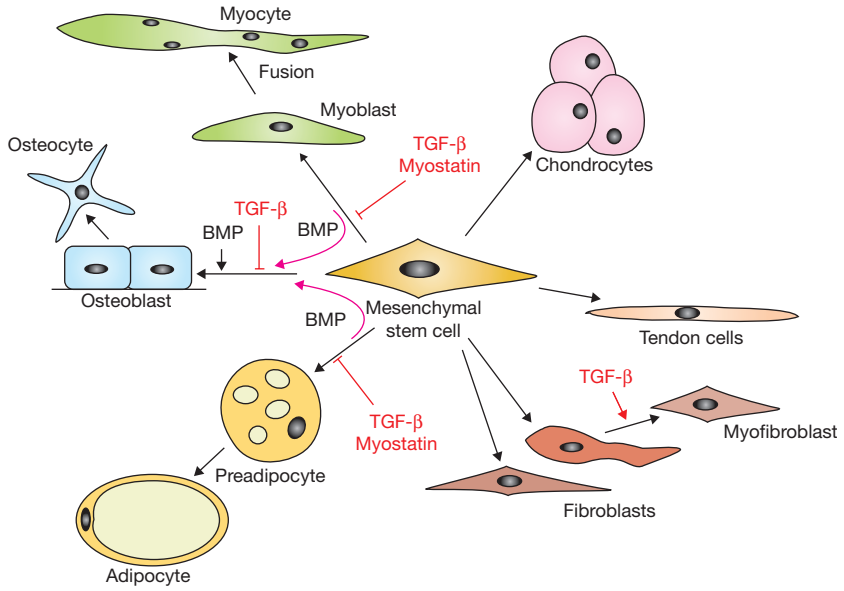

Figure 2 Roles of TGF-β family proteins in mesenchymal differentiation and their ability to redirect differentiation.

maturation[34]. TGF-β signalling may also help restrict the differentiation potential of cells. Dermal fibroblasts express the osteoblast transcription factor Runx2, without progressing along the osteoblast lineage. This inhibition is mediated by Smad3, as apparent from the ability of *Smad3*$^{-/-}$ dermal fibroblasts to display Runx2-activated osteoblast differentiation[35]. Other TGF-β family members, in particular the BMPs, stimulate the selection and progression of differentiation along defined mesenchymal lineages, namely differentiation into adipocytes, osteoblasts and chondrocytes (Fig. 2).

The importance of TGF-β family proteins in muscle cell differentiation is well illustrated by the effects of myostatin/growth development factor (GDF)-8, a TGF-β family member that is first expressed in the myotome layer during development and later primarily in muscle cells. Targeted *Gdf8* gene inactivation confers increased muscularity in mice; this is associated with increased cell proliferation and muscle cell hypertrophy[36]. In cattle, spontaneous mutations in the coding region of myostatin have been associated with increased muscle mass[37], whereas a mutation in the human *GDF8* gene has also been shown to correlate with gross muscle hypertrophy in a child[38]. Consistent with these phenotypes, myostatin inhibits the proliferation and differentiation of myoblasts, and *Gdf8*$^{-/-}$ myoblasts and satellite cells (muscle progenitor cells interspersed between myofibres) proliferate and differentiate more rapidly than wild-type cells[39,40]. Thus, myostatin should be considered an endogenous autocrine inhibitor of muscle differentiation and growth. Inhibitors of myostatin have the ability to increase muscle mass and reduce muscle wasting in model systems, making them potentially suitable for therapeutic applications in conditions resulting in muscular degeneration and wasting, such as muscular dystrophy[41].

Redirecting mesenchymal differentiation

In contrast to the inhibitory activities of myostatin and TGF-β on myogenic differentiation, exposure of myoblasts to BMPs redirects their differentiation towards the osteoblast lineage while inhibiting myogenic differentiation[42]. At the molecular level, BMP signalling activates the expression of Id1 (inhibitor of DNA binding 1 in skeletal myoblasts, whereas TGF-β does not[42]. BMP-induced Id1 expression in myoblasts inhibits the activity of myogenic basic helix–loop–helix (HLH) transcription factors, leading to their accelerated degradation. The induction of Id protein expression by BMPs is not restricted to mesenchymal cells but also occurs in embryonic stem cells as well as in neural progenitor cells, where it induces astroglial differentiation over the formation of neurons through the inhibition of neurogenic HLH transcription factors, thus mechanistically resembling the BMP-induced osteoblast differentiation of myoblast cells[43]. These findings illustrate a mechanism by which cells committed to a certain lineage through the expression of differentiation-specific HLH factors can be redirected along another differentiation pathway by BMP signalling.

After intramuscular injection of BMP, ectopic cartilage and bone tissue is formed, and in this context it is thought that BMP induces satellite cells and myogenic precursors to differentiate into chondrocytes or osteoblasts[44]. Inappropriate BMP signalling, resulting in ectopic osteogenesis, may also provide the cellular basis for fibrodysplasia ossificans progressiva, a rare genetic disorder of connective tissue characterized by progressive postnatal endochondral ossification of tendons, ligaments, fascia and striated muscle. In affected individuals, osteogenic lesions often arise spontaneously, but they can also be induced by surgery, trauma or intramuscular injections. Some evidence suggests that increased BMP signalling is at the basis of this disease as a result of either increased BMP-4 expression[45] or of an activating mutation in the gene encoding the BMP receptor ALK-2 (ref. 46).

In addition to their effect on progenitor cells, BMPs can also redirect cells that have initiated differentiation along the adipogenic lineage into osteoblast differentiation. A combination of BMP signalling and retinoic acid has been shown to redirect pre-adipocytes, as well as adipose stromal cells, into fully mature osteoblasts[47,48]. This raises the possibility that adipose stromal cells could be used for cell-based therapy in bone repair[49]. Consistent with these findings, adipose stromal cells have been used to repair skull defects in mice and man[50,51]. Whether differentiation from pre-adipocyte to osteoblast occurs during normal development or in response to injury is currently unknown. The coordinated signalling of BMPs and retinoic acid in redirecting the differentiation of these cells is consistent with their crosstalk in cartilage differentiation and bone formation[52]. Because retinoic acid upregulates BMP receptor expression[48], the redirection of differentiation may primarily be a consequence of BMP signalling.

Outlook and perspectives

As illustrated with the above examples, autocrine and paracrine signalling by TGF-β family members have critical roles in mesenchymal differentiation, both in lineage selection and progression and in redirecting epithelial cells into mesenchymal differentiation. Although required for normal embryonic development, these activities of TGF-β family proteins can be important in adult disease pathology. Perhaps

the most striking realization of these studies is that cells respond to TGF-β ligands with far more plasticity in their differentiation than previously thought. Manipulation of this plasticity may therefore be exploited for therapeutic purposes. Clearly, much research is still needed for a better definition of the extent of this plasticity and the limits of redirecting cells from one differentiation lineage to another. □

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