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The Discovery and Early Days of TGF-β: A Historical Perspective

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Transforming growth factors (TGFs) were discovered as activities that were secreted by cancer cells, and later by normal cells, and had the ability to phenotypically and reversibly transform immortalized fibroblasts. TGF-β distinguished itself from TGF-α because it did not bind to the same epidermal growth factor (EGF) receptor as TGF-α and, therefore, acted through different cell-surface receptors and signaling mediators. This review summarizes the discovery of TGF-β, the early developments in its molecular and biological characterization with its many biological activities in different cell and tissue contexts and its roles in disease, the realization that there is a family of secreted TGF-β-related proteins with many differentiation functions in development and activities in normal cell and tissue physiology, and the subsequent identification and characterization of the receptors and effectors that mediate TGF-β family signaling responses.

HISTORICAL CONTEXT

In the early 1970s, George Todaro and Robert Huebner, studying the transforming activity of C-type RNA tumor viruses, proposed the viral oncogene hypothesis, that is, that "oncogenes" were an important component of the virogene of RNA tumor viruses, whose expression was suppressed in "normal" cells, and that treatment of cells with carcinogens, mutagens, or radiation could activate expression of this viral information by inactivating a repressor (Todaro and Huebner 1972). A few years later, Michael Bishop and Harold Varmus, also studying the effects of RNA tumor viruses on malignant transformation, made the Nobel Prize–winning breakthrough that neoplastic transformation of a cell by the avian sarcoma virus was mediated by a single viral gene ([*src*] the "oncogene"), and most significantly that the product of the *src* gene, now known to be a cellular signaling mediator of many tyrosine kinase receptors, was a slightly modified analog of a normal cellular protein (Stehelin et al. 1976; Levinson et al. 1978). A few years later, they showed that the transforming retroviral oncogene, *v-src*, arose by transduction of the cellular *c-src* gene (Swanstrom et al. 1983).

Around the same time, based on results from a collaboration with the Nobel laureate Stanley

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Cohen, George Todaro suggested that murine and feline tumor viruses transform cells by inducing cells to secrete a molecule functionally related to epidermal growth factor (EGF), which acted on the same cells to affect their transformation (Todaro et al. 1976) through a process later termed autocrine secretion (Sporn and Todaro 1980). Along similar lines, the late Nobel laureate Robert Holley had proposed that one mechanism of acquisition of malignancy involved an increase in the availability of specific hormone (or growth factor) receptor sites on the cell membrane (Holley 1972) and that "transformed or malignant cells escape from normal growth controls by requiring less of such hormones or growth factors" (Holley 1975). The transforming growth factors (TGFs) described by De Larco and Todaro (1978) shared properties with the *v-src* gene, in that they were able to cause normal fibroblasts to form progressively growing colonies in soft agar (anchorage-independent growth), a property that was closely associated with the transformed phenotype in vivo (Todaro et al. 1979). In contrast with *v-src* and other oncogenes, however, transformation did not result from cell-intrinsic, genetic changes, but from secreted factors that did not affect the genotype. The term TGF was chosen for this activity because of the induction of a transformed phenotype, in both monolayer and soft agar cultures, of nontransformed cells, and because of the assumption, later proven to be incorrect (see below), that the factor was produced only by transformed (cancer) cells. This behavior was reversible on removal of the source of TGFs, leading to the suggestion that these factors acted as proximal effectors of transformation. Considering the highly visible discovery of oncogenes as the genetic basis of malignant transformation around that time, the notion of reversible transformation by secreted factors was met with skepticism.

DISCOVERY OF TGF-β

The TGF-β field began with three papers, one published in 1978 and two in 1981. First, De Larco and Todaro (1978) described the partial purification of polypeptide growth factors secreted by fibroblasts transformed by an RNA virus, Moloney sarcoma virus (MSV), which they called sarcoma growth factor (SGF). Although SGF bound to EGF receptors, it was distinguished from EGF (suggested to be the nontransforming product of "normal" cells) by its ability to transform normal fibroblasts in vitro in the soft agar colony-forming assay. That the secreted factor both bound to EGF receptors and could induce transformation was based on co-elution of these two activities using molecular sieve chromatography (De Larco and Todaro 1978). In 1981, however, it became apparent through the work in the Harold Moses laboratory, then at the Mayo Clinic (Moses et al. 1981), and the Michael Sporn and Anita Roberts laboratory at the National Cancer Institute (Roberts et al. 1981), that the EGF receptor competing activity could be separated from the soft agar colony-forming activity. Both groups pursued purification and characterization of factors with seemingly different transforming properties, dependent on the different cell lines used in their assays. These factors turned out to be the same TGF-β.

At the present time, TGF-β is seen as the prototype of a large family of secreted, dimeric growth factors and cytokines, encoded in humans and mice by 33 genes. TGF-β family proteins are now known to be critically involved in embryonic development, cell and tissue differentiation, tissue homeostasis in the adult, and in many disease states, as detailed in at least 70,000 publications at this point. This review will cover the early history of TGF-β in the context of general scientific knowledge at the time.

PURIFICATION AND CHARACTERIZATION OF TGF-β

Approach of the Sporn/Roberts Laboratory

In the late 1970s, the Sporn laboratory focused on the role of retinoids as chemopreventive agents. Based on the identification by the Todaro group of peptides thought to be proximal effectors of transformation, the two laboratories entered a collaboration to design a large-scale isolation of the transforming peptides with the

ultimate goal of obtaining insights into the mechanisms of chemoprevention attributed to the retinoids. Because previous attempts at purification of SGF were based on the use of serum-free medium conditioned by MSV-transformed cells whose quantity was rather limited, the first breakthrough was the identification of a method that could be used to extract SGF directly from tumor cells. Using an acid–ethanol extraction procedure, previously described for the isolation of insulin from tissues and blood (Davoren 1962), and the formation of colonies in soft agar of "normal" NRK-2B clone 49F (NRK) fibroblasts as an assay, SGF-like activity was isolated from a variety of both virally or chemically transformed cells and tumors (Roberts et al. 1980). In attempts to purify SGF to homogeneity by successive chromatographic steps, it was noted, as mentioned, that the soft agar colony-forming response of NRK cells was lost, but could be restored by adding EGF to the assay (Roberts et al. 1981; Anzano et al. 1982). This led to the important finding that SGF, as well as colony-forming activities with similar biochemical properties isolated from a variety of non-neoplastic mouse, bovine, and human tissues, were actually comprised of two synergizing activities. One of these provided EGF receptor binding and was subsequently assigned to TGF-α, a member of the EGF family of growth factors, whereas the other one, subsequently named TGF-β, did not bind to EGF receptors, but was required to induce phenotypic transformation in combination with EGF. An interesting anecdote is that the assignment of "α" and "β" to these two TGFs was based on Todaro's insistence that TGF-α, which he thought was uniquely expressed by tumor cells, represented the dominant transforming activity and that TGF-β, then known to be expressed in many normal tissues, served merely to modulate the process.

Advice from the late Nobel laureate Christian Anfinsen was to "think big" so that a purification scheme would ultimately yield sufficient quantities of material for amino acid sequencing and not simply show an "activity." This was especially relevant because the soft agar colony-forming assay was 100-fold more sensitive than the detection of TGF-β protein on a silver-stained gel. Based on this advice, the Sporn/Roberts laboratory used normal tissues, including bovine kidneys, human placenta, and platelets, all available in larger quantities than transformed cells, for purification of TGF-β. The formation of colonies of NRK cells in soft agar in the presence of EGF (typically a 7-day assay) was used to monitor the purification, with image analysis to quantify the number of colonies above a designated size limit. Purification from bovine kidneys (Roberts et al. 1983a) or human placenta (Frolik et al. 1983) required very large-scale preparations and several chromatography steps (Fig. 1), whereas purification from human platelets (Assoian et al. 1983), which contain ~100-fold greater activity, required only two chromatography steps to achieve homogeneity (Fig. 1). The colony-forming activity in the assay depended on the addition of EGF, confirming that these TGFs met the definition of a TGF-β used by this laboratory. The ED_{50} (half-maximal effective dose) of purified TGF-β in the NRK colony-forming assay was 2–3 pM in the presence of EGF. Significantly, the finding that TGF-β could be isolated from non-neoplastic tissues and especially from platelets (Childs et al. 1982; Assoian et al. 1983), strongly suggested normal physiological roles for this molecule, as subsequently shown in wound healing (Sporn et al. 1983) and development (Heine et al. 1987), and later in diseases such as fibrosis and cancer.

Approach of the Moses Laboratory

In the late 1970s, the Moses group was interested in the molecular changes associated with transformation of cells by chemical carcinogens. As was typical at the time, murine embryonic fibroblast cell lines served as model systems; methods for culturing nontransformed epithelial cells were not yet widely available. The Moses laboratory had shown that many of the phenotypic alterations observed in the chemically transformed AKR-MCA and C3H/MCA-58 cell lines could be accounted for by continuous growth factor stimulation (Moses et al. 1978, 1979). His group further found that the trans-

Scale of purification of TGF-β in the Sporn laboratory

	Bovine kidney	Human platelets
Starting material	2 kg	25 units
Acid/ethanol extraction	8 L	100 mL
Precipitation: ether/ethanol	32 L/16 L	400 mL/200 mL
Bio-Gel-P-60 column in 1 M acetic acid	Apply 2 L/80 L column 1 L fractions	Apply 10 mL/2 L column 5 mL fractions
Bio-Gel-P-60 column in 1 M acetic acid/8 M urea	No	0.5 mL/170 mL column 0.5 mL fractions
Cation exchange	Yes	No
HPLC Bondapak C18	Yes	No
HPLC Bondapak CN	Yes	No
Final yield	**6 mg TGF-β1**	**10 mg TGF-β1**

Amino-terminal sequencing: ALDTNYCFSSTEKNC .....
Amino acid composition (nine cysteines per chain)

Figure 1. Purification to homogeneity of transforming growth factor β (TGF-β) from bovine kidney and human platelets. Human platelets contain ~100-fold greater amounts of TGF-β than most other normal tissues. The scale of purification from bovine kidneys (or placenta) was large, requiring volumes of solvents unlikely to be used in laboratories today, and requiring four chromatography steps. In contrast, purification of TGF-β to homogeneity from human platelets was achieved using just two chromatography steps on Bio-Gel P-60. These large-scale purification schemes enabled the first determination of the amino-terminal sequence and amino acid composition of TGF-β1. HPCL, High-performance liquid chromatography.

formed cells had greatly diminished detectable EGF receptors relative to the nontransformed precursor cells, similar to the observations of De Larco and Todaro (1978) with MSV-transformed cells. As a result, the Moses laboratory pursued identification of SGF-like transforming activities by the chemically transformed cells. They used nontransformed AKR-2B fibroblasts, the progenitors of the chemically transformed AKR-MCA cells, as indicator cells, and this was different from the assays used by the Todaro and Sporn/Roberts laboratories, which used rat NRK fibroblasts. Soft agar–stimulating activity with a molecular sieve chromatography profile similar to that reported by De Larco and Todaro was detected in medium conditioned by the chemically transformed cells (Moses et al. 1981). Additional purification using ion exchange chromatography separated the EGF receptor–competing activity from soft agar growth–stimulating activity. However, in contrast to the findings of the Roberts group using NRK cells (Roberts et al. 1981), addition of EGF in the soft agar assay was not required to obtain maximum colony–stimulating activity of AKR-2B cells. This caused confusion leading to the assumption that the TGF identified by the Moses group might be different from that identified by the Sporn group, even to the extent that three types of TGFs were proposed to exist, that is, TGF-α, TGF-β, and TGF-γ (Roberts et al. 1983b). However, with exchange of reagents between the two laboratories, it was soon apparent that the requirement for EGF in the transformation assay reflected a difference among the reporter cell lines, with EGF needed for stimulation of colony formation by NRK but not for AKR-2B cells.

The Moses group proceeded to identify TGF-β activity in mouse embryos (Proper et al. 1982) and, more important, in serum (Childs et al. 1982). It was further shown that plasma lacked TGF-β activity and that the TGF activity in serum derived from platelets, which are an abundant source for purification of TGF-β (Childs et al. 1982). Platelets, as well as re-

combinant protein, are now the source of much of the commercially marketed TGF-β1.

Competitive Binding Assays Identify High-Affinity Cell-Surface Receptors

The first evidence for a high-affinity TGF-β receptor was provided by Joan Massagué in collaboration with Todaro (Massagué et al. 1982). Using preparations of SGF that were later shown to contain both TGF-β and TGF-α, they identified, by affinity cross-linking, a high-affinity 60-kD band, in addition to the 140- to 170-kD EGF receptor. Unlabeled EGF competed with ^{125}I-labeled SGF binding to the latter band, but not the 60-kD band. The first competitive binding assays with Scatchard analyses for TGF-β receptors were reported in 1984 by the Sporn/Roberts and Moses laboratories working independently (Frolik et al. 1984; Tucker et al. 1984a). Both groups found very high-affinity binding sites, with dissociation constants of 25–40 pM and ~10–30 receptors per cell. Subsequently, Massagué performed competitive binding assays with similar results. Using affinity cross-linking, his laboratory showed that there were three TGF-β binding species that are now known as the type I and type II receptors, and the type III (co)receptor, better known as betaglycan (Massagué and Like 1985). The development of competitive binding assays for TGF-β was critical in the demonstration that TGF-βs are inhibitors of cell proliferation, particularly in epithelial cells.

In addition to the type I and type II receptors, and the co-receptor betaglycan, other putative TGF-β receptors were reported, including a 70- to 74-kD band identified by ^{125}I-TGF-β affinity cross-linking in GH_3 pituitary cells, which was reported to interact with TGF-β, activin, and inhibin (Cheifetz et al. 1988). A 38-kD binding component was identified by similar means on choriocarcinoma cells (Mitchell et al. 1992). These and other putative TGF-β-binding "receptors" received little attention subsequent to the initial reports. On the other hand, the cell membrane TGF-β-binding component called TGF-β type V receptor was later identified as the low-density lipoprotein receptor-related protein (LRP-1) (Huang and Huang 2005). Additionally, the endothelial cell-surface protein endoglin was also shown to act as a TGF-β-binding co-receptor, of particular importance in endothelial cells (Cheifetz et al. 1992).

DISCOVERY OF THE GROWTH-INHIBITORY EFFECTS OF TGF-β

The discovery of growth inhibition by TGF-β began with a publication in 1978 by Robert Holley (Holley et al. 1978). Following receipt of the Nobel Prize in Physiology or Medicine in 1968 for work on transfer RNA and the genetic code, Holley moved to the Salk Institute and redirected his efforts to growth control. He showed that BSC-1 cells, derived from African green monkey kidney tissue, secreted a peptide growth inhibitor, and proceeded to purify this growth inhibitor from medium conditioned by the BSC-1 cells. By early 1984, the Moses laboratory had derived data indicating that purified platelet-derived TGF-β could inhibit proliferation of AKR-2B cells in monolayer culture. After witnessing at a seminar that the BSC-1 growth inhibitor presented itself on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as a 25-kD protein band under nonreducing conditions and a 12.5-kD band under reducing conditions, the Moses group hypothesized that the BSC-1 growth inhibitor may well be the same as TGF-β. A collaboration was established with Holley resulting in the exchange of reagents. It was shown that platelet-derived TGF-β and BSC-1 growth inhibitor had similar biological activities in stimulating colony formation of AKR-2B cells in soft agar, inhibiting proliferation of AKR-2B cells as well as BSC-1 and Mv1Lu cells in monolayer culture, and competing comparably for binding of TGF-β in the TGF-β receptor assay (Tucker et al. 1984b; Moses et al. 1985).

The Sporn/Roberts group at this same time had noted decreased proliferation of a number of cancer cell lines in soft agar with the addition of TGF-β, assuming that this decrease was the result of toxicity related to impurities in the TGF-β preparation. Following the presentation of some of the growth-inhibition data by Moses

at a national meeting in the spring of 1984, the Sporn/Roberts group concluded that the decreased cell proliferation was the result of growth inhibition by TGF-β (Roberts et al. 1985). They also showed that the effects of TGF-β on anchorage-independent growth of cells could be modulated by other growth factors. As an example, Myc-1 cells formed large colonies in the presence of EGF, and formation of these colonies was strongly inhibited by addition of TGF-β. In contrast, identical concentrations of TGF-β enhanced colony formation of these same cells, when in the presence of platelet-derived growth factor (PDGF), which by itself had almost no colony-forming activity. Moreover, in monolayer culture, TGF-β inhibited the growth-promoting effects of PDGF on these same cells, emphasizing the highly contextual effects of TGF-β (Roberts et al. 1985).

It was then also important to evaluate whether TGF-β could inhibit the proliferation of normal cells in culture. Primary cultures of human foreskin keratinocytes were shown to be growth inhibited by TGF-β in a reversible manner, and to be arrested in the G_1 phase of the cell cycle. In contrast, a squamous cell carcinoma cell line, derived from epithelial cells, was not inhibited by TGF-β (Shipley et al. 1986). Additionally, the Sporn/Roberts and Moses laboratories showed that several carcinoma cell lines were growth-inhibited by TGF-β, whereas others were not (Moses et al. 1985; Roberts et al. 1985). Numerous subsequent studies from several laboratories showed that TGF-β could inhibit a wide variety of epithelial cell types in culture, and that, in general, highly malignant carcinoma cells had lost the growth-inhibitory response. The notion that a growth factor could inhibit cell proliferation was at that time surprising and counterintuitive. Indeed, numerous growth factors had been identified in the 1970s and early 1980s, based on their abilities to stimulate cell proliferation, thus equating growth factors with growth stimulation. Additionally, transformation was inherently linked to increased cell proliferation. The notion of autocrine control of growth inhibition was novel.

The first demonstration that TGF-β could inhibit proliferation of epithelial cells in vivo was by Silberstein and Daniel (1987). They inserted slow-release pellets containing TGF-β into developing mouse mammary glands and observed marked inhibition of mammary ductal growth and morphogenesis. They further showed that this growth inhibition was reversible. Subsequently, systemically administered TGF-β1 was shown to inhibit the proliferative response of liver to partial hepatectomy (Russell et al. 1988). Additionally, transgenic mice overexpressing TGF-β1 in epithelial tissues also showed inhibition of proliferation in the mammary gland (Jhappan et al. 1993; Pierce et al. 1993) or skin (Cui et al. 1996; Fowlis et al. 1996). Numerous later studies involving transgenic expression of TGF-β, dominant-negative type II TGF-β receptors and conditional knockout of type II receptor expression supported the role of TGF-β as an endogenous negative regulator of proliferation of several cell types, including epithelial, endothelial, and hematopoietic cells.

By the late 1980s, many groups suspected that, even though TGF-β expression was shown to be up-regulated in cancers (Derynck et al. 1987), the TGF-β pathway may have a tumor-suppressive function because of its ability to inhibit cell proliferation. Thus, inactivation of the growth-inhibitory response to TGF-β would then initiate malignant transformation. However, solid evidence for tumor suppression by TGF-β signaling was not reported until 1995. Data supporting TGF-β's tumor suppressor role were derived from transgenic mouse studies (Pierce et al. 1995) and through identification of inactivating mutations in the TGF-β type II receptor in human colon carcinoma cells with microsatellite instability (Markowitz et al. 1995). Subsequent studies of genetically modified mice and mutations in genes encoding TGF-β signaling mediators in human cancers came to support a major role for the TGF-β pathway in tumor suppression (reviewed in Akhurst and Derynck 2001). Additionally, however, a substantial body of evidence arose that TGF-β signaling facilitates tumor progression through effects on the carcinoma cells and on host cells and tumor stroma (reviewed in Derynck et al. 2001; Massagué 2008; Ikushima and Miyazono 2010; Pickup et al. 2013).

COMPLEMENTARY DNA (cDNA) CLONING AND CHARACTERIZATION OF PRE-PRO-TGF-β

cDNA Cloning of TGF-β1

Searching for a new research area of interest, Rik Derynck, then at Genentech, became interested in the newly discovered TGF activity that was described to him by George Todaro over a pizza dinner at Frederick, Maryland, early in 1983. After all, the induction of reversible transformation by a secreted factor specifically made by tumor cells, even though controversial, was a remarkable observation, and, if indeed TGF exists, then one must be able to clone it. Although Derynck focused his efforts on the cDNA cloning of TGF-α (Derynck et al. 1984), Dave Goeddel, the head of the department, pointed him toward a recent paper from the Sporn/Roberts laboratory (Roberts et al. 1983a), biochemically showing that TGF-β was a dimeric protein, unlike TGF-α, with an amino-terminal sequence that was unrelated to TGF-α. Derynck first did not want to deal with it, being too busy with TGF-α cDNA cloning, but then contacted the Sporn group to affirm his interest in cloning TGF-β, which led to a formal collaboration. The Sporn/Roberts laboratory provided sufficient quantities of TGF-β isolated from human platelets (Assoian et al. 1983) for clostripain digestion, eventually resulting in the identification of a 55-residue-long contiguous amino-terminal amino acid sequence. Because the polymerase chain reaction had not yet been described (Mullis et al. 1986), a newly developed approach using long oligonucleotides as hybridization probes was used. As reliable amino acid sequence runs became available, two 44-residue-long oligonucleotides, designed based on a stretch of TGF-β sequence and the known bias of human codon usage, and a set of 16 14-mers complementary to all codons for amino acids 13–16 were used as hybridization probes against a human genomic DNA library. This led to the identification of an exon encoding amino acids 10–60 of mature TGF-β that then was used to screen a λgt10-based placenta cDNA library, and yielded a partial cDNA. Extensive screening of placenta and tumor cell cDNA libraries eventually led to a cDNA sequence of 2439 bp corresponding to the TGF-β precursor mRNA (Derynck et al. 1985). The deduced polypeptide sequence established that the purified biologically active TGF-β, now known as TGF-β1, was the carboxy-terminal 112 amino acid segment of a 390 amino acid precursor protein with an amino-terminal signal sequence and a long pro-sequence (Fig. 2). It was proposed, and subsequently verified, that the TGF-β monomer was cleaved from its precursor following a tetrabasic site, Arg-His-Arg-Arg. Later studies showed that furin, a member of the mammalian convertase family of endoproteases, is the likely protease required for processing TGF-β1 in vivo (Dubois et al. 1995). Subsequent cDNA cloning of TGF-β1 from mouse (Derynck et al. 1986), pig (Derynck and Rhee 1987), and monkey (Sharples et al. 1987) cells showed that the protein was highly conserved, that mature human and simian TGF-β1 differed by only one amino acid from murine TGF-β1, and that human and porcine TGF-β1 were identical. cDNA cloning of TGF-β and subsequent northern hybridizations also revealed that, unlike other growth factors that were typically expressed in specific cell types, TGF-β1 mRNA was synthesized by almost all normal and tumor cells examined, yet at higher levels by tumor cells (Derynck et al. 1985,1987), alluding to the broad spectrum of activities that were to be discovered. The cDNA cloning of TGF-α and TGF-β, first presented at a Cold Spring Harbor meeting in September 1984, led to the acceptance that TGFs did exist.

Concept of Growth Factor Latency

Even before TGF-β was cloned, it was seen as peculiar that TGF-β was secreted from cells in a biologically inactive form or complex that required activation by acidification (Lawrence et al. 1984; Pircher et al. 1984). This had initially not been noted because the isolation procedure started with the use of acid in the extraction, and subsequent purification steps were also performed under acidic conditions. Additional early studies showed that latent TGF-β could be activated not only by extremes of pH, but also

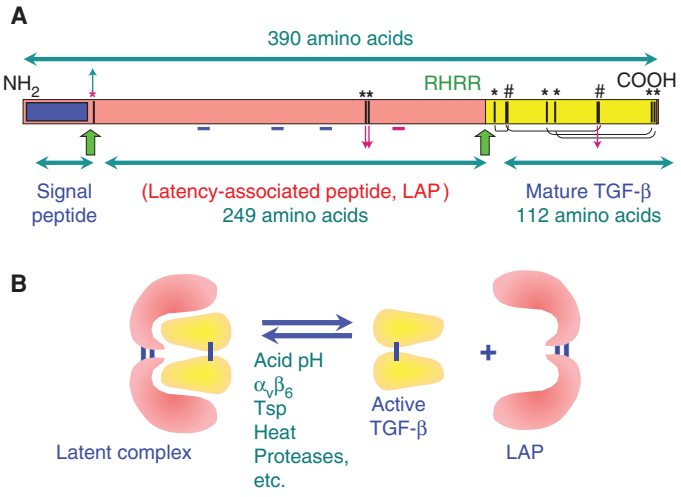

Figure 2. Structure and function of pre-pro-transforming growth factor β (TGF-β). (*A*) Complementary DNA (cDNA) cloning of TGF-β showed that it derives from a large precursor with a signal peptide, the large pro-segment (latency-associated protein [LAP]), and the carboxy-terminal mature biologically active protein (in dimeric form). Large green arrows show processing sites, black bars and asterisks show the position of cysteine residues, and # shows two adjacent cysteine residues. The pattern of cross-linking of the four intrachain disulfides is shown in brackets. Red arrows (down) show the position of the interchain disulfide bridges, whereas a green arrow (up) shows the cysteine that links LAP to latent TGF-β-binding protein (LTBP). A blue underscore shows the position of glycosylation sites and a red underscore shows the position of the Arg-Gly-Asp (RGD)-integrin-binding sequence present in the LAPs of TGF-β1 and TGF-β3 but not the TGF-β2 LAP. (*B*) TGF-β is secreted from cells in a biologically inactive form (latent TGF-β), with noncovalent association of the dimeric LAP with the dimeric mature carboxy-terminal 112 amino acid TGF-β. Latent TGF-β can be activated by a variety of treatments in vitro resulting in dissociation of the LAP protein from mature TGF-β, thus unmasking its receptor-binding epitopes.

by chaotropic agents, boiling, binding to α2-macroglobulin, or proteolysis using one of several proteases (Lawrence et al. 1985; O'Connor-McCourt and Wakefield 1987; Lyons et al. 1988; Sato et al. 1990). Moreover, because most cells can respond to TGF-β, physiological activation of this latent complex was increasingly seen as a key regulatory step in the control of its biological activity. The latency of TGF-β was subsequently revealed by Larry Gentry and Tony Purchio, and by Kohei Miyazono, then working with Calle Heldin, as well as Lalage Wakefield and Roberts, to result from noncovalent association of the mature TGF-β homodimer with the large dimerized pro-segment that has been renamed "latency-associated protein" (LAP), thus preventing binding of TGF-β to its receptors (Gentry et al. 1987; Miyazono et al. 1988; Wakefield et al. 1988). This "small latent complex" was also shown to bind to yet another and larger protein, latent TGF-β-binding protein (LTBP), a member of the fibrillin family of proteins, to direct extracellular localization of the latent complex (Miyazono et al. 1988, 1991; Wakefield et al. 1988; Kanzaki et al. 1990; Tsuji et al. 1990). The extensive sequence divergence among the three mammalian TGF-βs and the existence of four LTBPs suggest differences in extracellular targeting and activation mechanisms.

Expression of Recombinant TGF-β

Recombinant expression of TGF-β was reported 2 years after it was cloned (Gentry et al. 1987). Latent pre-pro-simian TGF-β was expressed in Chinese hamster ovary (CHO) cells, treated with methotrexate to amplify the coexpressed dihydrofolate reductase gene. One CHO clone showed TGF-β secretion exceeding by 50-fold the levels naturally secreted by cultured cells. The secreted recombinant TGF-β was latent, requiring acidification for biological activity.

This observation led to the hypothesis that the precursor sequences might play a role in the activation process. Studies of recombinant TGF-β expression confirmed the processing sites of the signal peptide and the mature TGF-β that were predicted from the cDNA cloning of the TGF-β precursor (Gentry et al. 1988). These studies also showed that the precursor was cross-linked by disulfide bonding and likely to function as a dimer, and that glycosylation of TGF-β was restricted to the precursor domain (Fig. 2). Subsequent complementation assays showed that the TGF-β prosequence was also required for proper folding, disulfide formation, dimerization, and secretion of mature TGF-β (Gray and Mason 1990), and thus acts as a chaperone. Because TGF-β was shown not to be glycosylated, attempts were made to express the mature form of TGF-β in bacteria (Tuan et al. 1996). Bacterially expressed mature TGF-β was inactive, confirming the need of the precursor domains to assure proper disulfide bonding of the mature TGF-β, and required renaturation using the glutathione-redox method to endow some biological activity.

IDENTIFICATION OF TGF-β HOMOLOGS

Three TGF-βs

Following the identification of platelets as the most concentrated source of TGF-β, R&D Systems (Minneapolis, MN) chose porcine platelets to generate commercially available TGF-β, which made sense considering that porcine and human TGF-β had identical sequences (Derynck and Rhee 1987). They worked with the Moses laboratory, then at the Mayo Clinic, to learn purification techniques based on the method published by Assoian et al. (1983). Surprisingly, they identified three peaks of activity, subsequently found to be homodimeric TGF-β1, homodimeric TGF-β2 and a heterodimer, TGF-β1:TGF-β2 (Cheifetz et al. 1987). Although TGF-β2 had the identical size and biological activity of TGF-β1 in growth inhibition assays, it had a distinct amino-terminal amino acid sequence and was not recognized by antibodies raised against TGF-β1 sequences. It also showed differential binding efficiencies to the then putative three TGF-β receptor types, identified as the 65-kD type I receptor, the 85-kD type II receptor, and the 280-kD type III receptor or betaglycan. As later confirmed, once these TGF-β receptors had been cloned, TGF-β2 bound only poorly to the type II TGF-β receptor, but bound to betaglycan with similar affinity as TGF-β1 (Cheifetz et al. 1987; Lopez-Casillas et al. 1993). Independent studies in other laboratories identified TGF-β2 as a cartilage-inducing activity from bovine bone (Seyedin et al. 1987), a T-cell suppressor activity made by human glioblastoma cells (Wrann et al. 1987), and a growth inhibitor secreted by BSC-1 monkey kidney cells (Hanks et al. 1988). Subsequent cDNA cloning showed that TGF-β2 derives from a 412 amino acids precursor, and that its mature 112 amino acid sequence is 71% identical to TGF-β1 (de Martin et al. 1987; Marquardt et al. 1987).

Shortly after the characterization of TGF-β2, cDNA cloning identified yet a third TGF-β, called TGF-β3, which was 72% identical to TGF-β1 and 76% identical to TGF-β2 (Derynck et al. 1988; ten Dijke et al. 1988). All nine cysteines in the 112–amino acid mature TGF-β forms were positionally conserved in the three TGF-βs. TGF-β3 was found to play a unique role in fusion of the palatal shelves (Proetzel et al. 1995) and, unlike TGF-β2, to bind the type II TGF-β receptor with similar affinity as TGF-β1. Although the three mature TGF-βs show 64%–82% sequence identity, their prodomains are only 28%–45% identical, yet some sequence features are conserved. The three prodomains have conserved *N*-linked glycosylation sites, and, with the exception of the TGF-β2 pro-domain, an Arg-Gly-Asp (RGD) potential integrin binding site is apparent. In addition, each pro-region has three cysteines, two of which are involved in interchain disulfide bonds necessary for dimerization of the pro-region in the small latent TGF-β complex, whereas the third cross-links to its binding protein LTBP (Fig. 2). Mammalian cells encode only these three TGF-βs, encoded by three distinct genes (Table 1). Although the term TGF-β isoforms is often used, these three TGF-βs are not to be

Table 1. Comparative features of the TGF-β isoforms

	Number of amino acids				Chromosomal locus		
	Precursor	LAP	Mature	Tetrabasic site	Human	Mouse	mRNA (kb)
TGF-β1	390	249	112	RHRR	19q13	7	2.5
TGF-β2	414,442[a]	281	112	RKKR	1q41	1	4.1,5.1,6.5
TGF-β3	412	279	112	RKKR	14q23-4	12	3.0

[a]TGF-β2 has a prominent alternative spliced variation encoded by the 5.1-kb mRNA and is expressed only in certain tissues. A 29-amino acid insertion results in a latency-associated protein (LAP) with three additional cysteine residues, suggesting a more complex secondary structure (Webb et al. 1988).

considered as isoforms derived from the same gene, but are encoded by different genes.

The TGF-β "Superfamily"

Shortly after the cDNA cloning of TGF-β1, and at the time that TGF-β2 and -β3 were identified, unrelated cDNA cloning projects in other laboratories revealed the existence of other large precursors with sequence homology with TGF-β in their carboxy-terminal sequences and conservation of the last seven of TGF-β's nine cysteines. Structural analyses later revealed that the positioning of these seven cysteines, which characterizes the class of TGF-β-related proteins, defines the "cystine knot" in the mature forms of these molecules (McDonald and Hendrickson 1993). The first two proteins so identified were the two polypeptides that form the disulfide-linked inhibin heterodimer, an inhibitor of follicle-stimulating hormone secretion (Mason et al. 1985). The alignment of the inhibin cDNA-encoded polypeptides, isolated by Tony Mason in the Seeburg laboratory at Genentech, with the TGF-β1 cDNA sequence followed a random evening encounter at Genentech, in which Mason discussed with Derynck the difficulties in cloning inhibin cDNAs that seemed to resemble similar frustrations with the TGF-β1 cDNA cloning. Half a year later, the cDNA-derived sequence for the Müllerian-inhibiting substance (MIS), which causes Müllerian duct regression in development, revealed again a large precursor sequence with a carboxy-terminal, TGF-β-related sequence (Cate et al. 1986), and the first role for a TGF-β-related protein in development. Again, half a year later, the DPP-C locus, which directs patterning in *Drosophila* was shown to encode a precursor with a carboxy-terminal TGF-β-related sequence, demonstrating the evolutionary ancestry of TGF-β proteins, and the first evidence that TGF-β-related proteins direct developmental patterning (Padgett et al. 1987). Later that year, Vg1 mRNA, which localizes to the vegetal hemisphere of *Xenopus* eggs, was also shown to encode a TGF-β-related protein as the carboxy-terminal fragment of a larger precursor, further expanding the TGF-β family, and further drawing attention to regulation of development, in this case mesoderm formation, by TGF-β-related proteins (Weeks and Melton 1987). Finally, the characterization, late in 1988, of the cartilage- and bone-inducing "bone morphogenetic protein (BMP)," isolated from bone, resulted in the characterization of cDNAs encoding the TGF-β-related BMP-2A (now known as BMP-2) and BMP-3, which induced cell differentiation, and formed the basis for the discovery of additional BMPs (Wozney et al. 1988).

The rapid succession of reports identifying TGF-β-related proteins, the revelation that they function in very diverse contexts and have multiple developmental roles in patterning, morphogenesis, and cell differentiation, and their evolutionary ancestry and conservation profoundly affected many fields, most notably developmental biology and cancer biology. These discoveries also generated enthusiasm for the notion that TGF-β-related proteins form a "superfamily" encoded by a "super gene family." However, considering the similar organization of all encoded protein sequences as large precursors with a signal peptide, a large pro-segment and a carboxy-terminal mature TGF-β-

related sequence, the TGF-β-related proteins constitute a family that, by analogy with other classes of proteins, does not meet the definition of a superfamily that groups proteins with divergent organization. We now know that the mouse and human genomes have 33 genes encoding precursor monomers of TGF-β-related proteins. With the existence of homodimeric and heterodimeric TGF-β-related proteins, the number and diversity of homo- and heterodimeric combinations with functional roles remains unclear. TGF-β family proteins play diverse roles in the control of cell proliferation, metabolism, and differentiation, and act as potent and often essential regulators of developmental processes. TGF-βs, BMPs, and activins (inhibin polypeptide homodimers) also emerged as regulators of disease pathogenesis and progression.

THE EXPANDING BIOLOGICAL ACTIVITIES OF TGF-β

TGF-β research in the latter half of the 1980s was characterized by discoveries of a plethora of biological effects on cells in culture. With the commercial availability of TGF-β, it had become apparent that most if not all cell types responded to TGF-β, while they also made and secreted TGF-β, thus allowing for autocrine stimulation. The many effects seen included regulation of cell proliferation, cell differentiation, extracellular matrix (ECM) production and remodeling, chemotaxis, lymphocyte function, and growth factor and hormone production. By the mid-1980s, it was widely accepted that TGF-β could either stimulate or inhibit cell proliferation depending on the cell type, and presence of other growth factors and culture conditions (discussed above). TGF-β also induced many other cell responses that appeared to also depend on the cell type and culture conditions. This led to an oversimplified and caricatural notion among some that TGF-β acted as a switch: It switched on what was off, and switched off what was on. In addition to being biologically confusing, this notion did not set an inviting stage to explore therapeutic modalities using TGF-β or TGF-β antagonists.

Given that TGF-β was expressed by many cell types found in all tissues examined and abundant in platelets, it was evident that the molecule must have physiological functions distinct from its transforming activity and roles in cancer. Early in vivo experiments in the Sporn/Roberts laboratory were based on the adage of Alexander Haddow (1972) that "the wound is a tumor that heals itself," restated by Dvorak (1986) as "tumors are wounds that do not heal," thus inviting the evaluation of a TGF in wounds. Release of TGF-β from Hunt–Shilling wire-mesh chambers implanted into the backs of rats profoundly stimulated a stromal response, including angiogenesis and ECM deposition (Sporn et al. 1983). Injection of TGF-β into the nape of the neck of newborn mice confirmed that TGF-β stimulated formation of granulation tissue, was chemotactic for inflammatory cells, and stimulated ECM production by fibroblasts (Roberts et al. 1986), thus demonstrating that it was active in multiple aspects of wound healing. These observations were confirmed and expanded in many follow-up studies using models of impaired healing (reviewed in Roberts 1995).

A molecular basis for these effects on wound healing and connective tissue deposition was provided by studies in cell culture (Fig. 3). TGF-β was shown to strongly induce the expression of ECM components, including fibronectin, certain collagens, chondroitin/dermatan

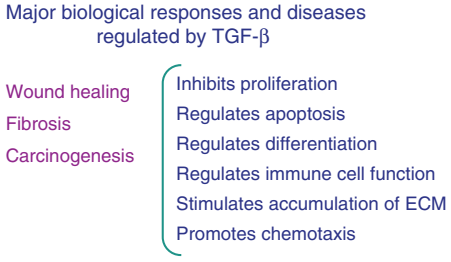

Figure 3. Roles of transforming growth factor β (TGF-β) in pathophysiology. TGF-β plays prominent roles in wound healing, fibrosis, and carcinogenesis, as well as a host of other diseases. Listed on the *right* are various TGF-β-dependent cellular mechanisms that contribute to its effects. ECM, Extracellular matrix.

sulfate, biglycan, decorin, osteopontin, osteonectin, tenascin, and thrombospondin (Massagué 1990). In addition, TGF-β stimulated the synthesis of inhibitors of matrix-degrading enzymes, including plasminogen activator inhibitor 1 (PAI-1) and a tissue inhibitor of matrix metalloproteinases (TIMPs) (Laiho et al. 1986; Overall et al. 1989). Conversely, TGF-β inhibited expression of matrix-degrading proteases such as collagenase, stromelysin, and plasminogen activator (Edwards et al. 1987; Kerr et al. 1988). Thus, the increase in inhibitors and decrease in matrix proteases caused increased ECM accumulation. TGF-β treatment was also shown to induce marked changes in integrin expression in several cell types resulting in altered cell binding to ECM components (Ignotz and Massagué 1987; Roberts et al. 1988; Heino et al. 1989). Further, TGF-β revealed itself as potently chemotactic for fibroblasts (Postlethwaite et al. 1987) and macrophages (Wahl et al. 1987), a function that likely contributes to the increased abundance of these cells at sites of increased TGF-β production such as wounds and cancers.

Around that time, collaborative studies between the laboratories of Sporn/Roberts and Toni Fauci at the National Institutes of Health revealed that TGF-β could inhibit the differentiation and functions of B and T lymphocytes as well as natural killer cells (Kehrl et al. 1986a,b; Rook et al. 1986). These unexpected findings with a factor that was thought to play a major role in cancers linked TGF-β to the immune system and provided the basis for the many subsequent studies that highlight the roles of TGF-β as a potent immunoregulator, controlling proliferation, differentiation, and function of most lymphocyte classes, as well as macrophages and dendritic cells, and a potent suppressor of immune surveillance (Letterio and Roberts 1998). These proposed roles of TGF-β were supported and in part elucidated by later studies using TGF-β1 knockout mouse (Shull et al. 1992; Kulkarni et al. 1993) that further characterized a role for TGF-β1 in autoimmunity (Letterio et al. 1994) and in immune tolerance (Letterio and Roberts 1998).

Additionally, steroidogenesis was reported to be inhibited by TGF-β in adrenocortical cells and Leydig cells, while it was stimulated in granulosa cells. TGF-β was also shown to stimulate expression of follicle-stimulating hormone by pituitary cells (Ying et al. 1986), whereas other studies revealed that TGF-β induces PDGF expression by fibroblasts (Leof et al. 1986). Consequently, TGF-β was now additionally seen as a mediator of hormonal control in different contexts, consistent with the discovery that the hormone inhibin is a TGF-β-related factor. Furthermore, it was now apparent that some activities of TGF-β occurred secondarily through the activation of expression of some hormones and growth factors.

Studies in the latter part of the 1980s also revealed that TGF-β has potent effects on differentiation of a wide variety of cell types. This stood in remarkable contrast with the "classical" growth factors, whose effects on cells were primarily seen to enhance proliferation, and were thought of primarily in the context of cancers. These effects on cell differentiation were especially remarkable considering TGF-β's link to cell transformation. TGF-β was often seen to inhibit cell differentiation, for example, of preadipocytes, skeletal muscle myoblasts, muscle satellite cells, and osteoblasts, conceptually resembling inhibitory effects of TGF-β on immune cell functions. On the other hand, TGF-β stimulated differentiation of prechondrobasts, intestinal epithelial cells, keratinocytes, bronchial epithelial cells, and osteoblasts under some conditions. These findings led to the notion that TGF-β was a differentiation factor, in addition to a growth inhibitor, as summarized around that time by Massagué (1990). These initial observations, together with the realization that defects in developmental patterning and tissue differentiation were associated with dysregulation of TGF-β-related proteins, set the stage for a wide range of studies that defined the developmental control of tissue differentiation by TGF-β and TGF-β family proteins.

Expanding these observations on cell differentiation, the Derynck laboratory reported, in 1994, that TGF-β induces epithelial cells in culture to undergo a reversible transition into cells with mesenchymal appearance (Miettinen et al. 1994). Although this was consistent with obser-

vations by Ray Runyan that TGF-β is required for transition of endocardial cells into mesenchymal cells during heart valve formation (Potts et al. 1991), these results were nevertheless met with surprise, if not apprehension, primarily because of the overall notion that differentiation could not be redirected, and certainly not by one factor. Epithelial–mesenchymal transition (EMT) had been shown to occur in development (Hay 1995), but the underlying signaling mechanisms were not known. The induction of EMT by TGF-β in cell culture was the basis for many subsequent studies characterizing mechanisms and roles of TGF-β-induced, Smad-mediated gene reprogramming during EMT, and complementary roles of non-Smad pathways. As the roles of EMT in development, starting with mesoderm induction and gastrulation, became more apparent, many studies gradually revealed the developmental control of EMT by TGF-β-related proteins (reviewed in Lamouille et al. 2014).

The increasing realization in the late 1980s and early 1990s that TGF-β controls cell differentiation was complemented by in situ hybridization and immunohistochemical studies showing expression of all three TGF-βs during embryogenesis, each of these in specific patterns, both spatially and temporally (Heine et al. 1987; Lehnert and Akhurst 1988; Pelton et al. 1989, 1990a,b, 1991; Millan et al. 1991). These observations suggested specific roles for the individual TGF-βs in processes such as angiogenesis, cardiogenesis, palate formation, and osteogenesis, and provided the basis for subsequent studies on developmental defects resulting from targeted inactivation of the individual TGF-β genes in mice (Shull et al. 1992; Kulkarni et al. 1993; Proetzel et al. 1995; Sanford et al. 1997). Although *Tgfb1*$^{-/-}$ mice highlighted the roles of TGF-β1 in the control of the immune system and inflammation (Shull et al. 1992; Kulkarni et al. 1993), as well as angiogenesis, vasculogenesis, and hematopoiesis (Dickson et al. 1995), inactivation of TGF-β2 and TGF-β3 expression primarily resulted in defects in organs that develop through epithelial–mesenchymal tissue interactions (Proetzel et al. 1995; Sanford et al. 1997). These observations also revealed that, despite their very similar activities in cell culture, the three TGF-βs have highly divergent developmental roles.

With TGF-β having been discovered as a factor that induces cell transformation, and the overall notion in the 1980s that growth factors act predominantly in cancers, thus contributing to cancer progression, many researchers aimed to elucidate roles of this newly discovered factor in cancer initiation and progression. As with other growth factors, much emphasis was on the control of cell proliferation by TGF-β, even though, and especially because, it rapidly became apparent that TGF-β has growth-inhibitory activities on epithelial, immune, and hematopoietic cells, which give rise to the bulk of cancers. As the other activities of TGF-β became apparent, researchers incorporated their results and views into a more complex picture of the roles of the apparently increased TGF-β signaling in cancers. Increased ECM protein expression in response to TGF-β was linked to increased ECM turnover in cancers. The chemotactic effects of TGF-β on fibroblasts and immune cells contributed to tumor stroma formation and the tumor microenvironment. Localized immunosuppression by TGF-β was seen to contribute to cancer growth and progression. TGF-β was also found to induce angiogenesis in cancers and to contribute to cancer-associated localized inflammation. Finally, EMT in response to increased TGF-β signaling was increasingly viewed as a key contributing event to carcinoma invasion and dissemination, and recently to the generation and properties of cancer stem cells (Derynck et al. 2001; Massagué 2008; Ikushima and Miyazono 2010; Katsuno et al. 2013).

Besides cancer, other pathological connections with dysregulated TGF-β production and responsiveness became gradually apparent. Most notably, the suppression of experimental glomerulonephritis by TGF-β antiserum (Border et al. 1990) provided the impetus for many subsequent studies highlighting the contributions of increased TGF-β signaling to different types of fibrosis. Again, increased TGF-β activity, TGF-β's abilities to activate ECM protein expression, its potent chemotactic activities

for fibroblasts and immune cells, and the induction of EMT by TGF-β became incorporated in the etiology and progression of fibrosis (Chapman 2011; Nieto 2011). In addition to fibrosis, several connective tissue and skeletal diseases were also seen to result from increased TGF-β signaling, which, in a number of cases, was associated with genetic mutations that lead to dysregulated TGF-β activation (Shore and Kaplan 2010; Doyle et al. 2012).

The sheer number and overwhelming variety of biological activities described for TGF-β during the 1980s was unprecedented in the growth-factor field, and attracted many investigators from different disciplines to the field. Although this generated rapid progress with new insights into the biology of TGF-β, one negative aspect of so many biological effects was that development of pharmacologic agents to target and modulate TGF-β actions by industry was discouraged for a number of years because of the potential plethora of side effects.

cDNA CLONING AND IDENTIFICATION OF TGF-β RECEPTORS AND SMADs

cDNA Cloning of the TGF-β Receptors

Once specific high-affinity receptors for TGF-β had been identified in 1984 (Massagué et al. 1982), the next goal was to characterize these with the ultimate aim to define the TGF-β signaling pathways that lead to the diversity of emerging biological activities. Using growth-factor-activated tyrosine kinase receptors as a reference point, it was rapidly concluded that the TGF-β receptors would act through a different class of receptors, because they induced growth inhibition, rather than growth stimulation, and many other responses hitherto not seen with receptor tyrosine kinases (RTKs). Furthermore, three types of receptors were consistently identified, with both the type I and type II receptors required for TGF-β responses, based on analyses of mutant cell lines (Laiho et al. 1991). This was again very different from RTKs. The task to purify or clone these receptors would, however, be challenging considering the very low levels of the TGF-β receptors at the cell surface (Frolik et al. 1984; Tucker et al. 1984a), when compared with average numbers for RTKs. Around 1987–1990, several laboratories started projects aimed at cDNA cloning of the receptors, using receptor purification or expression cDNA cloning approaches, in combination with radiolabeled ligand-binding assays.

The cDNA cloning of the first known receptor for a TGF-β family protein was achieved by Lawrence Mathews working in the laboratory of Wylie Vale (Mathews and Vale 1991). An expression cDNA cloning strategy in combination with ^{125}I-activin binding to transfected cells allowed him to isolate cDNAs for the activin type II receptor, now known as ActRII, noting that it was likely a transmembrane serine/threonine kinase. The cytoplasmic domain of this receptor showed extensive sequence similarity to the transmembrane protein daf-1 in *Caenorhabditis elegans*, which had been cloned the year before, and was proposed as the first transmembrane serine/threonine kinase receptor with a ligand still to be identified (Georgi et al. 1990). The identification of ActRII was rapidly followed by the cDNA cloning of a related activin receptor, ActRIIB (Attisano et al. 1992; Mathews et al. 1992), and of alternatively spliced variants (Attisano et al. 1992). A similar expression cloning approach as for ActRII, but using ^{125}I-TGF-β as ligand, resulted in the cDNA cloning of the TGF-β type II receptor, now known as TβRII, by the laboratories of Harvey Lodish and Bob Weinberg (Lin et al. 1992). Also, this receptor was shown to phosphorylate on serine and threonine. A few years later, the MIS binding type II receptor was cloned, based on its homology with previously isolated TGF-β family receptors (Baarends et al. 1994; di Clemente et al. 1994), and the type II BMP receptor BMPRII was cloned based on either its association with the type II TGF-β receptor in a yeast two-hybrid approach (Kawabata et al. 1995; Liu et al. 1995) or sequence homology using a polymerase chain reaction (PCR)-based strategy (Nohno et al. 1995; Rosenzweig et al. 1995). Five TGF-β family type II receptors are now known to be encoded by human cells.

cDNA cloning of the type I receptors was accomplished in large part using PCR-based

strategies with oligonucleotide primers for conserved sequences in the ActRII, TβRII, and/or Daf1 receptors (Ebner et al. 1993a; He et al. 1993; ten Dijke et al. 1993). This strategy resulted in the identification of type I receptors with overall similarity to the type II receptors, easily recognizable by their distinct Gly-Ser (GS)-rich sequence immediately upstream of the kinase domains (Wrana et al. 1994a). As it was initially not clear what ligands they would bind, Peter ten Dijke coined the term ALK for "activin receptor-like kinase" for these receptors (ten Dijke et al. 1993), although various other names were chosen by other laboratories. Seven type I receptors, designated ALK1-7, are now known to be encoded by human cells (ten Dijke et al. 2000; ten Dijke and Hill, 2004), whereas ALK-8 was found in zebrafish (Bauer et al. 2001; Mintzer et al. 2001). With five type II and seven type I receptors, and a much larger number of ligands, the assignment of the type I receptors to individual ligands was, and continues to be, complex and tedious. It was proposed that the type II and type I receptors formed a heteromeric complex (Wrana et al. 1992), thus requiring the ligand binding and function of a type I receptor to be assessed in combination with the type II receptor. Furthermore, it became apparent that, at least in the case of TGF-β and activin, the type I receptors only bound ligand when combined with the appropriate ligand-binding type II receptor, which provides ligand specificity (Attisano et al. 1993; Ebner et al. 1993b). In contrast, the type II or type I BMP receptors, when expressed individually, generally showed only a weak affinity for BMPs, and only bind BMPs efficiently when coexpressed in heteromeric receptor combinations (Miyazono et al. 2001, 2010). Using a type II/type I receptor combinatorial strategy, TGF-β was shown to signal primarily through the type I receptor that is now known as TβRI or ALK-5 (Franzén et al. 1993, Bassing et al. 1994). The functional interdependence of the type II and type I receptors was subsequently shown to result from ligand-induced, type II receptor-mediated phosphorylation of the GS domain in the type I receptor that leads to activation of the type I receptor kinase (Wrana et al. 1994b). This dependence for signaling activation complemented the extracellular interdependence of the type II and type I receptors for productive ligand binding. We currently know many of the functional combinatorial type II/type I receptor interactions and the corresponding ligands that activate these complexes, but, considering the complexity of homomeric and heteromeric ligands, additional assignments will be forthcoming.

Finally, the type III TGF-β receptors, betaglycan and endoglin, differ from the other TGF-β family receptors in the sense that they do not directly initiate TGF-β signaling, but rather control ligand presentation to the type I and type II receptors (Bernabeu et al. 2009). Although betaglycan is in vivo primarily expressed in mesenchymal cells, endoglin expression is restricted to endothelial cells. The proteoglycan betaglycan was cloned by Fernando Lopez-Casillas, working with Joan Massagué, following protein purification and based on peptide sequences that allowed the generation of oligonucleotides as hybridization probes (Lopez-Casillas et al. 1991), and by Xiao-Fan Wang then working with Bob Weinberg using an expression cDNA cloning strategy (Wang et al. 1991). cDNA cloning of the glycoprotein endoglin was reported at a time that endoglin was only known to be a cell-surface protein specifically expressed in vascular endothelium (Gougos and Letarte 1990) and, thus, preceded the cDNA cloning of the first TGF-β family receptors and the realization that it participates in TGF-β signaling. Endoglin was subsequently found to bind TGF-β, allowing it to play a role in ligand presentation to type II/type I TGF-β receptor complexes (Cheifetz et al. 1992), and to link genetically with hereditary hemorrhagic telangiectasia type I (McAllister et al. 1994).

Discovery and Characterization of the Smad Pathway

At the time that the TGF-β receptors were cloned, little was known about any downstream mechanisms, and of course the next big step was to elucidate how TGF-β induces the many diverse responses. TGF-β had been shown to induce Ras and Erk mitogen-activated protein

(MAP) kinase activation (Mulder and Morris 1992; Yan et al. 1994; Mucsi et al. 1996), but activation of this pathway was at odds with the substantial differences in responses when compared with RTKs that activate the Ras-Erk MAP kinase pathway. Additionally, TGF-β was shown to activate protein phosphatase 1 (Gruppuso et al. 1991) and to induce phosphorylation of the transcription factor cAMP response element-binding (CREB) protein (Kramer et al. 1991), but these findings again did not explain the major TGF-β responses. With the receptors in hand, several laboratories embarked on identifying associated proteins and kinase substrates for the TGF-β receptors. This led, within the next few years, to the identification of FKBP12 (Wang et al. 1996a; Chen et al. 1997) and the α subunit of farnesyltransferase (Ventura et al. 1996; Wang et al. 1996b) as associated proteins, and the translation initiation factor eIF2α/TRIP-1 (Chen et al. 1995) and Bα subunit of protein phosphase 2A (Griswold-Prenner et al. 1998) as kinase substrates, but these again were not seen as signaling effectors that mediate the predominant TGF-β responses. The identification by Matsumoto and colleagues of the "TGF-β-activated kinase 1" (TAK1) as a signaling mediator that is activated by TGF-β and BMP (Yamaguchi et al. 1995) generated considerable excitement. However, its identity as a MAPKK kinase that could activate MAP kinase pathways and the inability of TAK1 to activate TGF-β target gene responses raised the general question to what extent TAK1 contributed to TGF-β and BMP responses. Furthermore, TAK1 was subsequently also shown to be an effector of signaling in response to ligands that are not related to TGF-β and act through different receptor classes.

A major breakthrough toward the identification of TGF-β-activated signaling effectors came from genetic analyses of *Drosophila* and *C. elegans*. The *Drosophila decapentaplegic* (*dpp*) gene, which encodes the homolog of vertebrate BMP-2 and -4, was by then known to act through the type II receptor, Punt, and two type I receptors, Thickveins and Saxophone (Ruberte et al. 1995). A genetic screen for enhancers of weak *dpp* alleles in Bill Gelbart's laboratory at Harvard yielded "mothers against dpp" (*mad*) and *medea* as genes that encode downstream components required for *dpp* signaling (Raftery et al. 1995), and revealed sequence similarity between Mad and three *C. elegans* genes (Sekelsky et al. 1995). Parallel studies in *C. elegans* had identified *daf-4* as a BMP-2 and -4 homolog receptor (Estevez et al. 1993). Three genes, *sma-2, -3*, and *-4*, were shown to function in the same cells as *daf-4*, and to be required for *daf-4* function (Savage et al. 1996). The identification of Mad in *Drosophila* and Sma proteins in *C. elegans* defined a new family of signaling mediators downstream from BMP-related proteins, and prompted an intense search by several groups for homologs in mammals. Within a year, five Mad homologs were identified in different laboratories resulting in a diverse set of names (Derynck and Zhang 1996). One of these, initially named human Mad4, was identical to DPC4 ("deleted in pancreatic carcinoma, locus 4"), whose gene had just been identified as a putative tumor suppressor gene with inactivating mutations in pancreas cancer (Hahn et al. 1996). The nomenclature was soon resolved by agreement of the involved investigators to use the term Smad as a combination of the Mad and Sma names (Derynck et al. 1996).

The assignment of Smads as effectors of TGF-β/activin or BMP ligands and key aspects of the overall signaling mechanism progressed at a frantically competitive pace in 1995–1996. Individual Smads, when overexpressed, were shown to mimic effects of corresponding ligands (Baker and Harland 1996; Graff et al. 1996; Liu et al. 1996; Zhang et al. 1996), and dominant-negative mutants were found to inhibit ligand-induced responses (Zhang et al. 1996). Ligand-induced receptor activation induced Smad phosphorylation (Eppert et al. 1996, Hoodless et al. 1996; Zhang et al. 1996), Smad3 was found to interact with the type I, but not the type II receptor (Zhang et al. 1996), and Smad4 cooperated with Smad3 (Zhang et al. 1996). Additionally, a carboxy-terminal fragment of Smad1 or Smad4 was shown to have transcription activation potential (Liu et al. 1996), and activin or BMP-2/-4-induced nuclear accumulation of Smad2 or Smad1, respectively

(Baker and Harland 1996; Hoodless et al. 1996; Liu et al. 1996). These observations provided the basis for the model of Smad activation that conceptually paralleled the well-established model of signal transducers and activators of transcription (STAT) activation in response to cytokine receptors (Fig. 4). Thus, Smads were thought to interact with type I receptors and be activated through phosphorylation by the receptor complexes, form a complex with Smad4, translocate into the nucleus, and target regulatory gene sequences for direct transcriptional activation (Derynck and Zhang 1996). This model was rapidly refined (e.g., by the demonstration that the type I TGF-β receptor directly phosphorylates Smad2, and not Smad4) at two carboxy-terminal serines, thus leading to its activation (Hoodless et al. 1996; Macías-Silva et al. 1996), and that Smad4 cooperates with all receptor-activated Smads tested (Lagna et al. 1996; Zhang et al. 1997).

How Smad complexes could activate transcription was again pursued at a high pace by several laboratories. An early report found Smad2 to interact with the Mix2 promoter through association with the DNA-binding transcription factor FAST1 (Chen et al. 1996), now known as FoxH1. However, Mad was subsequently shown to bind DNA directly, to a GC-rich sequence in the vestigial promoter (Kim et al. 1997), and a PCR-based selection procedure defined the palindromic sequence GTCTAGAC as the consensus "Smad-binding element" for Smad3 and Smad4 (Zawel et al. 1998). Therefore, it was concluded that receptor-activated Smads were DNA-binding transcription factors, similarly to STATs. On the other hand, the association and cooperation of Smad2 with FoxH1 at target promoters (Chen et al. 1996; Labbé et al. 1998), the association and cooperation of Smad3 with c-Jun/c-Fos at overlapping promoter sequences (Zhang et al. 1998), and the cooperation of Smad3 with the transcription factor TFE3 in the control of PAI-1 expression (Hua et al. 1998), which were revealed around the same time, all emphasized the need for cooperation of Smad complexes with high-affinity DNA-binding transcription factors. These and other observations led to the now generally accepted model that Smads acti-

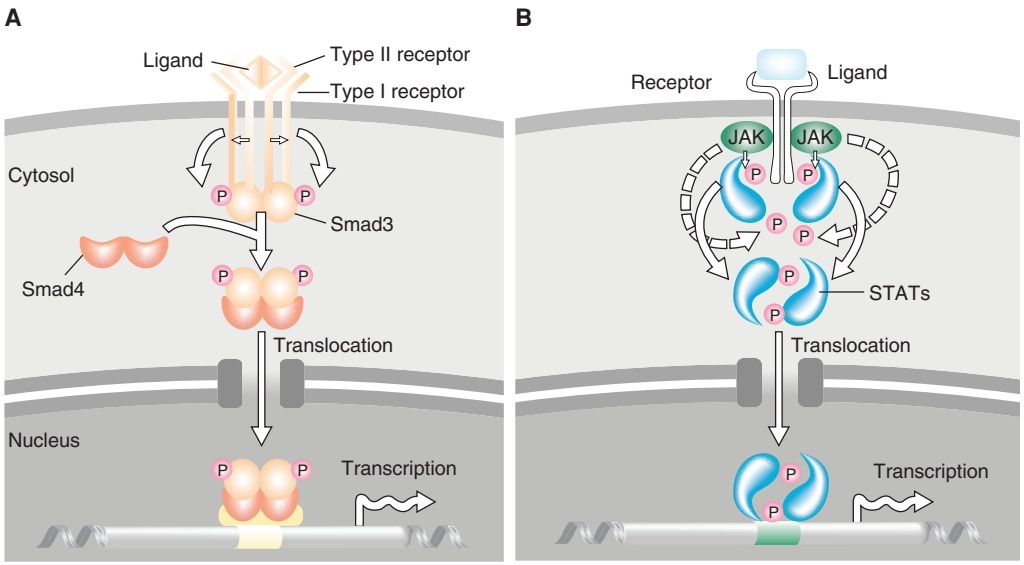

Figure 4. Initial working model of Smad signaling proposed in 1996. Following the identification of Smads as transforming growth factor β (TGF-β) family signaling effectors and several reports on how they might do so, this first model was proposed, based on these results and the perceived analogy with the established mode of signal transducers and activators of transcription (STAT) signaling. (From Derynck and Zhang 1996; reprinted, with permission, from the authors.)

vate target gene transcription, through physical association and functional cooperation with a variety of high-affinity DNA-binding transcription factors, thus enabling DNA binding to adjacent DNA sequences, and that Smad complexes use CBP or p300 as coactivators (Derynck et al. 1998). This model explained why, despite extensive efforts, no consensus TGF-β responsive DNA sequences could ever be found in TGF-β target genes, and illustrates the high versatility of Smad signaling dependent on the nature of the interacting transcription factor. This mode of cooperation also predicted direct cross-talk of Smads with other signaling pathways that target the Smad-associated transcription factors, thus providing a mechanistic basis for the context-dependence of the TGF-β responses. When this model was proposed in the context of an invited minireview for *Cell*, the editor, Ben Lewin, called Derynck to argue that this model was totally wrong, indicating that Smads were plain DNA-binding transcription factors with a DNA-binding consensus sequence (just like STATs), as just published in *Molecular Cell* (Zawel et al. 1998). After vigorous arguing, he very reluctantly agreed to have this model published. With an extensive body of ongoing research on many target genes in the next few years, Smads were shown to associate and cooperate with more than 100 DNA-binding transcription factors, depending on the nature of the target promoter, and with many co-repressors and coactivators that help define the amplitude of the TGF-β-induced transcription responses (Feng and Derynck 2005; Massagué et al. 2005).

Although we started understanding the mechanisms of TGF-β-induced, Smad-mediated activation of gene expression, these observations did not address how TGF-β family proteins induce repression of gene expression. With research on different target genes as model systems, it became subsequently apparent that also repression of gene expression was mediated by Smad complexes, thus fully attributing the control of gene expression by TGF-β family proteins to Smads. Smad3-mediated repression of myogenic transcription factor target genes was seen to result from direct physical interference by Smad3 with the formation of functional myogenic transcription factor complexes at E-box DNA sequences in target genes (Liu et al. 2001, 2004). In contrast, in TGF-β-induced repression of *c-myc* expression, Smad3/4 complexes were seen to associate with the DNA-binding E2F4/5 and DP1 transcription factors, and the co-repressor p107, thus enabling these complexes to bind at a composite Smad-E2F-binding site in the *c-myc* promoter, and to exert transcription repression through p107 (Chen et al. 2002). At the osteocalcin and Runx2 promoter, TGF-β-activated Smad3/4 complexes were seen to associate with Runx2 at a Runx2-binding DNA sequence and to directly recruit histone deacetylases (Alliston et al. 2001; Kang et al. 2005).

As these mechanistic insights were unfolding, two novel Smads were discovered that had a conserved transactivation domain that characterizes the Smads, but lacked the preceding amino-terminal Mad homology 1 (MH1) domain that is seen in all receptor-activated Smads and Smad4. These Smads, Smad6 and Smad7, with Dad as the *Drosophila* counterpart, were shown to associate with the type I receptors or other Smads, thus competitively preventing activation of the effector Smads, which is why they were named inhibitory Smads (Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997; Tsuneizumi et al. 1997; Hata et al. 1998). With Smad6 and Smad7 expression activated in response to several signaling pathways, it became apparent that the inhibitory Smads provide endogenous control of the level of Smad activation by TGF-β family proteins (ten Dijke and Hill 2004; Briones-Orta et al. 2011). Additional observations suggested that they may also directly repress transcription of target genes in the nucleus (Bai and Cao 2002; Grönroos et al. 2002).

With the overall mechanisms of Smad-mediated transcription control defined, at least in general terms, several laboratories have been focusing on the activation of non-Smad signaling pathways by TGF-β and TGF-β-related proteins. Indeed, TGF-β family proteins were shown to activate Erk, p38, and JNK MAP kinase pathways, as well as the PI3K-Akt-TOR pathway, and to act through TAK1, an upstream

Figure 5. Early contributors to the development of the transforming growth factor β (TGF-β) field. The picture taken in February 2006 shows (seated from *left*) Rik Derynck, Anita Roberts, Harold Moses, and (standing from *left*) Michael Sporn and Joan Massagué.

mediator of MAP kinase pathways (Derynck and Zhang 2003; Moustakas and Heldin 2005; Zhang 2009). The mechanisms through which TGF-β proteins induce activation of these pathways, or cross-talk with the Smad pathway, are being characterized, whereas other studies address how cross-talk between these pathways and the Smad pathway controls the cellular responses in cell differentiation and growth control and during development and in cancer.

SUMMARY

The pioneering efforts of the Todaro, Holley, Moses, Roberts/Sporn, Derynck, and Massagué laboratories (Fig. 5), resulting in the discovery and characterization of TGF-β1 in the early 1980s, have now led to the description of an almost unfathomable range of activities of TGF-β, and the identification of a large family of related growth and differentiation factors with important effects in development and adult physiology and disease pathogenesis. Many highly committed research groups have greatly contributed to our current mechanistic, structural, developmental, and physiological understanding of the biology of TGF-β and have made this into a highly exciting area of biology with substantial implications for our understanding of various diseases. The effects of TGF-β family proteins on many individual cell types and the very nature of Smad signaling, complemented by non-Smad pathways, emphasize and have made us appreciate the contextual nature of TGF-β effects at the cell, tissue, and organismal level. Certainly, 35 years after its discovery, the future of TGF-β research is bright and sure to yield exciting new insights and pharmacological approaches to manipulate this pathway for clinical benefit.

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