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From wavy hair to naked proteins: The role of transforming growth factor alpha in health and disease

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Abstract

Since its discovery in 1978 and cloning in 1984, transforming growth factor-alpha (TGF α, TGFA) has been one of the most extensively studied EGF receptor (EGFR) ligands. In this review, we provide a historical perspective on TGFA-related studies, highlighting what we consider important advances related to its function in normal and disease states.

Keywords

EGFR; epidermal growth factor receptor; TGFA; transforming growth factor alpha; Basolateral trafficking; Polarized epithelial cells; Ménétrier's disease; Autocrine singaling

INTRODUCTION

Transforming growth factor-alpha (TGF-α, TGFA) was the second member of the EGF receptor (EGFR) ligand family to be identified after the discovery of the prototypic member, EGF. TGFA activity was first discovered when researchers observed that sarcoma virus-transformed fibroblasts released a peptide growth factor activity that could block binding of EGF to cells, suggesting another growth factor competed with EGF for binding to cell surface EGFRs [1]. Later, this activity was partially purified from these transformed cells and induced growth in soft agar, the best *in vitro* correlate of tumorigenesis [2]. All three major peaks of activity (25, 12, and 7 kDa) competed with EGF for EGFR binding. This partially purified material was called "sarcoma growth factor" or simply "transforming growth factor". The discovery of transforming growth factors led to the concept of "autocrine signaling", that is, ligand is synthesized and released by a cell and then taken up by receptors on that same cell [3]. This novel concept helped explain growth factor

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independence, one of the defining properties of cancer cells: through autocrine stimulation, cancer cells are able to escape external growth controls [4].

A few years later, it was shown that the transforming growth factor activity consisted of two distinct peptide growth factors. The first bound to EGFR and was independently transforming when added to non-transformed fibroblasts; this was designated TGF-α. The second peptide, separated by HPLC, did not bind to EGFR and was not transforming on its own, instead it enhanced TGFA- or EGF-induced transformation of non-transformed fibroblasts [5]. This second peptide was named transforming growth factor-beta (TGF-β, TGFB1). Moreover, when added to epithelial cells, the two peptides exhibited distinct actions: TGFA stimulated growth, whereas TGFB1 was a potent growth inhibitor.

Rik Derynck cloned human *TGFA* in 1984 [6]. He showed it was an EGF homolog and bound to EGFR [6]. The *Tgfa* knockout phenotype resembled that of the *Egfr* knockout. In fact, the characteristic “waved” coat phenotype observed in *Tgfa* knockout mice had been reported much earlier (*wa1*) [7]. In complementation studies, the groups of David Lee and Ashley Dunn showed that *wa1* mapped to the *Tgfa* locus [8, 9]. *wa1* mice show reduced Tgfa expression, but the precise mutation has not been identified. *wa2* mice also show a similar coat phenotype; these mice have a mutation in the *Egfr* tyrosine kinase domain [10, 11].

We, and others, have previously reviewed TGFA in the context of EGFR and its cognate ligands as relates to normal physiology and selected disease states [12–19]. Here we review what we consider to be important advances in the study of TGFA. We highlight our work that led to the isolation of Naked2 (NKD2), a novel trafficking adaptor for TGFA that also acts as a negative regulator of WNT signaling. We also discuss the role for TGFA in the pathogenesis of a rare premalignant hyperplastic disorder of the stomach, Ménétrier’s disease.

TGFA: Gene and Protein Structure

Human *TGFA* is located on the short arm of chromosome 2 (2p13) spanning a 138.7 kb region [6]. The 4326 base *TGFA* transcript encodes a 160 amino acid peptide. A shorter transcript using an alternative in-frame splice site that removes three bases has been reported that encodes a 159 amino acid peptide lacking Val 32. Characteristic of the EGF ligand family, *TGFA* mRNA is spliced from six exons; all five introns are spliced out adhering to the consensus GT-AG boundary rule (Figure 1) [20]. In contrast, *EGF* is encoded by 24 exons, which encode nine EGF domains in its extracellular region. All the other ligands code for a single EGF peptide. *EREG* and *EPGN* are encoded by five exons each, where the fifth exon also codes for 3′ UTR. The sixth *TGFA* exon codes for a large 3.5 kb 3′ UTR that shares a high degree of sequence identity with mouse *Tgfa*, suggesting a conserved regulatory role [20]. Incidentally, exon 6 encodes only the two terminal valines; alternative splicing involving intron 5 and exon 6 removes a large proximal region of exon 6 resulting in both the terminal valines replaced with four or five amino acids [21]. These splice variants that lack large regions of 3′UTR regulatory sequences and terminal valines are observed in human keratinocytes, as well as cancer cell lines, and seem to be more efficient at imparting growth factor independence to CHO cells than longer TGFA transcripts [21].

The 160 amino acid pre-proTGFA begins with a signal sequence of 23 amino acids at the amino terminus that is removed co-translationally during translocation to the ER lumen [22]. ProTGFA undergoes two metalloprotease cleavages at the distal and proximal sites to the EGF domain, enabling release of the mature soluble peptide into the medium that then binds and activates EGFR. The 23 amino acid transmembrane domain is followed by a 39 amino acid cytoplasmic domain.

Several post-translational modifications have been reported in TGFA. Characteristic of the EGF domain, three disulfide bonds are formed within this region of TGFA that involve six conserved cysteine residues. TGFA is also N-glycosylated in its extracellular domain; N25 is the only potential N-glycosylation site that has the conserved signature of a glycosylation sequon (NxS/T) [23, 24]. TGFA O-glycosylation has not been reported. Cysteines at position 153 and 154 in the cytoplasmic domain are palmitoylated and this enhances the membrane association of TGFA [25]. Several detailed radiolabeling and pulse-chase experiments using different tagged and untagged TGFA constructs in various cell lines have shown consensus biosynthesis and processing of TGFA [17, 26, 27]. Nascent TGFA is synthesized as a 17–18 kDa peptide that is rapidly converted into a glycosylated 30 kDa form during transit from ER to Golgi. Within 30 minutes of appearance of the glycosylated cell surface form, cleavage proximal to the EGF domain leads to removal of the glycosylated region, resulting in a 17–18 kDa transmembrane form. This is followed by a second much slower (1–4 hr) cleavage, distal to the EGF domain that gives rise to two fragments: a 5.6 kDa soluble TGFA and a 13–15 kDa membrane-anchored remnant that contains juxtamembrane, transmembrane, and cytoplasmic domains.

TGFA in Physiological Processes

Much has been learned about the role of TGFA in normal and disease states from mouse models in which TGFA/Tgfa has been overexpressed or knocked out. These studies combined with other mechanistic studies show that TGFA is involved in a number of cellular signaling pathways. Some of these actions are summarized in Table 1 showing different stimuli affect TGFA levels, localization, and activity, indicating TGFA is an important integrator of cellular signaling and function. A common action of TGFA is increased proliferation through activation of EGFR downstream signaling, but it may also mediate other functions like mucous production and inhibition of gastric acid secretion as seen in the overexpression models that mirror Ménétrier's disease. Below we will discuss the phenotypes and information gathered from these studies.

TGFA transgenic studies: epithelial hyperproliferation, hyperplasia, and neoplasia—We first showed that TGFA expression was not just restricted to cancer cells, but is expressed by normal epithelial cells, in this case, cultured human keratinocytes [28]. Moreover, TGFA protein induced *TGFA* mRNA. We subsequently reported auto-and cross-induction of EGFR ligands [29, 30]. The biological significance, if any, of this feed-forward process remains to be determined. To better understand the role of TGFA *in vivo*, three groups simultaneously published results from overexpressing TGFA/Tgfa in transgenic mice.

In the first study, rat TGFA cDNA was cloned under the control of the mouse metallothionein promoter (MT-TGFA) [31]. Transcripts under the control of Zn^{2+}-inducible MT promoter are widely expressed in adult tissues and are active during development. These mice displayed uniform epithelial hyperplasia of liver, pancreas, stomach, small intestine, cecum, colon, and coagulation glands. In the pancreas, however, it promoted the proliferation of both epithelial acinar cells and stromal fibroblasts. It also induced dysplasia and hyperplasia of the coagulation gland epithelium (carcinoma *in situ*) and induced secretory mammary adenocarcinomas in the post-lactational mammary gland. Transgenic mice weighed less than their normal counterparts. Although several organs in transgenic mice were larger, reflecting *TGFA* expression in those organs, there was considerable weight loss in the muscle, connective tissue, bone and fat. One exception was the kidney; here transgene expression did not increase organ size. Interestingly, abnormal fetal development was not recorded in spite of *TGFA* transgene expressed in embryonic tissue, consistent with the broad metallothionein expression pattern.

In the second study, human TGFA cDNA was cloned under the mouse metallothionein 1 promoter. All of the transgenic mice displayed interstitial fibrosis and acinoductular metaplasia of the pancreas. These transgenic mice also showed reduced ability of mammary glands to penetrate fat pads. Multifocal yet well-differentiated neoplasms of the liver were observed with a high degree of penetrance [32]. Pancreatic transformation was also recapitulated in pancreas-specific TGFA transgenic mice, developed subsequently [33, 34].

In our transgenic mouse model, we expressed human TGFA under the control of the MMTV promoter [35]. Similar to the above two reports, we also observed mammary development abnormalities in transgenic virgin female mice, such as alveolar and terminal duct hyperplasia, lobular hyperplasia, cystic hyperplasia, adenoma, and adenocarcinoma. However, hyperplasia was only seen after four months of age, when the mice completed puberty, suggesting hormonal changes might affect TGFA signaling consistent with the other study where hyperplasia was seen in postlactational mammary glands [31]. In females, TGFA expression was largely restricted to mammary tissue. In males, TGFA expression was detectable in testis, seminal vesicles, salivary glands and lungs. Subsequently, MT-TGFA transgenic mice were noted to have increased gastric hyperplasia resembling Ménétrier's disease and increased intestinal epithelial proliferation that was coincident with local TGFA production [36, 37].

A year later, Fuchs and co-workers reported that K14 promoter-driven *TGFA* transgenic mice (where expression is largely limited to stratified squamous epithelia) exhibited skin-specific epidermal effects of TGFA overexpression [38]. Epidermal regions of moderate thickness with low hair follicle density were especially responsive to TGFA overexpression; TGFA induced stunted hair growth and epidermal thickening, due to the combined effect of hyperplasia and hypertrophy of keratinocytes. Psoriatic-like lesions and occasional skin papillomas were also found. Taken together, these transgenic mouse studies demonstrated that TGFA/Tgfa is a potent epithelial mitogen and, with sustained overexpression, it may act as a tumor-promoter/oncogene in certain contexts. Our experiments treating MMTV-*TGFA* mice with a tumor-initiating dose of the mammary gland carcinogen, dimethylbenzanthracene (DMBA), further supported that latter contention [39].

TGFA knockout studies—To further elucidate the role of Tgfa in the intact organism, two groups set out to knock out the gene in mice by homologous recombination. These studies, simultaneously published, showed strikingly similar phenotypes [8, 9]. More strikingly, these knockouts were remarkably similar to a mouse mutant *waved-1* (*wa1*) identified 60 years earlier [7, 40]. *wa1* is an autosomal monogenic recessive phenotype and mice display a particularly curly coat of hair. The waved phenotype was described "*as though the animals had been to the hairdresser and had had a permanent wave treatment*." The phenotype is visible after 7–8 days of life, becomes more distinct by week 7–8 and is replaced by a smoother coat after subsequent hair cycles. This wavy coat is preceded by curly or hooked whiskers visible within 4–5 days after birth. Both of the *Tgfa* knockout studies recapitulated the wavy coat and curly whiskers phenotype. Reappearance of a waved phenotype in crosses between *Tgfa* null and *wa1* homozygous mice confirmed that these genes lie within the same complementation group and that the *wa1* phenotype is result of an inactivating mutation in the *Tgfa* gene (the exact genetic nature of *wa1* is not known). *wa1* mice showed reduced *Tgfa* mRNA and protein expression. Transgenic and knockout mouse studies revealing the role of TGFA and other EGFR ligands in skin physiology and pathology has been extensively reviewed [40].

In addition to complete penetrance of the wavy coat characteristic, *Tgfa* null mice frequently showed other abnormalities. Defects in fertility or longevity were not observed and Mendelian ratios were maintained. A number of *Tgfa* nulls were born with partially open eyes, with superficial opacity, and reduced size; by week five, corneal inflammation was also seen in some eyes, showing infiltration of polymorphonuclear leucocytes, mononuclear cells, and occasional multinucleated cells. In contrast to the wavy coat phenotype that was truly recessive, the eye abnormalities were reported in a small fraction of heterozygotes. The partially open eyes in *Tgfa* null mice resembled the precocious opening of eyelids by chronic administration of TGFA or EGF; however, the mechanism is completely different. During the embryonic stages of eye development from embryonic day 13 (E13), the eyelid buds grow from opposite sides to contact and fuse by E16.5. The eyelids reopen around 12 days postnatally after epithelial cells apoptose at the junction. TGFA/EGF administration accelerated the eyelid reopening by inducing hyperproliferation and maturation of eyelids; *Tgfa* null mice, however, had underdeveloped eyelids, showing hypoplasia of orbicularis muscle and Meibomian glands, thus eyelids fail to fuse prior to opening. Mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*) transcriptional upregulation by TGFA/EGFR signaling is now known to govern eyelid development [41].

Interestingly, there is a *waved-2* phenotype that is strikingly similar to *wa1* [10]. *The waved-2* mutation results from a T→G point mutation in *Egfr*, causing a V743G substitution near the amino terminus of the tyrosine kinase domain that severely reduces the kinase activity of EGFR and mice display an *Egfr* hypomorphic phenotype [11]. Discovery of *waved-1* and *waved-2* alleles was followed by the characterization of their genetic basis almost 60 years later.

TGFA in Disease States

The earlier notion that TGFA is an embryonic protein and is only expressed in neoplastic states is clearly incorrect. Apart from the role of TGFA in skin, breast, pancreas, liver, and eye epithelia, highlighted from the above knockout and overexpression studies, numerous other studies have corroborated these roles and revealed additional locations of TGFA actions (Table 1) [13, 16, 18, 37, 42]. For example, in small and large intestines, TGFA is produced in a gradient along the crypt villus axis that gradually decreases from the villi to the crypts in small intestine, or crypt top to crypt bottom in the large intestine; non-epithelial components express little TGFA [43]. EGFR has a similar expression pattern with a decreasing gradient from villi to crypts. TGFA also assists in maintaining stem cell fate of human embryonic stem cells by increasing the expression of pluripotent markers like NANOG and SSEA-3 [44]. It is thus not surprising that TGFA is often dysregulated in hyperproliferative disorders of these tissues.

TGFA dysregulation plays a causal role in various epithelial cancers [14, 15, 45]. What has emerged from these studies is that TGFA is not only an epithelial-specific autocrine mitogen, but it also acts in a paracrine manner to modulate the tumor microenvironment, enabling cross-talk between a tumor and surrounding stroma and immune system [46, 47]. As an example, derivatives of KM12C colon cancer cells, expressing 10-fold higher levels of TGFA than parental KM12C cells, injected into the cecal wall of nude mice were able to recruit VEGF-producing macrophages. The resultant tumors had high vascular density and metastasized to the regional lymph nodes and liver compared to low TGFA-expressing tumors. A reverse action of stroma acting on epithelia is also observed that involves TGFA; here TGFB from fibroblasts modulates the oncogenic potential, in part, by regulating TGFA expression in epithelia [48, 49].

Ménétrier's disease—A rare hyper-proliferative premalignant disorder of the stomach was discovered by Pierre Ménétrier in 1888. Ménétrier's disease is characterized by abnormal overgrowth of surface mucous cells and reduction of parietal and chief cells in the gastric crypts, leading to appearance of abnormal gastric folds akin to cerebral convolutions. It is also accompanied by reduced gastric acidity, excess mucous secretion, and hypoproteinemia.

Endogenous TGFA and EGFR are expressed in the parietal and chief cells of the gastric mucosa [50]. During our TGFA studies in the GI tract, we observed that TGFA overexpression mimicked multiple features of Ménétrier's disease. For example, TGFA administration decreased gastric pH [42, 51]. We further showed that systemic TGFA administration in rats led to an increase in gastric mucin and could protect the stomach from injury induced by ethanol or acid [52]. Mouse models that overexpress TGFA in the stomach (*MT-TGFA*) recapitulated virtually all features of Ménétrier's disease, including increased mucin secretion and decreased parietal and chief cell numbers, accompanied by decreased gastric acidity [37]. Moreover, Ménétrier's disease patients showed increased TGFA immunoreactivity in their gastric mucosa [53]. Based on these parallels between TGFA action in stomach and Ménétrier's disease, we set out to seek compassionate-use approval for use of a monoclonal antibody against EGFR (cetuximab) in a Ménétrier's

disease patient [54]. Both the patient's symptoms and biochemical indicators showed remarkable improvement and a larger clinical trial was designed. Results from this trial showed that cetuximab was highly effective in alleviating the major symptoms of Ménétrier's disease and we recommend that it should be used as first-line medical therapy for Ménétrier's disease [55].

TGFA Regulation

TGFA-mediated activation of EGFR leads to upregulation of *TGFA* mRNA and protein in normal keratinocytes as well as colorectal carcinoma cell lines [28, 29]. In fact, the ligand upregulation phenomenon extended to other EGF family members and most ligands are able to auto-induce their expression, as well as cross-induce expression of other EGF-like ligands [30]. Enhanced expression is achieved by a combination of transcriptional and post-transcriptional pathways. Other control mechanisms work post-translationally, such as modifications and additions of molecules to the polypeptide that influence the activity of the protein and drive transit of the protein through the secretory pathway, ultimately to the cell surface. Interestingly, in addition to *TGFA* mRNA upregulation, TGFA autocrine activation of EGFR also induces TGFA cleavage [56, 57].

***TGFA* Transcriptional Regulation—**As shown in Figure 1, the *TGFA* gene encompasses a large genomic region, with large upstream and downstream non-coding spaces and particularly large intronic regions between exons 1–3. Human and rat *TGFA/Tgfa* gene and promoter studies showed absence of regulatory regions like canonical CAAT and TATA boxes and TPA-response elements that are recognized and regulated by the AP-1 transcription factor [6, 58, 59]. The *TGFA* promoter, however, can induce transcription in an orientation-independent manner and is interspersed by several AP2- and Sp1-binding consensus sequences [60]. High GC content, absence of a TATA box and presence of putative AP2 and Sp1 sites are several key features that the *TGFA* promoter shares with the EGFR promoter [61]. Lack of TATA box is commonly found in housekeeping genes and usually results in non-discrete initiation of transcription; human *TGFA* transcription, however, starts at a discrete site as a result of a nonconsensus TATA box within the *TGFA* core promoter [18, 59]. The 313 bp region immediately upstream of the transcription initiation site is able to respond to TPA or EGF [62]. A larger 1.1 kb 5′ region also confers responsiveness to retinoic acid, glucocorticoids, and thyroid hormone; some repressive elements are also present within 3.5 kb upstream region. The upstream 3 kb region is also responsible for estrogen regulation of *TGFA* expression [63].

TGFA Post-trancriptional Regulation—Given the large size of *TGFA* mRNA and disproportionately large 3′ UTR that contains multiple putative microRNA seed sequences, it is likely that *TGFA* expression is modulated post-transcriptionally (Figure 1). Consistent with this idea, miR-376c is shown to directly bind to the 3′ UTR of *TGFA* and regulate its expression and consequent EGFR expression [64]. miR-376c displays anticancer properties in a number of cancers including melanoma and osteosarcoma; it reduces melanoma proliferation and migration and its expression is lost in osteosarcoma lines [65, 66]. *TGFA* is also a target for miR-152, which acts as a tumor suppressor in prostate tissue, at least in part, by downregulating *TGFA* mRNA [67]. Predicted miR-152 seed sequences in the 3′ UTR of

TGFA were responsive to miR-152 regulation in a luciferase assay. *Drosophila* mir-8, *a* vertebrate miR-200 homolog, binds the 3′ UTR of *Spitz* (*TGFA* homolog) and regulates Spitz expression to ensure controlled proliferation of neuroepithelial cells during neurogenesis [68].

TGFA Cleavage—Since its cloning, the proximal and distal cleavage sites of TGFA have been known; a signature Ala-Val-Val sequence is present at both the distal and proximal cleavage sites with cleavage occurring between alanine and valine residues [6]. The groups of Rik Derynck and David Lee in 1989 expressed TGFA mutants with the proteolytic cleavage sites mutated to render the protease processing ineffective; the majority of these mutant TGFA species reside on the cell surface [22, 69]. The membrane-fixed TGFA species bind to and activate EGFR and impart features of transformation, like anchorage-independent growth. Later, it was shown that generation of soluble TGFA occurs via a two-step process. A distal cleavage removes the prodomain from the amino terminus. The second cleavage proximal to the membrane occurs at a slower rate (minutes vs hours) that led to the assumption that since TGFA resides longest on the cell surface, this must be the predominant mode of action of TGFA [70]. This was further supported by the observation that membrane-anchored TGFA activated EGFR and cell proliferation [26]. The concept of juxtacrine signaling was thus established, with its obvious advantage of allowing specific interaction between cells that are juxtaposed to each other; a soluble ligand would not be able to achieve that specificity. Although juxtacrine actions were first proposed for TGFA, it has become clear over time that majority of TGFA actions require ectodomain cleavage; among EGFR ligands the strongest evidence for juxtacrine mode of action exists for HBEGF [71, 72].

The discovery of the protease responsible for TGFA cleavage came from an unexpected observation: a germ-line mutation inactivating a protease belonging to the ADAM (a disintegrin and a protease) family. ADAM17, known to cleave tumor necrosis factor alpha (TNFα, TNF) displayed a phenotype closely resembling *Tgfa* or *Egfr* knockout [73]. *Tnf* knockouts are viable; however, *Adam17*-deficient mice showed increased lethality, along with open eyelids (failure of eyelid fusion), wavy hair coats, and curly whiskers. Moreover, 95% of TGFA release was blocked in *Adam17*-deficient mice. ADAM17 cleaves both the distal and proximal sites and thus can release the ligand independent of other proteases [74]. ADAM17 was also shown to activate EGFR via TGFA cleavage in cancers [75]. The striking resemblance of *Adam17*-deficient mice to the *Tgfa* or *Egfr* knockout mice is some of the strongest evidence that the majority of TGFA actions are mediated through soluble TGFA and not through juxtacrine actions by the transmembrane precursor [73].

ADAM17 is the major protease for TGFA; TGFA cleavage is reduced by almost 90% in *Adam17* null cells, which is comparable to the twenty-fold reduced cleavage of TGFA in fibroblasts from *Adam17*-deficient mice [76]. However, the fact that a small fraction of TGFA is still cleaved, suggests it may be a substrate for other metalloproteases. Thus, under physiological circumstances ADAM17 seems to carry out TGFA cleavage and subsequent EGFR activation. In disease states, however, a particular overexpressed metalloprotease might drive TGFA cleavage and subsequent EGFR activation. Additionally, although TGFA shedding is constitutive, it can be further increased by TPA/PMA stimulation [76]. In fact,

metalloprotease activation and subsequent ligand cleavage and receptor activation occur by a number of stimuli like GPCRs, UV, oxidative stress, and radiation [77–80]. This pathway involving metalloprotease-proligand-receptor, "EGFR signal transactivation" occurs in normal cells but is particularly exploited by a number of cancer cells to confer proliferative, anti-apoptotic, and invasive properties [79–82]. ADAM10, the main sheddase for EGF and betacellulin, interacts with and cleaves TGFA, but only at the distal site [83]. Apart from the ADAM family of metalloproteases, a member of the astacin family of metalloproteases, MeprinA, induces release of TGFA in human bronchial epithelial 16HBE14o cells and colon cancer Caco-2 cells [84, 85].

TGFA trafficking: First regulatory step in EGFR signaling—Prior to the cleavage of transmembrane TGFA by metalloproteases there exists another regulatory constraint: the availability of transmembrane precursors at the cell membrane. We have concentrated our efforts on this aspect in polarized epithelial cells because it is the major site of TGFA production. We have also learnt about TGFA trafficking in polarized cells from other research groups. What follows is a detailed account of proteins and factors that determine the membrane availability of TGFA, and its preferential localization at a particular cell surface.

The notion that trafficking of TGFA is a critical regulatory step came from an unexpected observation where removal/mutation of a terminal valine in the TGFA cytoplasmic domain abrogated PMA-induced TGFA cleavage [86]. Massagué and coworkers proposed that the single terminal valine was a "simple and specific determinant" that enabled cytosolic recognition which in turn governed specific cleavage on the extracellular side. This was also proposed as an example of "inside-out signaling" or transfer of information across the membrane. These experiments were performed in non-polarizing CHO cells; while the results were convincing, the actual mechanism discovered later was different from the proposed one.

Using a rat liver epithelial line, David Lee and coworkers showed that the terminal valine of TGFA is actually part of a PDZ target motif that is required for proper membrane localization. Maturation of PDZ motif deletion mutants is compromised and mutants became trapped in the ER. Thus, loss of membrane delivery compromised subsequent cleavage [87]. A number of studies have shown multiple proteins interact with the terminal TGFA PDZ target motif at different cellular locations during its transit through the secretory pathway. For example, PDZ-domain-containing proteins, Syntenin and Syntrophin-1α, interact with the TGFA PDZ target motif [88]. It should be noted that alternative splice variants of TGFA lack the critical PDZ target motif and, therefore, would not follow the trafficking and maturation pattern of wild-type TGFA [21].

Apart from PDZ-dependent interactions, TGFA also associates with proteins through its EGF-like domain (Figure 2). One such interaction is with human Cornichon (CNIH) at the ER [89]. Loss of the *Drosophila* homolog of CNIH, *Cni*, leads to reduced surface expression of *Gurken*, another *Drosophila* EGF-like ligand, indicating a cargo receptor function for *Cni* [90]. The Yeast homolog of CNIH (Erv14) interacts with COPII coat components; since TGFA is most likely transported from the ER to Golgi via COPII-coated vesicles, CNIH

may function as a cargo receptor and regulate TGFA export from the ER [91]. Both CNIH loss and overexpression compromises TGFA membrane trafficking. CNIH overexpression leads to decreased TGFA secretion, membrane localization and maturation, along with overexpression of the immature ER form of TGFA. Only immature TGFA interacts with CNIH; the fully glycosylated Golgi form of TGFA is unable to interact with CNIH. Moreover, CNIH also interacts with GRASP55 through TGFA, again indicating that the sequential handing over of TGFA through the secretory pathway drives its exocytic trafficking. GRASP65, a trans-Golgi protein that interacts with TGFA later in the secretory pathway, does not interact with CNIH.

Drosophila Rhomboid, a serine protease, regulates activity of Spitz through cleavage in the Golgi. RHBDF1, a human homolog of Rhomboid, also resides in the ER and cis-Golgi but is catalytically inactive. It interacts with the EGF-like domain of immature, unglycosylated forms of TGFA [92], Thus, glycosylation may determine the sequence, timing and extent of interaction, leading to dissociation from RHBDF1, making mature TGFA available to interact with other proteins during its maturation and trafficking. Consistent with this hypothesis, siRNA against RHBDF1 reduces GPCR-stimulated TGFA release, and overexpression increases TGFA release, while leaving TGFA production unperturbed [93].

Polarized trafficking of TGFA—Our studies concentrated on TGFA trafficking in polarized epithelial cells. Using MDCK cells, we showed that TGFA was localized to the basolateral surface and that it was rapidly cleaved and avidly captured as a local autocrine factor [27]. Local capture of TGFA is a recurring theme in normal and disease states that we have reviewed previously [13]. A bipartite motif consisting of a juxtamembrane region and a dileucine region within the 39 amino acid long cytoplasmic domain determines the basolateral sorting specificity; these residues are highlighted in Figure 2 [94]. The bipartite nature of the TGFA basolateral sorting motif is in contrast to short linear basolateral sorting motifs identified for EGF, AREG, and EREG, other EGFR ligands [95–98].

We and others have identified several proteins like Syntenin, PSD-95, and MAGI-3 that interacted with the PDZ target motif of TGFA, but were not required for the basolateral sorting specificity of TGFA (Figure 2) [99]. The MAGI3-TGFA interaction occurs early in the secretory pathway and increases the efficiency of basolateral TGFA release [99]. During these studies we also discovered that a human homolog of *Drosophila* naked cuticle, NKD2, interacts with the TGFA cytoplasmic domain [100]. Both regions in the bipartite basolateral sorting motif of TGFA assist interaction with NKD2. NKD2 overexpression in polarized MDCK cells enhanced the basolateral cell surface delivery of TGFA. NKD2 is myristoylated at its second amino acid, glycine, and expression of myristoylation-deficient mutants compromises the basolateral delivery of TGFA (Figure 4). In the presence of myristoylation-deficient G2A-NKD2, TGFA is packaged into exocytic vesicles, but fusion to the basolateral plasma membrane requires NKD2 myristoylation; G2A-NKD2-coated TGFA exocytic vesicles fail to fuse with the basolateral plasma membrane and accumulate at the basolateral corner of polarized epithelial cells instead [101]. The fusion of TGFA vesicles to the basolateral corner is in sharp contrast to the docking and fusion of other basolaterally targeted vesicles (like LDL Receptor-containing vesicles) at the apical junctional complex that is dependent on the Sec6/8-containing octameric exocyst complex

[102]. Incidentally, we also took advantage of accumulation of TGFA vesicles to the basolateral corner in presence of G2A-NKD2 to determine the molecular composition of these vesicles by isolating and characterizing them by fluorescence activated vesicle sorting (FAVS) [103].

Unlike palmitoylation of TGFA, which is a stable post-translational modification, the myristoylation of its cargo recognition and targeting (CaRT) protein, NKD2, is more transient. It is interesting to speculate that the TGFA-containing vesicle fusion could be modulated by altering the myristoylation state of NKD2. Another way of regulating TGFA trafficking may be regulation of NKD2 levels. NKD2 is a short-lived protein with a half-life of 1–2 hours. RNF25 is one of the E3 ligases that govern NKD2 degradation (Figure 4) [104]. Cytoplasmic NKD2 is constitutively degraded but TGFA association protects it from degradation. TGFA binds between residues 300–385 in NKD2, proximal to the binding region of NKD2 with RNF25 [100, 104]. Although TGFA and RNF25 binding regions in NKD2 are non-overlapping, their proximity may pose steric hindrance for RNF25 and TGFA to interact with NKD2 simultaneously. Thus, TGFA interaction stabilizes NKD2. Finally, in addition to regulating EGFR activity, NKD2 also antagonizes Wnt signaling by interacting with DVL1 and forming a co-degradation complex at the plasma membrane that is myristoylation-dependent [105].

CD9 binds to the EGF-like domain of TGFA and modulates basolateral trafficking of TGFA. CD9 overexpression leads to increased membrane localization of TGFA to sites of cell-cell contacts in non-polarized cells [106]. In polarized epithelial cells, CD9 localizes to both to apical and basolateral surfaces and mislocalizes TGFA to the apical surface [107].

Aberrant signaling of EGFR by mistrafficking of an EGFR ligand, EREG, induces transformation and mutations removing its basolateral sorting motif have been reported in human cancers [96]. Similarly, the basolateral sorting motif of TGFA is also mutated in human cancers [98]. Thus, aberrant EGFR signaling induced by mistrafficking of TGFA mutants might contribute to disease phenotypes. Alternatively, NKD2 loss or mutation might also lead to TGFA mistrafficking. Moreover, NKD2 loss would also induce signaling aberrations in canonical Wnt signaling pathways in addition to dysregulation of EGFR signaling. On a related note, EGFR mistrafficking itself has been observed and correlates with pathological conditions, such as autosomal recessive polycystic kidney disease, where EGFR mislocalizes to apical membranes [108]. EGFR mutants engineered to mistraffick to the apical surface also show aberrant cyst morphology that often correlates with transformation *in vivo* [109].

Endocytic trafficking of TGFA-EGFR complexes—Compared with EGF, TGFA binds with lower affinity to EGFR, yet is biologically more potent [110]. In fact, the broader question may be how different EGFR ligands signal differentially in spite of acting through the same receptor. Ligand affinity plays a role; EGF has the strongest affinity for EGFR, followed by HBEGF, TGFA, BTC, EPR, EPGN, and AREG [111, 112]. However, ligand affinity alone does not completely explain the biological potency of the ligands. In addition to the activation of downstream signaling pathways a parallel trafficking pathway is set in motion; it starts with the receptor-ligand complex being internalized into endocytic vesicles

that may be recycled back to the cell surface, or shunted down the progressively more acidic lysosomal degradation pathway [113]. Ligand-bound activated EGFR is ubiquitylated in its cytoplasmic domain by Cbl, a ubiquitin ligase; 1–2 short polyubiquitin chains conjugated via lysine 63 residues mark it for degradation [114]. One of the first clues that different ligands differentially affect the post-stimulation endocytic trafficking of EGFR came from TGFA and EGF comparisons [115]. It was shown that TGFA dissociated from EGFR at a much higher pH than EGF (6.5 vs 5.5); thus, TGFA-EGFR complexes dissociate, leading to receptor deubiquitylation and recycling before entering the lysosomal compartment. Recently, it has been shown that although both TGFA- and EGF-induced internalization of EGFR into EEA1-positive endosomes (early endosomes), only EGF stimulation led to colocalization of EGFR with Lamp1-positive endosomes (lysosomes) [116]. This recycling of TGFA-EGFR complexes results in a net higher activity when compared to EGF-EGFR complexes that undergo lysosomal degradation.

Concluding remarks

It is often underappreciated that all EGFR ligands are synthesized as transmembrane precursors. Using TGFA as a prototype, we have highlighted two regulatory steps: the delivery of TGFA to the cell surface and cleavage from the cell surface by active metalloproteases. Both of these steps are critical to TGFA-mediated EGFR activation and are dysregulated in a number of diseases. From the discovery of the “transforming growth factors” from retrovirally transformed cells to the identification of specific interacting proteins and how they affect the ultimate biology, TGFA studies have highlighted broadly applicable phenomena; autocrine regulation that leads cancer cells to escape external growth factor dependence is one example. TGFA-interacting proteins like NKD2, which also inhibits canonical Wnt signaling, provide exciting opportunities to understand the cross-talk between different pathways and further unravel the range of TGFA actions.

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Highlights

- TGFA is an EGFR ligand
- TGFA surface delivery and cleavage are two important regulatory steps
- Forced expression of TGFA acts as epithelial mitogen and carcinogen
- TGFA interaction with NKD2 is required for its basolateral delivery
- TGFA is dysregulated in hyper-proliferative epithelial disorders

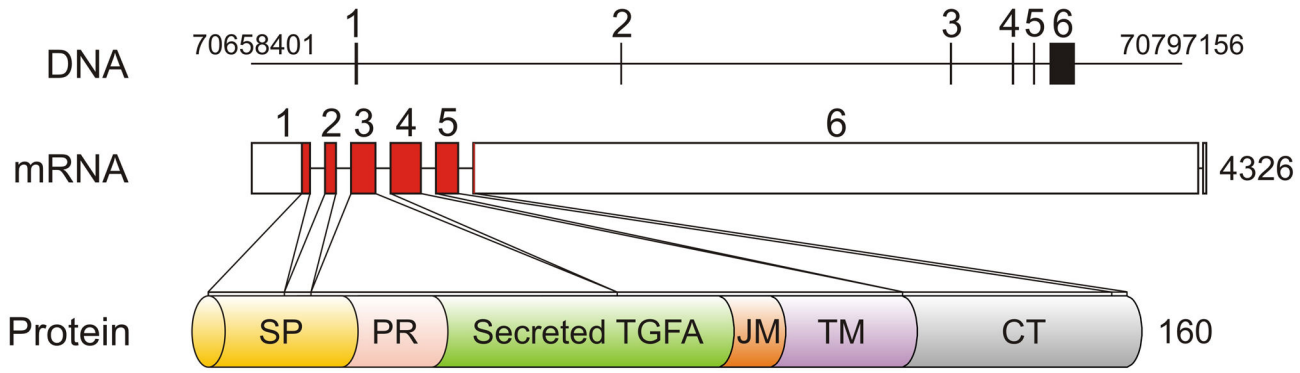

Figure 1. TGFA gene, mRNA, and protein structure

Human *TGFA* is located on 2p13 and spans a 138.7 kb region. *TGFA* exons are dispersed unevenly in the genetic locus; these are numbered and indicated by vertical marks. *TGFA* mRNA is 4326 bases long; all six exons code for part of the final protein sequence; coding region is indicated in red. The first 248 bases in exon 1 comprise the 5′ UTR. Exon 6 at 3.5 kilobases is the longest, but only the first eight bases are part of *TGFA* coding region, resulting in a large 3′ UTR that ends in a 12 base polyadenylation tail. TGFA protein is synthesized as a 160 amino acid precursor. A 23 amino acid long signal peptide (SP) is followed by a 16 amino acid pro-region (PR). The secreted EGF-like domain of TGFA (highlighted in green) is 50 amino acids, which is followed by a nine amino acid juxtamembrane region (JM). The transmembrane domain (TM) and cytoplasmic domain (CT) are 23 and 39 amino acids long, respectively.

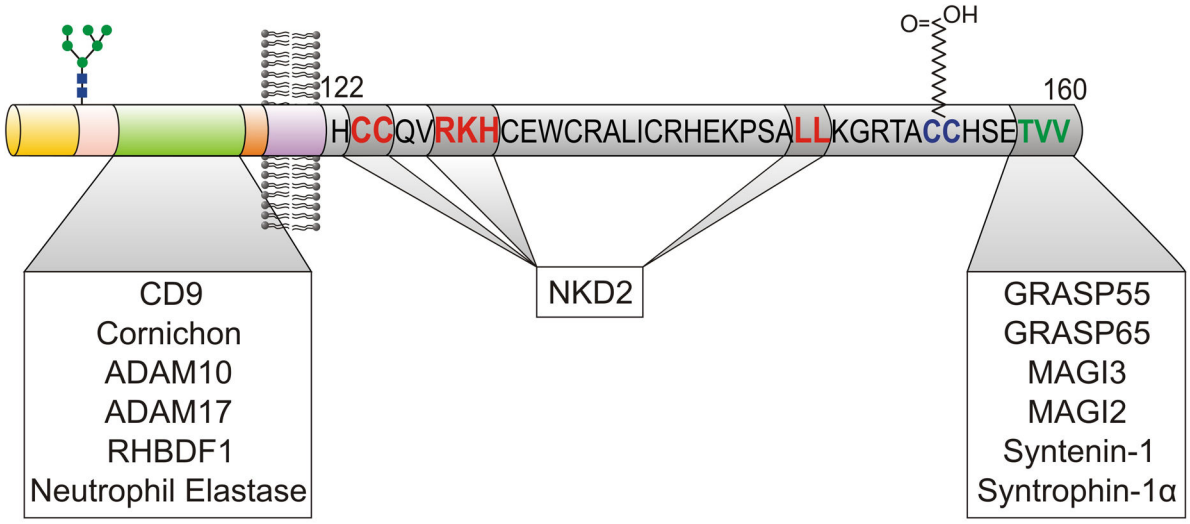

Figure 2. TGFA protein characteristics and interacting proteins

The color scheme and relative size of individual domains in TGFA protein are shown as in Figure 1 with the exception of the cytoplasmic domain that has been expanded to depict the sequence of the cytoplasmic tail. TGFA is N-glycosylated, most likely at position 25, which lies in the pro-region. Cysteines at 153 and 154 position (highlighted in blue) are palmitoylated. The EGF-like domain (green) interacts with multiple proteins listed underneath. Cytoplasmic domain residues that determine basolateral sorting specificity are highlighted in red and their interaction with the basolateral sorting adaptor, NKD2, is depicted below it. The cytoplasmic domain of TGFA culminates in a PDZ target motif and proteins interacting with this motif are listed underneath.

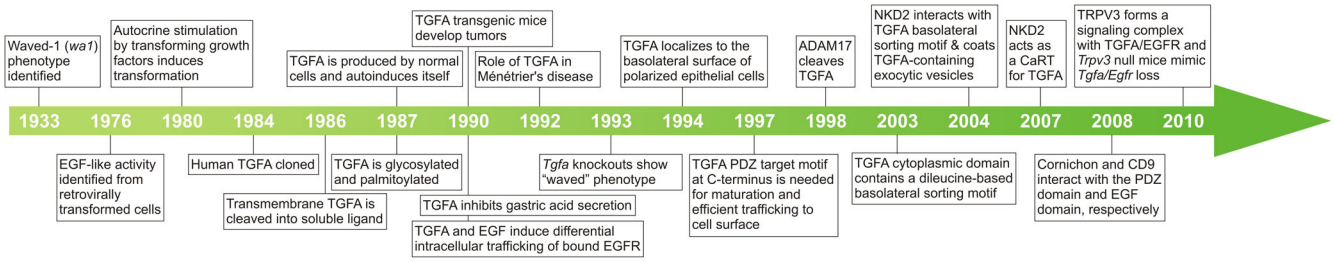

Figure 3.

Key discoveries that led to TGFA identification and its role in normal and disease states.

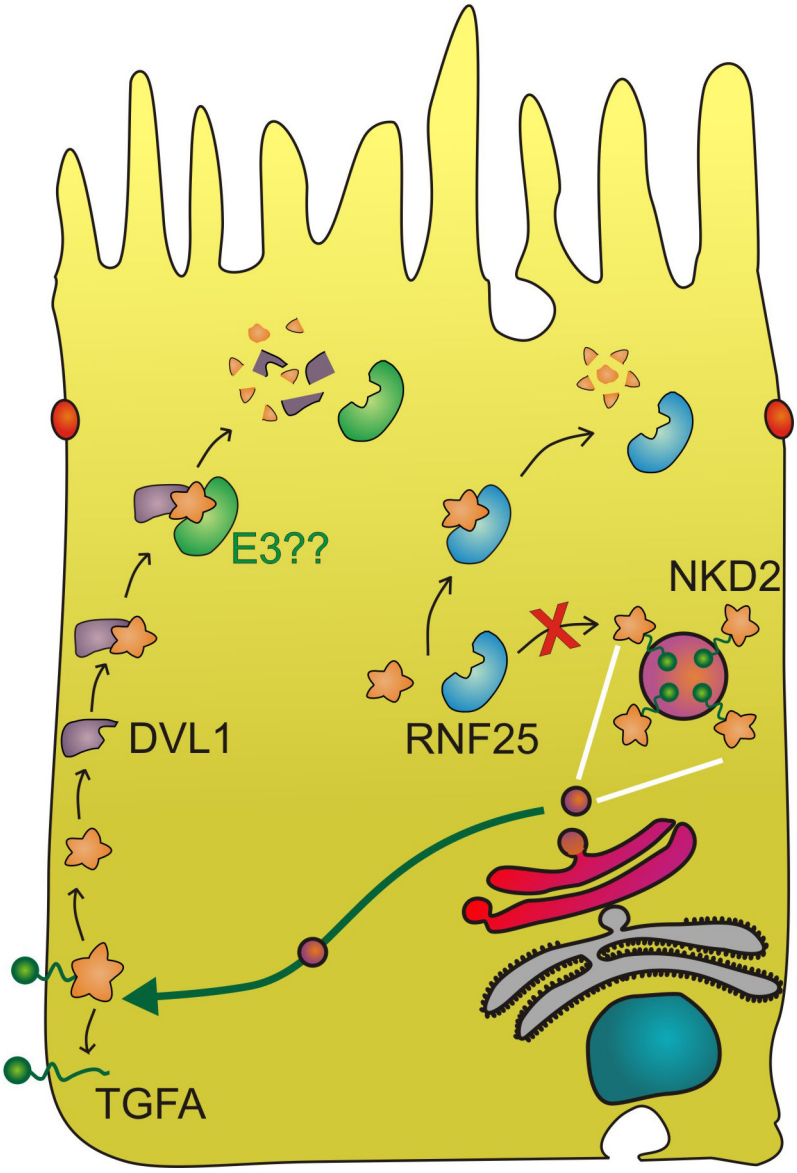

Figure 4. Proposed model for sequential action of NKD2 in basolateral targeting of TGFA and dampening of canonical Wnt signaling

Transmembrane TGFA is packaged into post-Golgi exocytic vesicles. NKD2 coats these exocytic vesicles and targets them to the basolateral corner of polarized epithelial cells, where they fuse to the plasma membrane in a NKD2 myristoylation-dependent manner. Cytosolic NKD2 undergoes proteasomal degradation that is mediated, at least in part, by the E3 ubiquitin ligase, RNF25. This association and subsequent degradation is impaired by NKD2 interaction with TGFA. After delivery of TGFA to the basolateral membrane, NKD2 dissociates from TGFA and interacts with DVL1, whereupon they are both degraded.

Table 1

Cellular stimuli that regulate TGFA actions through modulating its mRNA expression, ectodomain cleavage, or intracellular trafficking of the transmembrane precursor

Stimulus / Gene	Effect	System / Role	Reference
VHL	mRNA ↓	Renal cell carcinoma	[117]
HIV-1 Tat	mRNA ↑	Breast cancer cell line MDA-MB-468	[118]
Glucose	mRNA ↑	Arterial smooth muscle cells	[119]
Linoleic acid	Protein ↑	Lung & breast cancer cell line growth promotion	[120]
LPS	Cleavage	LPS transactivates EGFR through ADAM17-mediated cleavage of TGFA, which enhances MUC5AC expression in hepatothiliasis	[121]
PHD4	mRNA ↑	Promotes tumor angiogenesis in osteosarcoma	[122]
TSST-1	Cleavage	TSST-1 of *S. aureus* induces ADAM17-mediated TGFA shedding in human vaginal epithelial cells	[123]
PAR-2	mRNA ↑	ILK-mediated increased HIF-α protein levels increase TGFA expression in response to activated PAR-2	[124]
ROS	Cleavage	Bacterial LPS-induced ATP release that activated DUOX1 and released ROS. ROS activated ADAM17 that then cleaved TGFA	[125]
IL-13	Surface delivery	IL-13 mobilized intracellular TGFA to apical surface of human bronchial epithelial cells where it was cleaved by ADAM17 to activate autocrine/paracrine pathways	[126]
Estrogen	mRNA ↑	Breast cancer cell lines	[63]
EGF-like ligands	mRNA ↑	Keratinocytes, human colon cancer cell lines, RIE-1 cells	[28–30]
IL-6	mRNA ↑	Monocytoid cell line U-937-1	[127]
TGFB	mRNA ↑	Induces TGFA transcription via NADPH oxidase>ROS> NF-κB pathway	[128]
RHBDF1	Surface delivery	RHBDF1 regulates ER to Golgi transport of TGFA in response to gastrin-releasing peptide stimulation	[92, 93]
NKD2	Basolateral delivery	NKD2 coats TGFA-containing vesicles and delivers them to the basolateral corner of polarized epithelial cells	[100, 101]