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REVIEW

Converging physiological roles of the anthrax toxin receptors [version 1; peer review: 3 approved]

Oksana A. Sergeeva (ID), F. Gisou van der Goot

Global Health Institute, School of Life Sciences, EPFL, Lausanne, Switzerland

Abstract

The anthrax toxin receptors—capillary morphogenesis gene 2 (CMG2) and tumor endothelial marker 8 (TEM8)—were identified almost 20 years ago, although few studies have moved beyond their roles as receptors for the anthrax toxins to address their physiological functions. In the last few years, insight into their endogenous roles has come from two rare diseases: hyaline fibromatosis syndrome, caused by mutations in CMG2, and growth retardation, alopecia, pseudo-anodontia, and optic atrophy (GAPO) syndrome, caused by loss-of-function mutations in TEM8. Although CMG2 and TEM8 are highly homologous at the protein level, the difference in disease symptoms points to variations in the physiological roles of the two anthrax receptors. Here, we focus on the similarities between these receptors in their ability to regulate extracellular matrix homeostasis, angiogenesis, cell migration, and skin elasticity. In this way, we shed light on how mutations in these two related proteins cause such seemingly different diseases and we highlight the existing knowledge gaps that could form the focus of future studies.

Keywords

anthrax toxin receptors, CMG2, TEM8, HFS, ISH, JHF, GAPO

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Corresponding authors: Oksana A. Sergeeva (oksana.sergeeva@epfl.ch), F. Gisou van der Goot (Gisou.vanderGoot@epfl.ch)

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Introduction

In the early 2000s, two newly discovered proteins—capillary morphogenesis gene 2 (CMG2) and tumor endothelial marker 8 (TEM8)—were demonstrated to be anthrax toxin receptors (ANTXRs)[1,2]. Since then, much research has focused on their toxin-related pathogenic roles (most recently reviewed by Friebe *et al.*[3] and Sun and Jacquez[4]). However, both CMG2, encoded by the *ANTXR2* gene, and TEM8, encoded by the *ANTXR1* gene, also possess physiological roles in vertebrates, the study of which has not received nearly the same level of attention. What has helped to drive research into their endogenous roles is that both receptors are associated with recessive autosomal diseases: mutations in CMG2 lead to hyaline fibromatosis syndrome (HFS), whereas mutations in TEM8 result in growth retardation, alopecia, pseudo-anodontia, and optic atrophy (GAPO) syndrome[5–7]. The literature contains reports of some 350 HFS patients of whom 112 have been genotyped, possessing 56 different mutations. In parallel, 70 GAPO patients have been reported, 21 of which have genotype information, comprising 14 different mutations. Even though these diseases have grossly different symptoms, CMG2 and TEM8 share 62% sequence similarity, reasonably allowing for some overlapping structure/function relationship and ensuring that a better understanding of the function of one receptor can prompt progress on the other. Therefore, this review aims to synthesize the latest information on these ANTXRs with earlier established observations to better understand their physiological functions and provide open pathways for future research.

Anthrax toxin receptor structure and epistructure

Both CMG2 and TEM8 are type I transmembrane proteins consisting of an extracellular von Willebrand factor type A (vWA) that is also found in integrins and participates in receptor–ligand interactions, an extracellular immunoglobulin-like (Ig-like) domain, and a long cytoplasmic tail that is predicted to be largely unstructured and contains an actin–cytoskeleton interacting domain (Figure 1). The structure of the extracellular domains was analyzed by low-resolution cryo-electron microscopy, onto which the x-ray structure of the vWA domain[8,9] and a model of the Ig-like domain[10,11] were successfully docked. CMG2 and TEM8 have long cytosolic tails of 148 and 222 residues, respectively, which are predicted to be intrinsically unstructured like the cytoplasmic domains of many other signaling receptors[12]. These tails could allow sequential interaction with diverse partner molecules[13] and were also found to be the site of various post-translational modifications, such as S-palmitoylation[14], ubiquitination[14], and tyrosine-phosphorylation[15], all of which were found to be necessary for toxin-induced endocytosis[3]. The CMG2 and TEM8 tails are completely identical in certain segments but have no homology in others[16], pointing toward similarities and differences, respectively, in interacting partners. The most highly conserved portion between the two receptors, which is juxtamembrane, has homology with the actin-regulating Wiskott–Aldrich syndrome protein[17]. Relatedly, TEM8 was also consistently shown to interact with actin[18,19], although this interaction may be indirect and require adaptor proteins, such as those observed for integrins[20].
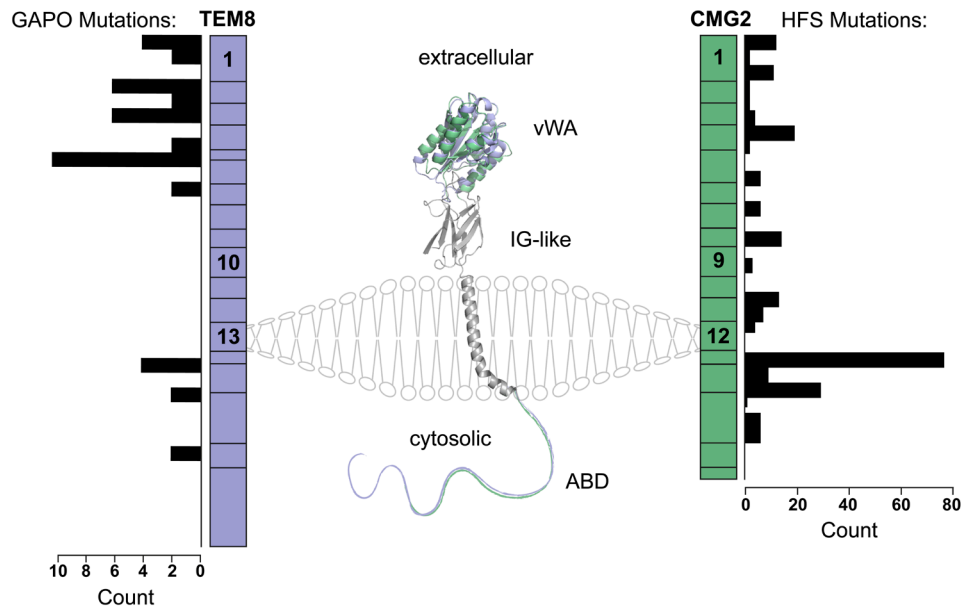

Figure 1. TEM8 and CMG2 are similar in gene and protein structure: mutations in the former lead to GAPO and those in the latter lead to HFS. Tumor endothelial marker 8 (TEM8) (blue) and capillary morphogenesis gene 2 (CMG2) (green) have similar exon schemes and protein structures. Crystal structures of the von Willebrand factor type A (vWA) domain of TEM8 (Protein Data Bank [PDB]: 3N2N[8]) and CMG2 (PDB: 1TZN[9]) are shown aligned. The immunoglobulin (Ig)-like and transmembrane domains have been modelled on CMG2 and are shown in gray[10]. The cytosolic tails, longer for TEM8 than for CMG2, are intrinsically disordered with a conserved juxtamembranous actin-binding domain (ABD). The number of reported occurrences of mutations in growth retardation, alopecia, pseudo-anodontia, and optic atrophy (GAPO) is depicted next to TEM8, and the corresponding number in hyaline fibromatosis syndrome (HFS) is shown next to CMG2. The number of HFS patients with mutations in CMG2 is almost an order of magnitude higher than those of GAPO/TEM8.

Receptors for anthrax toxin

The function of CMG2 and TEM8 as ANTXRs has been extensively studied. The surface residence time of ANTXRs is regulated by S-palmitoylation at three or four sites in their cytoplasmic tails, as shown for TEM8[14], and under "resting" conditions, TEM8 is associated with the actin cytoskeleton[15,21] (Figure 2A). As opposed to how integrins concurrently interact with their ligand and the cytoskeleton, upon binding of a ligand such as anthrax toxin protective antigen (PA), the interaction of TEM8 with the actin cytoskeleton is released. Upon ligand binding, PA oligomerizes into a heptameric or octameric complex, leading to clustering of the receptors[2,22,23]. This, in turn, triggers *src* family kinase-mediated phosphorylation of cytoplasmic tyrosines in the ANTXR tails[15,24] and subsequent recruitment of the adaptor protein β-arrestin, allowing an E3 ligase (Cbl for TEM8) to bind and facilitate ubiquitination[14,24]. Ubiquitinated ligand-bound ANTXRs are taken up by an adaptor protein 1 (AP-1)-dependent and clathrin-mediated endocytic route[24]. Upon arrival in sorting endosomes, in the presence of a multivalent ligand, ANTXRs are sorted into nascent intraluminal vesicles. The exact mechanism and molecular requirements for this sorting have not been investigated. Although a bird's-eye view of toxin-induced endocytosis is available, little is known about the physiological endocytic trafficking of ANTXRs, particularly whether they undergo endocytosis and recycling for re-utilization.

HFS-causing CMG2 mutations

HFS is now the unifying term for two diseases previously described in the literature: infantile systemic hyalinosis (ISH), which was named in 1986[25], and juvenile hyaline fibromatosis (JHF), first described in 1873 by Murray[26] and named in 1976[27]. Initially, these terms were thought to describe two different diseases with overlapping symptoms, including subcutaneous nodules, gingival hypertrophy, painful joint contractures, and persistent infections. ISH is, however, more severe with death occurring before the age of two because of protein-losing enteropathy. In the early 2000s, patients with both JHF and ISH were found to have mutations in CMG2[5,6,28], pointing to the disease causality. This allowed the classification of a unified syndrome with a symptom grading system, placing the previously named ISH and JHF on two ends of a continuum of disease severity[29,30]. We strongly encourage the community to adopt a single nomenclature—HFS instead of ISH or JHF—to ensure clarity in the literature.

HFS-causing mutations in CMG2 fall into four classes: (a) missense mutations in the vWA domain that specifically affect ligand binding; (b) missense mutations in exons 1 to 11 that affect folding/stability of the ectodomain, leading to protein degradation; (c) frameshift mutations that lead to premature stop codons, nonsense mutations, and those that affect splicing, leading to rapidly degraded mRNA; and (d) missense mutations in the cytosolic tail that do not affect protein abundance or localization but likely affect some aspect of CMG2 function[16]. Casas-Alba *et al*. recently published an exhaustive review on phenotype–genotype correlations of patients with HFS[31]. The authors used the aforementioned grading system[30] to support the general notion that missense mutations in exons 1 to 12 and nonsense and frameshift mutations lead to more severe
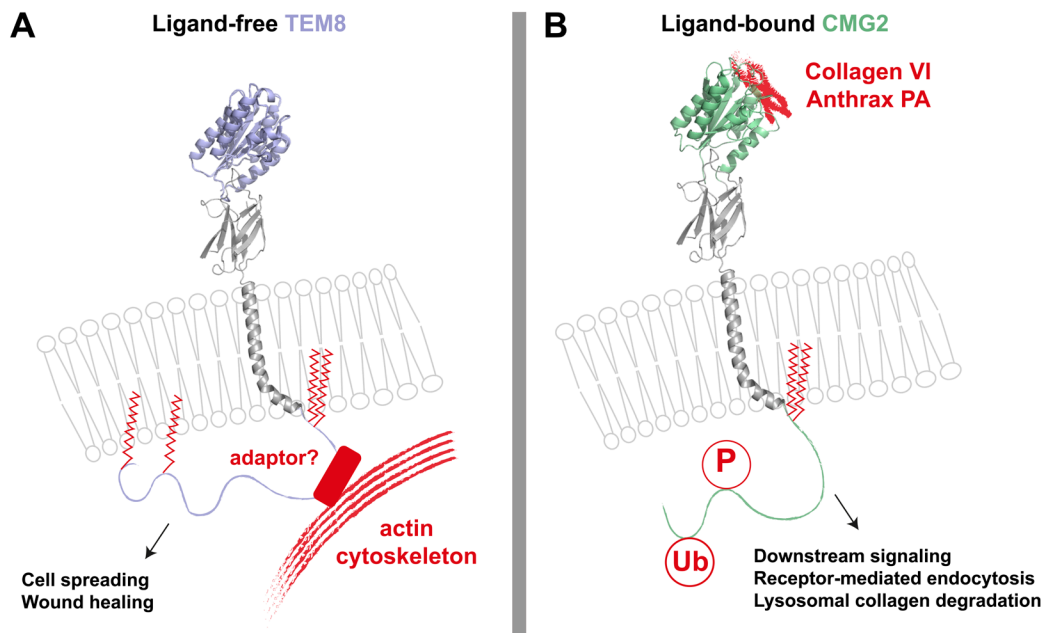
Figure 2. Depictions of ligand-free TEM8 and ligand-bound CMG2. (**A**) Ligand-free tumor endothelial marker 8 (TEM8) is palmitoylated and bound directly or indirectly to the actin cytoskeleton. Through this association, it plays a role in cell spreading and migration as well as wound healing. Red zigzag lines represent S-palmitoylation modifications of TEM8, which increase resident time of either receptor at the cell surface. (**B**) When bound to collagen VI (ColVI) or anthrax toxin protective antigen (PA), capillary morphogenesis gene 2 (CMG2) becomes phosphorylated ("P") and ubiquitinated ("Ub"). This allows CMG2 to signal downstream within the cell, endocytose the receptor–ligand complex, and degrade ColVI in the lysosomes.

forms of the disease than missense mutations in exons 13 to 17[31]. The gravest mutations largely affect protein abundance due to mRNA or protein degradation, whereas the milder mutations do not affect protein expression levels[16]. One exception is the missense mutations that prevent ligand binding, which still produce normal amounts of protein but are even more severe than mutations that lead to near-complete protein loss. This observation raises interesting possibilities. Either ligand-binding-deficient CMG2 not only loses its initial function but may gain a new pathogenic one or unliganded CMG2 has important signaling functions that need to be switched off by ligand binding. Upon mutation of the ligand binding site, sustained signaling could be detrimental.

GAPO-causing TEM8 mutations
The symptoms of GAPO were first described by Andersen and Pindborg in 1947[32] but the term was not coined until 1984[33]. The name of the disease itself, GAPO, describes its most characteristic symptoms: growth retardation, alopecia, pseudo-anodontia, and optic atrophy; however, since optic atrophy is seen in only a small fraction of patients, there is an appeal to rename the O to "ocular manifestations", as many patients do have other eye defects, such as glaucoma[34]. Although only a few cases of GAPO lead to death before adulthood[34–36], it is still a severe disease that often gives patients a geriatric appearance. The link between GAPO manifestation and mutations in TEM8 was found only in 2013[7], 10 years after the connection between CMG2 and HFS was discovered.

Recently, Abdel-Hamid et al. reported on seven new GAPO patients and their associated mutations[37], almost doubling the known mutations. It is still premature to identify any mutational hot spots in TEM8 like we observe in the juxtamembranous exon 13 of CMG2 for HFS (Figure 1). However, the mutant categories for GAPO will likely be similar to those of HFS, with missense mutations that map to the TEM8 cytosolic tail leading to milder phenotypes, as suggested by the description of a homozygous patient presenting with pseudo-anodontia and no other reported symptoms[38].

Converging physiological functions of anthrax toxin receptors
Extracellular matrix homeostasis and remodeling
What is clearest about the physiological roles of CMG2 and TEM8 is that they both interact with the extracellular matrix (ECM), although the specific ECM–protein interaction partners have been a point of contention in the literature. Early in vitro studies reported that CMG2 can bind collagen (Col) IV and laminin[17]. That experiment relied on testing five ECM proteins (those two and osteopontin, fibronectin, and albumin)[17], overlooking other potential ECM ligands. Recent and more comprehensive in vitro studies have instead indicated that CMG2 has a higher affinity for ColVI than ColIV or laminin[39]. TEM8 was also found to bind the cleaved C5 domain of ColVI(α3)[40]. In mice, the lack of CMG2 or TEM8 can lead to an accumulation of ECM in various tissues, and there is some controversy as to which components accumulate. In mice lacking CMG2, an accumulation of only ColVI[39] or both ColI and

ColVI[41] was observed, whereas in mice lacking TEM8, an increase in both ColI and ColVI was detected[42,43].

Significant insight into the physiological function of CMG2 and its involvement in HFS has come from the recent work of Bürgi et al., who analyzed HFS patient nodules to reveal that they are predominantly made up of ColVI[39]. The authors also showed that CMG2 is a cellular receptor for this non-fibrillar collagen and that it can mediate the degradation of ColVI in lysosomes (Figure 2B)[39]. Strikingly, they observed that whereas female mice lacking CMG2 have hypertrophic uteri and parturition defects (seen previously[41,44]), mice deficient for both CMG2 and ColVI were able to deliver pups normally[39]. Furthermore, this study did not implicate matrix metalloprotease inhibition as a mechanism for extracellular ColVI accumulation, as was proposed in earlier studies[41,43,45]. Thus, it appears to be the inability of CMG2 to control the abundance of ColVI in the extracellular space that leads to the formation of nodules in patients with HFS. Explaining other HFS symptoms, such as life-threatening protein-losing enteropathy in infants, will require further investigation.

The fact that CMG2 is a bona fide ColVI receptor is new, although the literature had previously hypothesized that the ANTXRs regulate collagen clearance, and CMG2 itself was already thought to be the cellular receptor responsible for collagen internalization and degradation[44]. Meanwhile, TEM8 was postulated to be involved in collagen degradation through an endocytosis-mediated pathway[46], and TEM8 recycling was posited to lead to ColI and ColVI clearance[47]. In one of the earlier reports of GAPO, Wajntal et al. theorized that the disease symptoms were due to ECM accumulation and hypothesized that GAPO was the result of an autosomal recessive defect in a gene responsible for ECM component breakdown[48]. However, TEM8 is unable to compensate for ColVI degradation in human fibroblasts upon silencing of CMG2 or in cells of patients with HFS, suggesting that even though TEM8 might have the ability to bind ColVI, it does not escort it to lysosomes, at least not in fibroblasts, as CMG2 does[39]. Since TEM8 was found to bind the C5 domain of ColVI, which is processed during maturation, TEM8 might have a different role in ColVI homeostasis.

Angiogenesis and cancer
Both CMG2 and TEM8 were originally discovered because of their connection to angiogenesis. In 2000, TEM8 was found to be the eighth most upregulated marker in the tumor endothelium[49], and in 2001, the gene encoding for CMG2 was uncovered as the second most upregulated gene in in vitro capillary morphogenesis in three-dimensional collagen matrices[17]. Consistent with these findings, anthrax PA was observed to prevent angiogenesis in a moderate yet significant manner[50]. Additionally, a heterozygous mutation in the transmembrane domain of TEM8 is associated with infantile hemangiomas, benign tumors arising from disorganized angiogenesis[51].

Since angiogenesis is a major hallmark of cancer, researchers have investigated the roles of CMG2 and TEM8 in cancer since their initial discovery. For TEM8, the literature is

consistent in that its overexpression results in more aggressive cancer, especially for osteosarcomas[52], gallbladder carcinomas[53], melanomas[42], and lung[54], breast[55,56], and colorectal[57] cancers. However, for CMG2, there are mixed reports of the effect of its overexpression on cancer progression; some show that lower CMG2 correlated with a more aggressive soft-tissue sarcoma[58] and breast cancer[59], and others reveal that higher CMG2 resulted in a worse survival rate in patients with gastric cancer[60] and glioblastoma[61]. Although these studies link these proteins to angiogenesis, mechanistic explanations of the cancer-related roles of ANTXRs are still lacking. Research has also focused on targeting CMG2 or TEM8 for cancer therapy[62], especially using anti-TEM8 antibodies[63,64], CMG2 vWA domain fragments[65], or anthrax toxin itself[66] for slowing cancer progression. Recently, Szot et al. demonstrated the potential of delivering antibody drug conjugates targeting TEM8 to tumor-associated stromal cells, thereby unexpectedly but effectively killing nearby cancer cells[67]. Meanwhile, Byrd et al. successfully used TEM8 chimeric antigen receptor T cells to target and regress xenograft tumors derived from patients with triple-negative breast cancer[68].

Actin cytoskeleton and cell migration
As mentioned earlier, both ANTXRs contain a putative actin-interacting peptide sequence in their cytoplasmic tails (juxtamembrane residues 355 to 420), and it has been shown that ligand-free TEM8 binds to the actin cytoskeleton[24] (Figure 2A). Furthermore, it was proposed that the actin cytoskeleton can regulate the affinity of TEM8 for its extracellular ligands[21,69]. More specifically, Go et al. showed that mutating tyrosine 383 to cysteine in TEM8 (mimicking an HFS mutation, Y381C) decreased actin–cytoskeleton interactions but increased anthrax-toxin binding[69]. This type of inside-out signaling is reminiscent of integrins. The fact that integrins are involved in cell adhesion, spreading, and migration has led to studies asking whether TEM8 could be involved in similar cellular processes. TEM8 was reported to affect cell adhesion and spreading by coupling the ECM with the actin cytoskeleton[19,47]. However, while integrins simultaneously bind extracellular ligands and the intracellular actin cytoskeleton, thus generating force, the mutually exclusive interaction of TEM8 with ligands and actin indicates that the contribution to spreading must occur via different mechanisms, which remain to be elucidated.

One process that combines angiogenesis, cell migration, and ECM remodeling is wound healing. In 2016, Wang et al. demonstrated that TEM8 expression is increased in acute or chronic wounds as compared with normal skin[70]. When they depleted TEM8 in keratinocyte cells, they saw decreased migration and proliferation[70]. However, the exact molecular role of TEM8 in regulating wound healing is still unknown.

Surprisingly, CMG2 has not yet been characterized to interact with the actin cytoskeleton. Instead, zebrafish Antxr2a, a CMG2 ortholog, was shown to be involved in positioning the mitotic spindle in a process that involves the small GTPase RhoA and its downstream effector mDia[71]. Consistent with a putative role of CMG2 in actin cytoskeleton rearrangements,

CMG2 knockdown led to decreased migration in human uterine smooth muscle cells while its overexpression led to increased migration[45].

Skin physiology and organ fibrosis
Both HFS and GAPO patients have issues with the skin: skin thickening in HFS and alopecia in GAPO. Although few histopathological analyses have been reported for patients with GAPO[37], the dermis of one patient with GAPO was shown to have abundant hyaline material with increased collagenous fibers[48], suggesting that, like HFS (the earliest histopathological analyses[72] to the latest[73]), GAPO is a disease of the connective tissue. This increased ECM could interfere in the development and cycling of the hair follicles[74] but this is still unproven.

An analysis of TEM8 knockout (KO) mice was consistent with patient molecular observations in that increased ECM is seen in organs that consequently develop fibrosis[42,43]. Whereas earlier reports did not observe gross changes in the TEM8 KO mice[42], more recent studies on a different KO mouse line indicate that mice rather accurately mimic the GAPO phenotype[43]. Hu et al. recently showed that fibroblasts isolated from older mice deficient in TEM8 have an increased expression of ColI, fibronectin, and the connective tissue growth factor (CTGF)[75]. They argue for a cell-autonomous mechanism wherein TEM8 targets CTGF to regulate ECM production, meaning that TEM8-lacking cells have higher CTGF, thus a higher ECM production that leads to fibrosis[75].

It is important to note that nodules develop months or years after birth in patients with HFS and that hair loss starts around age two for patients with GAPO[16,37]. Similarly, ECM accumulation was predominantly seen in older patients with GAPO[7]. In mice lacking CMG2, the uterine fibrosis phenotype was seen only in sexually mature mice[41]; similarly, progressive fibrosis was observed for mice deficient for TEM8[43]. This could mean either that the organism can temporarily buffer disease or that the accumulation of ECM reaches a threshold that causes these manifestations. One obvious implication is that CMG2 might partly compensate for TEM8 function and vice versa before the cell is overwhelmed and the disease symptoms arise. Interestingly, double-KO mice are viable but produce no pups when mated[76], suggesting that the receptors have non-redundant roles in fertility, embryonic development, or parturition.

Conclusions and Outlook
In recent years, significant progress has been made in understanding the functions of CMG2 and TEM8 in vertebrates. Specifically, the fact that these two receptors act as causative genes for strikingly different diseases was perplexing, as their main domains are conserved so they likely have similar physiological roles in the cell. The similarity in underlying molecular defects—both receptors contribute to ECM homeostasis, angiogenesis, cell migration, and skin pathology—has helped to explain this. However, the devil appears to be in the details that remain elusive: which collagen(s) do they bind, how do they (directly) interact with actin, can they endocytose ECM components for lysosomal breakdown without depleting the

extracellular environment of their ligand, which signaling cascades do they trigger, and in which cells do they primarily function? Furthermore, the role that these ANTXRs play at a molecular level in angiogenesis, cell migration, and wound healing remains to be elucidated. We hope that this review has provided sufficient evidence of the analogous nature of the ANTXRs to guide future research into these two receptors.

Abbreviations

ANTXR, anthrax toxin receptor; CMG2, capillary morphogenesis gene 2; Col, collagen; CTGF, connective tissue growth factor; ECM, extracellular matrix; GAPO, growth retardation, alopecia, pseudo-anodontia, and optic atrophy; HFS, hyaline fibromatosis syndrome; Ig, immunoglobulin; ISH, infantile systemic hyalinosis; JHF, juvenile hyaline fibromatosis; KO, knockout; PA, protective antigen; TEM8, tumor endothelial marker 8; vWA, von Willebrand factor type A

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   Department of Chemistry, Wichita State University, Wichita, KS, USA
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