R&D Systems Cytokine Bulletin
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R&D Systems BioBrief:
The TGF-β Superfamily
Phylogenetic Tree

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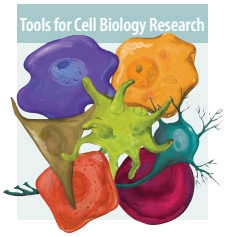

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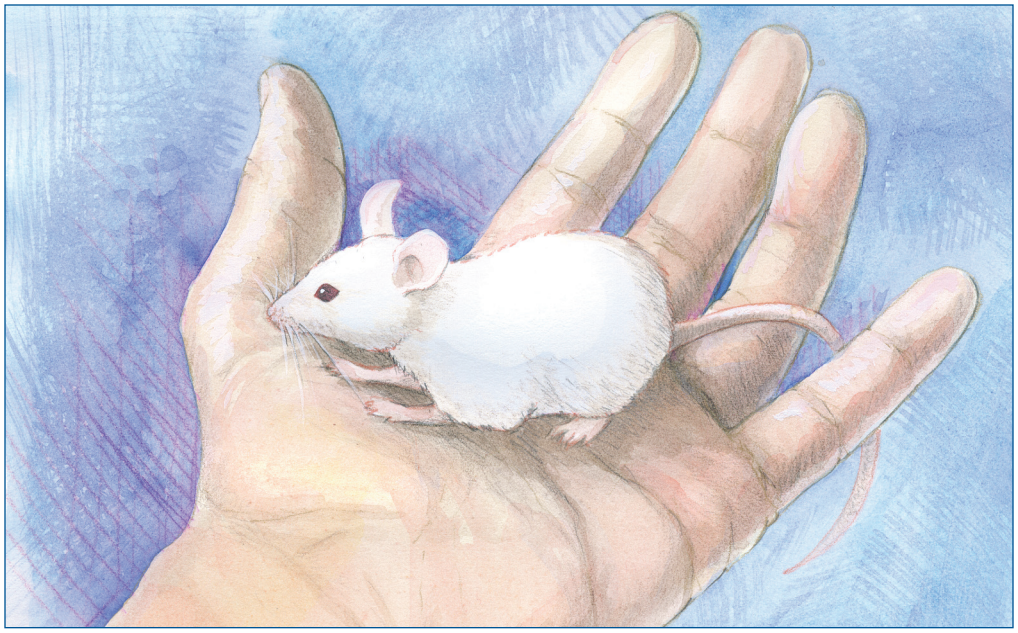

Using model systems to represent human biological processes can be challenging. This holds true in the study of mouse and human stem cells, as 75 million years of divergent evolution have produced differences in the responses to molecules that regulate stem cell fate. These include key interspecies differences in the activities of TGF-β family members.

TGF-β Superfamily Signaling in ES cells

Mice are Not Men

Although mouse embryonic stem (ES) cells were first isolated and characterized over 25 years ago, the discovery of their human counterpart in 1998 led to a strong surge of interest in identifying the molecular mechanisms that mediate self-renewal and broad differentiative capacity, the key characteristics of ES cells.[1-3]

Studies of the roles of TGF-β superfamily members in ES cell self-renewal and differentiation have highlighted the fact that despite their fundamental similarities, there appear to be significant differences between mouse and human ES cells. For example, bone morphogenetic protein (BMP) signals have been shown (in combination with leukemia inhibitory factor, LIF) to maintain mouse ES cells in an undifferentiated, pluripotent state.[4] In contrast, human ES cells can only maintain an undifferentiated phenotype by suppression of endogenous BMP signaling.[5]

Signaling through the activin receptor by activin or nodal has been shown to maintain human ES cells in the undifferentiated state by several groups.[6-9] The role of activin/nodal signaling in mouse ES cells is less clear. One report showed that although this pathway is active in undifferentiated mouse ES cells as assessed by phosphorylation and nuclear localization of smad 2/3, inhibition by a synthetic compound that prevents smad 2/3 phosphorylation had no effect on the undifferentiated state of the cells.[8] However, recent results from another group showed that mouse ES cell proliferation, but not pluripotency, was inhibited by the same synthetic Smad 2/3 inhibitor.[10]

Another TGF-β superfamily member that plays apparently opposite roles in mouse vs. human ES cells is growth and differentiation factor 3 (GDF-3). GDF-3 falls in the BMP branch of the superfamily, and has the greatest degree of homology to Vg1, a mesoderm inducer found in *Xenopus*. However, GDF-3 lacks the 4th canonical cysteine residue found in TGF-β superfamily members, a characteristic shared by GDF-9, BMP-15, and Lefty-A and -B.[11,12] GDF-3 is expressed in undifferentiated mouse and human ES cells, and is downregulated upon differentiation.[13] In human ES cells, ectopic expression of GDF-3 results in the maintenance of markers of pluripotency even when the cells are cultured in conditions that typically promote differentiation. Thus, increased GDF-3 levels appear to promote the undifferentiated state. Paradoxically, a similar effect is seen in mouse ES

℗ This symbol denotes references that cite the use of our products.

Signaling in ES Cells, continued on page 5.

BMP-1 Regulation of TGF-β Activity

TGF-β family responsiveness can be modulated by proteases that target TGF-β receptors and co-receptors. These proteolytic activities add to the regulatory complexity of TGF-β family signaling. For instance, thrombin cleavage of PAR1 leads to internalization of the co-receptor endoglin, and MMP-14 and MMP-16 mediate biglycan shedding.[1,2] Members of the BMP-1 protease family also play important roles in the regulation of TGF-β family activities. The highly conserved BMP-1/PCP subgroup of the astacin family includes BMP-1 and alternatively spliced mammalian tolloid (mTLD), and mammalian tolloid-like (mTLL) 1 and mTLL2. These enzymes contain an astacin protease domain followed by variable numbers of CUB and EGF-like domains. They cleave N-terminal to an aspartic acid residue in multiple extracellular matrix (ECM) components (collagens, laminins, small leucine-rich proteoglycans, and SIBLING proteins), lysyl oxidases, and growth factor-related molecules.[3]

TGF-β family proproteins are cleaved in the *trans*-Golgi between the N-terminal propeptide and the mature growth factor. For TGF-β1, -β2, -β3, GDF-8, and GDF-11, the prodomain is secreted in association with the growth factor and maintains the growth factor in an inactive state. BMP-1 family of proteases regulate the activation of these latent complexes by several mechanisms.

Large latent complexes consisting of TGF-β, latency-associated peptide (LAP), and latent TGF-β-binding protein (LTBP) are anchored to the ECM by the LTBP. A recent report by Ge *et al.*[4] describes how BMP-1 cleaves LTBP1 at two positions, leaving its central portion associated with TGF-β/LAP and severing the connection of the large latent complexes to the ECM (Figure 1A). This processing of LTBP1 is required for efficient MMP-2-mediated liberation of TGF-β from LAP. Knockout mice deficient in BMP-1, mTLD, and mTLL1 have greatly increased amounts of large latent complexes associated with the ECM and significantly reduced levels of active TGF-β. One of the many effects of TGF-β is to induce further expression of BMP-1, resulting in positive feedback regulation of TGF-β activity.

Certain TGF-β family members retain non-covalent associations with propeptides following cleavage from the latent protein. For instance, BMP-1 family proteases cleave at single positions within the non-covalently associated GDF-8 and GDF-11 prosegments, resulting in the release of active growth factors (Figure 1B).[5,6] BMP-1, mTLL-1, and mTLL-2 are comparably effective in this activity.[3] Prodomains of GDF-11 with a substitution of the Asp in the cleavage sites can associate with mature GDF-11 and block its activity.[6]

Other BMP homodimers and heterodimers are not secreted in complex with their prosegments, but are held in latent complexes by subsequent association with chordin. Activation of these complexes is achieved by BMP-1 mediated proteolysis at two sites within chordin (Figure 1C).[7] Chordin recognition is conferred by the first CUB domain of BMP-1, as mTLL-2 does not cleave chordin unless its first CUB domain is swapped with that of BMP-1.[8] PCPE-1, which enhances BMP-1 in the removal of the C-terminal propeptide of procollagen, does not affect chordinase activity.[8,9]

The activity of BMP-1 family proteases is required during embryonic development. Genetic knockout results in embryonic or perinatal lethal defects in skull, heart, and abdominal wall formation. In these experiments, the liberation of TGF-β from the large latent complexes is reduced, chordin processing is inefficient, and collagen fibrillogenesis is aberrant.[4,10,11]

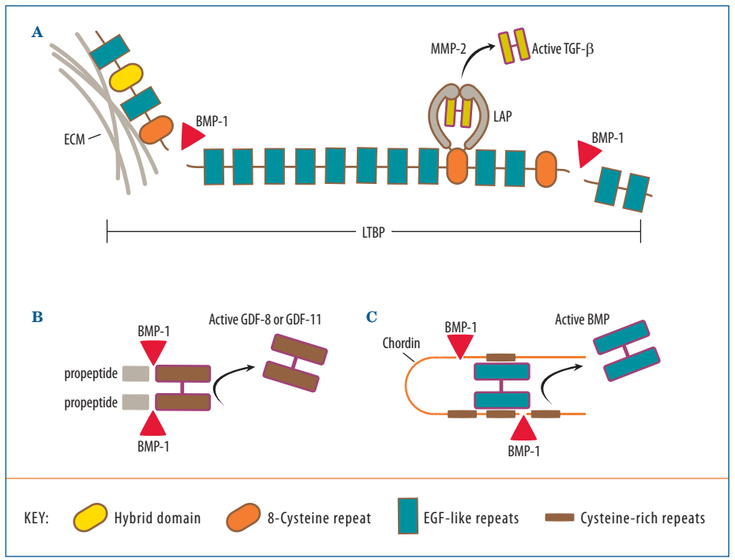

Figure 1. Three mechanisms for BMP-1-related activation of latent TGF-β family complexes. **A:** BMP-1 cleaves LTBP at two positions in a process required for efficient MMP-2-mediated liberation of TGF-β from LAP. **B:** BMP-1 family proteases cleave at single positions within the GDF-8 and GDF-11 propeptides, releasing the active growth factor dimers. **C:** Activation of BMP in complex with chordin is mediated by BMP-1 proteolysis at two sites within chordin. Figures adapted from references 1 and 2.

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Co-receptor Regulation of TGF-β Signaling

Transforming growth factor (TGF)-β superfamily cytokines play integral parts in cell differentiation and proliferation, and their signaling depends on an intricate spatiotemporal orchestration of protein interactions from membrane to nucleus. In general, a TGF-β ligand binds with a heteromeric receptor complex consisting of type I and type II serine/threonine kinase receptors that activate smad-dependent gene transcription.[1] Rather than a type I/type II receptor combination unique to each ligand, the signaling receptor complexes are composed from a finite group of seven type I (activin-like kinases; ALK) and five type II receptors. This redundancy allows for variation in response depending on ligand presence and/or accessibility. Participating in this regulation are co-receptors, proteins that enable, if not promote, ligand-receptor binding (Figure 1).

Betaglycan, also known as TGF-β RIII, associates with and presents TGF-β isoforms to the TGF-β RII (Type II) receptor.[2] Expression of betaglycan increases the sensitivity of TGF-β RII-expressing cells to TGF-β2 and equalizes the affinities across isoforms,[3] thus maximizing TGF-β signaling. Betaglycan also serves as a prerequisite co-receptor for inhibin,[4,5] a molecule known as an activin antagonist. Inhibin (bound to betaglycan) competitively interacts with activin type II receptors without the recruitment and activation of the type I receptor. Considering that both TGF-β and activin initiate the smad-2/3 pathway, betaglycan's role as a co-receptor can lead to opposing effects on smad-2/3-dependent processes. TGF-β signaling specificity is also carried out by endoglin (CD105), a transmembrane glycoprotein expressed on vascular endothelial cells that facilitates TGF-β1/3 binding to TGF-β RII with preferential recruitment of the type I receptor ALK-1.[6] ALK-1 activates smad-1 and indirectly inhibits TGF-β/ALK-5/smad-2/3 signaling.[7] Thus, endoglin appears to moderate a balance between smad-2/3-related cell growth inhibition and smad-1-associated cell proliferation during angiogenesis.[6]

EGF-CFC proteins are essential for vertebrate development by serving as co-receptors for the embryogenesis-related molecules nodal, vitellogenin (Vg)1, and growth differentiation factor (GDF)-1/3.[9-11] The EGF-CFC protein cripto permits nodal signaling by interacting with both ligand (EGF region) and the type I receptor ALK-4 (CFC region) to form a smad-2-activating Act RIIB-ALK 4-nodal-cripto complex. Cripto also can interact with activin/Act RIIB1[2] and TGF-β1/ TGF-β RII[3] complexes, preventing recruitment and activation of their type 1 receptors, ALK-4 and ALK-5, respectively. Cripto's initiation and disruption of TGF-β signaling highlight mechanisms for its multifunctional role in embryogenesis and tumorigenesis.

Recently, members of the repulsive guidance molecule (RGM) family of GPI-anchored proteins have been identified as specific co-receptors for the BMP subfamily.[14-17] Unlike TGF-β2/betaglycan and nodal/cripto, RGM-A-C are not obligate co-receptors for BMP signaling. Rather, they enhance BMP signal transduction through direct interaction with BMP-2/4 and ALK-3/6. This role may provide increased sensitivity to low ligand concentration such as those that may occur in morphogenetic gradients during embryogenesis. This may allow cells to respond at a lower threshold and/or exhibit a greater response. Whether regulation of BMP signaling contributes to RGM-A and RGM-B roles in the developing[18,19] and regenerating[20] central nervous system remains to be determined. However, RGM-C (hemojuvelin), as a BMP co-receptor, has been elegantly linked to iron metabolism *in vivo* and *in vitro*.[17] Further investigation is necessary to elucidate additional roles for the RGM family in TGF-β signaling.

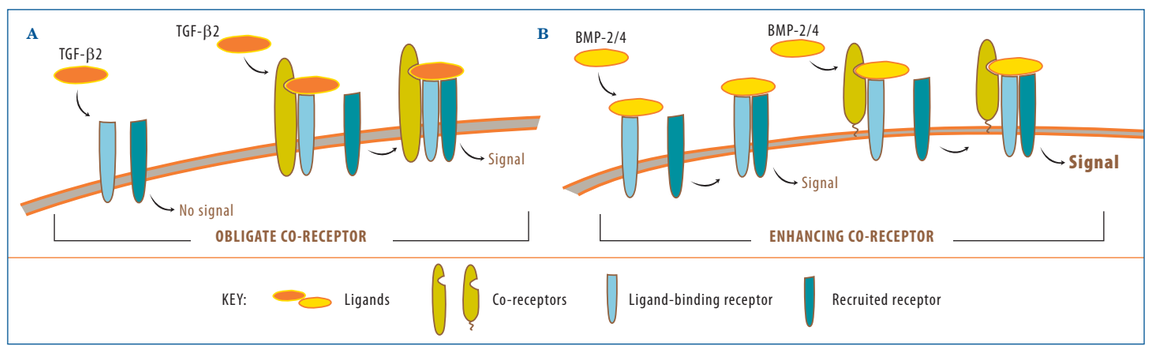

Figure 1. Co-receptors interact with TGF-β ligand and ligand-binding receptor (Type II for TGF-β2 and inhibin, Type I for nodal, BMP) to influence cellular response to ligand. **A:** Obligate co-receptors permit signal transduction by TGF-β2. **B:** Co-receptors for BMP enhance signal, providing increased sensitivity to low BMP concentration.

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The TGF-β Superfamily

The TGF-β superfamily contains molecules that encompass diverse functions during embryogenesis and adult tissue homeostasis. TGF-β ligands are initially synthesized as precursor proteins that undergo proteolytic cleavage. The mature segments form dimers via disulfide links (except for GDF-3, -9, BMP-15, and Lefty), which serve as the active ligand. Although homodimers are considered the standard form, there are natural heterodimers with biological activity. TGF-β ligand binds with a heteromeric receptor complex that consists of type I and type II serine/threonine kinase receptors. Upon phosphorylation by the constitutively-active type II receptor, type I receptor phosphorylates a receptor-activated smad (R-smad) protein initiating a signaling cascade that ultimately alters gene transcription. The type I – type II receptor combination, the activated smad pathway, and the cell-specific transcription factors may offer diversity in responses. Co-receptors and soluble or membrane-bound molecules regulate ligand access to the receptor complex, thereby fine-tuning TGF-β signaling.

Ligand	Type II R	Type I R	R-Smad	Co-Receptor	Regulating Molecules (sharing direct interaction with ligand)	
TGF-β	TGF-β RII	ALK-5 ALK-2 ALK-1	smad2/3 smad1/5/8	Betaglycan (TGF-β2✦) Endoglin (ALK-1 specific) CD109	α_2-macroglobulin BAMBI/NMA♦ biglycan cripto♦ decorin	KCP/Crim 2 LAP s-betaglycan s-TGF-β RII
Activin	Act RII/IIB	ALK-4 ALK-2	smad2/3 smad1/5/8		activin AC/BC/AE/CE BAMBI/NMA♦ cripto DAN FLRG	endoglin follistatin inhibin: betaglycan♦ KCP/Crim 2 s-Act RII/IIB
Inhibin	Act RII/IIB BMP RII/IIB	--	--	Betaglycan✦		
BMP	BMP RII/IIB	ALK-1 ALK-2 ALK-3 ALK-6	smad1/5/8	RGM-A/-B/-C	BAMBI/NMA♦ chordin: Tsg endoglin FLRG follistatin gremlin inhibin: betaglycan♦ KCP/Crim 2	nodal: BMP-7 noggin noggin: SOST PRDC s-ALK-3 SOST USAG-1
	Act RII/IIB	ALK-4 ALK-5 ALK-7 ALK-2	smad2/3 smad1/5/8			
GDF	BMP RII	ALK-5 ALK-6	smad2/3 smad1/5/8	Cripto (GDF-1/3)✦	DAN follistatin propeptide (GDF-8,11)	
	Act RIIB	ALK-4 ALK-5 ALK-6	smad2/3 smad1/5/8			
Nodal	Act RII/IIB	ALK-4 ALK-7	smad2/3	Cripto✦	BMP-7: nodal cerberus	DAN lefty
Lefty	Act RII/IIB	--	--	Cripto (+nodal)✦		
MIS	MIS RII	ALK-2 ALK-3 ALK-6	smad1/5/8			

Note: The GDNF family utilizes a receptor tyrosine kinase (RET) for signal transduction and is not included in the chart above.

Abbreviations:

MIS: Müllerian inhibiting substance
BAMBI: BMP and activin membrane-bound inhibitor
KCP: kielin/chordin-like protein
FLRG: follistatin-related gene
ALK: activin-like kinase
Tsg: twisted gastrulation
PRDC: protein related to DAN and cerberus
SOST: sclerostin
USAG-1: uterine sensitization-associated gene-1

Key: ✦ required ♦ membrane-bound s=soluble

RECENT CITATIONS
GDNF Family & Receptors

MOLECULE	ANTIBODIES			PROTEINS		
	Species	Catalog #	Ref.	Species	Catalog #	Ref.
Artemin	mouse	AF1085	2	mouse	1085-AR	1, 8
GDNF	human	AF-212-NA	4	rat	512-GF	4, 5
Neurturin				mouse	477-MN	8
GFRα-1	human	AF714	3	rat	560-GR	5
	rat	AF560	8, 9			
GFRα-2	human	AF613	3			
	mouse	AF429	6, 8			
GFRα-3	human	AF670	3			
	mouse	AF2645	2, 8			
GFRα-4	mouse	AF1677	7			
Ret	human	AF1485	3	mouse	482-RT	10
	mouse	AF482	7			

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   Sample Type: MiaPaCa2, T3M4, Colo-357, and SU86.86 cells (human pancreatic cancer)
2. Elitt, C. *et al.* (2006) *Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold*. J. Neurosci. **26**:8578.
   Sample Type: Mouse skin, DRG, and spinal cord sections
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   Sample Type: Rat sympathetic neuron lysates
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    Sample Type: Mouse esophagus and colon

Signaling in ES Cells, continued from page 1.

cells when GDF-3 levels are *decreased*. When GDF-3-deficient cells are cultured in the absence of leukemia inhibitory factor (LIF), markers of pluripotency remain high. These results may be explained by the proposed mechanism by which GDF-3 exerts its functions. Levine and Hemmati-Brivanlou present evidence that GDF-3 acts as a BMP antagonist by direct binding to BMP-4.[13] Since, as previously discussed, BMP signals can promote pluripotency in mouse ES cells and can cause differentiation in human ES cells, lower levels of a putative BMP antagonist such as GDF-3 in mouse might enhance pluripotency. Similarly, higher levels of GDF-3 might neutralize BMP actions in human ES cells, again favoring pluripotency over differentiation.

However, GDF-3 presents yet another paradox: a separate study in which GDF-3 null embryos were analyzed suggested that GDF-3 acts as a nodal agonist.[14] In this work, GDF-3 signaling *in vitro* was shown to be dependent on the nodal co-receptor, cripto, and injection experiments in *Xenopus* showed similar effects of nodal and GDF-3.

There are a number of potential explanations for the conflicts, perhaps the most likely of which invoke the capacity of TGF-β superfamily ligands, notably BMPs, to act as morphogens. Because morphogens are well known to exert different effects at different concentrations, variability in activity levels, delivery mechanisms, and experimental systems may be confounding the analyses. Either way, the influence of TGF-β superfamily members on stem cell phenotype is likely to attract considerable future study.

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FREQUENTLY ASKED QUESTIONS
R&D Systems Antibodies

What is the difference between AB##, AF##, BAF##, MAB## and other catalog prefixes for antibodies?

AB designated antibodies are protein G-purified fractions of polyclonal antibody: they contain the total IgG fraction and may include IgG not specific for the antigen. **AF** designated antibodies are protein G-purified fractions that have subsequently been affinity-chromatography purified against the antigen: AF antibodies contain only IgG specific to epitopes on the antigen. Antibodies that have the designation **MAB** are monoclonal antibodies. **BAF** and **BAM** prefixes designate biotinylated versions of the AF and MAB antibodies, respectively. **FAB** and **IC** prefixes indicate fluorochrome-labeled antibodies that are validated for flow cytometry. In particular, IC designates an intracellular flow cytometry application.

What is the molecular weight of IgG?

An IgG protein comprises two heavy chains that are approximately 50 kDa each and two light chains that are approximately 25 kDa each for a total molecular weight of approximately 150 kDa.

What epitope does the antibody recognize?

While we do not epitope map our antibodies, the immunogen used for antibody generation is listed on the technical data sheet. In most cases we use a mature, biologically active protein instead of a peptide to generate highly specific antibodies. This type of immunogen makes epitope mapping difficult.

Why should I reconstitute the antibody in PBS when the data sheet states that it is lyophilized from a PBS solution?

Our antibody production lots are usually highly concentrated and therefore lyophilized from a very small volume of PBS. This additional salt is usually insignificant when diluted to a working concentration in most applications. If the salt concentration is a concern, please contact Technical Service to acquire more information for your particular lot of antibody.

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R&D Systems will support any antibody that has been validated in-house for ICC *or* IHC (frozen or paraffin-embedded sections) regardless of which application is listed on the data sheet. Our technical specialists will work with any customer who encounters difficulties while using the validated antibody in ICC or IHC. Although we cannot guarantee that an antibody will work in all cells and/or tissues under all conditions, we can provide evidence that the antibodies do recognize the fixed antigen. In the event that the customer is unable to achieve successful staining, a product credit will be offered.

How do I decide which antibody is best for my application?

Our website features an Antibody Application field which lists all the validated applications for each antibody offered at R&D Systems. Simply enter your analyte of interest in the search box and click *Go*. After the search results appear, activate the *Antibody Application* check box. You may then refine your search by defining the parameters in the section titled *Narrow results by*. If you see multiple antibodies that may work for your application, please access the technical data sheets by clicking on the catalog number link to determine which antibody is best suited for that application. If you do not find an antibody for your application, please feel free to contact Technical Service. We have thousands of references citing the use of R&D Systems antibodies on file and would be happy to help determine if the application has been demonstrated in the literature.

R&D Systems offers stringent production and rigorous application testing to ensure exceptional quality. Our antibodies are validated for one or more of the following applications:

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TECHNICAL NOTES
The Quantikine® ELISA in Establishing a Role for Endoglin in Preeclampsia

Endoglin/CD105 is a transmembrane co-receptor that facilitates the binding of TGF-β1 or -β3 to TGF-β RII and the recruitment of the type I receptor ALK-1. Endoglin expression is upregulated in endothelial cells and syncytiotrophoblasts during development of the placenta in early pregnancy. Preeclampsia, a condition defined by increased blood pressure and proteinuria, occurs in 5% of pregnancies worldwide. This common and potentially life-threatening complication of pregnancy increasingly appears to be caused by placental insufficiency. Recently, it has been shown that increases in the soluble form of endoglin are exaggerated during and, significantly, prior to the onset of preeclamptic symptoms.[1]

The Endoglin/CD105 Quantikine ELISA Kit (Catalog # DNDG00) is a reliable means for detecting endoglin in biological fluids. Researchers from Harvard Medical School have recently used the kit to demonstrate a two- and three-fold endoglin increase in preterm and term pregnancy, respectively, compared to non-pregnant states.[1] Significantly, mild preeclampsia, severe preeclampsia, and HELLP syndrome (hemolysis, elevated liver enzymes, low platelets) are accompanied by a further three-, five-, and ten-fold increase in circulating endoglin, respectively, as detected by the Quantikine Kit. These elevated endoglin levels closely parallel increases in sVEGF R1/Flt-1 also assessed using R&D Systems Quantikine ELISA Kit (Catalog # DVR100B; Figure 1). The role of the placenta is demonstrated by the rapid decrease in the levels of endoglin 48 hours after delivery (Figure 2).

Serum or plasma placental growth factor (PIGF) and soluble VEGF R1 (sVEGF R1) also increase during normal pregnancy. In preeclampsia, the circulating PIGF increase is attenuated while increases in both sVEGF RI and endoglin are exaggerated. An additional report by the Harvard group has demonstrated that the combination of high sVEGF R1/PIGF ratio with high endoglin gives an adjusted odds ratio greater than 30-fold for the subsequent development of either preterm or term preeclampsia.[2] The combination of the two values is more predictive than either value alone.

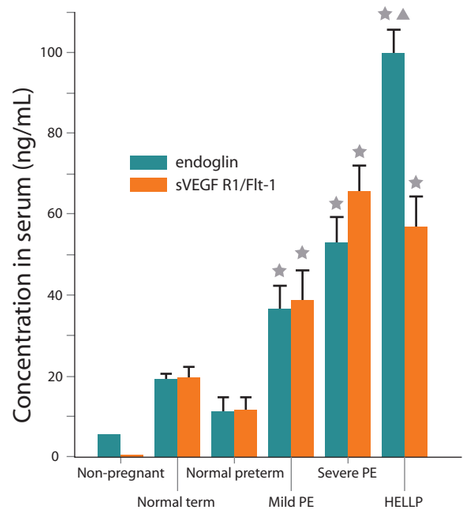

Figure 1. ELISA results for endoglin and sVEGF R1/Flt-1 in sera of individuals with varying degrees of preeclampsia (PE), control pregnancies, and four nonpregnant healthy volunteers. ★P < 0.05 compared to preterm controls, ▲P < 0.05 compared to severe preeclampsia. HELLP; hemolysis, elevated liver enzymes, low platelets. Figure adapted with permission from Nature Medicine.[1]

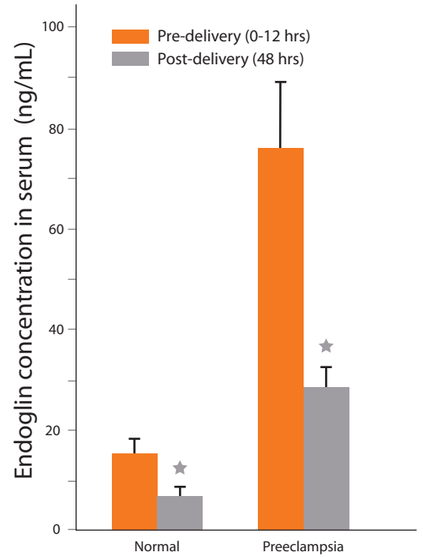

Figure 2. ELISA results for endoglin in a subset of pregnant individuals (normal, n = 6; preeclampsia, n = 11) with blood drawn before (0–12 h) or after (48 h) delivery. ★P < 0.05 as compared to pre-delivery samples. Figure adapted with permission from Nature Medicine.[1]

References

1. Venkatesha, S. *et al.* (2006) Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat. Med. **12**:642.
2. Levine, R. J. *et al.* (2006) Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N. Engl. J. Med. **355**:992.

NEW TOOLS: Cell-Based ELISAs

R&D Systems Cell-Based ELISAs are the first two-color immunoenzymatic assays to permit the simultaneous measurement of both phosphorylated and total proteins in the same microplate well without the lysis of cells. Normalizing the fluorescence signal derived from the phospho-protein to that of the total protein in the same well allows for the accurate correction of well-to-well variabilities such as differences in cell number.

Please see our website at **www.RnDSystems.com/go/CellBasedELISA**

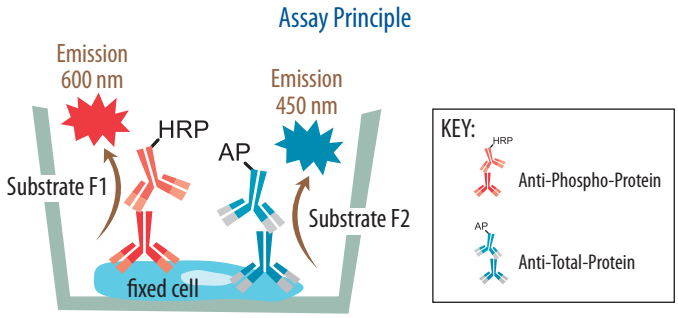

Figure 1. Cells are fixed and first incubated with anti-phospho and anti-total antibodies derived from different species. Alkaline phosphatase (AP)- and horseradish peroxidase (HRP)-conjugated secondary antibodies recognizing the different primary antibodies are then added, followed by incubation with two spectrally-distinct fluorogenic substrates that emit signals corresponding to the levels of phosphorylated and total proteins.

Advantages

> No lysate preparation

> Results with as little as 10,000 cells/well

> Measures total and phospho-proteins simultaneously in the same well

> Amenable to high-throughput screening of kinase inhibitors

> Correlates with Western blot

Current Cell-Based ELISA Kits Available

> Human Phospho-EGF Receptor (Y1068) (Catalog # KCB1095)

> Human Phospho-ERK1/ERK2 (T202/Y204) (Catalog # KCB1018)

> Human Phospho-PDGF Rβ (Y1021) (Catalog # KCB2316)

> Human Phospho-PDGF Rβ (Y751) (Catalog # KCB1767)

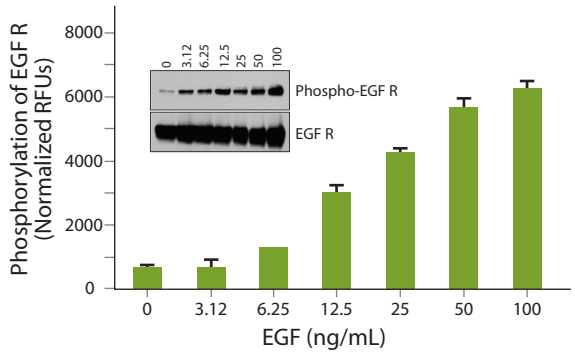

Figure 2. Phosphorylation of EGF R was determined in A431 epidermoid carcinoma cells using R&D Systems human Phospho-EGF R (Y1068) Cell-Based ELISA Kit (Catalog # KCB1095). Cells were cultured in 96-well plates and treated with increasing concentrations of R&D Systems human EGF (Catalog # 236-EG) for 5 minutes. After cell fixation, EGF R phosphorylation was determined and normalized to total EGF R in the same well. Values represent the mean ± the range of duplicate determinations. A comparison Western blot is also shown (inset).

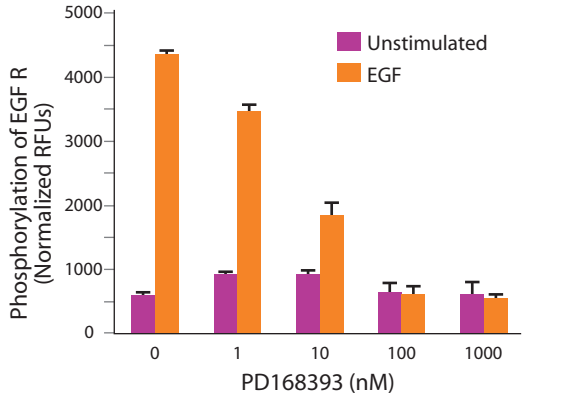

Figure 3. Effects of the EGF R tyrosine kinase inhibitor PD168393 on EGF-induced EGF R phosphorylation were measured using the human Phospho-EGF R (Y1068) Cell-Based ELISA Kit (Catalog # KCB1095). A431 epidermoid carcinoma cells were pretreated for 30 minutes with the indicated concentrations of PD168393. Cells were then incubated with no additions or with 50 ng/mL (5 min) EGF (Catalog # 236-EG) and fixed. Phosphorylation of EFG R was determined and normalized to total EGF R in the same well. Values represent the mean ± the range of duplicate determinations.

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