

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
HUMBERTO RUBIO CAMACHO

ASUNTO	Radicación	09 071 017
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	1 26 061
	Folios	022

Patente de Invención

☒

Patente de Modelo de Utilidad

7

Vía Nacional

1

Via PCT (Fase Nacional)

☒

Capítulo I

Capitolo II

7

No. Solicitud Internacional

PCT/US2007/02586
8

Fecha de Presentación Internacional

18/Diciembre/2007

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 58 a 127	09/Julio/2009
Reivindicaciones:	Folios 128 a 132	09/Julio/2009
Dibujos:	Folios 134 a 147	09/Julio/2009
Listado de secuencias:	Folios 42 a 57	09/Julio/2009
	Folios 172 a 188	21/Enero/2011
Prioridad:	US 60 875 682	18/Diciembre/2006

2. Análisis de la prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Antagonistas de ACTIVINA-ACTRII y usos para aumentar los niveles de glóbulos rojos".

El problema planteado en esta solicitud consiste en la necesidad de proporcionar composiciones alternativas y métodos para aumentar los niveles de glóbulos rojos en pacientes.

Para resolver este problema técnico la solicitud en estudio proporciona un método para aumentar los niveles de glóbulos rojos en un paciente humano, comprendiendo el método administrar a un paciente humano que lo necesite una cantidad eficaz de un polipéptido ActRII.

El objeto de la presente solicitud se relaciona con la clasificación internacional de patentes (CIP): A61K38/17; A61P7/06 y C12N15/62.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 1 – 14 y 16 – 22 no es patentable de acuerdo con el Art citado.

Se aclara al solicitante que a pesar de que la materia de las reivindicaciones 1 a 14 y 16 a 22 no es patentable el estado de la técnica ya anticipaba el objeto de la presente solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO2006012627	ACTRII receptor polypeptides, methods and compositions	02/Febrero/2006
D2	WO2004039948	ACTRIIB fusion polypeptides and uses therefor	13/Mayo/2004
D3	Trends in endocrinology and metabolism. Vol. 16, no. 2.	Antagonists of activin signaling: mechanisms and potential biological applications	1/Marzo/2005
D4	WO2005009460	Pharmaceutical composition comprising ACTIVIN A, ALK-4 or derivatives thereof for the treatment of ophthalmic disorders or cancer	03/Febrero/2005
D5	European Journal of Endocrinology (1998) 139 469–471	Activin: an endocrine or paracrine agent?	Noviembre/1998
D6	Mol Endocrinol. 1996 May;10(5):534-43.	Activin signaling through activin receptor type II causes the cachexia-like symptoms in inhibin-deficient mice.	Mayo/1996
D7	Mol Hum Reprod. 2007 Sep;13(9):675-83	Prevention of cachexia-like syndrome development and reduction of tumor progression in inhibin-deficient mice following administration of a chimeric activin receptor type II-murine Fc protein.	Septiembre/2007

D1
http://worldwide.espacenet.com/searchResults?DB=worldwide.espacenet.com&locale=en_EP&query=WO2006012627&ST=singleline&compact=false revela composiciones farmacéuticas que comprenden los antagonistas de activina-ActRII de la presente solicitud, para uso en promover el crecimiento de tejido y en particular en el tratamiento de caquexia (Reivindicaciones 1-59 y 86-87; pág. 5 línea 20; pág. 6, línea 27).

D2

http://worldwide.espacenet.com/searchResults?DB=worldwide.espacenet.com&locale=en_EP&query=WO2004039948&ST=singleline&compact=false revela un método para el tratamiento o prevención de un desorden degenerativo de un músculo, hueso o homeostasis de la glucosa, el cual comprende administrar una cantidad efectiva de una composición farmacéutica a un mamífero, donde la composición comprende un polipéptido de fusión ActRIIB comprendiendo (a) una primera secuencia de aminoácidos derivada del dominio extracelular de ActRIIB (b) una segunda secuencia de aminoácidos derivada de la porción Fc de un anticuerpo. El documento revela composiciones farmacéuticas que comprenden los antagonistas de activina-ActRII de la presente solicitud (todo el documento).

D3 Revela antagonistas del receptor de Activina tipo II y su uso en el tratamiento de caquexia, cáncer y cicatrización de heridas (Págs. 76-77).

D4

<http://patentscope.wipo.int/search/en/detail.jsf?docId=WO2005009460&recNum=1&maxRec=1&office=&prevFilter=&sortOption=&queryString=FP%3A%28WO2005009460%29&tab=PCTDescription> revela que la Activina A está implicada en numerosas enfermedades, entre ellas cáncer, labor pretérmino, enfermedades inflamatorias, infertilidad, anemias y enfermedades cardiovasculares (Pág. 6, líneas 19-24).

D5 Enseña que la Activina está implicada en funciones tan diversas como inducir la diferenciación de los eritrocitos y que en modelos de ratón transgénico en los cuales la subunidad α de Inhibina fue eliminada, los niveles de Activina se elevaron 10 veces y mediaron síndrome de caquexia por su acción sobre el receptor tipo II, implicando pérdida de peso, anemia, necrosis hepatocelular, letargia y un decrecimiento en el número de células parietales gástricas (Pág. 470, columna de la izquierda).

D6 Menciona la implicación de Activina A y de ActRII en anemia (Pág. 540, columna de la derecha, líneas 2-5).

D7 Enseña que la administración del antagonista de activina ActRII-mFc a ratones deficientes de inhibina previno la pérdida de peso, redujo los niveles de FSH y extendió el periodo de vida en comparación con los ratones control tratados con buffer de fosfato. Adicionalmente, los ratones tratados con ActRII-mFc fueron menos anémicos y sus hígados y estómagos fueron histológicamente normales.

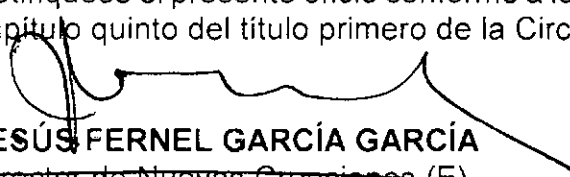
5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de la reivindicación 15 no es patentable, de acuerdo con el Art 14.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JESÚS FERNEL GARCÍA GARCÍA

Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha

Realizado por: Juanita Catalina Rico. 
Revisado por: Consuelo Leguizamón L. 

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
HUMBERTO RUBIO CAMACHO

ASUNTO Radicación 09 071 017
 Trámite 011
 Evento 378
 Actuación 519
 Oficio
 Folios 002

Patente de Invención ☒ Patente de Modelo de Utilidad
Vía Nacional ☐
Vía PCT (Fase Nacional) ☒ Cap. I ☒ Cap. II ☐
No. Solicitud Internacional PCT/US2007/025868
Fecha de Presentación Internacional 18/Diciembre/2007

Como resultado del examen de fondo realizado, con fundamento en el Artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

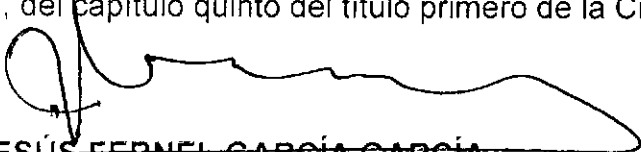
Solicitud de Suministro de Información

De acuerdo con el Art 46 D486, según el cual: "La Oficina Nacional competente podrá requerir el informe de expertos o de organismos científicos o tecnológicos que se consideren idóneos para que emitan opinión sobre la patentabilidad de la invención. Asimismo, cuando lo estime conveniente, podrá requerir informes de otras oficinas de propiedad industrial. De ser necesario, a efectos del examen de patentabilidad y a requerimiento de la oficina nacional competente, el solicitante proporcionará, en un plazo que no excederá de 3 meses, uno o más documentos relativos a una o más solicitudes extranjeras referidas total o parcialmente a la misma invención que se examina: ...b) copia de los resultados de exámenes de novedad o de patentabilidad efectuados respecto a esa solicitud extranjera...Si el solicitante no presentara los documentos requeridos dentro del plazo señalado en el presente artículo la oficina nacional competente denegará la patente"; y considerando que no ha sido posible para esta Oficina encontrar los documentos siguientes, atentamente se requiere su presentación:

Molecular Endocrinology. 1996 May;10(5):534-43. Activin signaling through activin receptor type II causes the cachexia-like symptoms in inhibin-deficient mice.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



~~JESÚS FERNEL GARCÍA GARCÍA~~

Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha

18 JUL. 2012

Realizado por: Juanita Catalina Rico 

Revisado por: Consuelo Leguizamón L. 



Antagonists of activin signaling: mechanisms and potential biological applications

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Activins are members of the transforming growth factor- β (TGF- β) superfamily that control many physiological processes such as cell proliferation and differentiation, immune responses, wound repair and various endocrine activities. Activins elicit these diverse biological responses by signaling via type I and type II receptor serine kinases. Recent studies have revealed details of the roles of inhibin, betaglycan, follistatin and its related protein follistatin-related gene (FLRG), Cripto and BAMBI in antagonizing activin action, and exogenous antagonists against the activin type I (SB-431542 and SB-505124) and type II (activin-M108A) receptors have been developed. Understanding how activin signaling is controlled extracellularly is the first step in providing treatment for wound healing and for disorders such as cachexia and cancer, which result from a deregulated activin pathway.

Introduction

The structurally related proteins of the TGF- β family control diverse cellular processes including cell proliferation, cell death, metabolism, homeostasis, differentiation, immune responses and endocrine function [1–4]. The disruption or deregulated activity of TGF- β superfamily members is often associated with pathological states such as aberrant cellular differentiation, proliferation and metabolism [5,6]. Activins are typical members of the TGF- β superfamily in that they possess a cysteine knot scaffold and are secreted as homo- or heterodimers of related β subunits. Although four β -subunit genes (β A, β B, β C and β E) have been described in human, only dimers of β A and β A (activin A), β B and β B (activin B), and β A and β B (activin AB) have been shown to be biologically active.

Activins signal by interacting with two transmembrane serine/threonine receptor kinases (type I and type II receptors; Table 1). Activin binds initially to its type II receptor, ActRII (for activin A) or ActRIIB (for activin B), which leads to the recruitment, phosphorylation and subsequent activation of the type I receptor, activin-like kinase 4 (ALK4). On activation, ALK4 binds and then phosphorylates a subset of cytoplasmic Smad proteins (Smad2 and Smad3; Table 1), which form part of the

post-receptor signal transduction system [7]. Access of activin to the cell is regulated by various extracellular binding proteins.

In this review, we focus on the recently identified mechanisms of action of the known endogenous activin antagonists. We also discuss recent advances in the development of exogenous activin receptor antagonists and their potential biological applications.

Activin antagonists

Inhibin and betaglycan

The activin β A and β B subunits can heterodimerize with the inhibin α -subunit to form inhibin A (α - β A) and inhibin B (α - β B). It is well established that inhibins are crucial for maintaining normal function in many tissues, particularly those of the reproductive axis. In these tissues, inhibin opposes most, but not all, of the actions of activin. As testimony to the importance of inhibin as an antagonist of activin, mice deficient in the inhibin α -subunit develop gonadal tumors and cachexia with severe weight loss and liver necrosis [8,9].

Inhibin antagonism of activin signaling is dependent on competition for binding to type II activin receptors [10,11]. Gray *et al.* [12] have shown that activin and inhibin share the same binding site on ActRII, supporting the idea that both proteins bind ActRII via their respective β -subunits. The affinity of inhibin for ActRII is, however, about tenfold lower than that of activin [10], and in some tissues and cells inhibin does not antagonize activin, suggesting that additional components are required for inhibin action [13].

Lewis *et al.* [13] recently identified betaglycan (a TGF- β type III receptor) as a co-receptor of inhibin. Betaglycan binds inhibin with high affinity, and this binding affinity increases about 30-fold in cells coexpressing ActRII and betaglycan. Affinity labeling experiments show that inhibin forms complexes with both recombinant and endogenously expressed betaglycan and ActRII, and that ALK4 is excluded from this ternary complex (Figure 1). Notably, expression of betaglycan can confer inhibin responsiveness in AtT20 corticotrope cells, which are normally refractory to this hormone [13].

Recent studies have shown that betaglycan protein and mRNA are expressed in rat brain, pituitary and gonads, supporting the idea that betaglycan has a modulatory role

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Table 1. Signaling pathway of activin and selected members of the TGF- β superfamily

Ligand	Type II receptor	Type I receptor	Co-receptor	Pathway Smad	Common Smad	Inhibitory Smad
Activin A	ActRII	ALK4 (ACTRIB)		Smad2	Smad4	Smad7
Activin B	ActRIIB	ALK7		Smad3		
Activin AB						
Inhibin A	ActRII		Betaglycan			
Inhibin B	ActRIIB					
	BMPRII					
BMP-2	ActRII	ALK2 (ActRI)		Smad1	Smad4	Smad6
BMP-4	ActRIIB	ALK3 (BMPRIA)		Smad5		Smad7
BMP-7	BMPRII	ALK6 (BMPRIIB)		Smad8		
TGF- β 1	TGF β RII	ALK5 (TGF β RI)	Betaglycan	Smad2	Smad4	Smad7
TGF- β 2		ALK1 (TSR1)		Smad3		
TGF- β 3						
Myostatin	ActRII	ALK4 (ACTRIB)		Smad2	Smad4	
	ActRIIB	ALK5 (TGF β RI)		Smad3		
Nodal	ActRII	ALK4 (ACTRIB)	Cripto	Smad2	Smad4	
	ActRIIB	ALK7		Smad3		

Abbreviation: TSR1, TGF- β superfamily receptor type 1.

on inhibin effects in these tissues [14,15]. Because betaglycan-null mice die from lethal proliferative defects in the heart and apoptosis in the liver soon after embryonic day 13.5, however, it has been difficult to confirm that betaglycan has an important role in inhibin action *in vivo* [16]. The field awaits the development of conditional knockout mice that could be used to focus on blocking betaglycan expression in pituitary gonadotropes or selected gonadal cell types.

Follistatin

Follistatin binds activin with high affinity (50–500 pM) to form biologically inactive complexes, thereby regulating processes as diverse as cell growth and the secretion of follicle-stimulating hormone (FSH) [17,18]. Follistatin consists of a 63-residue amino (N)-terminal segment, followed by three successive follistatin domains of 73–75

amino acids containing ten cysteine residues termed FS-1, FS-2 and FS-3. Alternative splicing generates two follistatin isoforms: FS288, which binds heparan sulfate proteoglycans with high affinity via residues in FS-1 [19] and is considered to be a local regulator of activin action; and FS315, which does not bind cell-surface proteoglycans and is the predominant circulating form of the protein.

In addition to its involvement in binding to the cell surface, FS-1 is also essential for suppressing activin biological activity. Keutmann *et al.* [20] have shown that deletion of FS-1 or the adjacent FS-2 abolishes activin binding, whereas deletion of FS-3 is tolerated. This finding complements earlier studies [21] that established that essential determinants for activin binding are located in the 63-residue N-terminal region that precedes the follistatin domains.

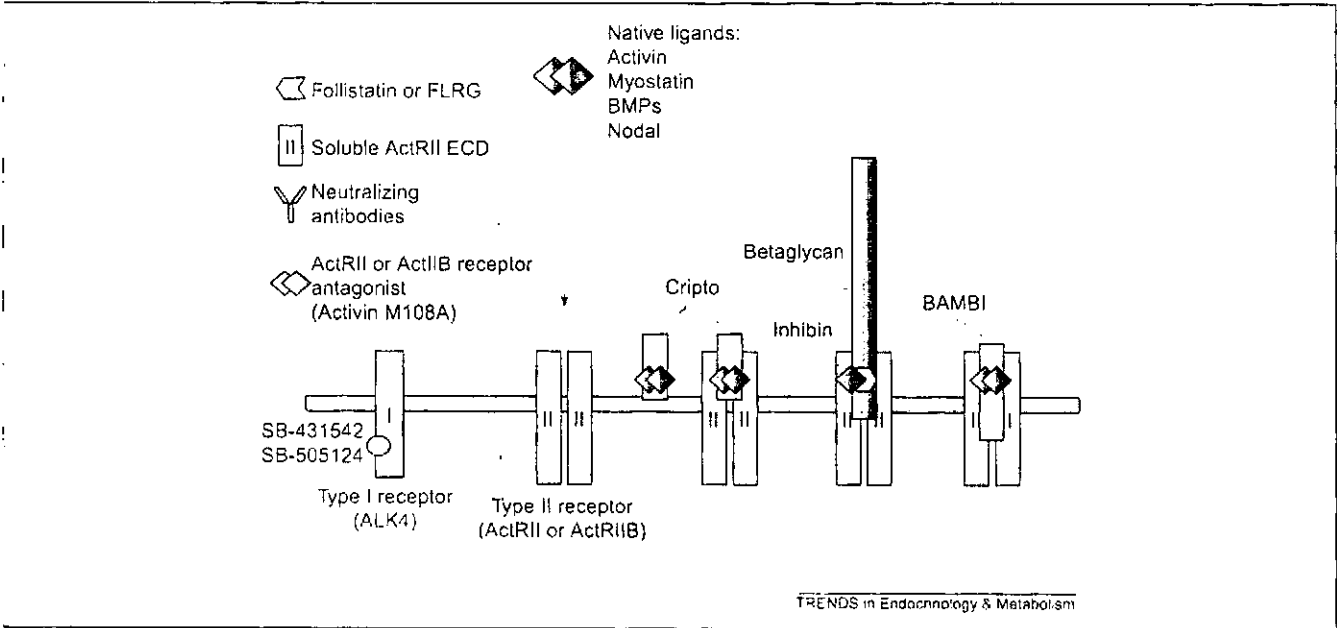


Figure 1. Model of antagonism of signaling by activin and related ligands. Activin, myostatin, Nodal and BMPs signal via ActRII or ActRIIB. The access of these TGF- β superfamily members to their type II receptors is blocked by extracellular binding proteins (follistatin and FLRG), membrane-bound pseudoreceptors (BAMBI and Cripto; for activin only, Cripto actually facilitates Nodal signaling) and structurally related molecules (inhibin and betaglycan). Recent studies using an antagonist of the ActRII and ActRIIB receptor (activin M108A), small molecule inhibitors of ALK4 (SB-431542 and SB-505124), neutralizing antibodies and the soluble ActRII ECD suggest that it will be possible to achieve pharmacological blockade of activin.

Surprisingly less is known about the regions of activin involved in follistatin binding. Fischer *et al.* [22] have recently shown that an activin A deletion mutant, termed *dest85-109-activin A*, shows low affinity for both receptors and follistatin, suggesting that this region either is directly involved in follistatin binding or lies close to the binding interface. Binding of follistatin to this region would mask residues that are important in the interaction of activin with both ActRII (Ser90, Leu92 and Lys102) and ALK4 (Met91, Ile105 and Met108) [23–25] (Figure 2).

Notably, follistatin seems to have an affinity for activin B that is about ten times lower than its affinity for activin A, suggesting that the determinants for follistatin binding differ between the two isoforms of activin [26]. Further mutagenesis studies are required to elucidate the activin–follistatin interaction in more detail. The generation of an activin mutant that retains high affinity for its receptor but has compromised follistatin binding would act as a superagonist and clearly would have clinical relevance.

Follistatin-related gene

Follistatin-related gene (FLRG), also known as follistatin-related protein or follistatin-like 3, is a recently characterized member of the follistatin family that binds activin with relatively high affinity (~850 pM) [27]. FLRG differs from follistatin in lacking the third follistatin domain and a consensus heparin-binding sequence. In this regard, FLRG is thought to act as a circulating activin-binding protein, much like FS315. Outside the 26 conserved cysteine residues, murine FLRG and follistatin share about 40% amino acid identity. Given that both proteins bind and neutralize activin, it seems likely that the

residues that are important for activin binding are located in these conserved regions.

Intriguingly, expression and regulation studies suggest that follistatin and FLRG might not be complete functional homologs. FLRG is highly expressed in placenta, testis, skin and cardiovascular tissue, whereas follistatin expression is considerably higher in pituitary and ovary [19]. Wankell *et al.* [28] have shown that follistatin and FLRG are both expressed during the wound healing process, but their distribution within the wound differs and distinct factors regulate their expression.

Cripto

Nodal, a member of the TGF- β superfamily that is involved in specification of the anterior–posterior and left–right body axes [29,30], has been shown to signal via ActRII or ActRIIB and ALK4. Unlike activins, however, Nodal requires additional co-receptors from the epidermal growth factor, Cripto, FRL-1 and Cryptic (EGF-CFC) protein family, such as Cripto, to assemble its signaling receptor complex [31,32]. Cripto independently binds Nodal via its EGF-like domain and ALK4 via its CFC domain [33] to promote Nodal signaling.

Whereas expression of Nodal is predominantly restricted to the embryo, Cripto is highly overexpressed in breast, pancreatic, colon and ovarian carcinomas [34], raising the possibility that Cripto might regulate the activity of other TGF- β superfamily members in malignant adult tissues [35]. Because activins have been shown to be tumor suppressors for breast, liver and kidney cells [36], two groups have recently examined the effects of Cripto on activin signaling. Gray *et al.* [37] and Adkins *et al.* [35] have demonstrated that activin signaling can be

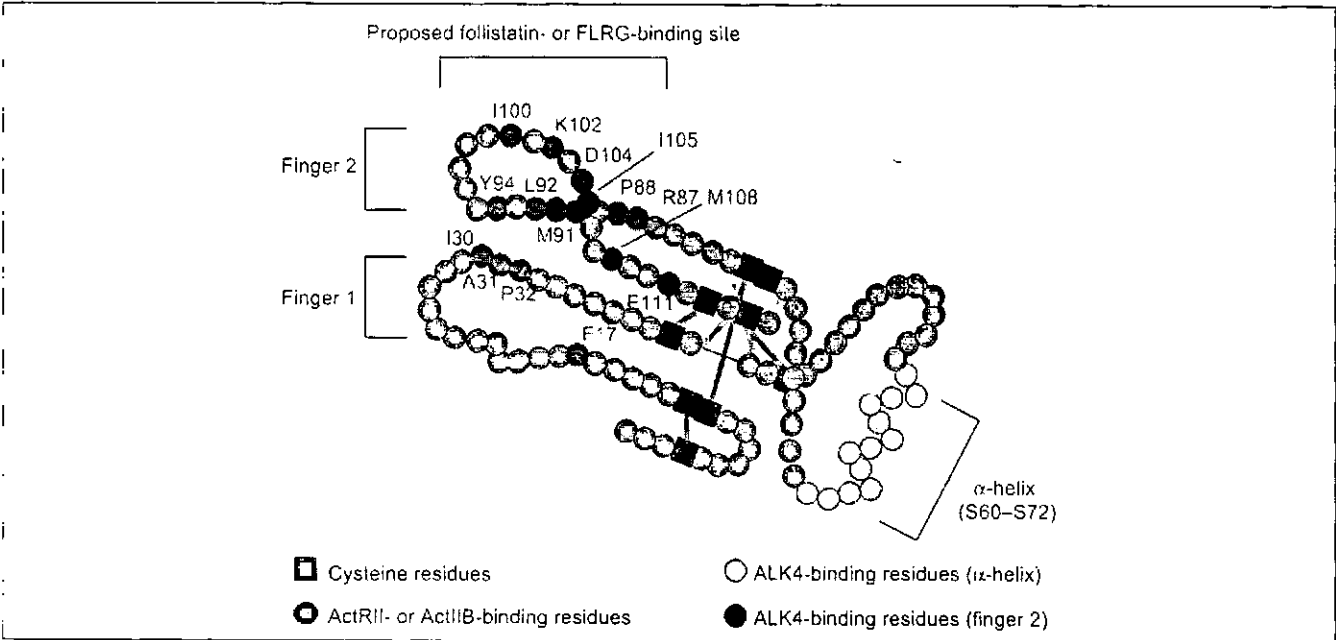


Figure 2. Diagram of the activin-A dimer, highlighting residues involved in interactions with its receptors and binding proteins. Activin contains a cystine-knot fold and a single disulfide bond that covalently connects the two chains of the dimer. In each monomer, two pairs of anti-parallel β -sheets stretch out from the cystine-knot core to form a short (finger 1) and long finger (finger 2). Residues present at the ActRII or ActRIIB interface (red) cluster in these finger regions. In the activin dimer, residues from both the long α -helix (green) and the concave surface of finger 2 (dark blue) have been proposed to form the ALK4-binding site. The important methionine residue at position 108 and the proposed follistatin-binding domain (residues 85–109) are indicated. The residues involved in the interaction between activin with Cripto are unknown. Figure adapted, with permission, from Ref. [61].

blocked by overexpression of Cripto in numerous cell lines (Figure 1).

Gray *et al.* [37] did not detect activin binding to Cripto in the absence of ActRII or ActRIIB, suggesting that activin, unlike Nodal, requires type II receptors to bind Cripto. Their results support a mechanism in which Cripto binding to the activin-ActRII (or activin-ActRIIB) complex inhibits subsequent recruitment of the signaling receptor ALK4. By contrast, Adkins *et al.* [35] demonstrated that soluble Cripto and activin B interact directly, but they did not detect an interaction between Cripto and activin A. In their model, Adkins *et al.* [35] propose that the independent binding of Cripto to ALK4 or activin B, or the formation of a complex of activin B, ALK4 and Cripto, is sufficient to inhibit activin signaling.

Although further experiments are required to decipher the molecular mechanisms of Cripto function, the work of these two groups [35,37] has clearly identified a novel extracellular regulator of activin signaling.

BAMBI

The transmembrane protein BAMBI, named after *Xenopus* bone morphogenetic protein (BMP) and activin membrane-bound inhibitor, has sequence similarity to type I receptors of the TGF- β superfamily (53% similarity to *Xenopus* ALK3) but lacks the intracellular kinase domain required for signaling [38]. BAMBI inhibits signaling by TGF- β superfamily ligands by stably associating, in a ligand-independent manner, with both type I and type II receptors [38]. It has been suggested that the action of BAMBI might be relevant to limiting the signaling range of BMPs and activins, which act as morphogens during early embryogenesis [38].

A homolog of BAMBI, referred to as non-metastatic gene A (*nma*), has been identified in mouse, rat, human and zebrafish. Inhibition of growth in response to TGF- β is restored in several human gastric carcinoma cell lines after treatment with *nma* antisense oligonucleotides [39]. This finding suggests that *nma* might play an important role by providing cancer cells with an escape mechanism from TGF- β -mediated growth control.

Recently, Loveland *et al.* [40] carried out an extensive analysis of BAMBI expression in juvenile and adult rat tissue, with a particular focus on reproductive organs. They demonstrated that the expression of BAMBI mRNA is regulated in male germ cells, a finding that correlates well with their previous observation that a decrease in activin activity has a role in gonocyte differentiation [40]. Interestingly, the absence of its mRNA in adult smooth muscle and its very low levels in the liver suggest that BAMBI does not regulate the growth inhibitory actions of myostatin and activin, respectively, in these tissues.

Activin type II receptor antagonist

Above, we have described the endogenous mechanisms whereby the activin signaling cascade can be modulated. There is, however, a growing need for the development and use of exogenous antagonists that can block activin signaling.

To this end, we and our co-workers [25] recently used a mutagenesis strategy to identify activin A mutants that could bind ActRII but not ALK4, and that would therefore

act as type II activin receptor antagonists. We found that the most important residues for activin biological activity and binding to ALK4 are residues Met91, Ile105 and Met108 on the concave face of finger 2 (Figure 2). These residues, in combination with Tyr35, form a pocket on the surface of activin A that presumably accommodates one or more of the hydrophobic residues on ALK4 (Leu40, Ile70, Val73, Leu75 and Pro77) that are known to be involved in activin binding [41].

Of the activin mutants generated, the Met108Ala variant showed the lowest signaling activity while retaining wild-type-like affinity for ActRII [25]. Unlike activin A, the Met108Ala mutant was unable to form a crosslinked complex with ALK4 in the presence of ActRII, indicating that its ability to bind ALK4 was compromised. These data suggested that the Met108Ala mutant might be capable of modulating the signaling of activin and related ligands that use ActRII or ActRIIB. Indeed, signaling in 293T cells in response to activin and myostatin, but not to TGF- β , was reduced in a dose-dependent manner by the Met108Ala variant, consistent with its proposed action as a selective antagonist of type II activin receptors [25].

Future studies should focus on the *in vivo* activity of the Met108Ala activin mutant. Would overexpression of Met108Ala in muscle lead to antagonism of myostatin and an increase in muscle mass? In a liver regeneration model, would treatment with Met108Ala block activin-induced growth suppression?

Activin type I receptor antagonist

Small-molecule inhibitors have the potential to be used as therapeutics to block signaling pathways that contribute to human diseases. Thus, there is considerable interest in two recently developed competitive inhibitors of the ATP-binding site of ALK5 (a TGF- β type I receptor) [42,43]. These molecules, called SB-431542 and SB-505124, also inhibit ALK4 and ALK7, which share similarity with ALK5 in their kinase domains (Figure 1). They have no effect on ALK1-, ALK2-, ALK3- or ALK6-induced Smad signaling, however, or on the activities of numerous other protein kinases, suggesting that they have a remarkable level of specificity [42,43].

Since its initial characterization, SB-431542 has been shown to inhibit both the TGF- β -induced proliferation of human osteosarcoma cells [44] and the TGF- β -induced promotion of malignant glioma cell proliferation, angiogenesis and motility [45]. It will be interesting to assess the effect of these small-molecule inhibitors in activin-dependent systems.

Neutralizing antibodies and the soluble ActRII extracellular domain

In addition to the recently characterized type I and type II receptor antagonists, several groups have used the extracellular domain (ECD) of ActRII and neutralizing antibodies to block activin actions (Figure 1). The ActRII ECD contains all of the structural determinants necessary for high-affinity ligand binding [46]. Treatment of cultured rat anterior pituitary cells with the ActRII ECD [46] or adenovirus-mediated overexpression of the ActRII ECD [47] attenuates secretion of

FSH in response to exogenous activin A or endogenous activin B. Similarly, antibodies specific for activin A [48] or activin B [49] block the activin-induced stimulation of FSH secretion from pituitary cells and the induction of erythrocyte differentiation.

Potential biological applications of activin antagonism

Cachexia

Mice deficient in the inhibin α -subunit develop sex-cord stromal tumors at an early age [8]. Tumor development is associated with a cachexia-like wasting syndrome, characterized by severe weight loss, hepatocellular necrosis around the central vein, and depletion of the parietal cells in the glandular stomach [9]. In inhibin-deficient mice with tumors, activins are increased tenfold in the serum. Coerver *et al.* [50] have shown that it is the increase in levels of activin signaling via ActRII that is responsible for the cachexia symptoms in these mice.

Notably, the related ligand myostatin has been also implicated in the induction of cachexia. Myostatin is expressed almost exclusively in cells of the skeletal muscle lineage and signals via activin type II receptors (Table 1). Zimmers *et al.* [51] have shown that systemic overexpression of myostatin in adult mice induces profound muscle and fat loss analogous to that seen in human cachexia syndromes. These studies suggest that activin and myostatin might be useful pharmacological targets for treating cachexia. Indeed, overexpression of follistatin has been shown to slow both activin- and myostatin-induced weight loss [51,52].

Cancer

As mentioned above, targeted deletion of the inhibin α -subunit in mice results in sex-cord stromal tumors at an early age [8]. It seems that the increase in activin in these mice is at least partially responsible for tumor progression, if not development [50,52]. When the inhibin-deficient mice are crossed with mice overexpressing follistatin, serum activin levels are lower and, although the mice still develop gonadal tumors, they do so at a significantly slower rate [52]. These studies suggest that modulating the levels of activin might have beneficial effects on the progression of gonadal tumors.

The benefits of blocking activin action in malignant tissues, however, are likely to depend on the cell context. Activin actually inhibits growth in many types of cell and acts as a tumor suppressor in the early stages of tumorigenesis. Many tumor cells escape the growth inhibitory effect of activin by acquiring mutations in activin receptors [53] or in Smad signaling molecules [54]. Thus, in most tissues activin inhibits the development of cancer and blocking its action would be detrimental. However, activin induces a proliferative response in fibroblasts, keratinocytes and additional gonadal cell types [55] and, in the absence of antagonists, it could be pro-oncogenic in these tissues.

Wound healing

Cutaneous wound repair is a complex process involving blood clotting, inflammation, new tissue formation and finally tissue remodeling. A series of papers by Werner and colleagues [56–58] has implicated activin in these wound

healing processes. Activin is strongly induced in granulation tissue and in suprabasal keratinocytes of the hyperproliferative epithelium after skin injury [56]. This finding led Munz *et al.* [57] to generate transgenic mice overexpressing the activin β A chain in the epidermis. After skin injury, histological characterization of the wounds revealed a marked increase in the amount of granulation tissue in the wounds of the transgenic mice. Conversely, overexpression of follistatin in the epidermis led to a significant delay in wound healing and a reduction in the formation of scar tissue [58].

These studies show that the levels of activin present in wound tissue are crucial for the outcome of the repair process. Thus, overexpression of activin might be useful for promoting wound healing, whereas inhibition of activin action by receptor antagonists or follistatin could prevent the excessive matrix deposition observed in human skin diseases characterized by either fibrosis or epithelial hyperproliferation.

Concluding remarks

There is currently great interest in developing clinical applications for members of the TGF- β superfamily and their antagonists. For example, recombinant human BMP2 has been tested successfully in trials for the treatment of open tibial fractures [59]. In addition, topical application of TGF- β 3 seems to be safe and beneficial in the treatment of pressure ulcers and is most effective at the earliest stages of therapy [60].

Clinical trials using activin or activin antagonists have yet to commence; however, the efficacy of these factors in animal disease models [51,52,57,58] suggests that they might also prove beneficial in treating human diseases. Follistatin and the activin Met108Ala receptor antagonist are particularly attractive drug candidates. Both are capable of differentially antagonizing several ligands of the TGF- β superfamily and thus could be useful therapeutic agents in tissue-specific settings.

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INVITED COMMENTARY

Activin: an endocrine or paracrine agent?

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Activin and inhibin are members of the transforming growth factor- β (TGF β) superfamily of proteins. Activins are protein dimers composed of two β subunits, including activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$) and activin AB ($\beta_A\beta_B$), whereas inhibin is a heterodimer composed of an α subunit in combination with one of the same β subunits to produce inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$) (1). Both activin and inhibin were initially isolated from follicular fluid and identified based on their opposing actions on follicle-stimulating hormone (FSH) secretion in pituitary cell cultures: inhibin inhibits whereas activin stimulates FSH release (1). More importantly, activin may be the primary physiological regulator of FSH, as demonstrated in a pituitary perfusion system (2). In this system, activin stimulates FSH β mRNA 50-fold compared with 3-fold stimulation by gonadotropin-releasing hormone (GnRH) treatment. Interestingly, this dramatic impact was not appreciated when activin was tested in plated cells, presumably because activin accumulation from local gonadotrope production unmasked the effect of exogenous activin (2). An unrelated glycoprotein hormone, follistatin, has also been isolated from follicular fluid and found to inhibit FSH secretion in an analogous fashion to inhibin. However, studies suggest that follistatin elicits its action solely through binding and neutralizing activin (3).

Systemic infusions of exogenous activin A in non-human primates (4, 5) result in a modest increase in circulating FSH, consistent with a possible endocrine role for activin. Direct studies are not yet possible in the human, however, and indirect models are required to examine the relationship between activin and FSH. The aging human is one such model, and is studied in this issue of *European Journal of Endocrinology* by Loria *et al.* (6). A selective increase in FSH secretion occurs across early reproductive aging in females and this increase in FSH occurs more consistently than the increase in luteinizing hormone in males over the age of 50 (7), suggesting a factor other than GnRH, i.e. activin or inhibin, elicits the selective FSH increase. Activin levels have been examined in this model using assays that utilize detergents to separate activin from follistatin and other binding proteins, thus measuring total activin A and not the free or biologically available form (8, 9). Total activin A levels are higher in the midfollicular phase of older compared with younger women of

reproductive age (10). However, follistatin in excess of activin (i.e. free follistatin) was detectable at low but similar levels in the two groups suggesting that all circulating activin is bound to follistatin. A larger study found an increase in total activin A (9) across a broad range of ages in males and females. The increase was accompanied by a parallel increase in follistatin (9), a significant portion of which was free follistatin, again suggesting that circulating activin is bound. In this issue, Loria and colleagues demonstrate an increase in total activin A levels across aging in males and an age-specific increase in females, using the largest number of subjects to date (6). They also show that this increase in circulating activin does not correlate with FSH levels. In contrast, the age-related increase in FSH is associated with a decrease in circulating inhibin in males and females (10–16), and inhibin levels correlate inversely with FSH (11, 13). Thus, the indirect model provides evidence that inhibin plays an endocrine role in the control of FSH in the aging human, whereas an endocrine role for activin is not apparent from these studies.

The results of Loria *et al.* and others are consistent with several lines of evidence which also argue against an endocrine role for activin in the reproductive axis. First, the broad expression pattern of activin β subunits in tissues as diverse as placenta, bone marrow, brain and endothelium (17) is unusual for the classic model of an endocrine hormone. Secondly, follistatin is coexpressed in the majority of activin-producing tissues (18) and binds activin with high affinity and low reversibility under physiological conditions (19) suggesting that tight local regulation results in little biologically available activin in circulating stores. Further, follistatin circulates in molar concentrations in excess of circulating activin as discussed above. Therefore, it appears that the entire circulating pool of activin is bound to follistatin and hence not biologically active under most circumstances. Finally, activin levels are measurable in the greatest quantities in normal humans during the third trimester of pregnancy, a time when circulating FSH levels are suppressed (20). Thus, the circumstantial evidence suggests that the primary role of circulating activin is not endocrine-mediated stimulation of FSH in the human.

Further, the increase in activin A which Loria *et al.* document across aging does not appear to be related to

gonadal changes. In females, activin is expressed and secreted by the granulosa cells; however, total activin A levels are similar in premenopausal, postmenopausal (6, 21) and in castrate women (21). In males, activin subunits are expressed in the Sertoli and Leydig cells (22) and one might expect a decrease rather than an increase in activin based on age-related changes. In addition, it is not clear why the increase in circulating activin A levels occurs only in males, with the exception of the slight increase in activin A in women between the ages of 40 and 50 seen in this study (6). One could postulate that testosterone suppresses activin and that the increase in circulating activin is related to the fall in testosterone levels with age (7) or that the fluctuating estradiol levels in the perimenopause are required to stimulate activin, but neither of these hypotheses is satisfying. Since both the β_A and β_B subunits are found in prostate epithelium (23) it is possible that the increase in circulating activin A results from prostatic hypertrophy in males. It is also possible that a portion of circulating activin may derive from the pituitary and that activin is transiently elevated during the time of greatest fluctuations in FSH levels, such as during the perimenopause. Regardless of the cause for these gender differences in activin levels with aging, the evidence to date leads away from the gonad as the primary source of circulating activin and contributions from other sources must be sought.

Activin β_A expression has also been demonstrated in multiple organs including the bone, bone marrow, adrenal, pancreas, pituitary, skin and even in atherosclerotic plaques (24). In addition, activin has been implicated in functions as diverse as inducing erythrocyte differentiation, increasing monocyte migration, stimulating insulin and growth hormone secretion, and enhancing osteoblast proliferation (18). Therefore, the increase in circulating activin across aging likely reflects the sum of changes at the local level in these tissues and may be analogous to the age-related changes in other cytokines such as interferon- α , interleukin-2 and TGF β (25). Activin could then be added to this list of cytokines which have been postulated to play a role in multiple manifestations of aging, from impaired immune function and wound healing to insulin resistance, decreased muscle mass and osteoporosis.

Studies have also demonstrated increased activin levels in patients with renal failure, liver disease and solid tumors (9, 21). Whether activin is responsible for any of the clinical symptoms in these disease states is not clear. However, in transgenic mouse models in which the α subunit of inhibin has been deleted, activin levels are elevated 10 fold and mediate a cachexia syndrome through their action at the activin type II receptor, involving weight loss, anemia, hepatocellular necrosis, lethargy and a decrease in gastric parietal cell number (26). The cachexia syndrome seen in these α inhibin knockout mice may be analogous to cancer-related cachexia syndromes in humans (26). It is

possible that the much less dramatic elevations in activin demonstrated by Loria *et al.* (6) may be responsible for an infinitely milder but similar syndrome in the aging human. However, it seems more likely that the accompanying increase in follistatin demonstrated in some studies (9) would prevent these consequences. Certainly these toxic effects do not occur in the third trimester of pregnancy when activin reaches the highest levels measurable under physiological conditions, possibly due to the accompanying increase in follistatin which renders it inactive (20).

The study by Loria and colleagues (6) provides convincing circumstantial evidence against an endocrine role for activin. It also adds further evidence that gonadally derived activin does not constitute the majority of circulating levels. But the study raises as many questions as it answers. It is clear that follistatin, activin B, and free activin levels must also be evaluated in the same population to assess whether the increase in activin represents biologically available activin and is truly gender specific. It will also be necessary to administer activin directly to humans to analyze its physiological effects. Fortunately, the work in this field is progressing rapidly, and with new assays being developed every day the answers will not be long in coming.

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Prevention of cachexia-like syndrome development and reduction of tumor progression in inhibin-deficient mice following administration of a chimeric activin receptor type II-murine Fc protein

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Inhibin is a secreted tumor suppressor, and inhibin α null mice develop gonadal sex cord-stromal tumors with 100% penetrance at an early age. Inhibin-deficient mice die of a severe wasting syndrome due to increased activin signaling through activin receptor type II. The current study was designed to assess the *in vivo* effects of an activin antagonist, a chimeric activin receptor type II fused to the Fc region of a murine IgG2a (ActRII-mFc), administered transiently to the inhibin-deficient mice. Results showed that the severe weight loss was prevented in the ActRII-mFc-treated mice, FSH levels were reduced, and an extended life span was observed for these mice compared with phosphate-buffered saline-treated controls. Although ActRII-mFc treatment did not seem to prevent the formation of gonadal tumors, tumors were smaller in the majority of experimentally treated mice and were characterized by the presence of variable numbers and sizes of cysts in contrast to the solid hemorrhagic tumors that typically developed in the controls. Moreover, the ActRII-mFc-treated mice were less anemic, and their livers and stomachs were histologically normal. In summary, this study demonstrated that *in vivo* administration of the activin antagonist, ActRII-mFc, not only prevents the cachexia-like symptoms in the inhibin-deficient mouse model, but also reduces tumor progression. These results support an essential role of activins in the cachexia-like syndrome development and implicate activins as growth-promoting factors in gonadal tumor progression. The current findings have potential implications in the design of new drugs or strategies for the treatment of ovarian and testicular tumors and other conditions where ligands signal through ActRII.

Keywords: activin receptor type II; inhibin; cachexia; tumor

Introduction

Activins and inhibins are members of the TGF- β superfamily that comprises a large group of structurally related polypeptide growth factors essential in normal physiologic and developmental processes (Massague, 1998; Pangas and Woodruff, 2000; Chang *et al.*, 2002; Pangas *et al.*, 2002; Brown *et al.*, 2003; Burdette *et al.*, 2005). Activins are homodimers and heterodimers of disulfide-linked β subunits that include activin A (β A: β A), activin B (β B: β B) and activin AB (β A: β B) (Risbridger *et al.*, 2001). Three additional activin β subunits (β C, β D and β E) have been recently identified (Oda *et al.*, 1995; Fang *et al.*, 1996; Lau *et al.*, 1996; Schmitt *et al.*, 1996). Although activin β C and β E are primarily expressed in adult liver (Fang *et al.*, 1997), they are not essential for liver function (Lau *et al.*, 2000). In contrast, activin β D was characterized in *Xenopus laevis* and represents a new dorsal mesoderm-inducing factor (Oda *et al.*, 1995). Activins signal through their type II receptors (ActRII and ActRIIB) and type I receptor (ActRI), leading to the phosphorylation and activation of the receptor-regulated SMADs (R-SMADs: SMAD2 and SMAD3)

(Mathews and Vale, 1991; Massague, 1992, 1998; Attisano *et al.*, 1993; Mathews, 1994; Matzuk *et al.*, 1995c; Derynck and Zhang, 2003). A heteromeric complex is formed between R-SMADs and the common SMAD (Co-SMAD: SMAD4), which then translocates to the nucleus to regulate activin-responsive gene transcription (Moustakas *et al.*, 2001; Chang *et al.*, 2002; Massague, 2000; Mehra and Wrana, 2002). In contrast, inhibins are heterodimers of disulfide-linked subunits of α and β [inhibin A (α : β A) and inhibin B (α : β B)]. In many cell types, inhibins have counter-regulatory roles to activins, acting as activin receptor antagonists (Xu *et al.*, 1995).

Although the initial role of activins and inhibins was found in the regulation of FSH secretion, where activins promote and inhibins inhibit FSH production (Pangas and Woodruff, 2000), additional diverse roles of activins and inhibins in cell proliferation and differentiation have been identified (Brown *et al.*, 2003). In contrast to their postulated roles in mesoderm induction in *X. laevis* (Smith *et al.*, 1990) and fish (Wittbrodt and Rosa, 1994), activins are not essential for mesoderm formation in mice (Matzuk *et al.*, 1995c). Instead, an

obligatory role of activins during craniofacial development has been unraveled in mammals (Matzuk *et al.*, 1995a,c). Our previous studies identified inhibin as a secreted tumor suppressor specific to the gonads by using embryonic stem (ES) cell technology to functionally inactivate inhibin α (*Inha*) *in vivo* (Matzuk *et al.*, 1992). Mice homozygous for the *Inha* null allele develop gonadal sex cord-stromal tumors at an early age with 100% penetrance (Matzuk *et al.*, 1992). The inhibin-deficient mice have elevated activin levels in the serum (Matzuk *et al.*, 1994, 1996) as well as overexpressed activin β A mRNA in the testis (Trudeau *et al.*, 1994). These mice succumb to a cachexia-like syndrome (Matzuk *et al.*, 1994), the primary cause of lethality. Activin signaling through ActRII directly causes the wasting syndrome since the cachexia-like symptoms were minimized in double homozygous mutant mice lacking both inhibin and ActRII (Coerver *et al.*, 1996).

The genetic studies described above provide powerful evidence that the activin–ActRII signaling axis is a critical mediator of many of the activin-induced pathological changes in the inhibin-deficient mice (e.g. depletion of gastric parietal cells and hepatocellular necrosis around the central vein). Nonetheless, these studies do not necessarily differentiate between effects that originate in the developing embryo and those effects that are subject to activin regulation in the postnatal animal and therefore amenable to treatment. We thus attempted to design a study by using an activin antagonist to probe the *in vivo* effects of activins on the development of the cachexia-like syndrome and gonadal tumors in inhibin-deficient mice. As previously reported, truncation of the serine/threonine kinase cytoplasmic domain of the ActRII is able to inhibit activin signaling in *Xenopus* (Hemmati-Briandou and Melton, 1992; De Winter *et al.*, 1996). Furthermore, the extracellular domain (ECD) of ActRII has the intrinsic ability to bind activins with high affinity and is functionally active in competing with cellular ActRII (Donaldson *et al.*, 1999; del Re *et al.*, 2004). Therefore, we selected a chimeric ActRII fused to the Fc region of murine IgG2a (ActRII-mFc) to use as an activin antagonist for the administration to inhibin deficient mice.

In the current study, we found that treatment with ActRII-mFc protected inhibin deficient mice from suffering the severe weight loss and early death by preventing the pathological changes in the livers and glandular stomachs observed in control mice. Furthermore, previously unidentified roles of activin signaling in gonadal tumor development in the absence of inhibin were revealed.

Materials and Methods

Gene targeting and generation of inhibin α (*Inha*) knockout mice

Deletion of the inhibin α gene in ES cells and generation of *Inha* null mice were described previously (Matzuk *et al.*, 1992). Mice were maintained on a mixed C57BL/6J⁺129SvEv genetic background. All animals used in this study were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Genotyping of *Inha* null mice was conducted by Southern blot (Matzuk *et al.*, 1992) using genomic tail DNA.

Construction, production and administration of ActRII-mFc

ActRII-mFc was constructed by joining the ECD of ActRII to the Fc region of a murine IgG2a and was generated by Acceleron Pharma, Inc., Cambridge, MA, USA. The ActRII-mFc protein has very high affinity (K_d 2×10^{-10} M) for activin A (unpublished data) (del Re *et al.*, 2004). Administration of ActRII-mFc to inhibin-deficient mice was as follows: (i) ActRII-mFc-treated group (experimental group): inhibin-deficient mice at 3 weeks of age ($n = 9$ for male and $n = 11$ for female) were injected with ActRII-mFc intraperitoneally (i.p.) (10 mg/kg body weight) in phosphate-buffered saline (PBS) twice a week; (ii) PBS-treated controls: inhibin-deficient mice ($n = 14$ per sex) were injected i.p. with 100 μ l of PBS at 3 weeks of age following the same injection schedule as the ActRII-mFc-treated group.

Body weights and survival rate

Body weights were measured and recorded twice a week for a period of 3–12 weeks (males) or 3–19 weeks (females), and the development of cachexia-like symptoms (weight loss, sunken-eye appearance, lethargy, pale periphery, kyphoscoliosis, etc.) was closely monitored (Matzuk *et al.*, 1994). Animals were sacrificed for further studies (morphology, histology, hormonal assays, etc.) when overt signs of wasting syndrome developed (i.e. body weight loss of $\geq 20\%$ of peak body weight, kyphoscoliosis, severe lethargy, etc.) or at 12 weeks in males or 19 weeks in females.

Histological analysis

Ovaries/testes, liver and stomach were collected from mice at the time of sacrifice and fixed in 10% neutral buffered formalin (NBF) overnight. Samples were then transferred to 70% ethanol and further embedded and sectioned for hematoxylin and eosin (liver and stomach) or periodic acid Schiff-hematoxylin (ovary and testis) staining using standard procedures of the Pathology Core Services Facility at Baylor College of Medicine.

Hematocrit analysis

Briefly, periorbital blood from isoflurane-anesthetized mice was collected into heparinized microhematocrit capillary tubes (Baxter Healthcare Corp., Deerfield, IL, USA) followed by 15 min spinning in a microhematocrit centrifuge. The value was then read on a microcapillary reader and recorded.

Serum activin and FSH analyses

Cardiac puncture was performed to collect blood samples from mice anesthetized by isoflurane inhalation at the time of sacrifice. Blood was allowed to clot at room temperature in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Serum was then separated by centrifugation and kept at -20°C until assayed. Total activin A was measured using a specific enzyme-linked immunosorbent assay (Knight *et al.*, 1996) according to the manufacturer's instructions (Oxford Bio-Innovations, Oxfordshire, UK) with modifications as described previously (O'Connor *et al.*, 1999). The average intra-plate coefficient of variation (CV) was 5.1% and the inter-plate CV was 6.6% ($n = 2$ plates). The limit of detection for activin A was 10 pg/ml.

Serum FSH was analysed by the Ligand Assay and Analysis Core at the Center for Research in Reproduction, University of Virginia. The limit of detection for FSH was 2 ng/ml (detailed information is at <http://www.healthsystem.virginia.edu/internet/err/ligand.cfm>).

Statistical analysis

Data were expressed as mean $\pm$ SEM and a Student's *t*-test was used to analyse the difference of means (testis/ovary/liver weights, hematocrit, FSH and activin A) between treatment (ActRII-mFc-treated) and control (PBS-injected) groups. Statistical significance was reported at $P < 0.05$.

Results

Cachexia-like symptoms were prevented and survival increased in inhibin-deficient mice treated with ActRII-mFc protein

Weight loss in inhibin-deficient mice starts after ~ 6 –7 weeks of age accompanied by several cachectic symptoms (i.e. dorsal kyphoscoliosis, a sunken-eye appearance, pale periphery, lethargy, etc.) due to increased activin signaling (Matzuk *et al.*, 1994). As expected, the above cachectic symptoms including early weight loss were observed in PBS-treated *Inha* null mice of both sexes (Fig. 1). However, ActRII-mFc treatment prevented the dramatic weight loss in both males (Fig. 1A) and females (Fig. 1B) during the respective 12- and 19-week experimental period. The survival curve showed that 71% (10/14) of PBS-treated inhibin-deficient male mice died by 12 weeks of age (9.5–12 weeks) due to the development of the cachexia-like syndrome, whereas 100% (9/9) of the ActRII-mFc-treated males survived past 12 weeks (Fig. 2A). In the PBS-treated inhibin-deficient

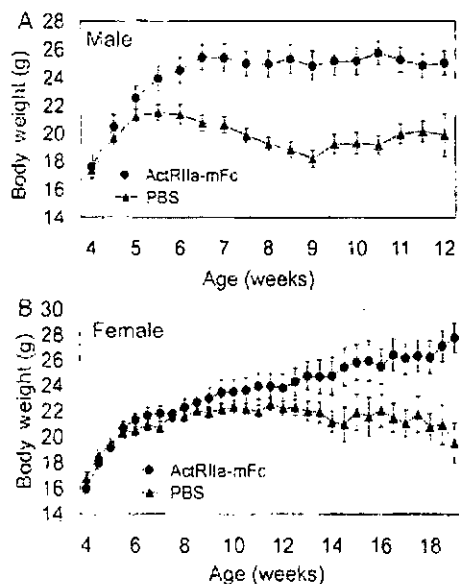


Figure 1: Weight curves of inhibin-deficient mice treated with ActRII-mFc and PBS. Weight loss was observed in PBS-treated males (A; $n = 14$) and females (B; $n = 14$) lacking inhibin, whereas no significant weight loss was observed in both male (A; $n = 9$) and female (B; $n = 11$) inhibin null mice following ActRII-mFc treatment during the respective 12- and 19-week experimental period. Data are shown as mean $\pm$ SEM.

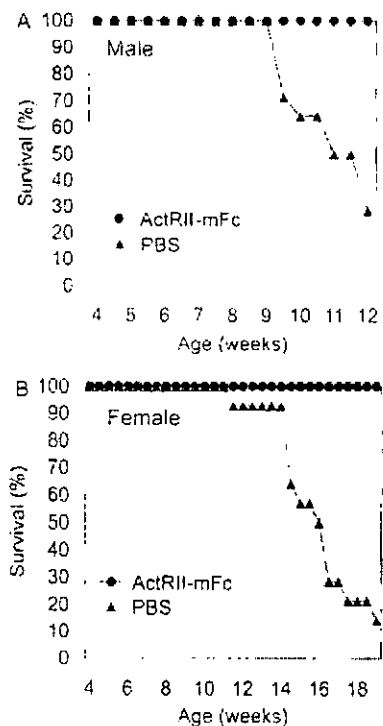


Figure 2: Survival curves of inhibin-deficient mice treated with PBS and ActRII-mFc. Mice that developed overt cachexia-like symptoms were sacrificed and mice were censored weekly to calculate the survival rate. Note the substantially extended life span of the inhibin-deficient mice treated with ActRII-mFc compared with PBS-treated controls. In the inhibin-deficient males (A), ~70% of PBS-treated mice ($n = 14$) died by 12 weeks of age, whereas all of the ActRII-mFc-treated males ($n = 9$) survived past 12 weeks. In the PBS-treated inhibin-deficient females (B; $n = 14$), ~80% died by 19 weeks of age, whereas all of the ActRII-mFc-treated female mice ($n = 11$) survived beyond 19 weeks.

female mice, 86% (12/14) died before 19 weeks of age (11.5–19 weeks), whereas 100% (11/11) of the ActRII-mFc-treated females survived beyond 19 weeks (Fig. 2B). Therefore, ActRII-mFc injection prevented the severe weight loss and substantially extended the life span of inhibin-deficient mice.

Reduction of tumor progression in inhibin-deficient mice following ActRII-mFc administration

To determine if the increased survival in ActRII-mFc-treated mice was caused by the attenuation of the inhibin-deficient tumor phenotype, gonads were examined grossly and histologically at the end time-points when both male (12 weeks) and female (19 weeks) mice were sacrificed for further analyses. In the inhibin-deficient males, although ActRII-mFc administration did not prevent testicular tumor formation, a pronounced effect of ActRII-mFc treatment on tumor growth/progression was found. Overall, the testicular tumors that developed in ActRII-mFc-treated mice were smaller than those in PBS-treated controls, as evidenced by comparison of average testis weights of ActRII-mFc-treated mice (12 weeks) with PBS controls (9.5–12 weeks) (Fig. 3F; $P < 0.05$) as well as analysis of testis histology (Fig. 3B–D). Morphological and histological analyses showed that PBS-treated control mice developed large, solid and hemorrhagic tumors as expected (Fig. 3A). In contrast, smaller testicular tumors were found in all of the experimental mice examined, and more seminiferous tubules were present in these testes, although with a block in spermatogenesis (Fig. 3B–D). Interestingly, testes from the majority of ActRII-mFc-treated mice appeared to be more cystic (Fig. 3C), and cysts were usually found in both testes with variable sizes and numbers.

In the females, tumor formation and development in the experimental mice appeared to be limited in some mice; there were minimal tumor foci in the ovaries which continued to be comprised of follicles and corpora lutea as well as cysts (Fig. 4A–C). In some experimental mice, ovarian follicles and corpora lutea continued to be present despite the development of solid tumors (Fig. 4D). In contrast, normal ovarian architecture was inevitably destroyed and replaced by tumors in the PBS-treated inhibin-deficient mice (Fig. 4E). However, exceptions were found in two ActRII-mFc-treated females that developed large hemorrhagic tumors (Fig. 4E), histologically indistinguishable from those observed in the PBS-treated control mice (Fig. 4F).

ActRII-mFc treatment abolished the activin-induced pathological changes in the livers and glandular stomachs of inhibin null mice

Based on the evidence that infusion of recombinant activin A caused similar liver and stomach pathology as that of inhibin-deficient mice which have highly elevated activin levels (Schwall *et al.*, 1993; Hully *et al.*, 1994), we expected that treatment of inhibin-deficient mice with ActRII-mFc would lead to normal morphology/histology of the livers and stomachs. Depletion of gastric parietal cells and hepatocellular death around the central vein are two histological hallmarks of cachectic inhibin-deficient mice (Matzuk *et al.*, 1994). In the PBS-treated control mice, the above pathological features were apparent in the livers (Fig. 5A: hepatocellular death and lymphocytic infiltration around the central vein) and stomachs (Fig. 5C: mucosal atrophy and depletion of parietal cells). However, ActRII-mFc treatment prevented these pathological changes in the livers (Fig. 5B) and stomachs (Fig. 5D) of inhibin-deficient mice of both sexes. Consistent with the normal liver histology, the weights of livers from ActRII-mFc-treated mice (12 weeks for males and 19 weeks for females) were increased

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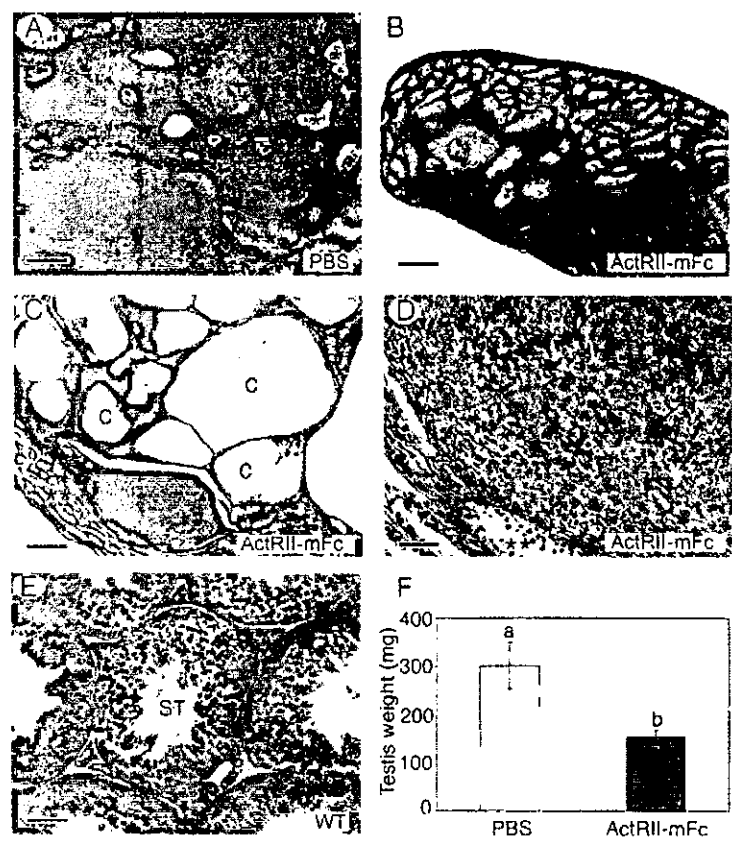


Figure 3: Histology of testicular tumors and testis weights from inhibin-deficient mice treated with PBS or ActRII-mFc. (A) Histopathology of a gonadal sex cord-stromal tumor from a 12-week-old inhibin-deficient male treated with PBS. Solid tumors (arrow heads) with hemorrhage were formed in the control mice. Note the presence of a few seminiferous tubules (**) that were depleted of germ cells. (B) Histopathology of a gonadal sex cord-stromal tumor from a 12-week-old inhibin-deficient male treated with ActRII-mFc. Note the overall testis size is small with more seminiferous tubules (**) present. A small tumor can be found within the testis (arrow head). (C) Histopathology of a gonadal sex cord-stromal tumor from a 12-week-old inhibin-deficient male treated with ActRII-mFc. Note the presence of seminiferous tubules (**), multiple cysts (c) and a small tumor (arrow head) within the testis. (D) High power view of the tumor (arrow head) shown in (C). Note the depletion of germ cells in the tubules (**). (E) Histology of testis from a 12-week-old WT male. Note the normal tubule structure and spermatogenesis. (F) Comparison of the testis weights between the PBS (9.5–12 weeks; $n = 12$) and ActRII-mFc-treated (12 weeks; $n = 5$) inhibin-deficient male mice. Data are shown as mean $\pm$ SEM, and bars without a common letter are significantly different at $P < 0.05$. Scale bars: A, 200 μ m; B and C, 100 μ m; D and E, 50 μ m.

compared with the PBS-treated controls (9.5–12 weeks for males and 11.5–19 weeks for females) (Fig. 5E and F; $P < 0.01$).

Hematocrit analysis

In addition to the severe weight loss and the pathological lesions in the liver and stomach, inhibin-deficient mice become anemic at the advanced cachectic stage of tumor development (Matzuk *et al.*, 1994, 1996). Hematocrit analysis showed that the inhibin-deficient female mice ($n = 7$) treated with ActRII-mFc had higher hematocrit compared with mice ($n = 9$) injected with PBS alone ($44.6 \pm 3.2\%$ versus $28.5 \pm 1.1\%$; $P < 0.01$). The hematocrit of inhibin-deficient male mice treated with ActRII-mFc ($38.7 \pm 3.2\%$; $n = 3$) was also higher compared with that of the cachectic inhibin-deficient mice at 7–12 weeks of age ($28.8 \pm 2.6\%$; $n = 7$) (Matzuk *et al.*, 1994).

Serum activin and FSH levels following ActRII-mFc treatment

Inhibins and activins are critical regulators of pituitary FSH production, and mice deficient in inhibin have elevated serum levels of activins (Matzuk *et al.*, 1994; Coerver *et al.*, 1996) and FSH (Matzuk *et al.*, 1992). In the current study, serum activin A was

measured to examine the potential effect of ActRII-mFc treatment on circulating activin levels (Fig. 6A). The results showed that PBS-treated inhibin-deficient mice have a dramatic elevation of serum activin A levels (24.55 ± 3.52 ng/ml for males and 35.31 ± 4.20 ng/ml for females) over adult WT males (0.05 ± 0.01 ng/ml) and females (0.04 ± 0.00 ng/ml) (Li *et al.*, 2007). Significantly higher serum total activin A levels were observed in ActRII-mFc-treated male (12 weeks; $P < 0.01$) and female (19 weeks; $P < 0.05$) mice compared with PBS-treated controls (9.5–12 weeks for males and 11.5–19 weeks for females), presumably due to the accumulation of circulating activin–ActRII-mFc complexes in the serum (Fig. 7). However, as an indication of potential attenuation of free/functional activins in the circulation, serum FSH was markedly reduced in the ActRII-mFc-treated males (12 weeks) in comparison with PBS-treated controls (9.5–12 weeks; $P < 0.01$), although statistical significance was not achieved in the experimental females (19 weeks; $P > 0.05$), due to low animal numbers and variations in the control group (11.5–19 weeks; Fig. 6B).

Discussion

Inhibin-deficient mice develop focally invasive sex cord-stromal tumors (Matzuk *et al.*, 1992), and the death was primarily attributed

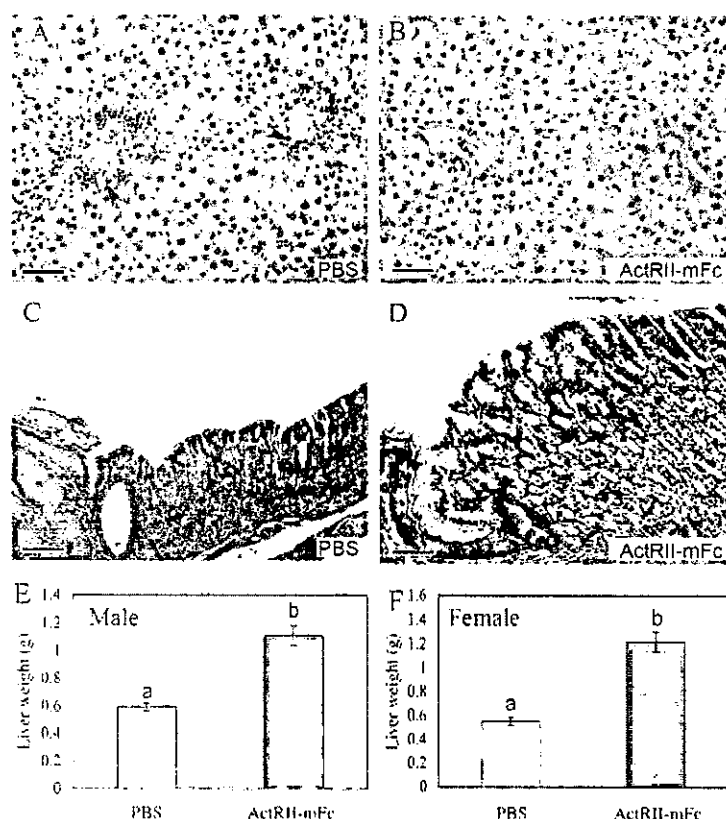


Figure 5: Analyses of livers and stomachs from inhibin-deficient mice treated with PBS or ActRII-mFc. (A) Histopathology of livers from a 15-week-old inhibin-deficient, PBS-treated female that has sex cord-stromal tumors. Focal hepatocellular death around the central vein and lymphocytic infiltration are noted (arrow heads). (B) Histology of livers from a 19-week-old inhibin-deficient female treated with ActRII-mFc. No pathological changes, including no hepatocellular necrosis, are noted and the architecture and cytology of the livers are basically normal. (C) Glandular stomach from a 10-week-old inhibin-deficient male treated with PBS. Note the mucosal atrophy and depletion of parietal cells in the glandular region (**). (D) Glandular stomach from a 12-week-old inhibin-deficient male mouse treated with ActRII-mFc. Note that the large, eosinophilic parietal cells are normally present in the glandular region (**). For orientation and comparison, the areas photographed in (C) and (D) show the junction between the squamous epithelium of forestomach (arrow head) and the glandular region (***) and were photographed at the same magnification ($\times 10$). (E), (F) Comparison of the liver weights from inhibin-deficient mice following PBS ($n = 14$ for males (9.5–12 weeks of age) and $n = 12$ for females (11.5–19 weeks of age)) and ActRII-mFc ($n = 8$ for males (12 weeks of age) and $n = 11$ for females (19 weeks of age)) treatment. Note the higher liver weights in the ActRII-mFc-treated mice of both sexes compared with the PBS-injected controls (E and F). Data are shown as mean $\pm$ SEM, and bars without a common letter are significantly different at $P < 0.01$. Scale bars: A and B, 50 μ m; C and D, 100 μ m.

roles of activins in gonadal tumorigenesis remain unknown. A number of studies have identified an antiproliferative role of activins in extra-gonadal cancer cell lines (Burdette *et al.*, 2005; Wang *et al.*, 1996) as well as tumor cells from a variety of organs including thyroid (Matsuo *et al.*, 2006) and adrenal (Beuschlein *et al.*, 2003) glands. In contrast, results of the current study provide novel *in vivo* evidence supporting a role of activins in stimulating gonadal tumor development. Although our results do not provide compelling evidence that activins are essential for tumor formation in inhibin deficient mice (since tumors are still present in most of the ActRII-mFc-treated mice), they do support the finding that activins act as tumor growth factors by showing that treatment of inhibin-deficient mice with ActRII-mFc appeared to reduce/suppress the tumor progression. Reduced testis weight and tumor size/foet as well as more testicular cysts were phenotypic characteristics of ActRII-mFc-treated males. Although more variable effects of ActRII-mFc injection were observed on the tumor development in the females, the majority of experimental female mice also had smaller tumors or demonstrated minimal tumor foet in their ovaries with variable numbers of cysts. Evidence corroborating the hypothesis that elevated activin levels positively affecting tumor growth and development also stems from *in vitro* studies indicating that activins function as autocrine factors in

stimulating the proliferation of gonadal tumor cell lines derived from inhibin-deficient mice (Shikone *et al.*, 1994) as well as ovarian clear cell adenocarcinoma cells (Mabuchi *et al.*, 2006).

The current findings are consistent with two of our genetic studies demonstrating that tumor development was compromised in inhibin-deficient mice with reduced/suppressed activins (Kumar *et al.*, 1999; Cipriano *et al.*, 2000). One study showed that gain-of-function transgenic mice overexpressing follistatin develop less aggressive tumors and have low circulating activin levels due to local binding of follistatin to activins (Cipriano *et al.*, 2000). In the other case, mice deficient in FSH have dramatically reduced activins in the inhibin-deficient background. These mice demonstrated minimal/milder cachexia-like symptoms and developed slower-growing tumors (Kumar *et al.*, 1999). Interestingly, FSH levels were decreased in inhibin deficient mice following ActRII-mFc treatment in the present study. However, the significance of reduction of FSH in the tumor development of ActRII-mFc-treated inhibin-deficient mice is not clear. More recently, we found that *Inha* and *Smad3* double knock-out males are protected from early tumorigenesis and associated cachectic symptoms, and SMAD3 deficiency also reduces ovarian tumorigenesis but to a much milder degree in comparison to males (Li *et al.*, 2007). Collectively, the above results suggest that elevated

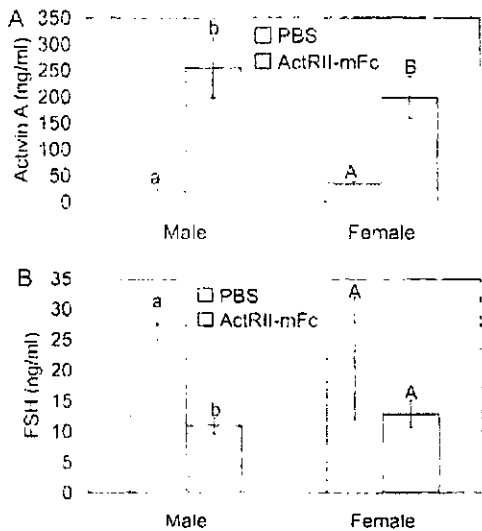


Figure 6: Serum activin A and FSH levels in inhibin-deficient mice treated with PBS or ActRII-mFc. (A) Comparison of serum activin A levels between the PBS ($n = 13$ for males (9.5–12 wks of age) and $n = 4$ for females (11.5–19 wks of age)) and ActRII-mFc-treated ($n = 8$ for males (12 wks of age) and $n = 7$ for females (19 wks of age)) inhibin-deficient mice. (B) Comparison of serum FSH between the PBS ($n = 10$ for males (9.5–12 wks of age) and $n = 5$ for females (11.5–19 wks of age)) and ActRII-mFc-treated ($n = 9$ for males (12 wks of age) and $n = 8$ for females (19 wks of age)) inhibin-deficient mice. Data are shown as mean $\pm$ SEM, and bars without a common letter (lower case for males and upper case for females) are significantly different at $P < 0.01$ (males) or $P < 0.05$ (females).

activins contribute to the fast growing and aggressive nature of gonadal tumors in inhibin-deficient mice. The mechanisms of tumor development and the downstream signaling pathways and proteins that mediate activin action *in vivo*, including the potentially redundant roles of SMAD2 and SMAD3 in the ovary, await further investigation. Analysis of activin levels in the present study was hampered by potential accumulation of activin–ActRII-mFc complexes. Activin levels are expected to decrease in inhibin-deficient mice treated with

ActRII-mFc, since ActRII can bind activins, and phagocytosis will be initiated by an interaction between the Fc region of the immunoglobulin and receptors primarily presented on effector cells (macrophages, monocytes, etc). However, higher serum total activin A levels were observed in ActRII-mFc-treated mice compared with PBS-treated controls. Of note, while formation of the activin–ActRII-mFc complex and subsequent phagocytosis reduce free activin levels, it might simultaneously stimulate the secretion of activin via a negative feedback mechanism (see Fig. 7 for details). The efficiency of ActRII-mFc clearance might also be compromised due to the binding of activins to the chimeric receptor as well as to potentially reduced phagocytosis in inhibin-deficient mice via unknown mechanisms. Therefore, the high serum activin A levels in ActRII-mFc-treated mice may reflect the accumulation of circulating nonfunctional activin–ActRII-mFc complex. As indirect evidence supporting the reduction of functional/free activins following ActRII-mFc treatment, serum FSH was markedly decreased in the experimental males, although statistical significance was not achieved in the females due to low animal numbers and variations in the control group.

Interestingly, our previous *ActRII* knockout mouse model revealed that absence of activin signaling through ActRII in the gonads has a minimal effect on the development of gonadal sex cord-stromal tumors *in vivo* (Coerver *et al.*, 1996). The seemingly anomalous results may be explained by the fact that both ActRII and ActRIIB are present in a variety of reproductive tissues including ovary and testis (Matzuk and Bradley, 1992; Wu *et al.*, 1994). Thus activin signaling through ActRIIB or other unidentified receptors in the gonads may contribute to mediate the tumor-promoting effect of activins in *ActRII* and *Inha* double knockout mice.

In summary, using the inhibin-deficient mouse as a model, we show that administration of a chimeric ActRII-mFc protein *in vivo* reduced tumor growth/progression and protected inhibin-deficient mice from suffering severe weight loss via preventing the pathological changes in livers and stomachs. These findings have important potential clinical implications in designing new drugs/strategies in alleviating or preventing the cachexia-like syndrome and gonadal tumor development as well as in alleviating other conditions in which TGF β superfamily signaling through ActRII causes disease in humans.

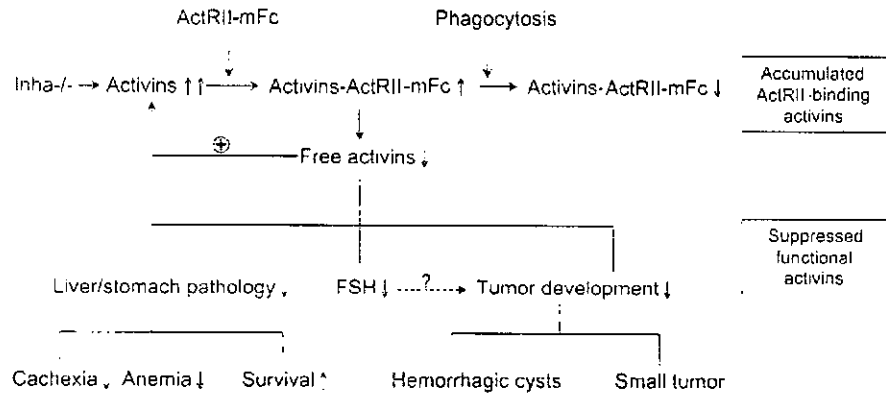


Figure 7: Hypothetical schematic of circulating activin changes in inhibin-deficient mice following ActRII-mFc administration and the effects on the cachexia-like symptoms and tumor development. Activin levels are elevated in the absence of inhibin. ActRII-mFc will bind free activin to form activin–ActRII-mFc complexes in the circulation, thus reducing free activin levels. Reduction of free activin levels may simultaneously stimulate the secretion of activins via a negative feedback mechanism. On one hand, more complexes may be formed due to the accelerated secretion of activins, leading to suppressed functional activins (free activins) in the circulation. On the other hand, the activin complex can be cleared through phagocytosis, the efficiency of which depends on that of the immune system. Consequently, total activin (free and bound) levels may be increased due to the accumulation of activins–ActRII-mFc complexes. Protection of ActRII-mFc-treated inhibin-deficient mice from the cachexia-like syndrome and the relatively normal liver and glandular stomachs are supposed to be the results of suppression of functional activin in the circulation, as indicated by the reduced FSH levels in the ActRII-mFc-treated male mice. Reduction of functional activins may also contribute to the slower-growing and less hemorrhagic tumors observed in ActRII-mFc-treated inhibin-deficient mice versus PBS-treated controls.

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