

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
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ASUNTO	Radicación	09-034961
	Trámite	11
	Evento	378
	Actuación	519
	Oficio	
	Folios	8

15860

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☒
		Capítulo II	☐
No. Solicitud Internacional	PCT/US2007/077845		
Fecha de Presentación Internacional	07/Septiembre/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 193 a 330	3/Abril/2009
Reivindicaciones:	Folios 331 a 336	3/Abril/2009
Dibujos:	Folios 338 a 399	3/Abril/2009
Listado de Secuencias:	Folios 400 a 609	3/Abril/2009
Prioridad:	US 60/825,063	8/Septiembre/2006
	US 60/951,175	20/Julio/2007

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 Wnt y su uso en el diagnóstico y tratamiento de trastornos mediados por Wnt".

Expediente 09-034961 1er Art 45

El problema planteado en esta solicitud consiste en la necesidad de suministrar un antagonista de Wnt con estabilidad *in vivo* mejorada para el tratamiento de trastornos mediados por Wnt.

Para resolver este problema técnico la solicitud en estudio proporciona un antagonista de Wnt que comprende el dominio Frizzled y un dominio Fc.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 38/17; C07K 14/705; C12N 15/62; G01N 33/50.

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

El objeto de las reivindicaciones 25 – 43 no es patentable de acuerdo con el Art citado.

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

El uso de las reivindicaciones 44 – 51 no es patentable, de acuerdo con el Art 14.

6. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque hace referencia a un uso, lo cual no se considera patentable, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1, 13 – 16, 20 y 21 utilizan las expresiones “un componente del dominio Frizzled”, “un dominio Fc”, “Fc es derivado a partir de una inmunoglobulina (...)”, “un enlazador”, “una secuencia de ácido nucleico que codifica (...)”, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda al solicitante que una polipéptido y un ácido nucleico se caracterizan por su secuencia de aminoácidos y polinucleótidos, respectivamente.

Las reivindicaciones 1 – 4 utilizan las expresiones “donde el antagonista de Wnt es activo *in vivo* por al menos 1 hora”, “donde el antagonista de Wnt es activo *in vivo* por al menos 5 horas”, “donde el antagonista de Wnt es activo *in vivo* por al menos 1 hora”, “donde el antagonista de Wnt tiene una vida media *in vivo* de al menos 1 día”, “donde el antagonista de Wnt tiene una vida media *in vivo* de al menos 2 días”, que son resultados a alcanzar y no definen a la molécula puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto, como la secuencia de aminoácidos.

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Unidad de Invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a 17 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**:

- 1° Un antagonista de Wnt que comprende hFrz1 (SEQ ID NO: 18 ó 50 ó 35) y un dominio Fc, o Frz1-Fc (SEQ ID NO: 76). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 2° Un antagonista de Wnt que comprende hFrz2 (SEQ ID NO: 19 ó 51 ó 36) y un dominio Fc, o Frz2-Fc (SEQ ID NO: 77). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 3° Un antagonista de Wnt que comprende hFrz3 (SEQ ID NO: 20 ó 52 ó 37) y un dominio Fc, o Frz3-Fc (SEQ ID NO: 78). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 4° Un antagonista de Wnt que comprende hFrz4 (SEQ ID NO: 21 ó 53 ó 38) y un dominio Fc, o Frz4-Fc (SEQ ID NO: 79). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 5° Un antagonista de Wnt que comprende hFrz5 (SEQ ID NO: 22 ó 54 ó 39) y un dominio Fc, o Frz5-Fc (SEQ ID NO: 75). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 6° Un antagonista de Wnt que comprende hFrz6 (SEQ ID NO: 23 ó 55 ó 40) y un dominio Fc, o Frz6-Fc (SEQ ID NO: 80). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 7° Un antagonista de Wnt que comprende hFrz7 (SEQ ID NO: 24 ó 56 ó 41) y un dominio Fc, o Frz7-Fc (SEQ ID NO: 81). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 8° Un antagonista de Wnt que comprende hFrz8 (SEQ ID NO: 25 ó 57 ó 42) y un dominio Fc, o Frz8-Fc (SEQ ID NO: 74). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 9° Un antagonista de Wnt que comprende hFrz9 (SEQ ID NO: 26 ó 58 ó 43) y un dominio Fc, o Frz9-Fc (SEQ ID NO: 82). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 10° Un antagonista de Wnt que comprende hFrz10 (SEQ ID NO: 27 ó 59 ó 44) y un dominio Fc, o Frz10-Fc (SEQ ID NO: 83). Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.
- 11° Un antagonista de Wnt que comprende sFRP1 (SEQ ID NO: 28 ó 60 ó 45) y un dominio Fc, o sFRP1-Fc (SEQ ID NO: 84). Reivindicaciones 1 – 4, 6, 9 y 12 – 24, parcialmente.
- 12° Un antagonista de Wnt que comprende sFRP2 (SEQ ID NO: 29 ó 61 ó 46) y un dominio Fc, o sFRP2-Fc (SEQ ID NO: 85). Reivindicaciones 1 – 4, 6, 9 y 12 – 24, parcialmente.
- 13° Un antagonista de Wnt que comprende sFRP3 (SEQ ID NO: 30 ó 62 ó 47) y un dominio Fc, o sFRP3-Fc (SEQ ID NO: 86). Reivindicaciones 1 – 4, 6, 9 y 12 – 24, parcialmente.
- 14° Un antagonista de Wnt que comprende sFRP4 (SEQ ID NO: 31 ó 63 ó 48) y un dominio Fc, o sFRP4-Fc (SEQ ID NO: 87). Reivindicaciones 1 – 4, 6, 9 y 12 – 24, parcialmente.

- 15° Un antagonista de Wnt que comprende sFRP5 (SEQ ID NO: 32 ó 64 ó 49) y un dominio Fc, o sFRP5-Fc (SEQ ID NO: 88). Reivindicaciones 1 – 4, 6, 9 y 12 – 24, parcialmente.
- 16° Un antagonista de Wnt que comprende hRor1 (SEQ ID NO: 33 ó 65) y un dominio Fc. Reivindicaciones 1 – 4, 7, 10, 13 – 17 y 19 – 24, parcialmente.
- 17° Un antagonista de Wnt que comprende hRor2 (SEQ ID NO: 34 ó 66) y un dominio Fc. Reivindicaciones 1 – 5, 8, 11 y 13 – 24, parcialmente.

No se guarda un concepto común inventivo porque los antagonistas de Wnt no están relacionados por un elemento estructural importante, esencial para la actividad común.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Septiembre 8 de 2006, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	<u>Nature, Vol. 423, Pág. 409-414</u>	A role for Wnt signalling in self-renewal of haematopoietic stem cells.	22/Mayo/2003
D2	<u>PNAS, Vol. 96, Pág. 3546-3551</u>	Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein.	30/Marzo/1999
D3	<u>US6900292</u>	Fc fusion proteins of human erythropoietin with increased biological activities	31/Mayo/2005

D1 <http://www.ncbi.nlm.nih.gov/pubmed/12717450> divulga un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled fusionado con IgG (Pág. 411, columna izquierda, párrafo 1).

D2 <http://www.ncbi.nlm.nih.gov/pubmed?term=10097073> divulga enseña un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled 8 fusionado con IgG (Pág. 3547, columna izquierda, párrafo 1).

D3 www.google.com/patents/US7030226 divulga fusiones de péptidos con el dominio Fc para incrementar la vida media del péptido (Resumen)

9. Examen de Patentabilidad (Art 14 D486)

Grupo Inventivo 8

Novedad (Art 16 D486)

La **reivindicación 1** consiste en un antagonista de Wnt, que incluye lo siguiente:
(a) un componente del dominio Frizzled, y
(b) un dominio Fc,
donde el componente del dominio Frizzled comprende un polipéptido derivado de una proteína seleccionada del grupo que consiste de (i) una proteína Frizzled (Frz), (ii) una Proteína Relacionada con Frizzled secretada (FRPs), y (iii) una proteína Ror, y
además donde el antagonista de Wnt es activo *in vivo* por al menos 1 hora.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un antagonista de Wnt, que incluye lo siguiente: a) un componente del dominio Frizzled, y	Un antagonista de Wnt, que comprende: el dominio rico en cisteína (CRD) de Frizzled y (Pág. 411, columna izquierda, párrafo 1)	Un antagonista de Wnt, que comprende: el dominio rico en cisteína (CRD) de Frizzled 8 y (Pág. 3547, columna izquierda, párrafo 1)
b) un dominio Fc, donde el componente del dominio Frizzled comprende un polipéptido derivado de una proteína seleccionada del grupo que consiste de (i) una proteína Frizzled (Frz), (ii) una Proteína Relacionada con Frizzled secretada (FRPs), y (iii) una proteína Ror. y además donde el antagonista de Wnt es activo <i>in vivo</i> por al menos 1 hora.	IgG (Pág. 411, columna izquierda, párrafo 1)	ii) IgG (Pág. 3547, columna izquierda, párrafo 1)

Las características **esenciales** del antagonista de Wnt de la reivindicación 1 habían sido divulgadas previamente en los documentos D1 y D2 porque:

D1 divulga un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled fusionado con IgG (Pág. 411, columna izquierda, párrafo 1).

D2 enseña un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled 8 fusionado con IgG (Pág. 3547, columna izquierda, párrafo 1).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en los documentos D1 o D2.

La reivindicación dependiente 2 y las reivindicaciones 3 – 17 no mencionan alguna característica que haga nueva al antagonista de Wnt de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

La **reivindicación 18** consiste en un antagonista de Wnt que comprende un polipéptido seleccionado a partir del grupo que consiste de Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2-Fc (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), y sFRP5-Fc (SEQ ID NO: 88).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2	D3
Un antagonista de Wnt que comprende un polipéptido seleccionado a partir del grupo que consiste de Frz8-Fc (SEQ ID NO: 74), Frz5-Fc (SEQ ID NO: 75), Frz1-Fc (SEQ ID NO: 76), Frz2-Fc (SEQ ID NO: 77), Frz3-Fc (SEQ ID NO: 78), Frz4-Fc (SEQ ID NO: 79), Frz6-Fc (SEQ ID NO: 80), Frz7-Fc (SEQ ID NO: 81), Frz9-Fc (SEQ ID NO: 82), Frz10-Fc (SEQ ID NO: 83), sFRP1-Fc (SEQ ID NO: 84), sFRP2-Fc (SEQ ID NO: 85), sFRP3-Fc (SEQ ID NO: 86), sFRP4-Fc (SEQ ID NO: 87), y sFRP5-Fc (SEQ ID NO: 88).	Un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled fusionado con IgG (Pág. 411, columna izquierda, párrafo 1).	Un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled 8 fusionado con IgG (Pág. 3547, columna izquierda, párrafo 1).	Proteínas de fusión de péptidos con el dominio Fc mejora la vida media del péptido <i>in vivo</i> (Columna 1, líneas 46-67)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 18 porque:

D1 enseña un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled fusionado con IgG (Pág. 411, columna izquierda, párrafo 1). D1 igualmente divulga que el dominio CRD de Frizzled inhibe la unión de proteínas Wnt al receptor Frizzled y que la presencia del dominio CRD inhibe el crecimiento de células madre hematopoyéticas (HSCs), lo cual es una evidencia de que Wnt modula la supervivencia y proliferación de HSC (Pág. 411, columna izquierda, párrafo 1).

La diferencia entre la invención, definida en la reivindicación 18 y el antagonista de D1 consiste en que el antagonista de la solicitud comprende la SEQ ID NO: 76.

D2 divulga un antagonista de Wnt que comprende el dominio rico en cisteína (CRD) de Frizzled 8 fusionado en el amino-terminal con IgG (Pág. 3547, columna izquierda, Expediente 09-034961 1er Art 45

párrafo 1). D2 también enseña que Wnts y la ruta de señalización Wnt está implicada en carcinogénesis en mamíferos y que una actividad incrementada de Wnt es un importante aspecto de carcinogénesis en melanomas humanos y cánceres colorectales (Pág. 3546, columna izquierda, párrafo 1). D2 igualmente divulga que el dominio CDR de Wnt contiene alguno o todos los determinantes de unión a Wnt porque es necesario y suficiente para conferir unión Wg a células transfectadas (Pág. 3546, columna derecha, párrafo 2).

La diferencia entre la invención, definida en la reivindicación 18 y el antagonista de D2 consiste en que el antagonista de la solicitud comprende la SEQ ID NO: 74.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a los antagonistas reclamados en esta solicitud porque no parecen presentar una mayor actividad moduladora de Wnt.

El problema técnico objetivo de esta solicitud consiste en la necesidad de compuestos antagonistas de Wnt alternativos a los ya conocidos en D1 o D2.

Teniendo en cuenta que los documentos D1 y D2 ya divulgaban antagonistas de Wnt que comprenden un dominio rico en cisteína (CRD) de Frizzled fusionado con Fc de IgG y que el documento D3 divulga que proteínas de fusión de péptidos con el dominio Fc mejora la vida media del péptido *in vivo* (Columna 1, líneas 46-67), la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, fusionar diferentes componentes del dominio Frizzled con un dominio Fc para obtener más antagonistas con la misma actividad. La persona del oficio medianamente versada en la materia se vería motivada a obtener fusiones del dominio CRD de Frizzled porque corresponde al dominio de unión al receptor y porque es bien conocido en el estado del arte que fusionar un péptido con el dominio Fc de Ig permite incrementar el tiempo de vida media del péptido *in vivo*¹.

De manera que la solución propuesta por esta solicitud en la reivindicación 18 y sus dependientes 19 y 20 y en las reivindicaciones 21 – 24, es obvia y no tiene Nivel Inventivo.

10. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	<u>Nature, Vol. 423, Pág. 409-414</u>	1 – 24	Novedad y Nivel Inventivo
D2	<u>PNAS, Vol. 96, Pág. 3546-3551</u>	1 – 24	Novedad y Nivel Inventivo
D3	<u>US6900292</u>	18 – 24	Nivel Inventivo

1 J. Dumont et al. Monomeric Fc fusions. Biodrugs, Vol. 20, Pág. 151-160. Publicado el 1 de Enero de 2006.
Expediente 09-034961 1er Art 45

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 a este requerimiento 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.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

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

11 SET. 2012

Elaborado por: Juan David Moncaleano P. 
Revisado por: Consuelo Leguizamón L. 

A role for Wnt signalling in self-renewal of haematopoietic stem cells

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Haematopoietic stem cells (HSCs) have the ability to renew themselves and to give rise to all lineages of the blood; however, the signals that regulate HSC self-renewal remain unclear. Here we show that the Wnt signalling pathway has an important role in this process. Overexpression of activated β -catenin expands the pool of HSCs in long-term cultures by both phenotype and function. Furthermore, HSCs in their normal microenvironment activate a LEF-1/TCF reporter, which indicates that HSCs respond to Wnt signalling *in vivo*. To demonstrate the physiological significance of this pathway for HSC proliferation we show that the ectopic expression of axin or a frizzled ligand-binding domain, inhibitors of the Wnt signalling pathway, leads to inhibition of HSC growth *in vitro* and reduced reconstitution *in vivo*. Furthermore, activation of Wnt signalling in HSCs induces increased expression of *HoxB4* and *Notch1*, genes previously implicated in self-renewal of HSCs. We conclude that the Wnt signalling pathway is critical for normal HSC homeostasis *in vitro* and *in vivo*, and provide insight into a potential molecular hierarchy of regulation of HSC development.

The HSC has the ability to perpetuate itself as well as to differentiate into mature blood cells of all lineages. In the mouse, long-term self-renewing HSCs make up approximately 0.007% of bone marrow and can be isolated by their expression of undetectable levels of lineage markers (such as B220, CD3, Mac-1), high levels of c-Kit and Sca-1, and low levels of Thy-1 (refs 1, 2). Although HSCs have been purified successfully and their phenotypic and functional properties well characterized^{1–4}, a fundamental question that remains is how their self-renewing growth is regulated. On the basis of a screen of genes expressed in HSCs—where we noted that members of the LEF-1/TCF family were expressed (K. Li, S. Cheshier and I.L.W., unpublished data)—and our previous finding that Wnt signalling can influence lymphocyte progenitor cell proliferation⁵, we have investigated whether Wnt signalling influences HSC development.

β -Catenin expression leads to self-renewal of HSCs *in vitro*

We first determined the effects of activating downstream components of the Wnt pathway on HSC function. We activated Wnt signalling in HSCs sorted via fluorescence-activated cell sorting (FACS) (c-Kit⁺ Thy-1.1^{lo} Lin^{−/lo} Sca-1⁺ (KTLS) cells) by retrovirally⁶ transducing them with constitutively active β -catenin⁷. Successful transduction of HSCs with retroviruses requires induction of cell cycle entry through the use of multiple growth factors, which can promote differentiation of stem cells *in vitro*. To minimize the pro-differentiation stimuli encountered by HSCs during infection before experiments of interest, we used HSCs from H2K-BCL-2 transgenic mice, which proliferate to steel factor (SLF) alone⁸. Sorted BCL-2 transgenic HSCs were infected with retroviruses encoding either β -catenin-IRES-GFP (β -catenin, internal ribosome entry site and green fluorescent protein) or IRES-GFP alone, and GFP expression was detected in 45–55% of HSCs, which persisted for the entire *in vitro* culture period (data not shown). GFP-positive (GFP⁺) HSCs were sorted to determine growth kinetics *in vitro* and the ability to reconstitute the immune system *in vivo*.

Short-term growth characteristics of HSCs expressing β -catenin or control vector were determined by cell cycle analysis⁹. In Fig. 1a,

whereas 34% of the HSCs infected with control vector were in S/G2/M phases of the cell cycle, 58% of the HSCs expressing activated β -catenin were in the same phases of the cell cycle (Fig. 1a). To determine whether activated Wnt signalling increased long-term growth, HSCs expressing β -catenin were grown *in vitro* in serum-free medium in the presence or absence of growth factors. Medium containing limiting amounts of SLF allowed the growth of β -catenin-transduced HSCs consistently for at least 8 weeks (Fig. 1b). During this period the GFP⁺ cells underwent eight–nine population doublings to generate at least 100 times the number of input cells. In contrast, HSCs infected with control vector showed minimal growth beyond a two-week period (Fig. 1b). On complete withdrawal of SLF during long-term culture, β -catenin-infected HSCs grew for at least 4 weeks, and in some experiments could be maintained and passaged for as long as 1–2 months. In contrast the control transduced HSCs did not survive beyond 48 h (data not shown).

To determine whether growth in response to activated β -catenin was accompanied by differentiation, the morphological characteristics of these cells were analysed at the end of a two-week period. This time point was chosen to be able to compare the differentiation status of control and β -catenin-transduced HSCs, as the lifespan of HSCs transduced with control vector was limited. Cells infected with control vector were found to have a myelo-monocytic appearance. In contrast, 65–75% of the β -catenin-transduced HSCs had a high nuclear to cytoplasm ratio (Fig. 1c). Consistent with this, although most (75–80%) of the HSCs infected with control vector were positive for lineage markers (Fig. 1d, left panel), only 5–10% of cells infected with β -catenin expressed high levels of lineage markers (predominantly Mac-1, an integrin expressed on fetal HSCs¹⁰ and regenerating HSCs¹¹). In fact, 60% of HSCs infected with β -catenin were lineage-negative and expressed high levels of c-Kit and Sca-1 (Fig. 1d, middle panel) and almost half of these also expressed low levels of Thy-1.1 (right panel). Thus, at least 30% of the cells in β -catenin-transduced cultures had retained the phenotype of HSCs; that is, c-Kit⁺ Thy-1.1^{lo} Lin[−] Sca-1⁺ (KTLS cells). This indicated that the expression of activated β -catenin maintained haemato-

poietic stem cells in an immature state, while simultaneously allowing these cells to proliferate, thus expanding the HSC pool 20–48-fold on the basis of the total numbers of cells generated (see Supplementary Information).

To determine whether β -catenin-transduced HSCs retained the functional characteristics of HSCs, and to determine whether there was indeed a functional expansion of HSCs, we tested the ability of β -catenin-transduced HSCs to give rise to sustained production of myeloid, B- and T-cell lineages in lethally irradiated mice when transplanted in limiting numbers^{1,2}. Specifically, HSCs were infected with control or β -catenin virus, cultured for 1 week *in vitro*, transplanted in varying doses down to 125 cells into irradiated mice, and analysed after 11 weeks. As shown in Table 1, 125 β -catenin-transduced HSCs were sufficient to reconstitute the haematopoietic system of all of the irradiated mice. The average level of chimaerism observed was 61% (range of 27.6–86.8%). Furthermore, each of the transplanted mice displayed clear reconstitution along myeloid, T and B lineages (Table 1 and Fig. 2). In contrast, mice receiving 125 HSCs transduced with control vector failed to reconstitute (Table 1). The reconstitution observed at limiting dilution suggested a functional HSC expansion of 8–80-fold in short-term cultures and 96–960-fold in long-term cultures (Supplementary Information). That β -catenin-transduced HSCs lead to higher levels of chimaerism was observed in five independent experiments using different doses of cells and different culture periods (up to 2 months), indicating that β -catenin prevents HSC differentiation while promoting proliferation, thus inducing self-renewal in serum-free medium.

The expansion of HSCs owing to activated β -catenin probably reflects upstream Wnt signals, as we have demonstrated recently that purified Wnt3a causes self-renewal in both BCL-2 transgenic¹² and wild-type HSCs (Supplementary Figs 1 and 2). Specifically, singly plated HSCs generate sixfold or more numbers of progeny in the presence of Wnt3a compared with control conditions¹² (Supplementary Figs 1 and 2). These daughter cells not only maintain an immature phenotype, but also display a 5–50-fold expansion of

HSC function as determined by transplantation analysis of the progeny of single HSCs after expansion *in vitro*¹² (Supplementary Figs 1 and 2).

HSCs *in vivo* normally signal through LEF-1/TCF elements

To determine whether the Wnt signalling pathway is physiologically relevant to HSCs, we tested whether HSCs *in vivo* use signals associated with the Wnt/ β -catenin pathway. HSCs were infected with LEF-1/TCF reporter driving expression of destabilized GFP (TOP-dGFP) or with control reporter with mutated LEF-1/TCF binding sites (FOP-dGFP), and then transplanted into lethally irradiated mice (L. Ailles and I.L.W., manuscript in preparation). Recipient bone marrow was examined after 14 weeks to determine whether donor HSCs demonstrated reporter activity. In the example shown, donor-derived HSCs infected with TOP-dGFP expressed GFP in 28% of the cells (Fig. 3a; range observed 4–28%, mean 11.8%), whereas HSCs from the recipient mouse were negative for GFP (Fig. 3b; range observed 2.3–3.2%, mean 2.7%). Moreover, HSCs transduced with the control reporter did not express GFP significantly, demonstrating that functional LEF-1/TCF binding sites were required for HSC expression of GFP (Fig. 3c). In all cases, no reporter activity was observed in the non-HSC myeloid progenitor fraction (Fig. 3a–c, thin line). As a control, we also tested whether the TOP-dGFP reporter was turned on in response to Wnt3a-mediated signalling in HSCs *in vitro*. Thus, HSCs transduced with either TOP-dGFP or FOP-dGFP were stimulated with Wnt3a, and the extent of GFP expression was monitored. As shown in Fig. 3e, Wnt3a-treated HSCs showed significant reporter activity, demonstrating that the reporter is turned on in response to Wnt stimulus, but not in control conditions. Increased reporter activity was observed when the reporter construct driving non-destabilized GFP was used. These data demonstrate that HSCs in their normal microenvironment respond to endogenous Wnt signalling during self-renewal and/or stimulation into cell cycle, and also support the interpretation that the Wnt3a stimulus that caused increased self-renewal¹² signals through the canonical Wnt pathway.

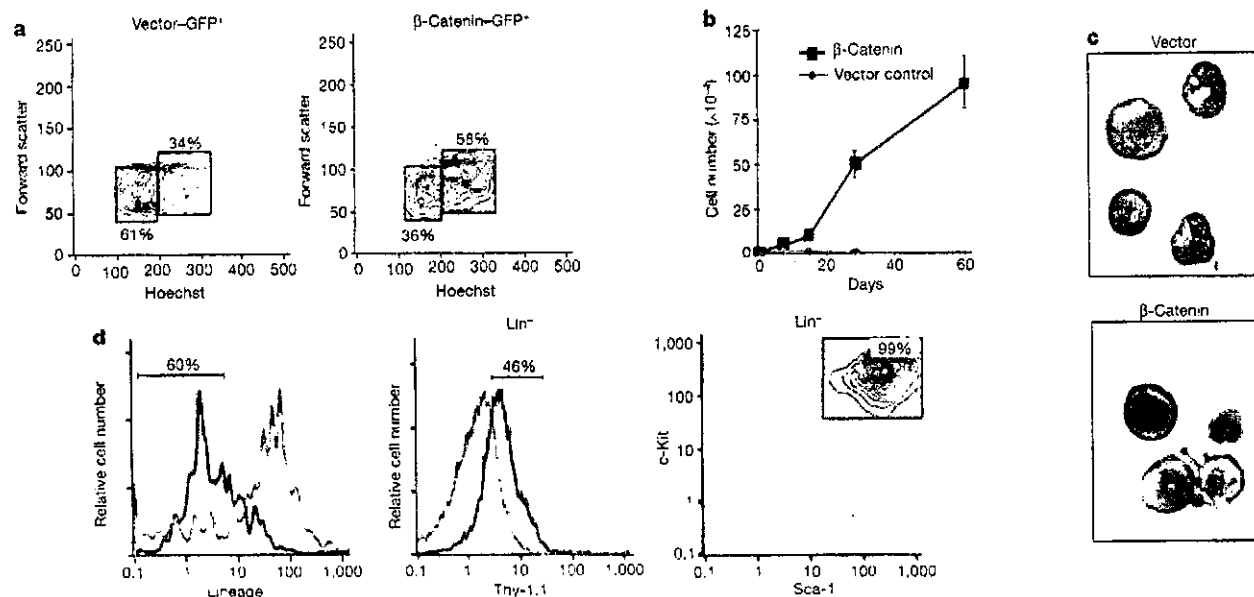


Figure 1 Activated β -catenin promotes growth of HSCs *in vitro* and maintains the immature phenotype of HSCs in long-term cultures. HSCs were infected with activated β -catenin-IRES-GFP or control GFP retrovirus, and subjected to cell cycle analysis after 60 h. **a**, β -Catenin-infected cultures display an increased number of blastogenic cells (right box, S/G2/M) compared with control. **b**, For long-term growth studies, 10,000 infected HSCs were plated in 1 ng ml^{-1} SLF and monitored over 60 days. Results are from

one of five experiments. **c**, Giemsa staining reveals myeloid characteristics in control cells and HSC morphology (high nucleus to cytoplasm ratio) in β -catenin-infected cells. **d**, Control cells (grey lines) are largely lineage-positive, whereas most β -catenin-infected cells (black lines) are lineage-negative (Lin⁻) or have low levels (left panel). β -Catenin-infected Lin⁻ cells have characteristics of HSCs, including low Thy-1.1 (middle panel), and high c-Kit and Sca-1 (right panel).

Table 1 Reconstitution efficiency of β -catenin- or vector-transduced HSCs

Transplantation		Cell type	Frequency of reconstitution (%)	Chimaerism (%)	
Number	Infection			Average	Range
125	Vector	PB	0 (2/3)	0	0
125	β -Catenin	PB	100 (3/3)	51.2	27.6–86.8
125	β -Catenin	B	100 (3/3)	58.1	37.9–78.8
125	β -Catenin	T	100 (3/3)	19.2	16.0–23.4
125	β -Catenin	M	100 (3/3)	15.0	2.6–25.7

HSCs infected with activated β -catenin or control vector were cultured for a week and cells were injected into groups of three to four lethally irradiated host mice along with 300,000 competing syngeneic bone marrow cells. Cells were isolated from peripheral blood and analysed by flow cytometry after 11 weeks. Average reconstitution rates and ranges of donor cells are shown for peripheral blood (PB), B cell (B), T cell (T) and myeloid (M) lineages. (See also Fig. 2.)

HSCs require intact Wnt signalling

To test whether Wnt signalling is required for normal HSC growth, we used a soluble form of the frizzled cysteine-rich domain (CRD) that inhibits the binding of Wnt proteins to the frizzled receptor^{13,14} (Supplementary Fig. 3). Wild-type HSCs were incubated with growth factors in the presence of IgG–CRD domain fusion protein or control IgG, and cell proliferation was monitored. The presence of the CRD domain inhibited growth of HSCs fourfold compared with control conditions (Fig. 4a). This inhibition provides direct evidence of a Wnt signal modulating HSC survival and proliferation, as soluble CRD acts at the level of Wnt binding the frizzled molecules. Because only HSCs were present, the Wnt signal is probably derived from some or all of the HSCs in the cultures, and is required despite the presence of multiple other growth factors. These results can be interpreted to mean that all HSC mitoses are the result of Wnt signalling, even if the primary signals are not Wnt.

We also inhibited Wnt signalling through an independent inhibitor by ectopically expressing axin in HSCs¹⁶. Axin increases β -catenin degradation and acts as an intracellular inhibitor of Wnt signalling^{16–19}. Live axin-infected wild-type HSCs were re-sorted 48 h after infection and plated in limiting numbers to assay growth in response to a combination of growth factors. Although control-infected cells proliferated 2.3-fold over 60 h, axin-infected cells showed a sevenfold reduction in the total growth response

(Fig. 4b). Axin had an inhibitory effect on growth of BCL-2 transgenic HSCs as well (data not shown), which suggests that expression of BCL-2 cannot protect cells from loss of Wnt signalling. To determine whether axin expression had an effect on cell survival, GFP⁺ cells were analysed at the end of the infection period using propidium iodide exclusion. Whereas 80% of the control-infected cells were negative for propidium iodide, only 38% of axin-infected HSCs were negative for propidium iodide (Fig. 4c), indicating that axin expression has significant effect on cell survival by blocking β -catenin function.

To determine whether Wnt signalling is required for haematopoietic stem cell responses *in vivo*, we injected axin- or control-transduced viable HSCs into lethally irradiated mice and analysed the level of reconstitution after 10 weeks. Mice transplanted with control-infected HSCs displayed on average sevenfold greater chimaerism (reconstitution range 5–11.6%) than mice transplanted with axin-infected HSCs (reconstitution range 0–1.8%) (Fig. 4e). A representative example of contribution from axin- or vector-infected HSCs in transplanted mice is shown in Fig. 4d. These data show that inhibition of the Wnt pathway reduces reconstitution, suggesting that Wnt signalling is required for normal development of HSCs *in vivo*. This finding, together with the finding

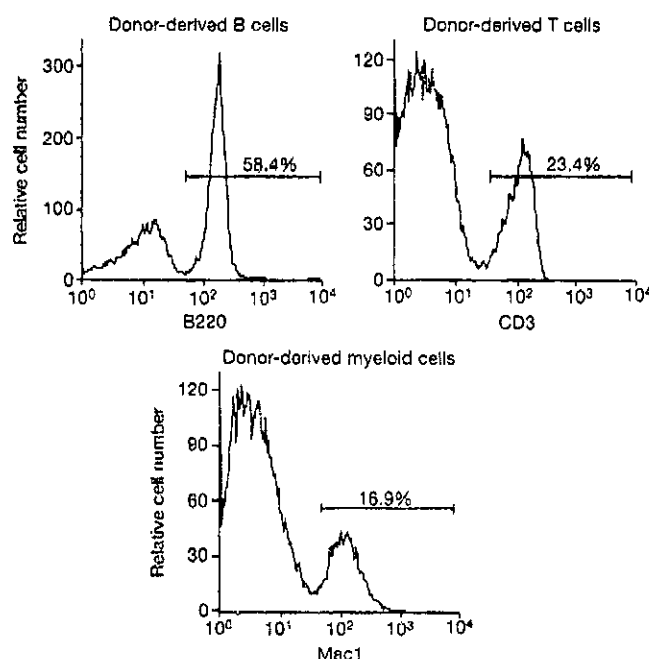


Figure 2 Activated β -catenin-transduced HSCs reconstitute lethally irradiated mice and give rise to multiple haematopoietic lineages. The diagrams show the contribution to each lineage as determined by FACS analysis in a representative sample.

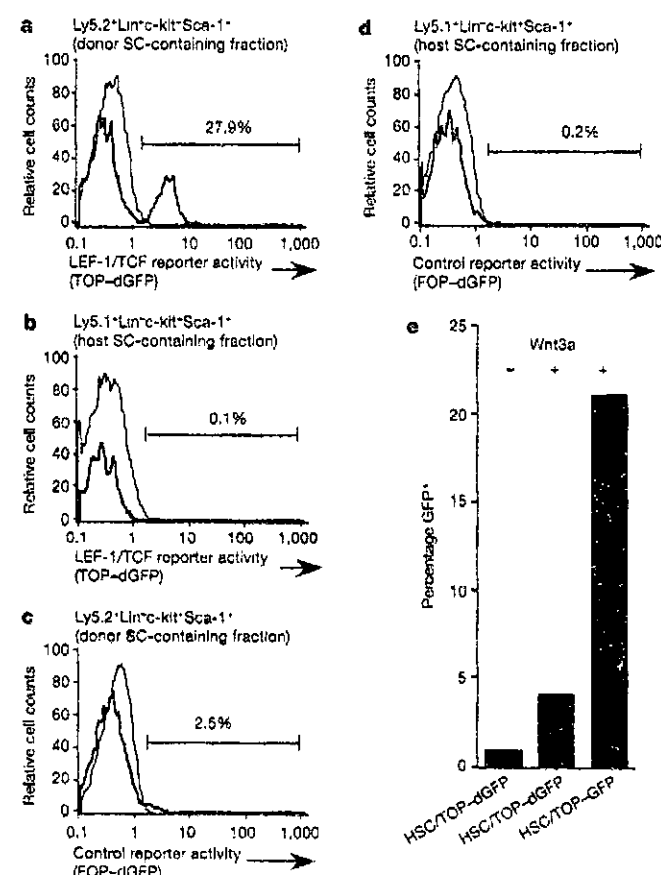


Figure 3 HSCs respond to Wnt signalling in native bone marrow microenvironment. HSCs were infected with a lentiviral reporter containing either LEF-1/TCF binding sites linked to destabilized GFP (TOP-dGFP), or mutated LEF-1/TCF binding sites linked to destabilized GFP (FOP-dGFP). Infected HSCs were transplanted into three lethally irradiated recipient mice, and analysed after 14 weeks. The data shown represent two independent experiments. **a, b**, GFP expression is shown in donor-derived (**a**) or host-derived (**b**) HSCs. **c, d**, Donor-derived HSCs carrying mutated LEF-1/TCF reporter (**c**) as well as the recipient mouse HSCs (**d**) are GFP negative. Expression of GFP in donor-derived Lin⁺ c-Kit⁺ Sca-1⁺ cells (non-HSCs) is shown by thin lines (**a–d**). **e**, HSCs infected with TOP-dGFP or TOP-GFP (a non-destabilized GFP) were stimulated *in vitro* with control medium or with 100 ng ml^{−1} Wnt3a, and the extent of GFP expression measured.

that HSCs respond to Wnt signalling *in vivo* (Fig. 3), indicates that Wnt/ β -catenin signalling is an important physiological mediator of HSC-derived haematopoiesis.

β -catenin upregulates HoxB4 and Notch1 in HSCs

We wished to determine whether Wnt signalling might be regulating HSC self-renewal by upregulating genes previously implicated in HSC self-renewal. To this end we tested upregulation of HoxB4 and Notch1 (refs 20, 21). By using real-time polymerase chain reaction (PCR) analysis on HSCs infected with either β -catenin or control vector, we found that HoxB4 was upregulated an average of 3.5-fold and Notch1 was upregulated 2.5-fold (Fig. 5a). In contrast, Gapdh expression was not differentially regulated as a consequence of β -catenin expression, and was used as a control (Fig. 5b). These data show that genes so far identified as regulators of HSC self-renewal may be related and perhaps act in a molecular hierarchy.

Discussion

Our study shows that components of the Wnt signalling pathway

can induce proliferation of purified KTLS bone marrow HSCs while significantly inhibiting their differentiation, thereby resulting in functional self-renewal. We find that expression of β -catenin in HSCs results in increased growth with significantly reduced differentiation *in vitro* for a period of at least many weeks. HSCs transduced with β -catenin give rise to sustained reconstitution of myeloid and lymphoid lineages *in vivo*, when transplanted in limiting numbers. We also find that Wnt signalling is required for the growth response of normal HSCs to other cytokines, as overexpression of axin leads to reduced stem cell growth both *in vitro* and *in vivo*. Furthermore, the inhibition of HSC growth with frizzled-CRD and the finding that Wnt3a causes expansion of HSCs supports the interpretation that the effects of β -catenin and axin reflect upstream Wnt activity. Finally, studies with HSCs containing a LEF-1/TCF reporter indicate that HSCs *in vivo* respond to endogenous Wnt stimulation. The expression of a number of Wnt proteins in the bone marrow⁵ and frizzled receptors in bone-marrow-derived progenitors and HSCs supports this possibility²².

Most growth factors that act on HSCs in culture induce no or limited expansion²³ or are unable to prevent differentiation²⁴. Thus, one of the most notable findings of our work is the induction of proliferation and the prevention of HSC differentiation by the Wnt signalling pathway. Other signals that increase proliferation of HSCs include Notch²⁹ and sonic hedgehog²⁵. Moreover, the cyclin-dependent kinase inhibitor p21^{Cip1/Waf1} (ref. 26) and the transcription factor HoxB4 (ref. 21) have been shown to be involved in regulating self-renewal of HSCs. Notably, Wnt signalling has been shown to interact with many of these pathways in a variety of organisms^{27, 30}, and our data show that both HoxB4 and Notch1 are upregulated in response to Wnt signalling in HSCs. This raises the possibility that the effects of Wnt signalling on HSCs are mediated through HoxB4 and/or Notch1. Whereas HoxB4 could act directly on these HSCs as demonstrated previously²¹, Notch1 action would require nearby Notch1 ligands.

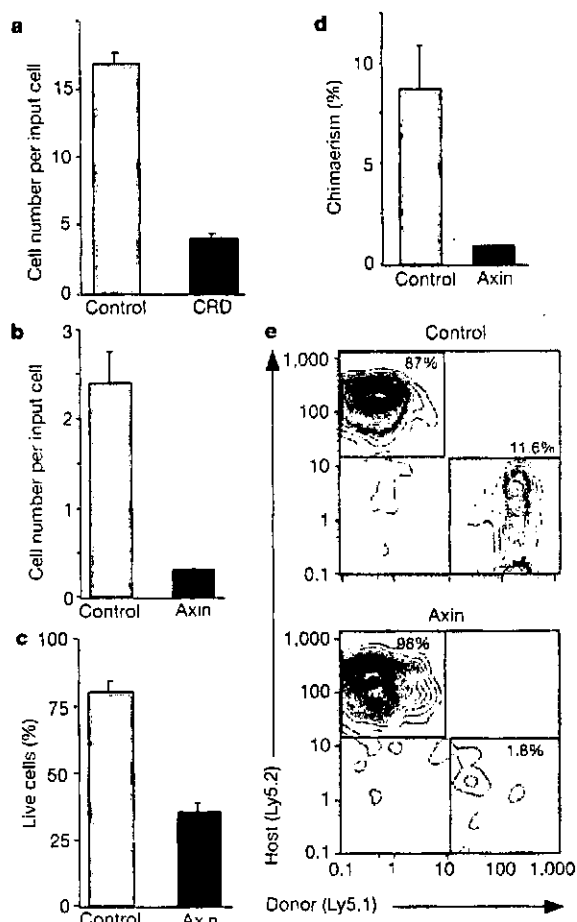


Figure 4 Inhibition of Wnt signalling reduces growth of HSCs *in vitro* and inhibits reconstitution *in vivo*. **a**, HSCs (20 cells per well) were cultured for 60 h in medium containing mitogenic factors and either IgG-CRD or control IgG. **b**, HSCs were infected with virus encoding axin-IRES-GFP or GFP alone. Growth of infected HSCs in the presence of mitogenic factors was monitored over 60 h. **c**, The number of live cells was determined by propidium iodide staining. **d**, **e**, The development of HSCs *in vivo* was determined by injecting 1,000 control or axin-infected cells per mouse into groups of four lethally irradiated, allelically marked (Ly5.2) host mice along with 300,000 competing syngeneic bone marrow cells. Cells were isolated from peripheral blood and analysed by flow cytometry after >10 weeks. Donor-derived (Ly5.1⁺) cells were monitored in the peripheral blood of hosts: analysis from a representative recipient and average reconstitution is shown.

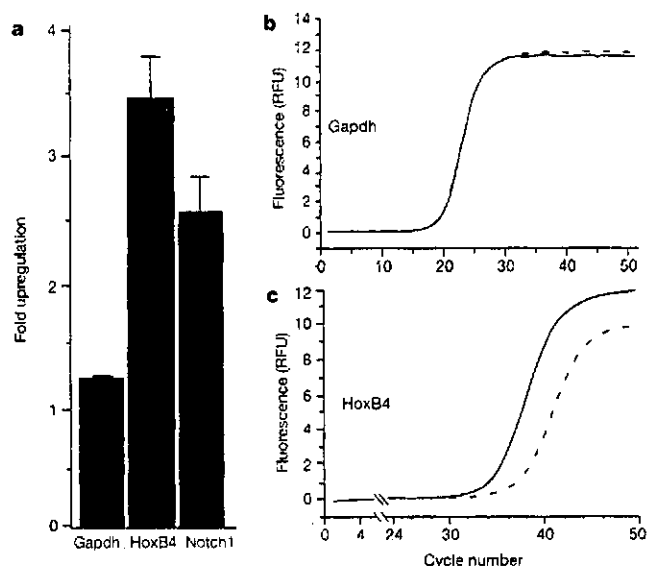


Figure 5 HSCs expressing β -catenin upregulate HoxB4 and Notch1. **a**, Purified wild-type HSCs were infected with activated β -catenin-IRES-GFP or control vector-IRES-GFP, and infected cells sorted based on GFP expression at 48 h. The RNA isolated from these cells was reverse transcribed and expression of HoxB4 and Notch1 was analysed by real-time PCR analysis. Results are averaged over five independent PCR reactions. **b**, **c**, Representative graphs of real-time PCR analysis demonstrating equal amounts of Gapdh (**b**) and differential amounts of HoxB4 (**c**) products from β -catenin-transduced HSCs (solid line) and control-transduced HSCs (dashed line). RFU, relative fluorescence units.

The ability of Wnt3a to induce expansion of HSCs is consistent with previous studies showing increased numbers of haematopoietic progenitors from mouse fetal liver and human bone marrow cells stimulated with Wnt-containing supernatants^{11,22}. These studies, although consistent with ours, are more difficult to interpret as they did not use purified HSCs, lacked *in vivo* reconstitution analysis, and did not provide evidence of a physiological requirement for Wnt signalling for HSCs. Components of the Wnt pathway have also been shown to promote proliferation of primitive cells in the skin^{33,34}, the gut^{35,36} and the brain³⁷, and to inhibit differentiation to a variety of lineages in embryonic stem cells³⁸, raising the possibility that Wnt signalling may be used as a general cue for self-renewal in stem and/or progenitor cells from diverse tissues. Its role as a self-renewal signal does not, however, preclude its involvement in differentiation of stem cells in certain contexts³⁹.

Our findings may have important implications for human haematopoietic cell transplantation. We have found that soluble Wnt3a protein induces proliferation of highly purified human bone marrow HSCs in the absence of any other growth factor (T.R., T. Miyamoto and I.L.W., unpublished observation). Induction of HSC growth by Wnt signalling may allow *in vitro* expansion of a patient's own or an allogeneic donor's HSCs, and could provide an increased source of cells for future transplantation. Finally, we have previously raised the hypothesis that self-renewal is a property that could be dangerous, as an adequate definition of cancer stem cells is poorly regulated self-renewal of a particular stage of a developmental lineage⁴⁰. The demonstration here that the Wnt/ β -catenin pathway may have a role in haematopoietic stem cell self-renewal leads us to propose that this pathway should be studied for a role in self-renewal of cancer stem cells. □

Methods

Mice

C57BL/Ka Ly5.1, Thy-1.1 (wild-type and BCL-2), C57BL/Ka Ly5.2, Thy-1.1, and AKR/J mice were used at 6–10 weeks of age. Mice were bred and maintained on acidified water in the animal care facility at Stanford and Duke University Medical Centers.

HSC isolation

We sorted HSCs from mouse bone marrow as described⁴¹. All cell sorting and FACS analysis was carried out on a FACSVantage (Becton Dickinson) at the Stanford shared FACS facility and the Duke Cancer Center FACS facility. Cells were sorted and reanalysed on the basis of expression of c-Kit, Sca-1, low levels of Thy-1.1, and low to negative levels of lineage markers (Lin).

Cell cycle analysis

Retrovirally transduced HSCs were collected from cultures and stained with Hoechst 33342 (Molecular Probes) at 37 °C for 45 min in Hoechst medium⁹. Cells were then washed and analysed by Flow cytometry to determine the cell cycle profile of GFP⁺ cells.

Viral production and infection

Virus was produced by triple transfection of 293T cells with murine stem cell virus constructs along with gag-pol and vesicular stomatitis virus G glycoprotein constructs. Viral supernatant was collected for three days and concentrated 100-fold by ultracentrifugation at 50,000g. For viral infection, 10,000 HSCs were sorted into wells of a 96-well plate and cultured overnight in the presence of SLF (30 ng ml⁻¹) for BCL-2 transgenic HSCs (used in Fig. 1, Fig. 2 and Table 1), or SLF (30 ng ml⁻¹) plus TPO (30 ng ml⁻¹) for wild-type HSCs (all other figures and Supplementary figures). After 12 h, concentrated retroviral supernatant was added to the cells at a 1:1 ratio. Cells were then incubated at 32 °C for 12 h and 37 °C for 36 h before GFP⁺ cells were sorted for *in vitro* and *in vivo* assays. Lentiviruses used were produced as previously described. Briefly, 293T cells were transfected with the transfer vector plasmid, the VSV-G envelope-encoding plasmid pMD.G, and the packaging plasmid CMVΔR8.74 (ref. 42). The supernatant was collected and concentrated by ultracentrifugation. All cytokines were purchased from R&D systems.

In vitro HSC proliferation assays

Freshly purified or virally transduced HSCs were plated at 1 to 20 cells per well in Terasaki plates. Cells were sorted into wells containing serum-free medium (X-vivo15, BioWhittaker) supplemented with 5 × 10⁻⁵ M 2-mercaptoethanol and the indicated growth factors. Proliferation was monitored by counting the number of cells in each well at defined intervals. For longer-term cultures, transduced HSCs were plated in 96-well plates in the absence or presence of SLF (1 ng ml⁻¹), and the number of cells generated was monitored by cell counting at defined intervals. For inhibition of growth by CRD or axin, cells were cultured in the presence of mitogenic factors (SLF (30 ng ml⁻¹), Flt-3L (30 ng ml⁻¹), interleukin-6 (10 ng ml⁻¹)).

In vivo analysis of HSC function

Virally transduced HSCs were cultured *in vitro* and injected retro-orbitally into groups of 4–6 congenic recipient mice irradiated with 9.5 Gy using a 200-kV X-ray machine, along with 300,000 rescuing host total bone marrow or Sca-1-depleted bone marrow cells. Host mice were given antibiotic water after irradiation. Transplanted mice were bled at regular periods to determine the percentage of the haematopoietic compartment contributed by donor cells. Donor and host cells were distinguished by allelic expression of CD45 (Ly5) or expression of the BCL-2 transgene.

Lentiviral reporter assays

The enhanced GFP (eGFP) or the d2-eGFP gene (destabilized, half-life of 2 h; Clontech) was cloned downstream of a LEF-1/TCF-responsive promoter, containing three LEF-1/TCF binding motifs and a TATA box⁴³. This cassette was then cloned into a self-inactivating lentiviral vector plasmid, and virus was produced as described above.

For *in vivo* assays, HSCs were transduced with reporter lentiviruses and cultured in X-Vivo15 with glutamine, 5 × 10⁻⁵ M 2-mercaptoethanol, and a cocktail of cytokines including 10 ng ml⁻¹ interleukin-11, 10 ng ml⁻¹ TPO, 50 ng ml⁻¹ SCF, 50 ng ml⁻¹ Flt-3L. Cells were incubated at 37 °C for 6 h overnight and transplanted into lethally irradiated congenic recipients. Lethally irradiated mice received 500 transduced HSCs along with rescue bone marrow. For analysis, haematopoietic progenitor cells were analysed for reporter activation 14–24 weeks after transplantation.

For *in vitro* assays, purified HSCs were sorted directly into medium (IMDM/10% FBS plus interleukin-11, TPO, SCF and Flt-3L as above) and plated at 500–1,000 cells per well in 96-well plates. Individual wells were transduced with the appropriate lentiviral reporter and stimulated with or without purified Wnt3a (about 100 ng ml⁻¹). Cells were collected 5 days later, stained with propidium iodide to exclude non-viable cells, and analysed for GFP expression.

Real-time PCR analysis

A total of 75,000 HSCs cultured in 96-well plates containing X-Vivo15, 5 × 10⁻⁵ M 2-mercaptoethanol and 100 ng ml⁻¹ SLF were infected with either β -catenin or control lentiviruses. After two days in culture, transduced cells were isolated on the basis of GFP expression. RNA was prepared using Trizol (Invitrogen) and linearly amplified using a modified Eberwine synthesis⁴⁴. Each amplified RNA was converted to the first strand and analysed for differential gene expression by real-time PCR. Complementary DNAs were mixed with FastStart Master SYBR Green polymerase mix (Roche), primers (Supplementary Information) and real-time PCR was performed using a LightCycler (Roche).

Received 12 December 2002; accepted 27 March 2003; doi:10.1038/nature01593.

Published online 27 April 2003.

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Supplementary Information accompanies the paper on www.nature.com/nature.

Acknowledgements We thank W. J. Nelson and A. Barth for providing the β -catenin construct; G. Nolan and L. Naldini for providing the retroviral and lentiviral packaging systems; F. Costantini for providing the axin construct; J. Nathans for the Pzd3-CRD IgG construct; H. Clevers and B. Vogelstein for providing the modified LEF-1/TCF elements used in the reporter constructs; A. Carlton for help with proliferation assays; and P. Pelicani for generation of retroviral constructs. We are grateful to L. Ierabek and P. Shabi for laboratory management and technical help; L. Hidalgo for animal care; S. Smith and V. Braunstein for preparation of antibodies; M. Cook for cell sorting; and M. Kiang for use of the Light Cycler. D.C.S. is a fellow of the American Cancer Society, L.A. is a fellow of the Canadian Institute of Health Research and R.N. is an investigator of the Howard Hughes Medical Institute. This work was supported by NIH grants awarded to L.L.W., and funds from the Cancer Research Institute, Leukemia Research Foundation and the NIH awarded to T.R.

Competing interests statement The authors declare competing financial interests; details accompany the paper on www.nature.com/nature.

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Biochemical characterization of Wnt-Frizzled interactions using a soluble, biologically active vertebrate Wnt protein

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Contributed by Jeremy Nathans, January 20, 1999

ABSTRACT Biochemical studies of Wnt signaling have been hampered by difficulties in obtaining large quantities of soluble, biologically active Wnt proteins. In this paper, we report the production in *Drosophila* S2 cells of biologically active *Xenopus* Wnt8 (XWnt8). Epitope- or alkaline phosphatase-tagged XWnt8 proteins are secreted by concentrated S2 cells in a form that is suitable for quantitative biochemical experiments with yields of 5 and 0.5 mg per liter, respectively. Conditions also are described for the production in 293 cells of an IgG fusion of the cysteine-rich domain (CRD) of mouse Frizzled 8 with a yield of 20 mg/liter. We demonstrate the use of these proteins for studying the interactions between soluble XWnt8 and various Frizzled proteins, membrane anchored or secreted CRDs, and a set of insertion mutants in the CRD of *Drosophila* Frizzled 2. In a solid phase binding assay, the affinity of the XWnt8-alkaline phosphatase fusion for the purified mouse Frizzled 8-CRD-IgG fusion is ≈ 9 nM.

The Wnt proteins define a large family of extracellular signaling molecules found throughout the animal kingdom. Wnts play a central role in central nervous system, renal, placental, and limb development in vertebrates; early embryonic cell fate decisions and cell migration/polarity in *Caenorhabditis elegans*; and segment polarity and muscle specification in *Drosophila* (reviewed in refs. 1–3). Wnts and the Wnt signaling pathway also have been implicated in mammalian carcinogenesis. Although no alterations in Wnt genes have been identified in human cancers, recent studies have shown that increased activity of the Wnt signaling pathway, mediated by stabilization of β -catenin, is an important aspect of carcinogenesis in human melanomas and colorectal cancers (4–7).

Current evidence indicates that the Frizzled family of integral membrane proteins act as Wnt receptors. The founding member of this family was identified in *Drosophila* based on its role in tissue polarity in the adult cuticle (8). Many additional family members have been identified in both vertebrates and invertebrates, and all have at the amino terminus a conserved extracellular cysteine-rich domain, referred to as the cysteine-rich domain (CRD), that spans ≈ 120 amino acids and contains 10 invariant cysteines, followed by seven putative membrane spanning domains (9–11). The identification of Frizzled proteins as Wnt receptors arose from experiments in cell culture showing that Frizzled-2, a second Frizzled family member from *Drosophila*, confers wingless (Wg) binding and Wg-dependent stabilization of Armadillo (the *Drosophila* orthologue of β -catenin) (12). Additional evidence for this inference comes from genetic interactions between pairs of Wnt and Frizzled genes in both embryonic and larval development in *C. elegans* (13–15), from RNA injection experiments in *Xenopus* embryos in which a mammalian Frizzled protein was shown to alter the subcellular localization (16) or to change the developmental effects of a coexpressed Wnt (17, 18),

and most recently from genetic studies in *Drosophila* that show that Frizzled and Frizzled-2 function redundantly in the embryo as Wg receptors (refs. 19–21; P. Bhanot, R. Nusse, J.N., and K. Cadigan, unpublished work). Tissue culture and *Xenopus* embryo experiments also provide evidence for specificity in Wnt and Frizzled interactions: some, but not all, Frizzled proteins confer Wg binding in transfected cells (12), and axis induction by *Xenopus* Wnt5A (XWnt5A) is conferred by only one of seven Frizzled proteins examined (17).

An additional level of complexity in Wnt signaling has emerged with the discovery in vertebrates of a family of secreted proteins that possess a CRD resembling those present in the Frizzled proteins; the members of this family are referred to as secreted Frizzled related proteins (sFRP) (22–28). Among Frizzled proteins, the CRD contains some or all of the Wnt-binding determinants because it is both necessary and sufficient for conferring Wg binding to transfected cells (12). sFRPs also can bind to Wnts, presumably via the CRD, when either protein is artificially anchored to the plasma membrane (23, 26). In *Xenopus* embryos, one sFRP, sFRP-3/FRZB, is localized to Spemann's organizer, and injection of RNA encoding any of several sFRPs inhibits the bioactivity of coinjected XWnt8 (22–24, 27, 28). These and other data suggest that the sFRPs normally act as competitive inhibitors of Wnt-Frizzled binding.

Despite the advances noted above, the biochemical and structural properties of Wnts and their interactions with Frizzled and sFRP proteins and with extracellular matrix molecules remain poorly defined. The principal challenge is to prepare the requisite quantities of biologically active Wnts in soluble form. Wnts exhibit a low efficiency of secretion and a propensity to adhere to the extracellular matrix (ECM) (29–32), and, as a result, soluble Wnts thus far have only been produced in small quantities and in impure form. For example, biologically active Wg has been produced in the conditioned medium of transfected *Drosophila* Schneider (S2) cells (33), and biologically active Wnt-1 (34) and Wnt-5A (35) have been produced in the conditioned medium of transfected mammalian cells, but none of these preparations have been used for quantitative binding or structural studies.

In this paper, we report the production in *Drosophila* S2 cells of biologically active XWnt8. Epitope- or alkaline phosphatase-tagged XWnt8 proteins are secreted in a form that is suitable for quantitative biochemical experiments. Conditions also are described for the production of an IgG fusion of the CRD of mouse Frizzled 8 (mfz8) in milligram quantities. We demonstrate the use of these proteins for studying the interactions between soluble XWnt8 and various Frizzled proteins, membrane-anchored and

Abbreviations: AP, human placental alkaline phosphatase; CRD, cysteine-rich domain; ECM, extracellular matrix; fz, Frizzled; GPI, glycosylphosphatidylinositol; PI-PLC, phosphoinositide-phospholipase C; sFRP, secreted Frizzled-related protein; Wg, Wingless; X-phos, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, nitroblue tetrazolium; h, human; m, mouse; D, *Drosophila*; X, *Xenopus*.

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secreted CRDs, and a set of insertion mutants in the CRD of *Drosophila* Frizzled 2.

MATERIALS AND METHODS

DNA Constructs. The XWnt8-myc protein contains a carboxyl-terminal myc epitope tag (EQKLISEEDL) inserted between amino acids 339 and 340 (36). The XWnt8—human placental alkaline phosphatase (AP) construct contains the coding region of XWnt8-myc, without the carboxyl-terminal 19 amino acids, fused upstream of the AP catalytic domain in a modified version of pAPtag2, a vector from which the alkaline phosphatase signal sequence has been removed (37). Glycophosphatidylinositol (GPI)-anchored and myc-tagged CRDs were constructed as described in ref. 26 by using a modified pCIS vector (38) in which a DNA segment encoding a myc epitope (39) flanked by glycine spacers (GGGMEQKLISEEDLNGGG) is followed by a segment encoding the carboxyl-terminal 40 amino acids of decay-activating factor (40). To construct the CRD-myc-GPI expression plasmids, PCR products engineered to contain an optimal Kozak sequence around the initiator ATG and encompassing the indicated codons were inserted in-frame and upstream of the myc-GPI cassette: *Drosophila* fz2 (Dfz2), 1–270 (12); mzf3, 1–157; mzf4, 1–181; human fz5 (hfz5), 1–172; mzf6, 1–153; mzf7, 1–185; mzf8, 1–173 (11). Insertion of three codons (5'GGTTCGGGA encoding gly-ser-gly and containing a unique *Bsp*E1 site) into each of 23 locations in the Dfz2 CRD was accomplished by PCR amplification with pairs of mutagenic and flanking primers followed by replacement of the wild-type DNA segment with the amplified DNA segment between the *Bam*HI site at codon 58 and either the *Bst*EII site at codon 171 or the *Pst*I site at codon 270. The mzf8CRD-IgG fusion was generated by fusing codons 1–173 of mzf8 upstream of the hinge region of the human IgG heavy chain gene (41). All DNA segments generated by PCR were sequenced to rule out spurious mutations.

Production of XWnt8-myc and XWnt8-AP in S2 Cells. Conditioned medium containing XWnt8-myc or XWnt8-AP was produced in S2 cells stably transfected with the corresponding cDNA under the control of a metallothionein promoter. For protein production, cells were concentrated 4-fold and were incubated at 25°C for 24 hr in serum-free *Drosophila* Expression System medium (Invitrogen) containing 0.5 mM CuSO₄. Control medium was produced by CuSO₄ induction of untransfected S2 cells. The media were centrifuged at 100,000 × g at 4°C for 1 hr to remove aggregates and were concentrated 20-fold by ultrafiltration.

Production of mzf8CRD-IgG Fusion Protein. Mzf8CRD-IgG and IgG were produced in 293 cells that were transiently transfected by using Lipofectamine (GIBCO/BRL). One day after transfection, cells were transferred to serum-free DMEM/F-12, and the secreted protein was harvested after an additional 24 hr. Control conditioned medium was obtained from untransfected 293 cells. For some experiments, mzf8CRD-IgG and IgG were affinity purified by using protein A Sepharose (Amersham Pharmacia) with a low pH elution. Protein concentrations were estimated by visual comparison with a BSA standard after SDS/PAGE and Coomassie blue staining.

β-Catenin Stabilization Assay. C57MG cells were grown in 35-mm wells in DMEM supplemented with 10% fetal bovine serum and 10 μg/ml insulin and were ~70% confluent at the time of the assay. Cells were washed once with serum-free DMEM/F-12 and were incubated at the indicated temperature with 0.8 ml of S2-conditioned medium that was diluted with an equal volume of serum-free DMEM/F-12. Heparin (molecular weight = 6,000; Sigma) was added as indicated. After a 3-hr incubation, the conditioned medium was removed, and cells were harvested by incubation at 4°C for 5 min in PBS containing 5 mM EDTA and were collected by centrifugation. The cells were resuspended and homogenized in 100-μl lysis buffer (10 mM Tris-HCl, pH 7.5/140 mM NaCl/5 mM EDTA/0.5 μg/ml leupeptin/0.5 μg/ml aprotinin/0.5 μg/ml antipain), followed by centrifugation at 800 × g for

2 min. The resulting supernatant was further centrifuged at 100,000 × g for 30 min at 4°C, and the proteins in the high speed supernatant (containing cytoplasmic β-catenin) were separated in a SDS/7.5% polyacrylamide gel and were immunoblotted by using anti-β-catenin mAb 15B8 (Signa).

Phosphoinositide-Phospholipid C (PI-PLC) Release of GPI-Anchored Proteins. COS cells in 35-mm wells were transfected with 2 μg of plasmid DNA and 4 μl of FuGene 6 transfection reagent (Boehringer Mannheim). Twenty-four hours after transfection, the cells were harvested, were centrifuged at 800 × g for 2 min, and were resuspended in 100 μl of PBS containing 5 mM EDTA and 10 units of PI-PLC (Boehringer Mannheim). After a 1-hr incubation at 37°C with gentle rotation, the supernatant containing the released proteins was separated from the cells by centrifugation at 800 × g for 2 min. The cell pellet was resuspended in 100 μl of solubilization buffer (20 mM Hepes, pH 7.2/150 mM NaCl/1 mM EGTA/1 mM EDTA/10% glycerol/1% Triton X-100), was incubated on ice for 10 min, and was centrifuged at 4°C for 2 min at 800 × g to remove nuclei and cell debris.

Binding of XWnt8-AP and Antibodies to Transfected Cells. For staining under nonpermeabilized condition, live transfected COS cells growing on coverslips were incubated at room temperature for 1 hr with anti-myc mAb 9E10 (39) diluted 1:1,000 in culture medium or with XWnt8-AP-conditioned medium diluted with an equal volume of DMEM/F-12. After binding, cells either were washed three times with DMEM/F12 and were fixed in 2% methanol-free formaldehyde/PBS or were washed three times with binding buffer (0.5 mg/ml BSA in Hank's balanced salt solution with 20 mM Hepes, pH 7.0), were fixed for 30 sec in 60% acetone, 3% formaldehyde in 20 mM Hepes (pH 7.0), and were washed twice with binding buffer. For anti-myc staining, the fixed cells were incubated at room temperature with Texas Red-conjugated anti-mouse antibody (Vector Laboratories) diluted 1:200 in 5% goat serum in PBS, followed by three washes in PBS. For XWnt8-AP staining, the fixed cells in 20 mM Hepes (pH 7.0), 150 mM NaCl were incubated at 65°C for 100 min, were washed once in AP buffer (0.1 M Tris-HCl, pH 9.4/0.1 M NaCl/5 mM NaCl) and were stained at room temperature overnight with 5-bromo-4-chloro-3-indolyl-phosphate (X-phos) (165 μg/ml)/nitroblue tetrazolium (NBT) (330 μg/ml) in AP buffer. For anti-myc staining under permeabilized condition, the transfected cells were fixed in 60% acetone, 3% formaldehyde in 20 mM Hepes (pH 7.0) before antibody incubation.

Solution Binding Assay. Twelve micrograms of IgG or mzf8CRD-IgG were prebound to protein A Sepharose beads and were incubated for 16 hr at 4°C with 500 μl of 4-fold-concentrated XWnt8-myc-conditioned medium. The beads were separated by centrifugation into bound and unbound fractions and then were washed over 10 minutes at 4°C with three changes of Hank's balanced salt solution with 20 mM Hepes (pH 7.0).

Solid Phase Binding Assay. One-hundred microliters of 293 cell conditioned medium containing 2 μg of Mzf8CRD-IgG or IgG alone was used to coat each well of a 96-well tray at 4°C overnight. The unoccupied sites in the wells were blocked at 4°C for 4 hr with 200 μl of 2 mg/ml BSA in wash buffer [Hank's balanced salt solution with 20 mM Hepes (pH 7.0)]. After three washes in wash buffer, XWnt8-AP-conditioned medium diluted in 2 mg/ml BSA in wash buffer was added to the coated wells. After incubation at 4°C for 20 hr, the wells were washed five times with 200 μl of wash buffer. Two-hundred microliters of 150 mM p-nitrophenyl phosphate in 1 M diethanolamine (pH 9.8), 1 mM MgCl₂ was added to each well, and the change in absorbance at 405 nm was measured.

RESULTS

Production of XWnt8-myc and XWnt8-AP in S2 Cells. Our attempts to produce a soluble vertebrate Wnt protein for biochemical experiments have focused on XWnt8 because Moon and colleagues (36, 42) have observed that addition of a myc

epitope tag near the XWnt8 carboxyl terminus, a construct referred to here as XWnt8-myc, is compatible with biological activity in *Xenopus* embryos. As an expression system, we have chosen *Drosophila* Schneider (S2) cells, which, as noted above, have been used successfully to produce bioactive Wg (33) as well as a variety of other secreted vertebrate proteins (43). Fig. 1A shows that S2 cells can secrete both XWnt8-myc and a fusion of XWnt8-myc and the catalytic domain of human placental alkaline phosphatase (XWnt8-AP). A comparison of the anti-myc immunoblot signals from XWnt8-myc and XWnt8-AP and from a myc-tagged derivative of the *Escherichia coli* maltose binding protein indicates that XWnt8-myc and XWnt8-AP are secreted from concentrated S2 cells with yields up to ≈ 5 and ≈ 0.5 mg/liter, respectively.

The biological activity of XWnt8-myc was tested by applying S2-conditioned medium containing XWnt8-myc or control S2 conditioned medium to C57MG cells and monitoring the stabilization of cytoplasmic β -catenin (Fig. 1B; ref. 44). In these experiments, we observed significant β -catenin stabilization if the incubation was performed at 23°C but little or no stabilization at 37°C. XWnt8-myc activity was enhanced by the inclusion of heparin, with a maximal effect at 0.1–0.5 μ g/ml. The temperature sensitivity of XWnt8-myc activity on C57MG cells could reflect either instability of XWnt8-myc and/or associated proteins from S2 cells or a temperature-dependence of β -catenin turnover in C57MG cells. XWnt8-myc is also active in stabilizing Armadillo in *Drosophila* clone 8 cells, an imaginal disc cell line, but the degree of stabilization is severalfold lower than that obtained with similarly prepared S2 conditioned medium containing Wg (data not shown). XWnt8-myc produced in transiently transfected 293 cells was secreted with an efficiency ≈ 10 -fold lower than that observed for S2 cells (Fig. 1A) and was inactive in the β -catenin stabilization assay and in Frizzled binding assays (data not shown).

Cell Surface Binding of XWnt8-AP to Frizzled Proteins and Frizzled CRDs. The XWnt8-AP fusion protein described above provides a convenient and quantitative probe to investigate the binding of Wnt and Frizzled proteins. When conditioned medium containing XWnt8-AP was incubated at room temperature with

live COS or 293 cells and subsequently was visualized with a X-phos/NBT tetrazolium histochemical reaction, little or no cell-associated AP activity was observed, indicating that this fusion protein does not bind significantly to either plasma membrane proteins or the ECM produced by these two cell lines. This behavior is in contrast to that of Wg produced by S2 cells, which adheres to the ECM of a variety of cultured cells. Specific binding of Wg to cell surface Frizzled proteins only has been observed after heparitinase pretreatment to decrease ECM binding (12). Transient transfection of COS or 293 cells with Dfz2, mzf4, hzf5, mzf7, mzf8, and Xfz8 conferred cell surface XWnt8-AP binding whereas transfection with mzf3 and mzf6 did not, a pattern identical to that observed previously for Wg (Table 1 and Fig. 2B; ref. 12). Binding experiments with control conditioned medium from untransfected S2 cells shows no alkaline phosphatase staining of COS cells transfected with Dfz2, mzf4, or hzf5, indicating that the observed Frizzled binding activity derives from XWnt8-AP. In these experiments, absence of XWnt8-AP binding could reflect a low affinity between ligand and receptor or a defect in the stability, folding, or surface localization of the Frizzled protein. Therefore, to measure the surface localization of each receptor protein in a uniform manner and also to assess the binding of XWnt8-AP to the various Frizzled CRDs in the absence of the Frizzled membrane spanning domains, we constructed a set of myc-tagged and GPI-anchored derivatives of each CRD (Fig. 2).

Incubation of XWnt8-AP with transfected cells expressing CRD-myc-GPI constructs from Dfz2, mzf3, mzf4, hzf5, mzf6, mzf7, and mzf8 reveals the same relative efficiency of XWnt8-AP

Table 1. Semiquantitative assessment of XWnt8-AP binding to different Frizzled and CRD-myc-GPI proteins based on X-phos/NBT staining of live, transiently transfected COS cells

Frizzled	Full-length protein	CRD-myc-GPI
mzf3	—	—
mzf4	++	++
hzf5	++	++
mzf6	—	—
mzf7	+	+
mzf8	++	++
Xfz8	++	ND
Dfz2	++	++
1. Dfz2 (66GSG67)	—	—
2. Dfz2 (68GSG69)	—	—
3. Dfz2 (73GSG74)	—	—
4. Dfz2 (80GSG81)	—	—
5. Dfz2 (85GSG86)	++	+
6. Dfz2 (89GSG90)	—	—
7. Dfz2 (92GSG93)	—	—
8. Dfz2 (96GSG97)	—	—
9. Dfz2 (102GSG103)	++	++
10. Dfz2 (106GSG107)	++	++
11. Dfz2 (111GSG112)	+	—
12. Dfz2 (114GSG115)	—	—
13. Dfz2 (126GSG127)	+	+
14. Dfz2 (128GSG129)	++	++
15. Dfz2 (130GSG131)	+	—
16. Dfz2 (137GSG138)	—	—
17. Dfz2 (141GSG142)	—	—
18. Dfz2 (144GSG145)	++	—
19. Dfz2 (152GSG153)	++	++
20. Dfz2 (160GSG161)	++	+
21. Dfz2 (165GSG166)	—	—
22. Dfz2 (172GSG173)	++	+
23. Dfz2 (174GSG175)	++	++

The three amino acid insertion mutants are referred to by the codon numbers flanking the gly-ser-gly insertion. For example, 66GSG67 refers to the insertion of the nine nucleotides encoding gly-ser-gly between codons 66 and 67. ++, strong binding; +, weak binding; —, undetectable binding. ND, not determined.

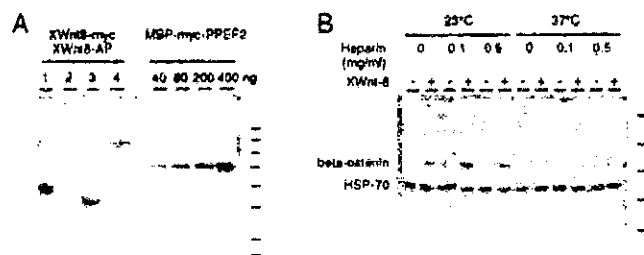


FIG. 1. Secretion of biologically active XWnt8-myc by S2 cells. (A) Immunoblot using anti-myc mAb. (Left) Lanes: 1, 32 μ l of 40-fold concentrated conditioned medium from 293 cells transfected with XWnt8-myc; 2, 32 μ l of 4-fold concentrated conditioned medium from untransfected S2 cells; 3, 32 μ l of 4-fold concentrated conditioned medium from S2 cells transfected with XWnt8-myc; 4, 32 μ l of 80-fold concentrated conditioned medium from S2 cells transfected with XWnt8-myc-AP. (Right) Dilution series of a purified fusion protein used as an immunoblot standard: the fusion protein contains the myc epitope flanked by the *E. coli* maltose binding protein at the amino terminus and an ≈ 15 -kDa segment of a ser/thr protein phosphatase at the carboxyl terminus (57), a configuration that eliminates problems in quantitation that might arise from proteolytic cleavage of an epitope fused to either of the two termini. Molecular mass standards are 194, 120, 87, 64, 52, 39, 26, and 21 kDa. (B) XWnt8-myc stabilizes β -catenin in C57MG cells at room temperature, and this activity is enhanced by heparin. Conditioned medium from untransfected S2 cells (—) or XWnt8-myc transfected S2 cells (+) in the presence of the indicated concentration of heparin was applied to C57MG cells at either 23 or 37°C as indicated. Cytosolic proteins were prepared, and β -catenin was visualized with mAb 15B8. To control for equal loading, HSP-70 was visualized with mAb BRM-22 (Sigma). Molecular mass standards are 194, 120, 87, 64, 52, and 39 kDa.

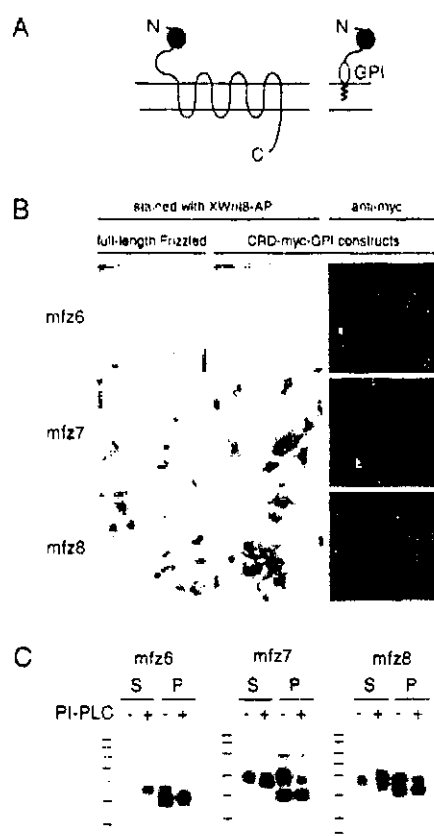


FIG. 2. Binding of XWnt8-AP to full-length Frizzled proteins and the corresponding GPI-anchored CRDs on the surface of transfected COS cells. (A) Schematic diagram showing the location of the CRD (filled ball) in a full-length Frizzled protein and in a CRD-myc-GPI construct. Horizontal lines represent the membrane, and zigzag lines represent the lipid component of GPI. N, amino terminus; C, carboxyl terminus. (B and C) XWnt8-AP binding and surface localization assays for three Frizzled proteins that show undetectable binding (mfz6), intermediate binding (mfz7), and strong binding (mfz8). (B) Light microscopy of representative samples of COS cells transiently transfected with the indicated full-length Frizzled (left column) or the corresponding Frizzled CRD-myc-GPI construct (center and right columns). Live cells were stained with XWnt8-AP (left and center columns) or with anti-myc mAb and a fluorescent secondary antibody (right column). The cells remained intact during the binding reaction as determined by the failure of the anti-myc mAb to bind myc-tagged Dishevelled (a cytoplasmic protein) under these conditions; fixation and permeabilization with acetone and paraformaldehyde before incubation with the anti-myc mAb led to intense staining of myc-tagged Dishevelled (data not shown). (C) PI-PLC release of GPI-anchored CRDs from the surface of live cells. Transfected COS cells were incubated in the absence (-) or presence (+) of PI-PLC, and, after centrifugation, the released protein was recovered from the supernatant (S) and the cells were recovered from the pellet (P). Proteins were resolved by SDS/PAGE and were visualized with anti-myc mAb immunoblotting. Molecular mass standards are 194, 120, 87, 64, 52, 39, 26, and 21 kDa.

binding as seen with the corresponding full-length Frizzled proteins. When live cells transfected with each CRD-myc-GPI construct were immunostained with anti-myc mAbs, similar levels of cell-surface CRD-myc-GPI protein were observed in each case (Fig. 2B and data not shown). As an independent measure of plasma membrane localization of the CRD-myc-GPI proteins, live cells expressing each of the seven constructs were treated with PI-PLC to cleave the GPI anchor and release the surface-accessible protein into the medium. Anti-myc immunoblotting of the CRD-myc-GPI proteins showed that, in each case, three major electrophoretic species are observed, presumably reflecting different patterns of glycosylation. The protein released by PI-PLC treatment corresponds to the lowest mobility species,

suggesting that this species is the only one present at the cell surface. The cell surface CRD-myc proteins were released by PI-PLC treatment with yields that range from 10 to 30% of the total CRD-myc-GPI protein, suggesting that in each case a significant fraction of the CRD protein is able to fold correctly and move through the endoplasmic reticulum-Golgi-plasma membrane pathway (Fig. 2C and data not shown; ref. 45). Because each of the different CRD-myc-GPI proteins is available for binding, these experiments demonstrate that XWnt8-AP binds efficiently to the CRDs of Dfz2, mfz4, hzf5, mfz7, and mfz8 but binds poorly or not at all to the CRDs of mfz3 and mfz6.

XWnt8-AP Binding to Insertion Mutants of Dfz2. To begin to define the regions of the CRD that mediate Wnt binding, we measured the binding of XWnt8-AP to live cells that had been transfected with a series of 23 insertion mutants in the CRD of Dfz2 (Fig. 3A). Insertional mutagenesis was performed with a three-codon cassette that codes for gly-ser-gly, an insertion that would be predicted to sterically disrupt binding if located at any point on the ligand-binding surface. Failure to bind XWnt8-AP also could reflect a defect in the folding or stability of the CRD. To minimize the probability that the tripeptide insertion might cause a structural defect, the 23 insertions were placed at least one amino acid from any cysteine and within those regions that are most hydrophilic. Given the predicted flexibility of the gly-ser-gly tripeptide (46), we presume that most insertions within surface loops would be compatible with a correctly folded CRD.

Each of the 23 insertion mutants was studied in the context of the full-length Dfz2 protein and in a CRD-myc-GPI construct. After transfection, the efficiency of plasma membrane localization was determined for each CRD-myc-GPI mutant by immunostaining of live cells with anti-myc mAbs and by PI-PLC digestion of live cells followed by immunoblotting as described above (Fig. 3B and C). Of interest, all 23 mutants resemble wild-type Dfz2-CRD-GPI in showing robust cell surface immunostaining and significant release of the low mobility electrophoretic species on PI-PLC treatment (corresponding to 10–30% of the total Dfz2-CRD protein), indicative of a high degree of plasma membrane localization. On the assumption that passage through the endoplasmic reticulum-Golgi-plasma membrane pathway reflects correct folding (45), this result implies that the native CRD structure can tolerate insertions at each of these positions. This observation raises the possibility that the CRD may be a relatively extended structure with a high surface to volume ratio.

Different Dfz2 insertion mutants differ markedly in XWnt8-AP binding when assayed on the surface of live transfected cells (Table 1 and Fig. 3B). Mutants 15 and 18 (130GSG131 and 144GSG145) bind XWnt8-AP when they are present in the context of the full-length Dfz2 sequence but fail to bind as GPI-anchored CRDs, suggesting the possibility that stabilizing interactions may occur between the membrane-embedded domain of the Frizzled protein and the CRD or the CRD-Wnt complex. Among the Dfz2CRD-GPI mutants, 5 bind with a strength indistinguishable from that of the wild type, 4 bind weakly, and 14 fail to bind. Binding and nonbinding mutants are distributed throughout the length of the CRD with nonbinding mutants showing significant clustering.

Quantitative Binding of XWnt8-AP to mfz8CRD-IgG. The binding of soluble XWnt8-AP to membrane-anchored CRDs suggests that these proteins also might interact if the CRD is produced as a secreted molecule. To test this idea, we produced the mfz8 CRD as an amino-terminal fusion with a human IgG heavy chain (mfz8CRD-IgG) in transfected 293 cells. In this expression system, mfz8CRD-IgG accumulates to ≈ 20 mg/liter and can be purified to apparent homogeneity with protein A Sepharose (Fig. 4A).

In an initial experiment, we determined the efficiency of coprecipitation of XWnt8-myc with mfz8CRD-IgG or with the IgG backbone alone (Fig. 4B). This experiment indicates that at least 90% of the secreted XWnt8-myc is competent to specifically

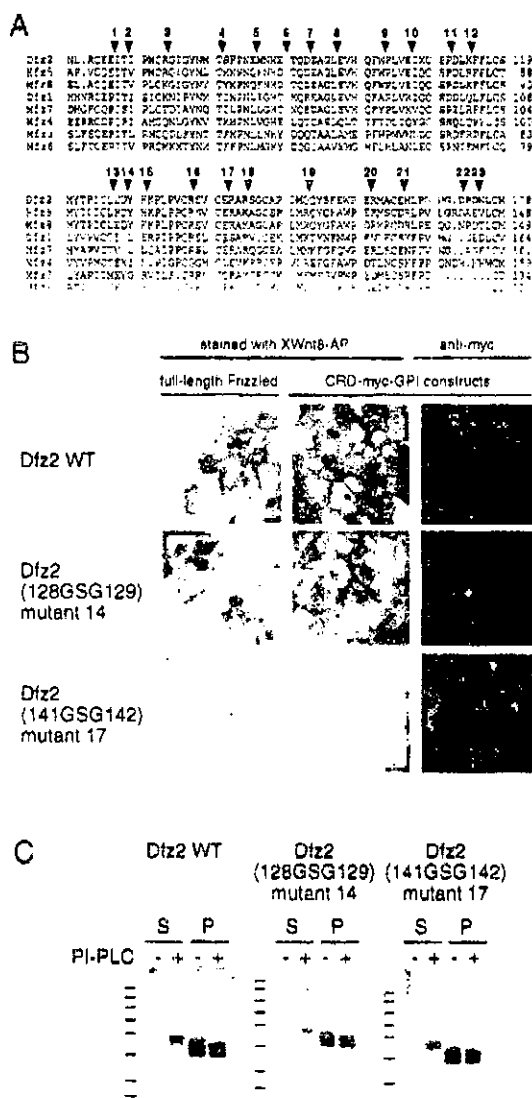


FIG. 3. Binding of XWnt8-AP to tripeptide insertion mutants within the Dfz2 CRD. For the wild type and for each mutant, the experiments were performed with both full-length Dfz2 and the corresponding GPI-anchored CRDs displayed on the surface of transfected COS cells. (A) Alignment of CRDs from a subset of Frizzled proteins showing the locations of the 23 gly-ser-gly insertion mutations. (B) Light microscopy of representative samples of COS cells transiently transfected with the indicated full-length Dfz2 (left column) or the corresponding Dfz2 CRD-myc-GPI construct (center and right columns). Examples are shown for wild-type Dfz2, Dfz2 mutant 14 (128GSG129), which shows strong binding and Dfz2 mutant 17 (141GSG142), which shows no detectable binding. Live cells were stained with XWnt8-AP (left and center columns) or with anti-myc mAb and a fluorescent secondary antibody (right column), as described for Fig. 2. (C) PI-PLC release of GPI-anchored Dfz2 CRDs from the surface of live cells, as described for Fig. 2. Molecular mass standards are 194, 120, 87, 64, 52, 39, 26, and 21 kDa.

bind mzf8CRD-IgG. These reagents then were used to develop a solid phase enzyme-linked binding assay in which varying concentrations of soluble XWnt8-AP were incubated with immobilized mzf8CRD-IgG or IgG in the wells of a 96-well tray (Fig. 4C). Four independent experiments using either crude conditioned medium containing mzf8CRD-IgG, which is ~80% pure, or Protein A Sepharose purified mzf8CRD-IgG yielded similar results. When redrawn as a Scatchard plot and fit to a single line, the binding data indicate an average affinity of 9 ± 2 nM ($n = 4$). However, close examination of the Scatchard plots suggests that two or more classes of sites may better represent the binding

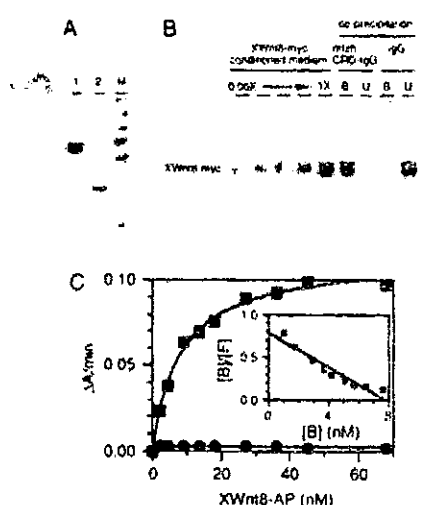


FIG. 4. Binding of XWnt8-myc and XWnt8-AP to mzf8CRD-IgG. (A) Coomassie-stained gel with mzf8CRD-IgG (lane 1) or the IgG fusion partner alone (consisting of the hinge and F_c regions) (lane 2) secreted from transfected 293 cells and purified by using protein A Sepharose. Molecular mass standards are 194, 120, 87, 64, 52, 39, 26, 21, and 15 kDa. (B) Binding efficiency of XWnt8-myc with IgG or mzf8CRD-IgG prebound to protein A Sepharose beads. Equal fractions of the bound (B), unbound (U), or starting material (1X) were analyzed by SDS/PAGE and anti-myc immunoblotting. A 2-fold dilution series of the starting material is shown at left. Greater than 90% of XWnt8-myc was bound to mzf8CRD-IgG, with little or no binding to IgG. Molecular mass standards are 194, 120, 87, 64, 52, 39, 26, and 21 kDa. (C) Solid phase binding of XWnt8-AP to IgG (circles) or mzf8CRD-IgG (squares). The rate of hydrolysis of p-nitrophenyl phosphate (measured as the rate of change in absorbance at 405 nm) is plotted against XWnt8-myc concentration. *Inset* shows a Scatchard plot of the binding data fit to a single straight line, which yields a calculated K_d of 8 nM. B, bound; F, free.

data; under an assumption of two sites, the estimated affinities of the two sites would be predicted to differ by ≈ 10 -fold.

DISCUSSION

Production of Soluble Wnt and Frizzled Derivatives. Producing soluble, biochemically well behaved Wnts has been a long-standing goal in this field but has been problematic. Producing secreted Frizzled or sFRP CRDs, the domain implicated in Wnt binding, either alone or as fusion proteins with IgG or AP, also has proven difficult because most are poorly secreted (A.R., J.-C.H., and J.N., unpublished data). The several examples described in the present report represent that minority of constructs and host-vector systems for which we have observed good yields of apparently native proteins. In the case of XWnt8, this could reflect the presence in S2 cells of putative chaperones such as the porcupine protein (47, 48), as yet uncharacterized proteins in the conditioned medium that might associate with XWnt8, or the low temperature (23°C) at which S2 cells are grown. The efficient secretion of mzf8CRD-IgG from transfected 293 cells—~20 mg/liter—may reflect a serendipitous choice of the fusion point between the CRD and the IgG heavy chain in this construct.

Structural and Functional Mapping of the CRD. The analysis of 23 tripeptide insertion mutants in the Dfz2 CRD identifies a set of discontinuous segments along the linear sequence where insertion results in loss of XWnt8-AP binding. These are presumed to be sites of direct or close contact with the XWnt8-AP ligand. Other regions where tripeptide insertion fails to diminish XWnt8-AP binding can be eliminated as sites of close contact. The loss of XWnt8-AP binding caused by several tripeptide insertions in the carboxyl-terminal two-thirds of the CRD is at odds with the results of Lin *et al.* (49) in which coimmunoprecipitation was observed between cotransfected Wnt-1 and various sFRP-3 derivatives, including deletion derivatives lacking large parts of the CRD. This discrepancy may be related to the

significant endoplasmic reticulum retention that is characteristic of both Wnts and sFRPs, which suggests that much of the association observed between these proteins in cotransfected cells represents aggregation of misfolded proteins. These conflicting data serve to emphasize the importance for binding experiments of starting with biochemically well behaved proteins.

Specificity of Wnt-CRD Binding. The available data for Wnt-Frizzled and Wnt-sFRP binding at the surface of transfected cells (refs. 12, 23, and 26 and this report) indicates that some Wnts can bind to multiple Frizzled and/or sFRP targets. For example, a *Drosophila* Wnt, Wg, binds with similar efficiency to a subset of mammalian Frizzled proteins and to *Drosophila* Frizzled and Frizzled-2 (12). Here, we show that a *Xenopus* Wnt, XWnt8, binds with similar efficiency to *Drosophila* fz2 and the same set of mammalian Frizzled proteins to which Wg binds.

One explanation for this apparent promiscuity in binding is that semiquantitative cell surface binding assays do not reveal small but biologically significant differences in affinity. This possibility can be investigated by extending the quantitative enzyme-linked binding assay described here to cell-surface measurements and by determining the binding affinities of additional pairs of Wnts and CRD-IgG fusions. It is also possible that additional factors associate with Wnts, Frizzled proteins, or sFRPs *in vivo* to modify and/or narrow the specificity of their interactions. These factors might include heparin, other ECM components, coreceptors, plasma membrane lipids, and additional secreted proteins. The plausibility of these more complex interactions is supported by the well known affinity of Wnts for heparin and other ECM components (e.g., ref. 31), the ability of heparin and other ECM components to modulate Wnt signaling *in vitro* (ref. 50 and this report), the affinity of the carboxyl-terminal domain of at least two of the sFRPs for heparin (ref. 22; A.R., J.-C.H., and J.N., unpublished work), and the recent discovery of Wnt inhibitory factor-1, a secreted Wnt-binding protein that lacks a CRD (51). *In vivo* evidence for the importance of the ECM in Wnt signaling comes from the effects of mutations in proteoglycan biosynthetic genes in *Drosophila* (52–54).

Despite the evidence for additional cofactors, the *in vitro* binding experiments with mFz8CRD-IgG show that affinities of <10 nM are obtained with the CRD alone. These experiments imply that the Frizzled transmembrane domains, plasma membrane lipids, ECM, and putative coreceptors do not play an obligatory role in Wnt-Frizzled binding. In particular, the solid phase binding assay shows that heparin does not alter the affinity of XWnt8-AP for mFz8CRD-IgG, although it enhances β -catenin stabilization induced by XWnt8-myc treatment of C57MG cells. These data suggest that the role of heparin in Wnt signal transduction may not involve a direct effect on receptor–ligand binding, a possibility that would be in contrast to the direct role of heparin in fibroblast growth factor dimerization and receptor binding (55, 56). We note that the experiments reported here do not rule out the possibility that other proteins in the S2 conditioned medium may associate with XWnt8 and participate in the binding reaction.

Wnt, Frizzled, and sFRP proteins are all members of large families. To date, 15 Wnt, 9 Frizzled, and 5 sFRP genes have been identified in mammals, and several Wnt and Frizzled genes have been identified in *Drosophila* and in *C. elegans*. The broad and overlapping patterns of expression seen for many of these genes raises the possibility of multiple Wnt-Frizzled and Wnt-sFRP interactions. There is currently little quantitative information regarding the affinity and specificity of interactions among individual members of these protein families *in vitro*, and, in most cases, it is not yet known which family members interact in a biologically meaningful manner *in vivo*. The *in vitro* interactions observed among several Wnt, Frizzled, and sFRP proteins from diverse vertebrate and invertebrate species indicate that the relevant protein–protein interfaces have been conserved during evolution and suggests that many pairs of family members may have the potential to interact.

The authors thank Dr. Randy Moon for the XWnt8-myc plasmid; Dr. Peter Klein for Xfz8; Dr. Brian Seed for the human IgG cDNA; Dr. John Flanagan for the AP cDNA; Dr. Ingrid Caras for the decay activating factor cDNA; and Drs. Phil Beachy and Dan Leahy for comments on the manuscript. This work was supported by the Howard Hughes Medical Institute.

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Monomeric Fc Fusions

Impact on Pharmacokinetic and Biological Activity of Protein Therapeutics

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Abstract

The delivery of therapeutic proteins by noninvasive routes of administration has been a challenging goal, hence current modes of delivery generally require injections. However, we have recently shown that a naturally occurring receptor, the neonatal Fc receptor (FcRn) can be utilized to carry aerosolized therapeutic proteins conjugated to a portion of its respective ligand (Fc domain of immunoglobulin G) across epithelial cells of the lung to effectively deliver biologically active molecules to the bloodstream. First-generation dimeric Fc fusion molecules were successfully transported by the pulmonary route and biologic activity was demonstrated in both non-human primates and human volunteers. Continuing efforts to improve transport efficiency have led to the development of an alternate configuration of Fc fusion proteins with improved characteristics. These second generation Fc fusion molecules are monomeric with respect to the therapeutic protein and dimeric with respect to the Fc region, and have been termed Fc fusion 'monomers'. Several different Fc fusion monomers have demonstrated improved transport efficiency, achieving high bioavailabilities for pulmonary delivery in non-human primates. While the traditional dimeric Fc fusion molecule generally increases the half-life compared with the unconjugated effector molecule, the monomer configuration has been shown to result in an even greater extension of the circulating half-life, which improves pharmacokinetic parameters for protein therapeutics, whether administered by pulmonary delivery or injection. Finally, many of the Fc monomer fusions have enhanced biologic activity compared with the dimeric configuration. Because of these many advantages, the monomer configuration promises to be an enabling advance to achieve clinically relevant, noninvasive delivery with potentially less frequent administration regimens for a broad range of protein therapeutics. In addition,

molecules that are comprised of heterodimeric subunits or multi-subunit complexes can also be constructed as Fc fusions that result in a molecule with enhanced pharmacokinetics and greater bioactivity. Several examples of novel Fc fusion proteins, both monomer and heterodimer are described herein.

The Fc domain of immunoglobulins has been utilized to form fusions with molecules such as cytokines, growth factors, enzymes or the ligand-binding region of a receptor or adhesion molecule,^[1-5] which have proven to be useful research tools and, in some cases, therapeutic agents (e.g. etanercept, alefacept, abatacept). A naturally occurring receptor called the neonatal Fc receptor (FcRn) has been shown to transport high molecular weight Fc fusion molecules noninvasively by the pulmonary route in non-human primates and humans.^[6,7] In an effort to improve the pharmacokinetics of these Fc fusion molecules, we modified the traditional Fc fusion construct (dimeric with respect to the effector molecules) to create novel Fc fusion configurations. Several examples of these enhanced molecules are described below.

1. Neonatal Fc Receptor (FcRn)-Mediated Transport and Protection of Immunoglobulin

More than 40 years ago, Brambell and colleagues^[8] published a hypothesis that linked the transmission of passive immunity (i.e. transmission of antibodies from mother to young) with the protection of IgG from catabolism. He postulated that a single protein receptor might mediate both processes and also demonstrated that it was the Fc domain of IgG that was involved in both the transport of IgG as well as the protection from degradation.^[9] Brambell^[9] also suggested that the receptors involved in maternofetal transfer of IgG were saturable, based on data generated by Fahey and Robinson^[10] that showed increased metabolism of IgG with hyperimmunization.

This receptor was isolated, characterized, and cloned nearly 30 years later from the brush borders of intestinal epithelial cells of the neonatal rat and was called FcRn.^[11,12] The receptor is comprised of two subunits, one which is structurally related to the major histocompatibility complex (MHC) class I antigens and the other subunit is β_2 -microglobulin (β_2m). Further studies by others provided direct evidence confirming the role of FcRn in regulation of IgG catabolism^[13-15] and the transport of IgG in mice.^[16]

Site directed mutagenesis of the Fc domain of IgG identified the CH2 and CH3 domains as the regions critical for the control of IgG catabolism.^[17] Later, crystal structures for the complex of the Fc fragment of IgG and FcRn were determined for both rat and human,^[18,19] and the residues important for this interaction were

more precisely identified. Further mapping of the binding sites on FcRn revealed Ile 253, His 310, and His 435 as residues important to the regulation of IgG half-life.^[20]

2. Expression of FcRn

FcRn was initially isolated from the intestinal epithelium of newborn rats, where it is expressed at relatively high levels during the first few weeks of life and is responsible for the absorption of immunoglobulin from maternal milk.^[11,21] At the time of weaning, expression in the intestine decreases precipitously and remains low throughout adult life. The receptor is also expressed in the yolk sac of rats, where maternal transfer of immunoglobulin takes place during gestation.^[22]

FcRn is expressed in the syncytiotrophoblast of the placenta, where it mediates maternofetal transfer of immunoglobulin during gestation in humans.^[23] In contrast to rodents, expression in humans remains relatively constant throughout life in the epithelial cells lining the intestine as well as the lung and kidney.^[24-26] Further examination of expression in the lung showed that expression of FcRn is most prominent in the conducting airways, with less receptor being found in the peripheral lung regions in humans and non-human primates.^[6]

FcRn is also expressed in the endothelial cells lining the vasculature, the site at which IgG protection most likely occurs.^[27,28] IgG binding to FcRn occurs intracellularly in acidic endosomes where FcRn expression is localized. IgG bound to FcRn is shuttled back to the plasma membrane of the endothelial cells and released back into the circulation, whereas IgG that has not interacted with FcRn appears to be degraded in lysosomes.^[29,30]

The transport pathway involving FcRn has since been utilized to deliver therapeutic proteins noninvasively by the pulmonary route in humans and non-human primates through conjugation of the effector molecule to the Fc domain of IgG.^[6,7]

3. Pulmonary Delivery of a Prototype Fc Fusion Protein

3.1 Prototype Molecule Erythropoietin-Fc (EpoFc)

A prototype molecule was created by recombinantly fusing full-length human erythropoietin (Epo) to the Fc domain (hinge-

CH2-CH3) of IgG1 and was called EpoFc. Epo was selected as the model molecule because it is a well characterized protein that stimulates red blood cell production and elicits a readily measured biologic response.^[31] In addition, the fusion molecule was approximately 112 kDa in size, which we thought would sufficiently test the transport system. The protein was produced recombinantly in Chinese hamster ovary cells. This molecule bound to the Epo receptor with an affinity not significantly different from that of unconjugated Epo and bound to FcRn with slightly less affinity compared with IgG.^[6] EpoFc had slightly less biologic activity as measured *in vitro* in a TF-1 (human erythroleukemia) cell proliferation assay.^[6]

3.2 Pulmonary Delivery of EpoFc Dimer in Non-human Primates

Because FcRn expression is greatest in the central lung regions with very little expression in the alveolar regions,^[6] delivery of EpoFc was targeted to the upper airways of non-human primates. This was accomplished by administering aerosolized protein (particle size 4–6 μ m mass median aerodynamic diameter) to intubated anesthetized animals that were allowed to breathe spontaneously. The lung-deposited dose of EpoFc was ~0.3 mg/kg, which was calculated based on a deposition fraction of 15%. Confirmation of delivery to the targeted lung regions and estimation of delivered dose were determined from studies using co-administration of a radioactive tracer followed by gamma scintigraphy. EpoFc was readily measured with maximum serum concentrations (C_{max}) of 800–1000 ng/mL (figure 1), which is much greater than the concentration of Epo that is necessary to achieve a biologic response.^[32] To show that this uptake was specifically mediated by FcRn, three mutations (I253A, H310A, and H435A)^[20,33] were introduced into the Fc domain of EpoFc such that it no longer bound to FcRn (the mutant construct was termed 'EpoFc/IHH'). Only poor absorption from the lung was observed for the mutant molecule, thereby demonstrating that uptake was mediated by FcRn (figure 1).

Because the standard method for administration of macromolecules to the lung was to target the deep lung regions,^[34,35] delivery of EpoFc to this area of the lung was also studied. This was accomplished in the non-human primates by forced deep inhalation including a breath hold using a respirator. In addition, aerosols with a small particle size (~2 μ m) were created to favor deposition to the alveoli. Using this method of delivery, uptake was again poor and resembled that of the mutant protein (figure 1). These results were consistent with absorption

from the sites in the airways where expression of FcRn is greatest.^[6] Absorption of macromolecules in the upper airways differentiates this method of delivery from approaches that target drugs to the deep lung regions.^[36] Uptake of EpoFc at doses necessary to produce a biologic response was highly reproducible, and bioavailability was ~5–10% compared with intravenous administration.

3.3 Pulmonary Delivery of EpoFc Dimer in Humans

A clinical study was performed to determine if the FcRn pathway functioned similarly in humans.^[7] Single doses (3, 10, or 30 μ g/kg) of EpoFc were administered to healthy volunteers by inhalation. The subjects were asked to breathe normally targeting 10–30% vital capacity and approximately 15 breaths per minute in order to favor deposition to the central lung regions. While most subjects achieved the targeted breathing patterns, there were some outliers. Despite this variability in breathing patterns, consistent deposition to the lung was achieved with 70% of the total dose deposited in the central airways and 30% deposited in the peripheral lung regions for all three dose groups. Concentrations of EpoFc in the serum were dose-dependent and serum concentration versus time curves were similar for different individuals within each dose group (figure 2). In addition, a biologic response (i.e. an increase in circulating reticulocytes) was evident in the highest dose group (EpoFc 30 μ g/kg). The results of the clinical study

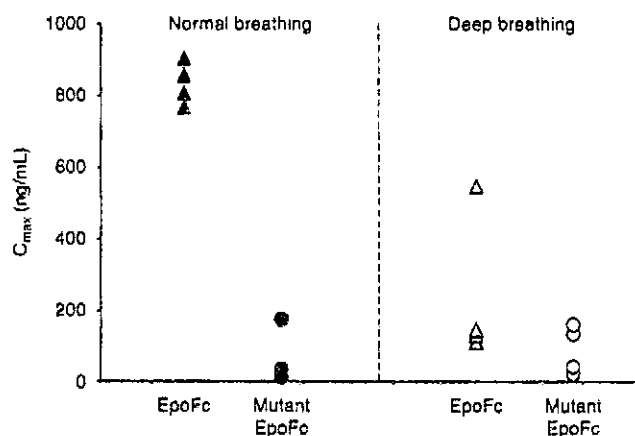


Fig. 1. Effects of breathing pattern on absorption of erythropoietin (Epo)Fc from the lungs of non-human primates. Approximately 0.3 mg/kg of either EpoFc or mutant EpoFc/IHH (a mutant construct with I253A, H310A, and H435A mutations) was deposited into the lungs of monkeys that were breathing normally (EpoFc, $n = 4$; EpoFc/IHH, $n = 3$) or breathing deeply (EpoFc, $n = 4$; EpoFc/IHH, $n = 4$). EpoFc and EpoFc/IHH in serum from each animal were measured using an Epo-specific ELISA. The maximum serum concentrations (C_{max}) for each animal are shown in the graph (reproduced from Bitonti et al.^[6] Copyright [2004] National Academy of Sciences, USA, with permission).

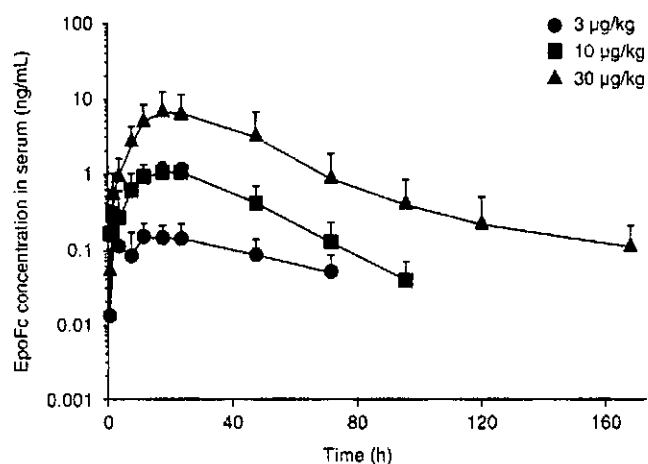


Fig. 2. Pulmonary delivery of erythropoietin (Epo)Fc to human volunteers. Healthy volunteers were administered a single dose of EpoFc by inhalation using the Aeronex[®] Pro nebulizer to aerosolize the protein. Lung deposited doses were 3 µg/kg, 10 µg/kg, or 30 µg/kg. The number of subjects per group were 7, 6, and 8, respectively. Serum concentrations of EpoFc were measured by an Epo-specific ELISA (reproduced from Dumont et al.,¹⁷ with permission of Mary Ann Liebert, Inc.).

demonstrated that the preclinical model described in non-human primates was predictive of the transport pathway in humans.

4. Monomeric Fc Fusions

4.1 Rationale for Fc Fusion Protein Monomers

Although the pulmonary delivery of large proteins was demonstrated in non-human primates and humans, we thought that the efficiency of transport could be improved to increase the bioavailability. Airway mucus provides a protective environment for the airways and can be a substantial barrier for the absorption of molecules through the lung.^[37-39] It is known that size can affect the ability of molecules to penetrate the mucus layer. In fact, there is an inverse relationship between molecular weight and the diffusion of particles through mucus, which is most apparent at molecular weights >30 kDa^[40] Also, it has been shown that charge can affect endocytosis in epithelial cells, which is an important step for uptake through the lung.^[41] Because EpoFc is a relatively large protein (112 kDa) that is highly glycosylated, efficient uptake may be limited. In addition, it was possible that there might be some steric hindrance introduced by two molecules of Epo linked to dimeric Fc, which could adversely affect binding to the respective receptors.

4.2 EpoFc Monomer

To reduce both size and charge of the EpoFc fusion protein, a new molecule was created that was monomeric with respect to Epo linked to dimeric Fc (figure 3). *In vitro* bioactivity, i.e. stimulation of TF-1 cell proliferation, was not significantly different compared with the EpoFc dimer (table I), but there was approximately a 2-fold increase in binding affinity to FcRn.^[6]

Pulmonary absorption of the EpoFc monomer was compared with the dimer after an equivalent dose in non-human primates.^[6] Uptake was dose-dependent and was markedly improved for the monomer compared with the dimer (figure 4, table I). The result was a 4- to 5-fold increase in the C_{max} , a 6- to 10-fold increase in the area under the serum concentration-time curve (AUC), and an increase in the circulating half-life of the monomer (25 hours) compared with the dimer (16 hours). As a result, the bioavailability of EpoFc monomer was >30% (compared with intravenous administration), which was a significant improvement compared with 5–10% observed for the dimer. We believe that bioavailability of this magnitude for a therapeutic protein of this size is unprecedented.

In addition, a biologic response (i.e. an increase in circulating reticulocytes) was achieved with a lung-deposited dose as low as 5 µg/kg for the EpoFc monomer, while in comparison the EpoFc dimer produced a more modest increase in reticulocyte levels at a higher dose (10 µg/kg).^[6]

4.3 Interferon (IFN)β-Fc Monomer

Interferon (IFN)β is a glycoprotein with a molecular weight of approximately 22 500Da that is used in the treatment of multiple sclerosis (MS).^[42] Although the mechanisms by which IFNβ exerts its effects in MS are not well understood, IFNβ is known to

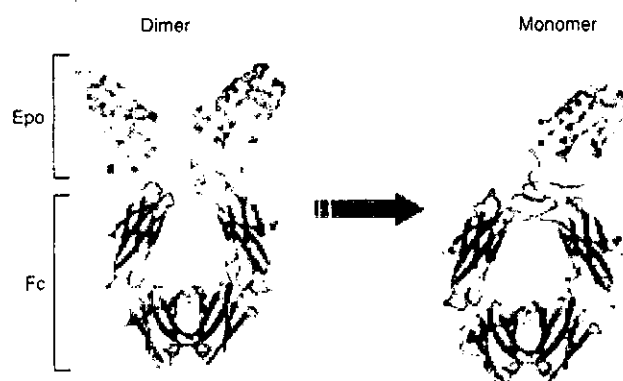


Fig. 3. Schematic diagram for erythropoietin (Epo)Fc dimer and monomer. The dimer is comprised of two molecules of Epo joined to dimeric Fc and the monomer has a single molecule of Epo linked to dimeric Fc.

Table I. Summary of bioactivity and pharmacokinetics for dimeric and monomeric Fc Fusion proteins

Species (route)	Pharmacokinetics					In vitro bioactivity	
	construct	dose (μg/kg)	C _{max} (ng/mL)	AUC (ng • h • mL ⁻¹)	t _{1/2} (h)	assay	result
EpoFc^[6]							
Monkey (pulmonary)	Dimer	20	17	557	16	TF-1 proliferation assay ^a (EC ₅₀)	0.07nM
	Monomer	20	86	5 279	25		0.09nM
IFNβ-Fc							
Monkey (pulmonary)	Dimer	20	3.8	123	11	CPE assay ^b (specific activity)	0.45 × 10 ⁶ IU/nmol
	Monomer	20	22.3	987	27		1.22 × 10 ⁶ IU/nmol
IFNα-Fc^c							
Monkey (pulmonary)	Dimer	15	107	18 760	107	CPE assay ^b (specific activity)	2.3 × 10 ⁵ IU/nmol
	Monomer	15	102	17 325	87		5.2 × 10 ⁵ IU/nmol
FIXFc							
Neonatal rat (oral)	Dimer	1370	590	9 570 000	14	Not determined	
	Monomer	1000	5830	121 250 000	19		

a Growth of human erythroleukemia cells were measured for 48 hours. Values represent the concentration of drug that stimulated growth by 50% of maximum (EC₅₀).

b Antiviral activity was measured in a standard CPE assay using human lung A549 cells and encephalomyocarditis virus.

c Construct with 15-amino-acid linker.

AUC = area under the serum or plasma concentration vs time curve; C_{max} = maximum serum concentration; CPE = cytopathic effect; EC₅₀ = concentration of drug that stimulates growth to 50% of maximum; EpoFc = erythropoietin-Fc; FIXFc = factor IX-Fc; IFN = interferon; IU = international units; $t_{1/2}$ = half-life in serum or plasma; TF-1 = human erythroleukemia cells.

regulate expression of several hundred genes,^[43] some of which have been used to measure bioactivity, including oligoadenylate synthetase, neopterin, and $\beta 2\text{m}$.^[44,45] In addition, interferons are known to protect cells from viral infection, and this type of assay is commonly used to determine the *in vitro* activity of interferon preparations.^[46]

Similarly to EpoFc, both dimeric IFN β -Fc (two molecules of IFN β fused to a single Fc dimer) and monomeric IFN β -Fc (one molecule of IFN β fused to a single Fc dimer) were created. *In vitro* cytopathic effect assays demonstrated an approximately 3-fold increase in antiviral activity for IFN β -Fc monomer compared with IFN β -Fc dimer (table I), suggesting that steric hindrance between the two molecules of IFN β fused to Fc may be responsible for decreased activity of the IFN β -Fc dimer.

Pulmonary uptake of an equivalent dose of IFN β -Fc monomer was compared with IFN β -Fc dimer in non-human primates. Pulmonary uptake was markedly improved for the monomer compared with the dimer (figure 5), indicated by a 6-fold increase in the C_{max} , an approximately 2-fold increase in the half-life and an 8-fold increase in AUC (table I). The improved pharmacokinetic

parameters of IFN β -Fc monomer were mirrored by improved biologic response compared with IFN β -Fc dimer in the same non-human primates. Serum neopterin levels were measured in each case, and IFN β -Fc monomer was found to stimulate neopterin approximately 2-fold higher than IFN β -Fc dimer.

4.4 IFN α -Fc Monomer

IFN α is an 18 kDa natural cytokine that is widely prescribed for the treatment of hepatitis C, hepatitis B, and several types of cancer.^[47] Standard administration of IFN α involves subcutaneous injection, given three times weekly because of the relatively short half-life of a few hours for this protein.^[48] A few studies have demonstrated uptake of IFN α after pulmonary delivery;^[49,51] however, frequent administration was still required because of the relatively short half-life. Although pegylation of IFN α (Pegasys®, PEG-Intron A®)¹ extends the half-life of the molecule allowing for once-weekly administration, these improved molecules still require injection.

1 The use of tradenames is for identification purposes only and does not imply endorsement.

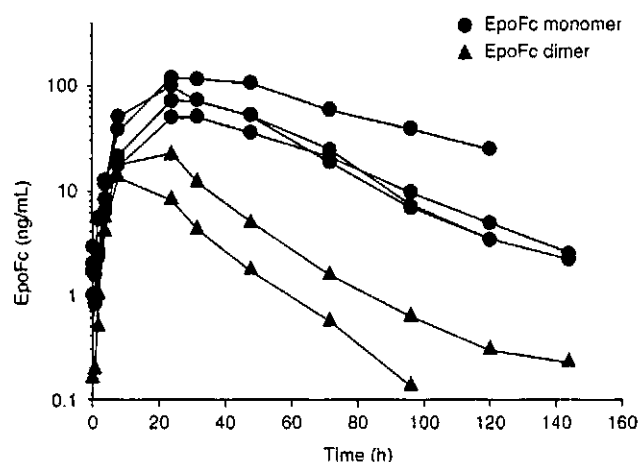


Fig. 4. Pulmonary delivery of erythropoietin (Epo)Fc dimer and EpoFc monomer in non-human primates. Approximately 20 $\mu\text{g/kg}$ of either EpoFc dimer or EpoFc monomer were aerosolized with an Aeroneb[®] Pro nebulizer, and the aerosol was delivered directly into the lungs of cynomolgus monkeys through an endotracheal tube, using shallow breathing maneuvers. Blood was drawn at selected times after administration, and the serum concentrations of the EpoFc monomer and EpoFc dimer were measured with an Epo-specific ELISA. The number of animals per group were EpoFc monomer, $n = 4$, and EpoFc dimer, $n = 2$ (reproduced from Bitonti et al.,^[6] Copyright [2004] National Academy of Sciences, USA, with permission).

The activity of IFN α is commonly assessed *in vitro* by measuring the reduction in cytopathic effect by viral challenge after interferon treatment.^[46]

Interestingly, the bioactivity of the IFN α -Fc fusions in *in vitro* antiviral assays was found to be dependent on the length of the linker between the IFN α and Fc regions (table II). Increasing the length of the linker from 0 to 8 amino acids resulted in a 2-fold increase in specific activity, and increasing the length of the linker further from 8 to 15 amino acids increased the specific activity by an additional factor of five (table II), thus indicating that there may have been some steric hindrance.

The monomer configuration of the IFN α -Fc was created for all three linker constructs in an effort to relieve this putative steric hindrance. *In vitro* assays revealed a 10-fold increase in specific activity when comparing the monomer to the dimer with no linker, and a similar 10-fold increase when comparing the 8-amino-acid linker constructs (table II). There was only an approximately 2-fold increase in specific activity when comparing the monomer to the dimer with the 15-amino-acid linker, indicating that much of the steric hindrance was already relieved by the longer linker, such that conversion to the monomer configuration had less of an effect. Note that this steric hindrance was specific to the interaction of IFN α -Fc with IFN receptors; the monomer and dimer interacted comparably with FcRn *in vitro*.

Pulmonary absorption of the IFN α -Fc dimer in non-human primates was robust, with bioavailability in excess of 50% versus intravenous administration (unpublished data). Not only was the uptake much greater compared with unconjugated IFN α , but also the serum half-life ($t_{1/2}$) was much longer (100 hours vs 5 hours). Uptake of the IFN α -Fc monomer was compared with the dimer and the resulting pharmacokinetic parameters (i.e. C_{max} , AUC, and $t_{1/2}$) were similar after inhalation of the same dose in non-human primates, irrespective of linker length (table I). Pharmacokinetics of intravenous IFN α -Fc monomer has not yet been performed, therefore, pulmonary bioavailability has not been calculated. However, the bioavailability of the monomer is expected to be similar to the dimer since the pulmonary pharmacokinetic parameters are alike. Thus, in the case of IFN α , the monomer configuration had no effect on the efficiency of pulmonary delivery, but resulted in a significant increase in the *in vitro* efficacy compared with the dimer.

4.5 Factor IX (FIX)-Fc Monomer

Factor IX (FIX) is a clotting factor that plays a central role in the coagulation cascade.^[52,53] A deficiency in FIX results in the bleeding disorder known as hemophilia B, and is treated by intravenous injections of either recombinant or plasma-derived FIX protein. The FIX protein undergoes extensive post-translational modifications, and is reported to have a molecular weight of approximately 55 kDa, making it the largest protein we have

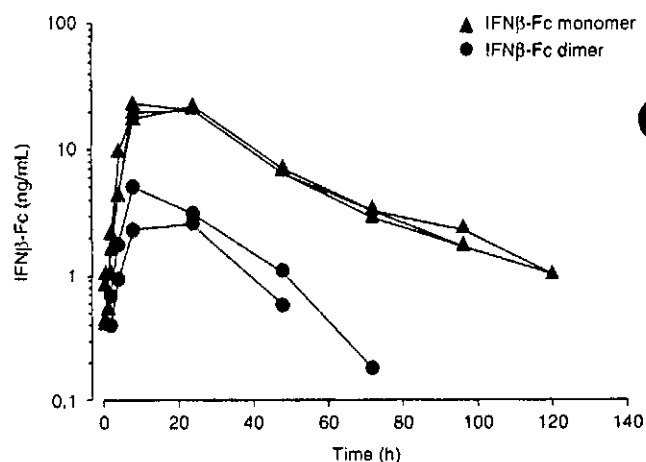


Fig. 5. Pulmonary delivery of interferon (IFN) β -Fc dimer and IFN β -Fc monomer in non-human primates. Approximately 20 $\mu\text{g/kg}$ of either IFN β -Fc dimer ($n = 2$) or IFN β -Fc monomer ($n = 3$) was aerosolized with an Aeroneb[®] Pro nebulizer and the aerosol delivered directly into the lungs. Blood samples were drawn at various times and the serum concentrations of IFN β -Fc dimer or IFN β -Fc monomer were measured with an IFN β ELISA.

Table II. *In vitro* antiviral activity of interferon (IFN) α -Fc dimer and monomer^a

	Dimer			Monomer		
Linker length ^b	0	8	15	0	8	15
IU/mg $\pm$ SD	2.5×10^5 $\pm 0.8 \times 10^5$	4.9×10^5 $\pm 3.0 \times 10^5$	2.5×10^6 $\pm 1.1 \times 10^6$	3.4×10^6	6.3×10^6 $\pm 1.6 \times 10^6$	7.3×10^6
IU/nmol	2.2×10^4	4.5×10^4	2.3×10^5	2.4×10^5	4.5×10^5	5.2×10^5
n	3	10	13	2	3	2

a Antiviral activity was measured in a standard cytopathic effect assay using human lung A549 cells and encephalomyocarditis virus.

b Number of amino acids between the IFN α and Fc sequences.

IU = international units; n = number of assay replicates; SD = standard deviation.

examined in both the monomer and dimer Fc fusion configurations.

FIXFc monomer and dimer were produced recombinantly in a manner similar to the other constructs. FcRn-mediated delivery for the two proteins was examined in 10-day-old rats after a single oral dose. At that age, there is high expression of FcRn in the intestine, no secretion of stomach acid, and relatively few digestive enzymes present, which can be neutralized with the addition of soybean trypsin inhibitor. These studies demonstrated significantly enhanced delivery of the monomer compared with the dimer, with a 10-fold increase in the C_{max} and a >12-fold increase in AUC, after a similar molar dose (figure 6, table I). In addition, the half-life of the monomer (19 hours) appeared to be extended compared with that of the dimer (14 hours) in these animals. Further comparisons of the monomer and dimer after intravenous administration in adult rats and dogs demonstrate a 2- to 3-fold greater half-life of the FIXFc monomer relative to the dimer (unpublished data). Overall, both the half-life and FcRn-mediated uptake of the FIXFc monomer were greater than for the FIXFc dimer, again displaying the superiority of the monomer over the dimer configuration.

5. Other Novel Fc Fusion Proteins

Fc monomers have significantly improved properties compared with their dimeric counterparts when the effector molecule is naturally a monomer. However, this technology is unlikely to be useful for proteins that require a heterodimeric partner for full biological activity. Cytokines typically act as multi-component systems to mediate desired immunological responses and, therefore, forming Fc fusion proteins with two independent and potentially synergistic cytokines may result in more effective therapeutics. It has been shown that heterodimeric Fc fusion proteins of interleukin (IL)-2/IL-12 and IL 4/granulocyte-macrophage colony

stimulating factor resulted in biological activity similar to the synergy normally seen with the combined application of the individual cytokines.^[54]

Cytokines are also known to be involved in the initiation and perpetuation of human diseases, and can be blocked by administration of soluble receptors. For example, blocking tumor necrosis factor (TNF) α with etanercept, a homodimer of the ectodomain of the TNF α receptor fused to human Fc, is used in the treatment of rheumatoid arthritis.^[55] However, for cytokines that use multi-component receptor systems, disease treatment with soluble receptors is limited since monomeric receptors often exhibit low affinity for cytokines or even function as agonists. Cytokine traps consisting of the extracellular domains of two distinct cytokine receptor components (e.g. IL-6 receptor- α and gp130) fused as a heterodimer to human Fc has been shown to constitute one of the most potent blockers of cytokine action reported.^[56]

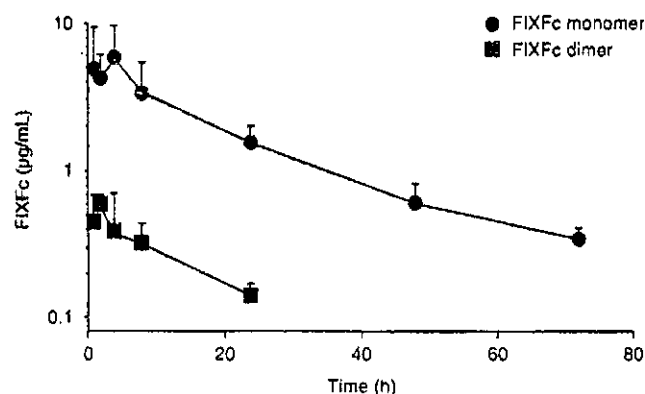


Fig. 6. A comparison of the pharmacokinetics of factor IX (FIX)Fc dimer with FIXFc monomer after a single oral dose in neonatal rats. Ten-day old rats were given approximately equimolar doses (~ 10 nmol/kg) of FIXFc dimer or FIXFc monomer orally. Blood was collected at various time points and the protein in the plasma measured with a FIX:Fc sandwich ELISA. The number of animals per time point was 4.

5.1 Follicle Stimulating Hormone (FSH)-Fc Heterodimer

Follicle stimulating hormone (FSH) is a common therapy in the treatment of male and female infertility and requires daily subcutaneous or intramuscular injections for several days in women and up to several months in men.^[57,58] In females, FSH binds to specific receptors on granulosa cells of the ovary and is responsible for the selection and growth of ovarian follicles. FSH bioactivity is typically measured as an increase in ovarian weight in response to FSH treatment in female rats.^[59] FSH is a noncovalently linked heterodimer of α and β subunits.^[60] FSH subunit assembly is critical for the biological activity of the protein^[61] as well as for the protection of the β subunit, which is unstable when not complexed with the α subunit.^[62]

In order to stabilize the FSH heterodimer, we generated an Fc fusion protein with an FSH α subunit fused to one arm of an Fc dimer and an FSH β subunit fused to the other arm, thus creating an FSH-Fc heterodimer molecule.^[63] When injected into 21-day-old female rats, FSH-Fc heterodimer significantly increased ovarian weight compared with control animals and with animals given an equivalent dose of recombinant FSH (figure 7). In addition, oral uptake of heterodimer FSH-Fc in neonatal rats revealed a terminal half-life for this molecule of ~69 hours, which is much longer than the half-life reported for recombinant FSH in rats (11.4 hours).^[64] Since it is unlikely that heterodimer FSH-Fc will inherently have more activity than recombinant FSH, the increased activity is probably due to the long terminal half-life in rodent circulation.

FSH-Fc heterodimer was also administered through the lung in two cynomolgus monkeys in a manner similar to other Fc fusion proteins. Serum levels of FSH-Fc heterodimer were measured using an FSH ELISA, and it was shown that high maximal serum concentrations of FSH-Fc heterodimer could be achieved.^[63] In

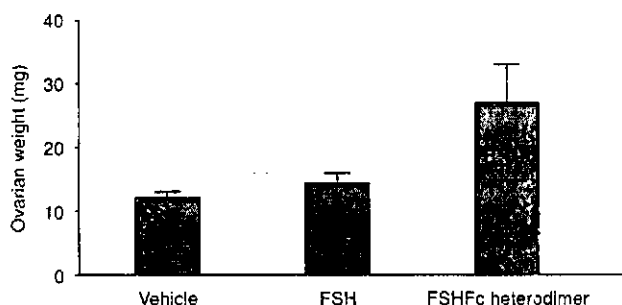


Fig. 7. Effect of follicle stimulating hormone (FSH) or FSHFc heterodimer on ovarian weight in female rats. Twenty-one-day-old female rats were treated with a single subcutaneous dose of 1 nmol/kg recombinant FSH or FSHFc heterodimer. Ovarian weight was measured 72 hours after administration. The number of rats per group was 10 (reproduced from Low et al.,^[63] by permission of Oxford University Press).

addition, the half-life of FSH-Fc heterodimer in non-human primate serum was much longer (~200 hours) than the half-life of recombinant FSH (~24 hours) in humans^[65] and non-human primates.^[66,67] Thus, an obvious advantage of FSH-Fc heterodimer over recombinant FSH is the potential for a greatly reduced administration frequency, as well as a method of administration that could significantly improve the tolerability of current treatments.

6. Conclusion

Various proteins have been conjugated to the Fc domain of an immunoglobulin and shown to retain biologic activity. These molecules typically include a disulfide-linked homodimer of Fc (comprised of the hinge, CH2 and CH3 domains of IgG1) with a molecule of choice linked recombinantly to each arm of Fc. The result is a structure that is dimeric with respect to both Fc and the target molecule. We have investigated the transport of such molecules across epithelial cell barriers, such as the lung, utilizing the naturally occurring receptor FcRn. The Fc portion of these fusion proteins binds to FcRn, which functions to both transport and protect the protein from degradation. The prototype molecule EpoFc retained biologic activity similar to unconjugated Epo, and was transported after inhalation in both monkeys and humans; however, bioavailability was less than optimal (5–10% compared with intravenous administration). Creation of a molecule that is monomeric with respect to Epo and linked to dimeric Fc resulted in marked improvement in the pharmacokinetics, with bioavailability in excess of 30%, which is similar to the bioavailabilities obtained with subcutaneous injection of unconjugated Epo. As a result, it is estimated that once-weekly administration of monomeric EpoFc by the pulmonary route of administration would be sufficient for the treatment of anemia.

Other effector molecules were constructed as monomeric Fc fusion proteins, and in each case the bioactivity, pharmacokinetics, or both attributes were clearly improved. For example, construction of the IFN β -Fc monomer resulted in both improved pharmacokinetics and bioactivity, whereas the IFN α -Fc monomer showed greater bioactivity, but there was essentially no change in pharmacokinetics. However, it should be noted that IFN α -Fc already possessed good transport properties as a dimer with pulmonary bioavailability of 50% compared with intravenous administration and a half-life that was approximately 20 times longer than the unconjugated molecule.

In addition, molecules that are heterodimeric in nature can also benefit from linkage to Fc, resulting in improved efficacy and/or

pharmacokinetics. This can be particularly advantageous for a molecule that is noncovalently linked (e.g. FSH), with the Fc moiety functioning to both stabilize the complex and extend the half-life of the molecule through protection by means of its interaction with FcRn.

The modifications of Fc fusion molecules described here represent a fundamental advance in both pulmonary delivery and construction of Fc fusion proteins. As a result, administration of monomeric or heterodimeric Fc fusions by inhalation or by parenteral routes are a feasible approach to treatment either by noninvasive means or with less frequent administration.

Acknowledgments

The authors are associated with Syntonix Pharmaceuticals, Inc., a company focused on the development of long-acting biopharmaceuticals for therapeutic use. No other sources of funding were used to assist in the preparation of this article.

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