

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒ Capítulo I ☒ Capítulo II ☐

No. Solicitud Internacional PCT/EP2007/09880
Fecha de Presentación Internacional 15/Noviembre/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 141 a 305	12/Mayo/2009
Reivindicaciones:	Folios 306 a 309	12/Mayo/2009
Dibujos:	Folios 311 a 318	12/Mayo/2009
Listado de Secuencias:	Folios 319 a 453	12/Mayo/2009
Prioridad:	EP 06124350.7	17/Noviembre/2006

2. Análisis de la Prioridad

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

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3. Objeto de la invención

Título de la solicitud: "Moléculas de enlace de lingo y uso farmacéutico de las mismas".

El problema planteado en esta solicitud consiste en la necesidad de suministrar moléculas de enlace para el tratamiento de lesiones del sistema nervioso central.

Para resolver este problema técnico la solicitud en estudio proporciona una molécula que se enlaza a la proteína LINGO.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/395; C07K 16/28.

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 1 – 4 no se considera una invención, de acuerdo con el Art citado, porque hace referencia a moléculas que se unen a la proteína LINGO de acuerdo con la SEQ ID NO: 1, SEQ ID NO: 2, ó SEQ ID NO: 3 o un epítipo, las cuales corresponden a material biológico existente en la naturaleza.

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

El objeto de la reivindicación 21 no es patentable de acuerdo con el Art citado.

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

El uso de las reivindicaciones 17 – 19 no es patentable, de acuerdo con el Art 14.

7. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque hace referencia al 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 – 4 utilizan las expresiones "que es capaz de enlazarse con la proteína (...) con una constante de disociación <1,000 nM", "la cual se enlaza con una o más de las secuencias (...)", "la cual es capaz de desinhibir la mielina de médula espinal en una concentración (...)", "la cual es capaz de aumentar la longitud de neurita promedio (...)", 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 requiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto, como la secuencia de aminoácidos de las

cadenas pesada y ligera, la secuencia de las regiones variables de las cadenas pesada y ligera o la secuencia de las 6 regiones determinantes de complementariedad.

Las reivindicaciones 6 y 7 carecen de concisión porque utilizan la expresión “es cuando menos el 50 por ciento homóloga a (...)”, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda al solicitante que una secuencia no se caracteriza por un porcentaje de identidad porque no existe soporte en la descripción que todas y cada una de las posibilidades generan una proteína con la misma actividad. Se requiere al solicitante eliminar este término del alcance del capítulo reivindicatorio.

Las reivindicaciones 8, 10 y 12 – 16 carecen de concisión porque utilizan las expresiones “una cadena pesada humana”, “una cadena ligera humana”, “un polinucleótido que codifica una molécula (...)”, “un vector de expresión que comprende (...)”, “un sistema de expresión”, “una célula huésped”, lo cual dificulta determinar la extensión del objeto que se busca proteger. Se recuerda al solicitante que un polipéptido y un polinucleótido se caracterizan por sus secuencias de aminoácidos y nucleótidos, respectivamente. Así mismo, se sugiere al solicitante definir el tipo de sistema de expresión y célula huésped.

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para la célula huésped que comprende el sistema de expresión al que se hace referencia en la reivindicación 16.

Se requiere al Solicitante hacer las aclaraciones necesarias.

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: Noviembre 17 de 2006, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	<u>WO2006002437</u>	Treatment of conditions involving demyelination	5/Enero/2006
D2	<u>Molecular and Cellular Neuroscience, Vol. 33, Pág. 311-320</u>	LINGO-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury	29/Septiembre/2006
D3	<u>US20050214288</u>	Antibodies against NOGO receptor	29/Septiembre/2005

D1 <http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=2006002437&KC=&FT=E&locale=en> EP divulga una molécula de enlace para el tratamiento de la desmielinización que es capaz de enlazarse con la proteína LINGO-1, que puede ser un anticuerpo (Pág. 4, párrafo 10).

D2 <http://www.sciencedirect.com/science/article/pii/S1044743106001849> divulga la molécula de enlace LINGO-1-Fc que es capaz de enlazarse con la proteína LINGO-1 (Resumen y Pág. 318, *Experimental Methods*).

D3[http://worldwide.espacenet.com/publicationDetails/biblio?CC=US&NR=2005214288&KC=&FT=E&lo](http://worldwide.espacenet.com/publicationDetails/biblio?CC=US&NR=2005214288&KC=&FT=E&locale=en)
[cale=en](http://worldwide.espacenet.com/publicationDetails/biblio?CC=US&NR=2005214288&KC=&FT=E&locale=en) EP divulga una molécula de enlace anti-LINGO-1 que es capaz de enlazarse con la proteína LINGO-1 (Pág. 2, párrafo 11).

9. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La **reivindicación 5** consiste en una molécula de enlace de acuerdo con cualquiera de las reivindicaciones 1, 2, 3, ó 4, la cual comprende una o más secuencias de aminoácidos seleccionadas a partir del grupo que consiste en las SEQ ID NOs: 12 a 17 ó en las SEQ ID NOs: 18 a 23.

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

Características esenciales	D1	D2	D3
Una molécula de enlace de acuerdo con cualquiera de las reivindicaciones 1, 2, 3, ó 4, la cual comprende una o más secuencias de aminoácidos seleccionadas a partir del grupo que consiste en las SEQ ID NOs: 12 a 17 ó en las SEQ ID NOs: 18 a 23.	Una molécula de enlace que es capaz de enlazarse con la proteína LINGO-1 (Pág. 4, párrafo 10)	Una molécula de enlace que es capaz de enlazarse con la proteína LINGO-1 (Resumen)	Una molécula de enlace que es capaz de enlazarse con la proteína LINGO-1 (Pág. 2, párrafo 11)

Los documentos D1, D2 y D3 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 enseña una molécula de enlace para el tratamiento de la desmielinización que es capaz de enlazarse con la proteína LINGO-1, que puede ser un anticuerpo (Pág. 4, párrafo 10) y una composición farmacéutica que comprende el anticuerpo (Pág. 85, párrafo 246).

La diferencia entre la invención, definida en la reivindicación 5 y la molécula de D1 consiste en que la molécula de la solicitud comprende una o más secuencias de aminoácidos seleccionadas a partir del grupo que consiste en las SEQ ID NOs: 12 a 17 ó en las SEQ ID NOs: 18 a 23.

D2 divulga la molécula de enlace LINGO-1-Fc que es capaz de enlazarse con la proteína LINGO-1 y una composición que comprende la molécula recombinante
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(Resumen y Pág. 318, *Experimental Methods*).

La diferencia entre la invención, definida en la reivindicación 5 y la molécula de D2 consiste en que la molécula de la solicitud comprende una o más secuencias de aminoácidos seleccionadas a partir del grupo que consiste en las SEQ ID NOs: 12 a 17 ó en las SEQ ID NOs: 18 a 23.

D3 enseña una molécula de enlace anti-LINGO-1 que es capaz de enlazarse con la proteína LINGO-1 (Pág. 2, párrafo 11).

La diferencia entre la invención, definida en la reivindicación 5 y la molécula de D3 consiste en que la molécula de la solicitud comprende una o más secuencias de aminoácidos seleccionadas a partir del grupo que consiste en las SEQ ID NOs: 12 a 17 ó en las SEQ ID NOs: 18 a 23.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la molécula de enlace reclamada en esta solicitud porque no parecen presentar una mayor actividad moduladora de la actividad de la proteína LINGO-1 según lo establecido en la descripción.

El problema técnico objetivo de esta solicitud consiste en la necesidad de moléculas de enlace de la proteína LINGO-1 alternativa a las ya conocidas en los documentos D1, D2 o D3.

Teniendo en cuenta que los documentos D1, D2 y D3 ya divulgaban moléculas de enlace de la proteína LINGO-1 para el tratamiento de lesiones del sistema nervioso central, como desmielinización, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, generar más líneas celulares para la producción de moléculas con la misma actividad.

De manera que la solución propuesta por esta solicitud en la reivindicación 5 y sus dependientes 6 – 11 y en las reivindicaciones 12 – 16 y 20, es obvia y no tiene Nivel Inventivo.

10. Conclusión

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

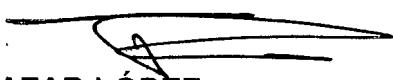
Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2006002437	5 – 16 y 20	Nivel Inventivo
D2	<u>Molecular and Cellular Neuroscience, Vol. 33, Pág. 311-320</u>	5 – 16 y 20	Nivel Inventivo
D3	US20050214288	5 – 16 y 20	Nivel Inventivo

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
26 AGO. 2012

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

Results

LINGO-1-Fc inhibits LINGO-1 binding to NgR1

Previously we have shown that AP-LINGO-1 binds to NgR1 with nanomolar affinity (Mi et al., 2004). To determine if LINGO-1-Fc can act as an inhibitor of this interaction, we tested whether LINGO-1-Fc can inhibit AP-LINGO-1 binding to NgR1 in an ELISA competition assay. Preincubation of NgR1 coated ELISA plates with LINGO-1-Fc resulted in a significant reduction in AP-LINGO-1 binding to NgR1 (Fig. 1a).

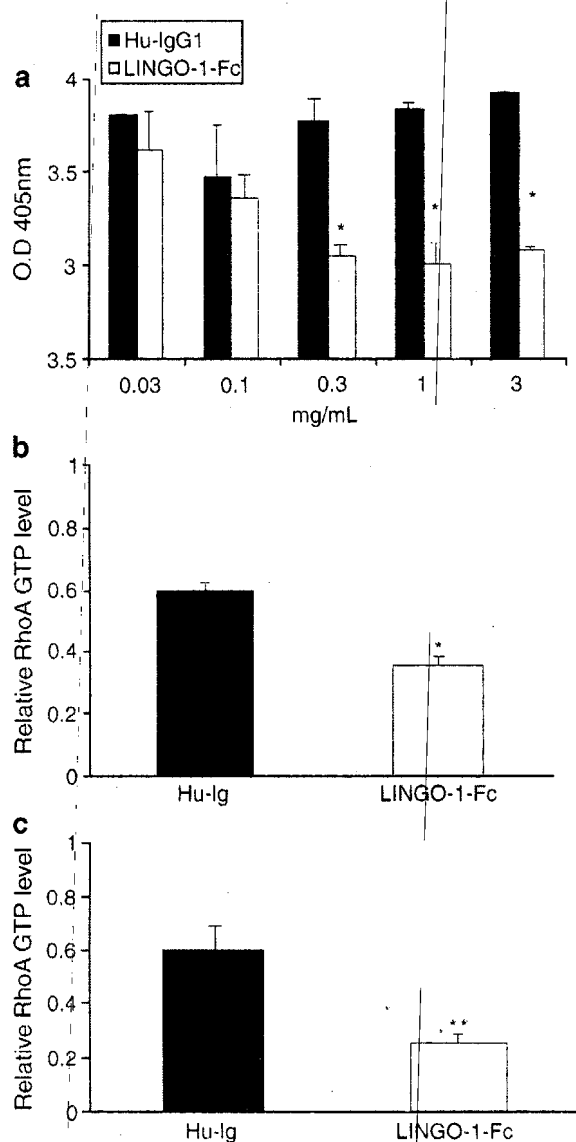


Fig. 1. LINGO-1-Fc inhibits LINGO-1 binding to NgR1 and reduces RhoA activation after spinal cord injury. (a) LINGO-1-AP binding to NgR1 in an ELISA format was reduced by co-incubation with LINGO-1-Fc, mean $\pm$ SEM, ($n=3$), $*P<0.05$, unpaired Student's t -test. (b–c) RhoA-GTP isolated from tissue homogenates of injured spinal cord 3 days after transection was immunoprecipitated and detected by immunoblotting with anti-RhoA antibody. Total RhoA and Rho-GTP levels were quantified by densitometry and the Rho-GTP level was normalized to total RhoA bands. Total RhoA represents only 5% of the total sample used for RhoAGTP analysis. (b) Lateral hemisection of the spinal cord. (c) Dorsal hemisection ($*P<0.05$, $**P<0.01$, unpaired Student's t -test).

LINGO-1-Fc treatment inhibited RhoA activation after spinal cord injury

We and others have observed that Rho-GTP levels are low in uninjured spinal cord homogenates and increase in tissue surrounding the site of transection after SCI (Dubreuil et al., 2003) (data not shown). RhoA activation inhibits axon regeneration after SCI (Dubreuil et al., 2003), and we determined whether LINGO-1-Fc inhibits RhoA activation. After SCI, spinal cord tissue homogenates from LINGO-1-Fc-treated rats had lower RhoA-GTP levels than control injured cord homogenates measured 3 days after injury in both lateral and dorsal hemisection models (Figs. 2b–c).

LINGO-1-Fc improves functional recovery after SCI

To determine the effect of LINGO-1 inhibition on axonal regeneration *in vivo*, we tested the effect of LINGO-1-Fc treatment in two models of spinal cord injury. Lateral hemisection of the cord at cervical level C7 severed the right rubrospinal tract (RST) projecting down the cord from the red nucleus (RN). RST axons undergo marked atrophy after transection, limiting their spontaneous regenerative response (Ruitenberg et al., 2004). Functional recovery after lateral hemisection was assessed using the vertical exploration test in a Plexiglas cylinder 4 weeks after SCI (Yick et al., 2004). Forelimb function was expressed as the percentage of wall exploration initiated by the right (impaired) or left (normal) forelimb alone or both forelimbs together (Fig. 2a). Normal rats explored the cylinder wall using a single forelimb or both forelimbs together. Animals subjected to lateral hemisection of spinal cord rarely used the forelimb ipsilateral to the injury, leading to an asymmetry of forelimb use. Treatment with LINGO-1-Fc promoted the use of both forelimbs as compared with the other treatment groups. In addition, the LINGO-1-Fc-treated animals used their impaired forelimb alone to explore the wall more frequently compared to the other treatment groups. Treatment with PBS or a Human Ig control protein did not lead to spontaneous recovery of forelimb function. In a separate experiment, SCI was induced by dorsal hemisection at thoracic vertebra 7. The dorsal and dorsolateral components of the corticospinal tract (CST) were completely interrupted and the ventral portion of the CST left intact. Hindlimb function was quantified using the Basso–Beattie–Bresnahan (BBB) open field scoring method (Basso et al., 1995), and all animals sustained marked functional deficits after SCI, with almost complete hindlimb paralysis the day after surgery. Control animals receiving continuous intrathecal infusion of Human Ig recovered substantial function over the 4 week duration of the experiment, attaining a mean BBB score of 10.8 ± 0.88 (Fig. 2b). Continuous intrathecal infusion of LINGO-1-Fc for 4 weeks after spinal cord transection resulted in significantly improved BBB scores at 3 and 4 weeks, reaching a mean BBB score of 13.1 ± 1.01 (Fig. 2c). The difference between 11 and 13 on the BBB locomotor rating scale reflects recovery from no forelimb–hindlimb coordination (11) to frequent forelimb–hindlimb coordination (13). A BBB score of 14 or greater reflects the ability of rats to display consistent coordinated hindlimb–forelimb movement and represents a threshold of recovery that is biologically meaningful (M. Basso, personal communication). The frequency with which LINGO-1-Fc-treated animals attained a score of 14 or more was greater than for control animals, 3 weeks (28.6% vs. 12.5%) and 4 weeks

LINGO-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury

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LINGO-1 is a CNS-specific protein and a functional component of the NgR1/p75/LINGO-1 and NgR1/TAJ(TROY)/LINGO-1 signaling complexes that mediate inhibition of axonal outgrowth. These receptor complexes mediate the axonal growth inhibitory effects of Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) via RhoA activation. Soluble LINGO-1 (LINGO-1-Fc), which acts as an antagonist of these pathways by blocking LINGO-1 binding to NgR1, was administered to rats after dorsal or lateral hemisection of the spinal cord. LINGO-1-Fc treatment significantly improved functional recovery, promoted axonal sprouting and decreased RhoA activation and increased oligodendrocyte and neuronal survival after either rubrospinal or corticospinal tract transection. These experiments demonstrate an important role for LINGO-1 in modulating axonal outgrowth *in vivo* and that treatment with LINGO-1-Fc can significantly enhance recovery after spinal cord injury.

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Introduction

Spinal cord injury (SCI) results in damage to axonal tracts that control motor and sensory function. Axons in the spinal cord regenerate poorly, limiting functional recovery. The weak regenerative capacity of CNS axons has been attributed, at least in part, to the inhibitory components of myelin ensheathing the axon (Filbin, 2003). The components of myelin with axonal growth inhibitory properties include Nogo-A (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000), myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte-myelin glycoprotein (OMgp) (Wang et al., 2002a) and ephrin-B3 (Benson et al., 2005). Nogo, MAG and OMgp bind to the Nogo-66 receptor (NgR1) and exert

their actions through signaling complexes containing p75 (Wang et al., 2002b), and LINGO-1 (LRR and Ig domain containing Nogo receptor interacting protein) (Mi et al., 2004) or LINGO-1 and TAJ/TROY (Park et al., 2005; Shao et al., 2005). The relative role of these factors in preventing regeneration of axons after SCI is currently an active area of research.

The inhibitory effect of myelin proteins on neurons is mediated by RhoA (Neideröst et al., 2002; Winton et al., 2002), an intracellular regulator of the cytoskeleton (Hall, 1998), activated after SCI (Dubreuil et al., 2003; Madura et al., 2004). RhoA activation correlates with the inability of axons to elongate, whereas RhoA inactivation results in neurite outgrowth (Lehmann et al., 1999; Neideröst et al., 2002) and promotes axonal regeneration after SCI (Dergham et al., 2002; Fournier et al., 2003). Neurons overexpressing dominant negative transmembrane forms of LINGO-1, TAJ(TROY) or p75, or neurons from TAJ(TROY) or p75 knockout mice have reduced levels of activated RhoA and enhanced neurite outgrowth in the presence of the myelin inhibitors (Mi et al., 2004; Park et al., 2005; Shao et al., 2005; Wang et al., 2002a; Zheng et al., 2005). In addition, co-incubation of cerebellar granule neurons with a soluble LINGO-1-Fc construct blocks Rho activation and reverses the growth inhibitory effects of myelin inhibitors (Mi et al., 2004). Together, these data demonstrate that disruption of the NgR1/p75/LINGO-1 and/or NgR1/TAJ(TROY)/LINGO-1 signaling pathways represents a potential point of therapeutic intervention to block the actions of myelin inhibitory proteins mediating their effects via these pathways.

To investigate the role of LINGO-1 *in vivo* after SCI, we tested if treatment with a LINGO-1 antagonist, LINGO-1-Fc, could improve functional and histological outcome after either lateral or dorsal hemisection of the spinal cord. Treatment with LINGO-1-Fc improved forelimb and hindlimb function, promoted axonal sprouting and decreased RhoA activation in injured spinal cord. These data are the first to demonstrate a functional role for LINGO-1 *in vivo* and strongly suggest that LINGO-1 could be a novel target for the treatment of SCI.

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Available online on ScienceDirect (www.sciencedirect.com).

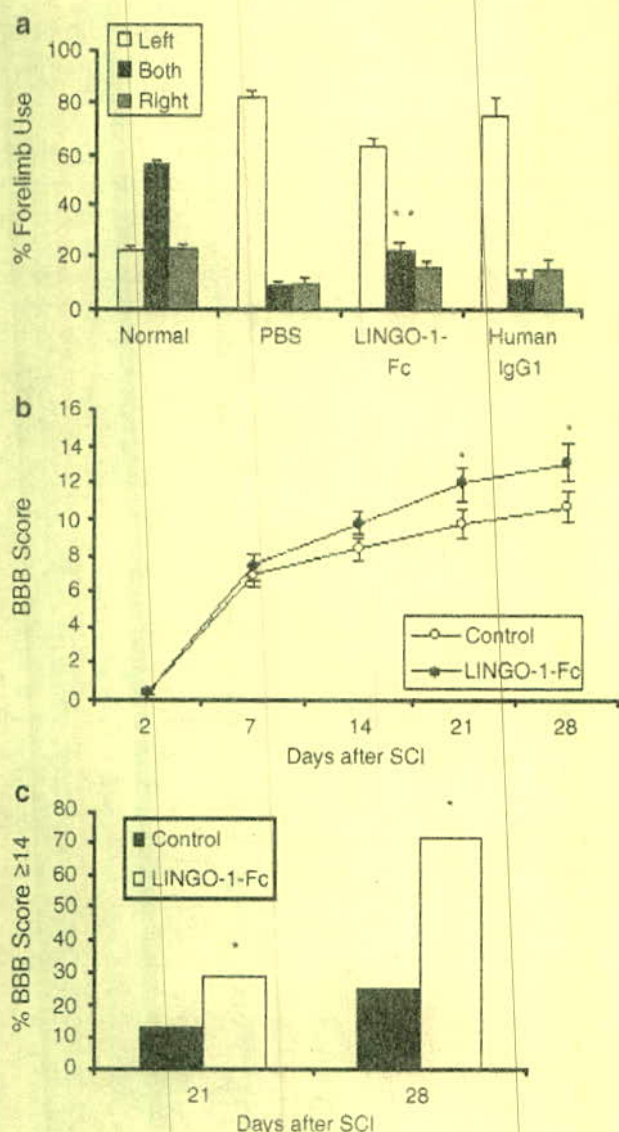


Fig. 2. LINGO-1-Fc treatment improved functional recovery after spinal cord injury. (a) Forelimb function was quantified using the vertical exploration test 4 weeks after lateral hemisection. Paw placements were counted 20 times for each rat. LINGO-1-Fc-treated ($n=8$), PBS-treated ($n=8$) and Hu-Ig-treated ($n=5$) controls ($P<0.01$, ANOVA, Bonferroni post hoc test). BBB locomotor score was recorded the day after surgery (day 2) and weekly thereafter for 4 weeks after dorsal hemisection. (b) LINGO-1-Fc-treated rats ($n=7$) compared to controls ($n=8$), mean $\pm$ SEM, $*P<0.05$, two way repeated measure ANOVA, Tukey's post hoc test. (c) Frequency of consistent plantar stepping and hindlimb–forelimb coordination was expressed as the proportion of rats that attained a BBB score of 14 or higher 3 and 4 weeks after SCI (mean $\pm$ SEM, $*P<0.05$, Chi-squared test).

(71.4% vs. 25.0%) after SCI (Fig. 2c). Together, these results clearly demonstrate that treatment with LINGO-1-Fc promotes the recovery of function after SCI.

LINGO-1-Fc promotes axonal regeneration and plasticity

Axonal integrity is the anatomical correlate of functional recovery (Weidner et al., 2001; Raineteau and Schwab, 2001,

Raineteau et al., 2002). After lateral hemisection of the spinal cord, RST axons were labeled by retrograde transport of the marker FluoroGold (FG). The tracer was injected distal to the transection site at thoracic level T2, labeling the neuronal cell bodies in the RN of axons that project beyond the site of injury and occasional ipsilateral uncrossed RST fibers ($<1\%$ of fibers). In PBS and Ig-treated control animals, almost no neurons ($<1\%$) in the RN were labeled with FG 4 weeks after injury (Figs. 3a–b).

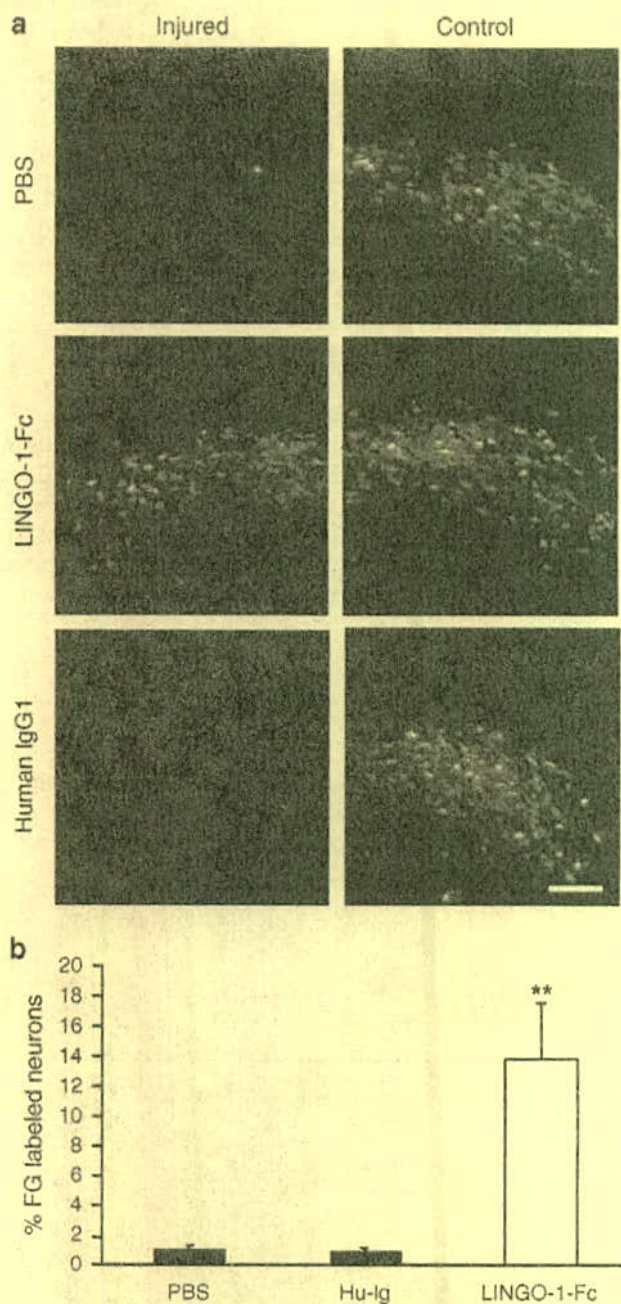


Fig. 3. LINGO-1-Fc treatment promotes axonal regeneration after spinal cord lateral hemisection. (a) Immunohistochemistry showing FG-labeled neurons in the RN in LINGO-1-Fc-treated rats compared to PBS ($n=8$) or Hu-Ig ($n=5$)-treated controls. Scale bar = 200 μ m. (b) Number of FG-labeled RST neurons in the midbrain after SCI (mean $\pm$ SEM, $**P<0.01$, ANOVA, Tukey–Kramer multiple comparison test).

In contrast, after LINGO-1-Fc treatment, $10.5\% \pm 3.2\%$ of RN neurons were labeled with FG (Figs. 3a–b). After dorsal hemisection of the spinal cord, CST axons were labeled by anterograde tracing down the spinal cord by injecting biotin dextran amine (BDA, 10,000 Da) into the motor cortex 2 weeks prior to termination of the study. The majority of CST axons could be traced projecting via the dorsal column with $<5\%$ of labeled axons projecting via the ipsilateral and ventral CST. A greater number of BDA-labeled axons were observed 10 mm caudal to the site of transection in LINGO-1-Fc-treated rats (15.7 ± 5.9 axons per section) compared to controls (2.4 ± 1.1 axons per section) (Figs. 4a–b). The majority of axons projecting below the lesion site were observed in the ventral CST and adjacent gray matter (Fig. 4c). In LINGO-1-Fc treated rats (Fig. 4d) but not controls (Fig. 4e), BDA-labeled axons projected from the ventral CST into the gray matter of the ventral horn, and a small proportion of these axons contacted neurons in the lumbar motor neuron pool in LINGO-1-Fc-treated rats (Figs. 4f and h).

After transection of the CST, axons proximal to the lesion degenerate and retract or “dieback” from the site of injury (Stirling et al., 2004). In control spinal cord injured rats, BDA-labeled axons appeared fragmented and dystrophic and had clearly retracted from the transection site (Figs. 5a and b). In LINGO-1-Fc-treated rats, BDA-labeled axons appeared healthy, projecting into and around the lesion site (Figs. 5c–e). Axonal dieback was quantified as the distance between the furthest projecting BDA-labeled axon/field, and the edge of the lesion and dieback was reduced by 62% in LINGO-1-Fc-treated rats compared to controls (Fig. 5f). The marked improvement in axonal integrity following LINGO-1-Fc treatment prompted us to analyze the effect of this treatment on the survival of neurons and oligodendrocytes after SCI. Four weeks after SCI, significantly more neurons stained positively for β III-tubulin (Figs. 5g and h) and oligodendrocytes stained positively with the

CC1 antibody (Figs. 5i and j) in the spinal cord tissue surrounding the lesion site in LINGO-1-Fc-treated animals compared to controls (LINGO-1-Fc: 18.3 ± 2.3 vs. control: 10 ± 1 neurons/mm²; LINGO-1-Fc: 788.9 ± 54.5 vs. control: 469.4 ± 100.3 oligodendrocytes/mm²). The number of oligodendrocytes staining positively with the CC1 antibody in LINGO-1-Fc-treated spinal cords was comparable to that observed in the same region of the cord from uninjured animals (795.8 ± 154.2 cells/mm²). Spinal cords were double labeled with CC1 antibody and anti-GFAP antibody to ensure selective staining of oligodendrocytes with CC1 antibody and no co-localization was observed (data not shown).

LINGO-1-Fc reduced apoptotic death of oligodendrocytes and neurons after SCI

Pronounced apoptotic cell death occurs after SCI in both neurons and oligodendrocytes (Liu et al., 1997; Springer et al., 1999; Li et al., 2000; Casha et al., 2001; Beattie et al., 2002) and is mediated by caspase-3 activation (Springer et al., 1999; Li et al., 2000). We observed extensive activated caspase-3 positive cell staining both rostral and caudal to the site of transection 3 days after SCI (Figs. 6a–b), and DNA fragmentation was confirmed using TUNEL staining (Fig. 6c). Activated caspase-3 staining was co-localized in neurons (NeuN positive cells, Fig. 6a) and oligodendrocytes (CC1 positive cells, Fig. 6b). The vast majority of activated caspase-3 positive cells were identified as oligodendrocytes which is consistent with previous findings (Beattie et al., 2002). The number of TUNEL positive cells counted 2.5 mm rostral and caudal to the site of transection was significantly less in LINGO-1-Fc-treated animals (11.5 ± 3.5 cells per mm²) compared to controls (30 ± 8.7 cells per mm²) 3 days after SCI (Figs. 6c–d). Jun N-terminal kinase (JNK) is phosphorylated as a downstream consequence of Rho activation

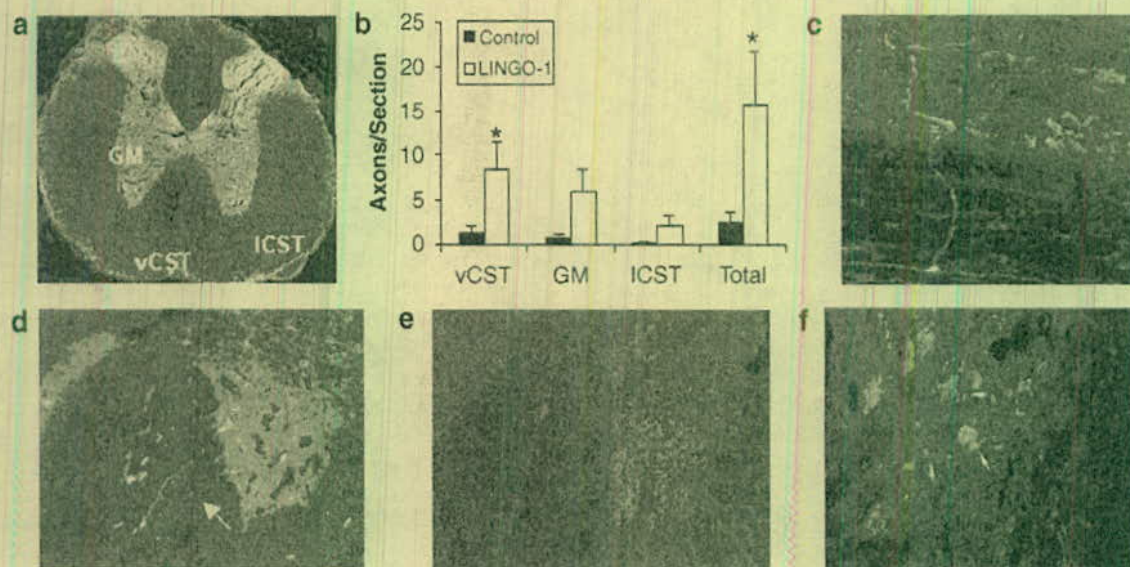


Fig. 4. LINGO-1-Fc treatment promoted axonal regeneration after spinal cord dorsal hemisection. (a–b) The number of BDA labeled axons per section was counted in the ventral CST (vCST), gray matter (GM) and lateral CST (lCST) 10 mm caudal to the lesion (mean $\pm$ SEM, * $P < 0.05$, unpaired Student's *t*-test, $n = 8$ control, $n = 15$ LINGO-1-Fc). (c) BDA-labeled axons sprouted from the vCST into the adjacent GM. (d–f) Examples are shown in longitudinal sections (c, 100 $\times$), and cross-section caudal (d–f, 200 $\times$) to the lesion site. BDA labeled axons projecting from the vCST were more frequently observed in LINGO-1-Fc treated rats (d) than in controls (e). In some instances BDA+ sprouting axons contacted neurons (vGLUT1+ cells) in the ventral horn observed here 10 mm caudal to the site of transection in a LINGO-1-Fc-treated rat (f, 200 $\times$).

(Marinissen et al., 2004) and is a mediator of apoptotic cell death. We therefore measured total and phosphorylated-JNK levels in spinal cord tissue 3 days after SCI and found that phosphorylated

JNK levels were significantly lower in lesioned spinal cord tissue from LINGO-1-Fc-treated rats compared to Hu-Ig-treated controls (Figs. 6e–f).

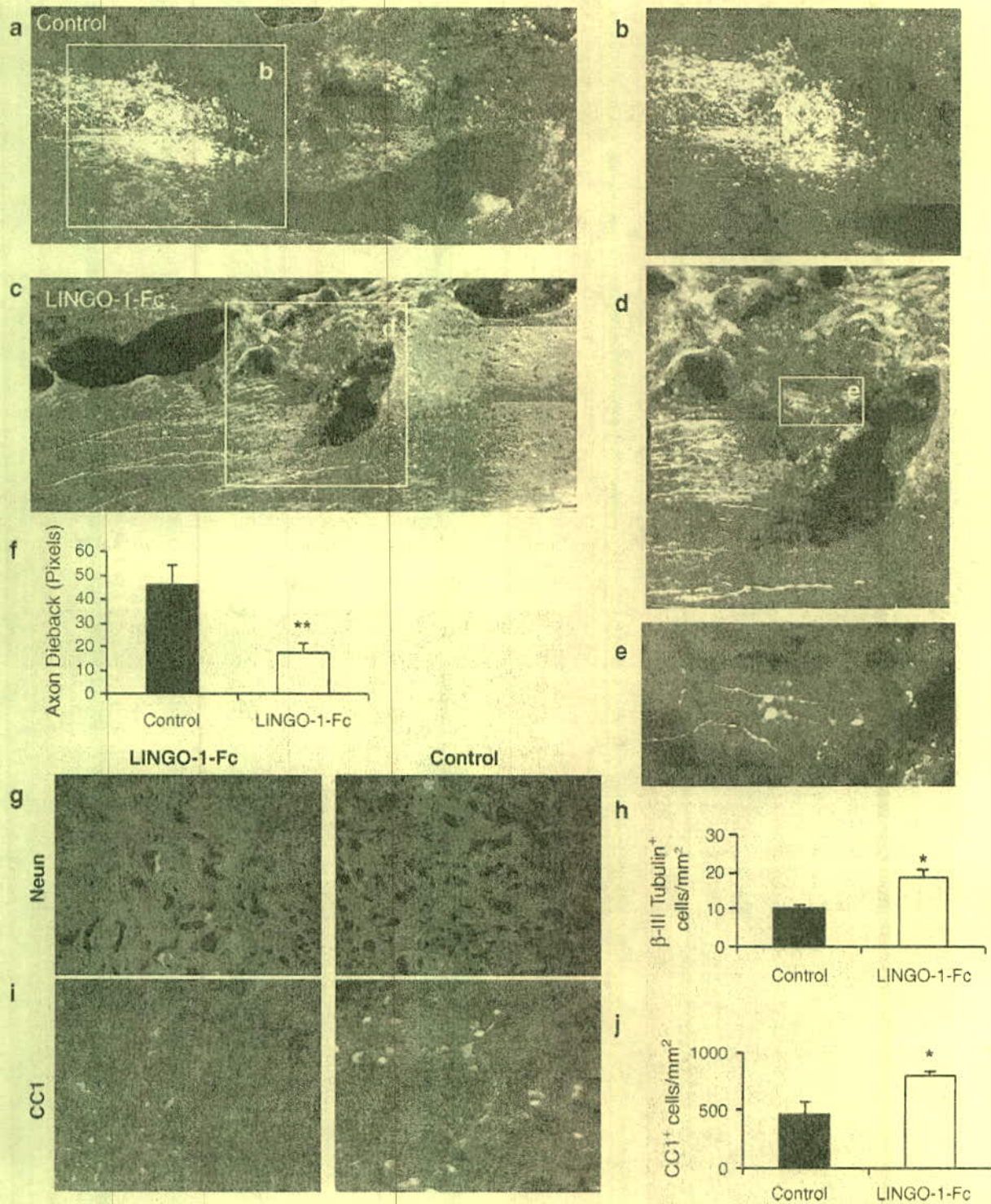


Fig. 5. LINGO-1-Fc treatment reduced axonal dieback and promoted oligodendrocyte and neuronal survival in the injured spinal cord. (a–b) BDA-labeled CST axons retracted from the lesion site in control spinal cord injured animals. (c–e) In LINGO-1-Fc-treated rats, axons projected into the lesion site. (f) Axon dieback, quantified as the distance in pixels from the furthest projecting axon per field to the edge of the lesion, was significantly reduced in LINGO-1-Fc-treated rats (mean ± SEM, $n = 14$ control, $n = 13$ LINGO-1-Fc-treated, $**P < 0.01$, unpaired Student's *t*-test). The number of neurons (NeuN+ cells, $n = 4$ controls, $n = 6$ LINGO-1-Fc-treated) (g, h) and oligodendrocytes (CC1+ cells, $n = 3$ per group) (i, j) was increased in spinal cord tissue 2.5 mm caudal to the lesion site (mean ± SEM, $*P < 0.05$, unpaired Student's *t*-test).

Discussion

We report that LINGO-1, a component of the NgR1 signaling complex (Mi et al., 2004), mediates axonal growth inhibition *in vivo* since intrathecal or local administration of a decoy protein, LINGO-1-Fc, which binds to NgR1 and inhibits RhoA activation, significantly promoted axonal sprouting and functional recovery in two models of SCI. LINGO-1-Fc treatment also increased neuronal and oligodendrocyte cell survival in the injured cord demonstrating a functional role for endogenous LINGO-1 in SCI pathology.

Axonal regeneration after SCI is inhibited in part by the effects of Nogo-A, MAG and OMgp (Filbin, 2003). Pharmacological inhibition of these ligands signaling through NgR1 significantly enhanced recovery after SCI; inhibition of Nogo-A using a neutralizing antibody (Bregman et al., 1995; Thallmair et al., 1998, Merkler et al., 2001) or intrathecal delivery of a peptide inhibitor of NgR1 improved outcome after SCI (GrandPré et al., 2002), as did systemic administration of the same peptide antagonist (Li and Strittmatter, 2003). Similarly, intrathecal delivery of the ectodomain of NgR1 fused to an Fc domain (NgR-Ig) (Li et al., 2004) or transgenic expression of NgR1 (residues 1–310) specifically in

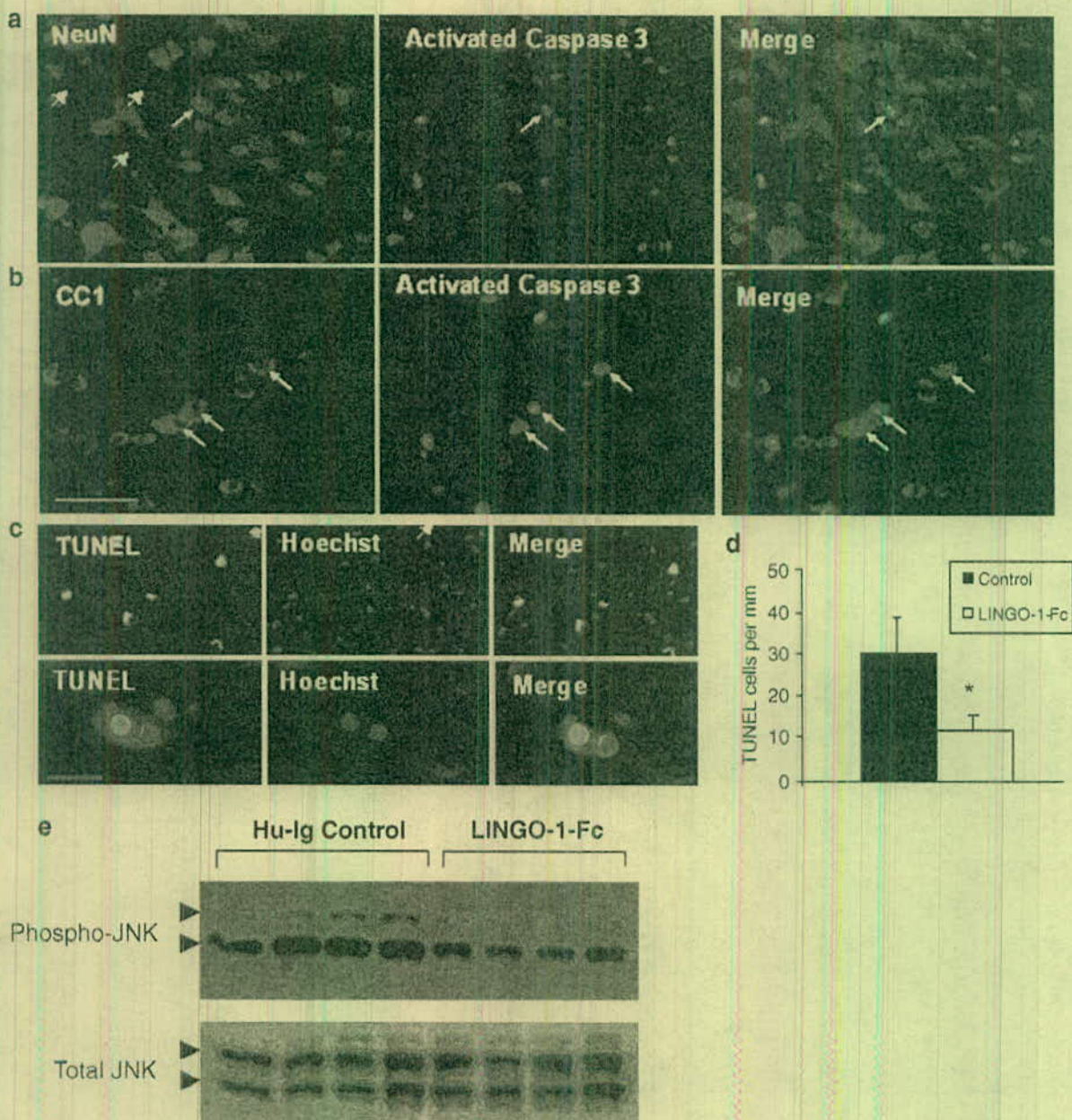


Fig. 6. LINGO-1-Fc treatment increased neuronal and oligodendrocyte survival. Activated caspase-3 staining was co-localized in neurons (NeuN+ cells) (a) and oligodendrocytes (CC1+ cells) (b) 2.5 mm caudal to the lesion 3 days after dorsal hemisection of the spinal cord by immunohistochemistry (scale bar = 20 μ m). (c) TUNEL+ cells surrounding the lesion site confirm cell death and the number of apoptotic cells measured 3 days after SCI were quantified (Scale bar = 6.25 μ m). (d) TUNEL+ cells in control and LINGO-1-Fc treated rats (mean $\pm$ SEM, * P < 0.05, unpaired Student's *t*-test). The level of phosphorylated JNK in spinal cord tissue from LINGO-1-Fc-treated rats compared to controls (e, f) (n = 4 per group, mean $\pm$ SEM, ** P < 0.01, unpaired Student's *t*-test).

astrocytes (Li et al., 2005) conferred significant improvement in functional and histological recovery after SCI. The present data are the first to describe the efficacy of NgR1 pathway inhibition, specifically LINGO-1 antagonism, in the rubrospinal tract and show that LINGO-1-Fc improved anatomical and functional recovery to an extent comparable to that observed with other antagonists of myelin inhibitory proteins (Bregman et al., 1995; Thallmair et al., 1998; Merkler et al., 2001; GrandPré et al., 2002; Li et al., 2004; Li et al., 2005). In fact, under the same conditions that LINGO-1 Fc induced sprouting in the rubrospinal tracts, NgR1-Fc was ineffective (unpublished findings). Together, these findings demonstrate that targeting LINGO-1 is a valid approach to blocking axonal growth inhibition *in vivo* and provide further support for the hypothesis that blockade of the NgR1/p75/LINGO-1 and/or NgR1/TAJ(TROY)/LINGO-1 receptor complexes is beneficial after SCI.

Plasticity in the corticospinal motor system after injury involves sprouting from intact collateral fibers, synaptic reorganization, reorganization of the motor cortex representation and the formation of new intraspinal circuitry (Fouad et al., 2001; Raineteau and Schwab, 2001; Raineteau et al., 2002; Bareyre et al., 2004). Spontaneous plasticity in the ventral CST after SCI specifically correlates with functional recovery after dorsal hemisection of the cord (Weidner et al., 2001). Recent work demonstrates that experience-driven plasticity of visual cortex is limited by myelin and NgR, indicating a role for NgR in physiological plasticity (McGee et al., 2005). We show that LINGO-1-Fc treatment enhances plasticity in the injured spinal cord by promoting sprouting from intact collateral axons. Whether LINGO-1-Fc promotes cortical and local synaptic reorganization or intraspinal circuitry remains to be determined.

Both the corticospinal and rubrospinal tracts are descending motor pathways and have overlapping functional properties (Kennedy, 1990). However, in contrast to the high degree of spontaneous functional recovery observed after dorsal hemisection of the rat spinal cord in untreated animals, little or no spontaneous recovery in forelimb asymmetry is observed after lateral hemisection of the rat spinal cord (Yick et al., 2004). This may be due to the susceptibility of these axons to atrophy after transection and/or their reduced inherent capacity to regenerate (Ruitenberg et al., 2004). Despite the lack of spontaneous recovery after RST transection, LINGO-1-Fc treatment significantly improved the symmetry of forelimb use in this model. Together, our observations show that LINGO-1-Fc treatment can enhance the regenerative capacity of the injured spinal cord, in the presence or absence of functionally relevant plasticity, resulting in a meaningful improvement in recovery.

Rapid and sustained RhoA activation occurs after SCI (Dubreuil et al., 2003) in response to environmental cues such as the inflammatory mediator tumor necrosis factor (Neumann et al., 2002). In neurons, RhoA inactivation results in axonal elongation (Luo et al., 1997) and regeneration (Lehmann et al., 1999). To elucidate the mechanism of action of LINGO-1-Fc on functional recovery and axonal regeneration, we investigated its effects on RhoA inactivation. Inhibition of RhoA activation by LINGO-1-Fc after SCI is consistent with the effect of LINGO-1-Fc on RhoA activation in cerebellar granule neurons in culture (Mi et al., 2004). Since LINGO-1-Fc can block LINGO-1/NgR1 interactions, we conclude that LINGO-1-Fc treatment acts to increase axonal regrowth via inhibition of signaling by the NgR1/p75/LINGO-1 and NgR1/TAJ(TROY)/LINGO-1 receptor complexes and consequently RhoA inactivation after SCI.

In addition to its expression on neurons and an essential function in NgR1 signaling, LINGO-1 is expressed on oligodendrocytes and has recently been ascribed a role in the negative regulation of myelination (Mi et al., 2005). Both neurons and oligodendrocytes undergo apoptosis after SCI, contributing to secondary tissue damage (Liu et al., 1997; Springer et al., 1999; Li et al., 2000; Beattie et al., 2002). Unlike previous published studies in SCI with soluble NgR1, a Nogo-derived peptide inhibitor or anti-Nogo antibodies (Bregman et al., 1995; Merkler et al., 2001; GrandPré et al., 2002; Li et al., 2004), we provide the first evidence for a cell survival effect following treatment with an inhibitor of NgR1 signaling, suggesting an additional, and possibly unique, mechanism of action of LINGO-1-Fc. Published reports demonstrated that reduced cell death promotes functional recovery and axonal regeneration or sprouting after SCI by inhibition of the CD95/FasL pathway (Demjen et al., 2004) or by administration of the tetracycline derivative minocycline (Stirling et al., 2004). These findings illustrate that local neuronal and oligodendrocyte survival are an important consideration in the goal of promoting recovery after SCI. Whether other treatments promote survival or whether this is a unique property of LINGO-1-Fc remains to be determined. In addition to improved cell survival, we observed a significant reduction in axonal dieback in LINGO-1-Fc-treated rats. Axons appeared to be in an active growth state projecting into and around the lesion site, and this observation suggests that LINGO-1-Fc acts directly on the axon to improve axonal integrity after injury.

RhoA can regulate apoptotic cell death pathways (Jimenez et al., 1995; Aznar and Lacal, 2001). Overexpression of RhoA can induce apoptosis after serum deprivation (Jimenez et al., 1995; Aznar and Lacal, 2001), and caspase-3 is required for this process (Jimenez et al., 1995). Activation of the JNK pathway can also occur in response to Rho activation (Marinissen et al., 2004). Inactivation of RhoA *in vivo* by treatment with the RhoA antagonist C3-05 protects neurons and oligodendrocytes from apoptotic cell death after SCI and results in improved functional recovery that was attributed in part to the neuroprotective effect of the RhoA antagonist (Dubreuil et al., 2003). We demonstrate reduced RhoA activation, reduced caspase-3 activation and JNK phosphorylation and reduced TUNEL staining in LINGO-1-Fc-treated spinal cord indicating that LINGO-1-Fc may protect cells from apoptotic death possibly via inhibition of RhoA activation.

TAJ (TROY) and p75, additional components of the LINGO-1/NgR1 signaling complex, can also mediate cell death via activation of the JNK pathway (Eby et al., 2000; Bhakar et al., 2003). Here we demonstrate inhibition of JNK phosphorylation in injured spinal cord tissue after LINGO-1-Fc treatment, providing an alternative mechanism for LINGO-1-Fc-mediated reduction in apoptotic cell death via inhibition of TAJ or p75 signaling.

In summary, we have demonstrated that a LINGO-1 antagonist can be used therapeutically to treat SCI in two different animal models of disease. LINGO-1-Fc treatment increases axonal plasticity and sprouting, reduces oligodendrocyte and neuronal cell death and attenuates RhoA activation culminating in improved functional recovery, defining an important role for LINGO-1 in SCI pathology. These studies provide a strong rationale that LINGO-1 is a potential and novel target for therapeutic intervention after SCI.

Experimental methods

Recombinant Human LINGO-1-Fc

LINGO-1-Fc (residues 1–532 of Human LINGO-1 fused to the hinge and Fc region of Human IgG1) was expressed in CHO cells, protein A purified and characterized as described previously (Mi et al., 2004). Human IgG1 was used as a control (Protos Immunoresearch, San Francisco, CA). LINGO-1-Fc and Human Ig were formulated in PBS. AP-LINGO-1 (residues 34–532 of Human LINGO-1 fused to His-tagged human placental AP) was purified by sequential chromatography steps of TMAE fractogel and Ni-NTA agarose (Mi et al., 2004).

LINGO-1/NgR binding

ELISA plates were coated overnight at 4°C with 10 µg/ml soluble NgR1 (human NgR1 residues 27–344) (Shao et al., 2005) in 100 mM sodium bicarbonate (pH 9.2), blocked with 1% BSA, 0.1% ovalbumin and 0.1% nonfat dry milk in Hank's balanced salt buffer plus 25 mM HEPES (pH 7.0) for 2 h at 37°C and incubated at room temperature for 1 h with various concentrations of LINGO-1-Fc or Human IgG1 control in blocking buffer with 0.1% BSA. AP-LINGO-1 (6 µg/ml) was added to the wells directly (final concentration 3 µg/ml) and incubated at 4°C overnight. Bound AP-LINGO-1 was quantified with 4-nitrophenylphosphate (NPP). Each sample was analyzed in triplicate.

Spinal cord injury

All procedures were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Biogen Idec Institutional Animal Care and Use Committee or the University of Hong Kong Animal Ethics Committee.

Lateral hemisection was induced in adult Sprague–Dawley rats (200–250 g) anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) as described previously (Yick et al., 2004). Briefly, a dorsal laminectomy was performed at cervical vertebra C7, the dura mater opened and a right lateral hemisection performed using spring scissors, completely interrupting the rubrospinal tract (RST) components. LINGO-1-Fc was administered locally using a gel foam sponge soaked with 20 µg/10 µl LINGO-1-Fc protein placed at the site of injury. After 3 days, a cohort of animals were euthanized and spinal cord tissue was removed to quantify RhoA activation. Functional recovery was quantified after 4 weeks then animals were anesthetized with a lethal dose of ketamine/xylazine and trans-cardially perfused with saline followed by 400 ml 4% PFA in 0.1 M PBS. Brains and spinal cords were removed and post-fixed in PFA overnight then cryoprotected in 30% sucrose. Tissues were cut into 30 µm sections and mounted on gelatin-coated slides for histological analysis.

Dorsal hemisection was induced in anesthetized female Long Evans rats (7 weeks old, Charles River) modified from methods described previously (Li et al., 2004). Briefly, rats were given pre-operative analgesia (Buprenorphine/Buprenex, 0.05 mg/kg s.c., Reckitt Beckinset Healthcare Ltd, UK), tranquilized (Midazolam, 2.5 mg/kg i.p., Abbott Labs, Chicago IL) and anesthetized using Fluothane (2% in O₂, Baxter, Deerfield, IL). A dorsal hemisection was performed at T7, completely interrupting the main dorsomedial and the dorsolateral corticospinal tract (CST) components using a microscalpel to transect the cord at a depth of 1.8 mm from the surface of the cord. Immediately after CST transection, an intrathecal catheter was inserted into the subarachnoid space at T7 and connected to a primed mini-osmotic pump (Alzet model 2004, Alza Corp) inserted into the subcutaneous space. Mini-osmotic pumps delivered Human IgG isotype control protein (5 mg/ml) or PBS (*n*=18) or soluble Human LINGO-1-Fc fusion protein (25 µM, *n*=15) at a rate of 0.25 µl/h. Postoperative care comprised analgesia (Buprenorphine/Buprenex, 0.05 mg/kg s.c.) every 8–12 h for 3 days and antibiotic treatment (Ampicillin, Bristol Myers Squibb, 100 mg/kg s.c. twice daily) for 7 days after surgery. Bladders were expressed manually twice a day for the

duration of the study (4 weeks) or until return of function. On completion of the study, rats were anesthetized and trans-cardially perfused with heparinized saline followed by 4% paraformaldehyde (PFA) and spinal cords removed for histological analysis. To quantify apoptotic cell death after SCI, animals were euthanized 3 days after SCI and spinal cords fixed in PFA and paraffin embedded for subsequent sectioning and histological analysis.

Axonal tract tracing

FluoroGold (FG, 6% w/v, Fluorochrome) was used to label the RST axons projecting to the RN. Two days prior to termination of the study, animals were anesthetized with ketamine/xylazine (80 mg kg⁻¹/8 mg kg⁻¹) and a dorsal laminectomy performed at T2 and 0.5 µl FG was injected into the spinal cord using a Hamilton syringe. Two weeks after dorsal hemisection, animals were re-anesthetized using Fluothane (2% in O₂) and an incision made in the scalp. The area around the skin incision was injected with a local anesthetic, Marcaine (Abbott Labs), and the left sensory-motor cortex exposed via a craniotomy and 7 µl 10% biotin dextran amine (BDA, 10,000 Da Molecular Probes) in PBS, stereotactically injected at 12 predetermined points across the motor cortex 0–3 mm posterior to Bregma, 0–3 mm lateral to the midline at a depth of 1 mm below the surface of the cortex. The first injection point was at the level of Bregma and 1 mm lateral to the midline. Each additional injection point was 1 mm lateral or 1 mm posterior to the previous injection site forming a grid of 12 points (3×4 rows). Injections were performed using a micro-infusion pump (World Precision Instruments) connected to a nanoliter injector (World Precision Instruments) and a glass micropipette. BDA was infused at a rate of 5 nl/s, 650 nl/site. The micropipette was left in the tissue for 2 min before moving to the next injection site. After micropipette removal, the craniotomy was covered with gel foam and the scalp stapled closed. In some instances, BDA tracing was performed bilaterally.

Functional recovery

After lateral hemisection, the use of forelimbs during spontaneous vertical exploration was examined 1 month after different treatments as described previously (Liu et al., 1999) with minor modifications. Rats were placed in a clear Plexiglas cylinder (15 cm in diameter and 30 cm high) and encouraged to use their forelimbs for vertical exploration for 5 min. The following behaviors were scored: (1) independent use of the left (unimpaired) or right (impaired) forelimbs for contacting the wall of the cylinder; and (2) simultaneous use of both forelimbs to contact the wall of the cylinder. The assessments were made by two investigators blinded to the treatments. The vertical exploration behavior was expressed in terms of (1) percentage of unimpaired forelimb use relative to the total number of both limb use (including independent and simultaneous use of both forelimbs); (2) percentage of impaired forelimb use relative to the total number of both limb use; and (3) percentage use of both forelimbs simultaneously relative to the total number of both limb use. The differences between groups were tested by one-way ANOVA followed by Bonferroni post hoc analysis.

After dorsal hemisection, animals were scored using the open field BBB scoring system (Basso et al., 1995). Rats were evaluated the day after CST transection (day 2) and weekly thereafter for 4 weeks with observers blinded to the treatment regimen.

RhoA assay

Injured spinal cords were rapidly removed 3 days after transection and 5 mm blocks of tissue taken each side of the epicenter of the lesion. Tissue was lysed in 50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, plus protease inhibitors and processed as described previously (Ren et al., 1999). GTP-bound and total RhoA proteins were detected by Western blotting using an anti-RhoA mAb (Santa Cruz).

Histological analysis

FluoroGold labeled RST neurons were counted on alternate sections through the rostro-caudal extent of the RN. The number of FG-labeled neurons on the lesioned side was expressed as a percentage of the total number of the FG-labeled neurons on the contra-lateral intact side.

The number of BDA-labeled axons caudal to the injury site was counted on PFA-fixed cryoprotected (30% sucrose) spinal cord sections (50 μ M). Axons were visualized by fluorescence microscopy using streptavidin conjugated Alexa-594 (Molecular Probes). Axons were counted on every eighth section (i.e. every 400 μ m) throughout the cord, a minimum of six sections per animal were counted. All histological analysis was performed in a blinded manner. Axonal dieback was quantified using Open Lab software, as the distance in pixels from the furthest projecting axon terminal field to the edge of the lesion in a minimum of 3 sections per rat.

Apoptotic cell death was assessed immunohistochemically by staining for activated caspase-3 (Cell Signaling Technologies) and TUNEL (Promega) on 10 μ m sections cut from paraffin embedded spinal cord tissue harvested 3 days after injury. Sections were double labeled with markers for neurons (NeuN, Chemicon) and oligodendrocytes (CC1 antibody, Calbiochem). Four weeks after SCI, the CC1 antibody and the anti- β -tubulin (Sigma) antibody were used to identify oligodendrocytes and neurons respectively on PFA-fixed cryoprotected 40 μ M spinal cord sections and cell counts consistently performed in the dorsal column and dorsolateral white matter on cross-sections taken 5 mm caudal to the lesion site. A minimum of 3 sections per rat were counted. All analyses were performed in a blinded manner.

Measurement of JNK phosphorylation by Western blot

JNK and phospho-JNK levels were measured by Western blot of protein extracted from a 5 mm block of injured spinal cord tissue encompassing the lesion, 3 days after injury and treatment with Hu-Ig or LINGO-1-Fc ($n=4$ per group) using selective antibodies to JNK and phospho-JNK (Cell Signaling Technologies). Protein levels were quantified by densitometry, and phospho-JNK levels were normalized to total JNK levels for each sample.

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