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REPORT

Tenascin-R distinct domains modulate migration of neural stem/progenitor cells in vitro

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Abstract Extracellular matrix (ECM) molecules constitute a "niche" that modulates the migration, proliferation, and differentiation of neural stem/progenitor cells (NSPCs). The glycoprotein Tenascin-R (TN-R) is an ECM molecule, comprising multiple domains. Either the whole TN-R molecule or its distinct domains has been demonstrated to play a very important role in the developing central nervous system. However, little is known about the effect of the TN-R domain on NSPCs, especially NSPC migration. In the present study, we first show that both TN-R domains epidermal growth factor-like repeat (EGFL) and fibronectin type III (FN) 6–8 can inhibit the NSPCs migration from neurospheres in vitro. Furthermore, both the EGFL and FN6–8 domains affect the distribution of neurons generated from neurospheres, indicating that EGFL and FN6–8 domains inhibit the motility of neurons generated from neurospheres. These results suggest that TN-R has an inhibitory effect on NSPCs migration.

Keywords Tenascin-R · Neural stem/progenitor cells · Migration · Distribution

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Neural stem/progenitor cells (NSPCs) exist mainly in the subventricular zone and the hippocampal subgranular zone in the adult mammalian central nervous system (CNS), where they have the capacity for self-renewal and can generate major classes of CNS cell types, which are neurons, astrocytes, and oligodendrocytes (Alvarez-Buylla et al. 2002; Song et al. 2002). Mounting evidence has demonstrated that the NSC "niche" plays an important role in the NSPC migration, proliferation, and differentiation, such as cell–cell interactions, somatic cell signaling, neurotrophins, chemokines, and especially extracellular matrix (ECM) molecules (Song et al. 2002; Doetsch 2003; Kearns et al. 2003; Imitola et al. 2004; Tate et al. 2004; Butovsky et al. 2006). For example, Tenascin-C inhibits neurogenesis from neural stem cells without altering the number of glial cells, shifting the balance of neural stem cell differentiation towards a glial fate (Garcion et al. 2004).

Tenascin-R (TN-R) is an ECM glycoprotein that contains a cysteine-rich amino-terminal region, epidermal growth factor-like repeats (EGFL), a region consisting of fibronectin type III (FN) homologous repeats, and a fibrinogen-like domain (FG) at the carboxy-terminus (Fig. 1*A*) (Norenberg et al. 1996). TN-R is mainly expressed in CNS, predominantly by differentiating oligodendrocyte, as well as some interneuron in the spinal cord, retina, cerebellum, and hippocampus (Pesheva et al. 1989; Bartsch et al. 1993; Fuss et al. 1993; Wintergertst et al. 1993; Jones and Jones 2000). TN-R is a multifunction molecule; depending on its mode of presentation, it inhibits growth cone advance when presented as a sharp substrate boundary and promotes neurite outgrowth when offered as a uniform substrate. TN-R bands to voltage-gated sodium channels at nodes of Ranvier to modulate its function (Srinivasan et al. 1998; Xiao et al. 1999). TN-R initiates

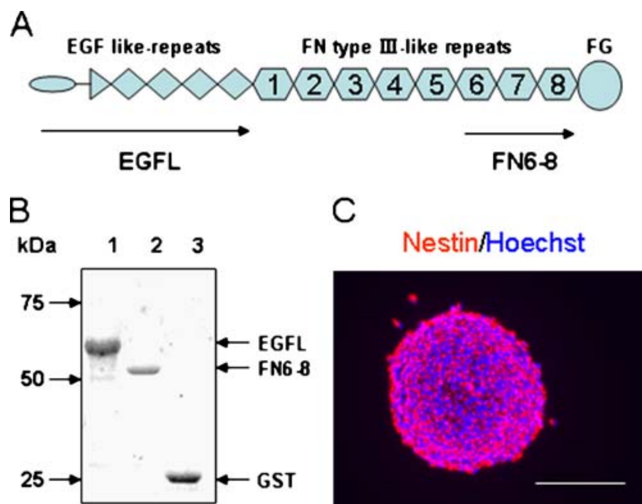

Figure 1. (*A*) Structure of TN-R, the following domains are: EGF-like repeats (*oval and rhombuses*), FN type III-like repeats (*hexagons*), and the FG knob (*circle*). *Arrows* represent the domains of TN-R included in the GST fusion proteins used in our experiments. (*B*) GST-fusion proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue: EGFL (*lane 1*), FN6–8 (*lane 2*), and GST (*lane 3*). (*C*) Fluorescent overlap image of identification of NSPCs; the neuroshperes were positive for nestin, a neural stem cell marker, and nuclei were stained with Hoechst33342.

both the detachement of neuroblast from chains and radial migration in the adult mouse olfactory bulb (Saghatelyan et al. 2004). Distinct domains of TN-R have different functions, e.g., the EGFL domain is antiadhesive for microglia while the FN6–8 domain promotes the adhesion of microglia (Liao et al. 2005). This paper explores the effects of TN-R distinct domains EGFL and FN6–8 as an ECM molecule on NSPC migration and neuron migration from neurospheres.

First, we generated and purified the recombinant EGFL and FN6–8 domains of TN-R as fusion proteins with GST as described (Xiao et al. 1996). The plasmid of pGEX-EGFL and pGEX-FN6–8 were kindly given by Dr. Xiao Z. C. (Singapore General Hospital, Singapore). The two fusion proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli 1970) (Fig. 1*B*).

In our present study, all animal work was conducted under the institutional guidelines of the Animal Ethics Committee of China Pharmaceutical University. All experiments were also conducted in accordance with the guide for the care and use of laboratory animals of the Institutional Animal Care and USA Committee and followed the internal guiding principles for animals of the National Institute of Health. For the culture of NSPCs, Sprague–Dawley rats (E13, 14 d) were stripped of meninges, and the subventricular zone was taken out under a surgical microscope and dissociated into a single cell mechanically. Cells were seeded into a six-well plate, maintained in DMEM/F12 medium containing B27, bFGF (20 ng/ml), EGF (20 ng/ml) (all from Invitrogen, Carlsbad, CA), at 37°C incubator with 5% CO_2. Neurospheres formed for 3–5 d were dissociated gently into single cells, seeded in a new six-well plate. Neurospheres from 3–5 passages were used for the experiment. The neurospheres were positive for nestin, a marker for NSPCs (Fig. 1*C*).

To study the effect of the two TN-R domains on the NS/PC migration, neurospheres formed for 5 d were harvested for a radial cell migration assay with or without recombinant GST protein and GST fusion protein EGFL or FN6–8 (100 μg/ml, respectivly). Neurospheres were seeded onto the 24-well plates precoated by poly-L-lysine and treated with EGFL, FN6–8 or GST [phosphate-buffered saline (PBS) as control]. The observed migration was not chain migration but primarily individual cell migration (Fig. 2*A*). Migration assay was performed according to previous methods (Tate et al. 2004; Kearns et al. 2003). Migration distance was defined as the distance between the neurosphere edge and the farthest migratory cell boundary. Measurements were performed by Image Plus Pro software. Quantification was carried out from 10–15 randomly selected neurospheres per well (two wells per group) for at least three independent experiments. Data were obtained at 8 and 24 h after neurospheres were seeded. At 8 h, the migration distance of the EGFL and FN6–8 group was significantly lower than that of the PBS group. At 24 h, the migration level of all groups increased; however, migrations of the EGFL and FN6–8 groups were still lower than the PBS group (Fig. 2*B*), indicating that TN-R could inhibit the NSPC migration for neurospheres via its EGFL and FN6–8 repeats.

As neural stem cells are able to differentiate into neurons, astrocytes, and oligodendrocytes, we focus on the neurons distribution among all the migratory cells from

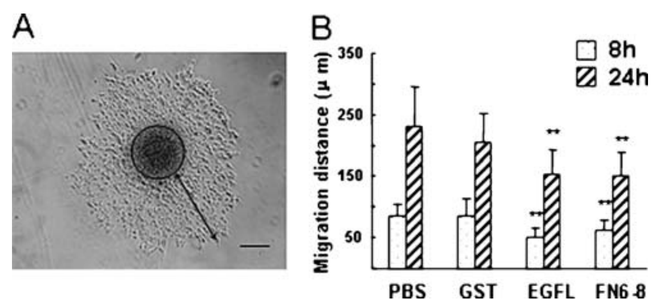

Figure 2. Both TN-R domains EGFL and FN6–8 inhibited NSCs migration from neurospheres. (*A*) Schematic drawing shows the migration distance from the neurosphere edge to the farthest migratory front cell. (*B*) Quantification analysis of NSPC migration distance at 8 and 24 h after being seeded onto poly-L-lysine coated plates with EGFL, FN6–8, GST (100 μg/ml, respectively), and PBS, showed as mean ± SD, analyzed by one-way ANOVA; *double asterisks*, $P<0.001$ vs PBS group. n=15–20. *Scale bar*=50 μm.

neurospheres under the two domains of TN-R: EGFL and FN6–8. The immunocytochemistry method was used for the observation. Neurospheres were seeded onto glass coverslips (precoated by poly-L-lysine) in four-well plates and stimulated with EGFL, FN6–8, or GST (100 μg/ml, respectively, PBS as control). After 72 h, neurospheres were washed with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature, then permeated with 0.1% Trinton X-100 and blocked with 10% goat serum for 1 h followed by incubation with primary antibody against βIII tubulin (Tuj 1:500, StemCell) for neuron, glia fibrillary acidic protein (GFAP 1:500, DAKO) for astrocyte, for 2 h at room temperature followed with second antibody labeled with Cy2 or Cy3. Nuclei were labeled with Hoechst33342 (10 ug/ml, Molecule Probe); after mounting in fluorescent mounting medium (Beyotime, China), neurons were identified by double-label validation (Hoescht plus TuJ) for each count. As observed under a fluorescent microscope, the neuron numbers near the migration boundary in the EGFL and FN6–8 groups (Fig. 3*A* and *B*) seemed lower than in the GST and PBS groups (Fig. 3*A*,*C*, and *D*), and it means that neurons from neurosphere in the EGFL or FN6–8 group seemed to distribute more uniformly among all the migratory cells than the neurons in the GST and PBS groups.

For quantification of the neurons distributing among the whole migratory cells, three concentric circles were defined as follows (Fig. 3*B*): circle 1 is along each sphere edge, circle 2 is 200 μm away from circle 1, and circle 3 is 100 μm away from circle 2. Neurons in the area between circle 1 and circle 2 were termed as inner-neurons (I), and those between circle 2 and circle 3 were outer-neurons (O). Then, the distribution ratio of neurons was defined as: distribution ratio = outer-

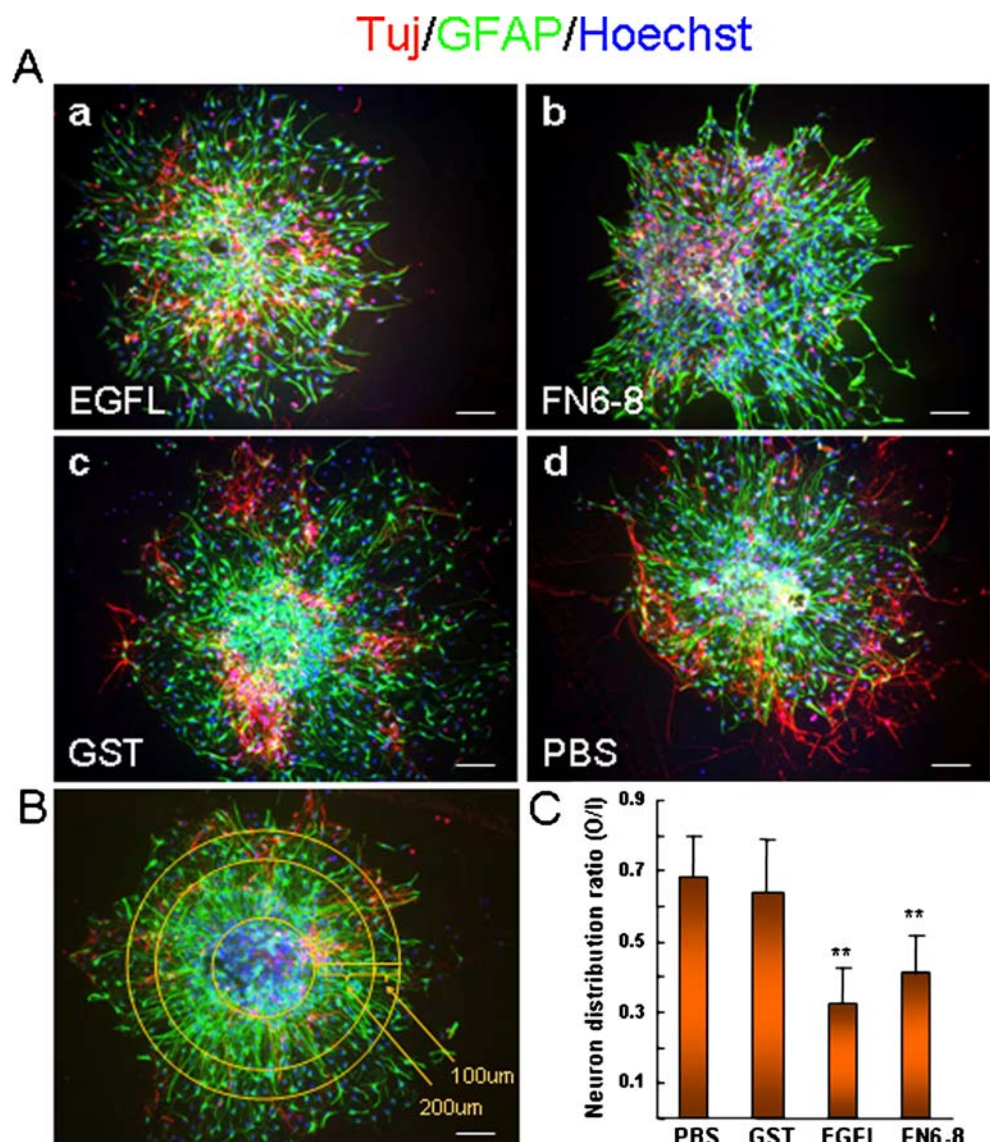

Figure 3. Both TN-R domains EGFL and FN6–8 inhibited neuron migration from neurospheres. Neurospheres were seeded onto poly-L-lysine coated coverslips with or without proteins, and after 72 h, immunostained for neurons (*Tuj*), astrocytes (*GFAP*), and nuclei (*Hoechst33342*). (*A*) Fluorescence overlap micrographs show the morphology of neurons and astrocytes migrated from neurospheres cultured with EGFL (*a*), FN6–8 (*b*), GST (*c*) (100 μg/ml, respectively), and PBS (*d*). (*B*) Schematic drawing shows the three defined concentric circles with a distance of 200 and 100 μm from each other, respectively. Neurons in the outer area (O, 100 μm) and inner area (I, 200 μm) were counterstained with Hoechst33342. (*C*) Quantification analysis of neuron distribution ratio, showed as mean ± SD, analyzed by one-way ANOVA. *Double asterisks*, $P<0.001$ vs PBS group. $n=15$–20. *Scale bar*=50 μm.

neurons numbers (O)/inner-neurons numbers (I). Measurements were performed with Image Plus Pro software and 10–15 spheres per well (two wells per group) were selected randomly for three independent experiments. The quantification analysis showed that the distribution ratio of neurons in the outer area to the inner area was significantly decreased in both EGFL and FN6–8 domain groups compared with the PBS group (Fig. 3*C*); this result indicated that EGFL and FN6–8 domains could influence the distribution of neurons among cells migrated from neurospheres and inhibit the neurons' migration from neurospheres.

TN-R is a glia-derived molecule with multiple domains in the CNS; several previous studies have identified distinct TN-R domains that confer different effects on neuronal cell functions, such as neuronal cell adhesion, neurite outgrowth, and modulation of sodium channels (Xiao et al. 1996, 1997). Some experiments in vivo have demonstrated that in TN-R-deficient mice functional recovery after spinal cord injury was better than that of wild-type mice, and cortical and hippocampal neuronal excitability were also enhanced in mice deficient in TN-R; these findings suggest that TN-R is involved in the regulation of certain inhibitory mechanisms in the intact brain (Saghatelyan et al. 2001; Gurevicius et al. 2004; Apostolova et al. 2006).

A recent study has shown that TN-R is detectable exclusively surrounding the rostral migration stream (RMS), but not within the RMS itself, indicating that TN-R may be involved in directing neuroblasts into their prospective target areas (Saghatelyan et al. 2004). Furthermore, many studies have shown that transplanted NSPCs will migrate toward damaged areas of the brain directed by chemokines but cannot migrate long distances in an intact brain (Kelly et al. 2004; Abrous et al. 2005; Belmadani et al. 2006), which suggests that some inhibitory mechanisms should restrict NSPCs. In the present study, we have shown that both EGFL and FN6–8 domains inhibited the NSPC migration from neurospheres. These observations illustrate that TN-R plays an important role in the inhibitory mechanisms to modulate the NSPCs motility.

Our results showed that both TN-R domains inhibited neural stem cell migration. Both domains have the same effect on the neural stem cell; this is a little different with previous studies that TN-R domains that confer different effects on neuronal cell functions. The EGFL domain is antiadhesive for microglia and neuronal cells while the FN6–8 domain promotes the adhesion of microglia and neuronal cells; all these may be related to both domains that bind different receptors expressed on the microglia and neuron. The result of both TN-R domains inhibited neural stem cell migration did not show that both TN-R domains bound the same receptors; perhaps both TN-R domains bound different receptors because mounting evidence has shown that different receptors have similar functions, such as different cytokines all stimulated microglia migration or promote astrocyte proliferation via different receptors (Giulian et al. 1988; Sawada et al. 1990; Guillemin et al. 1996). Therefore, detailed signaling pathways of both domains affecting neural stem cell migration remain for further study

As was shown in our experiment, neurons and astrocytes were generated from the neurospheres. On the one hand, both the EGFL and FN6–8 domains could inhibit the neurons' migration from neurospheres; on the other hand, these observations also suggested that TN-R possibly restricted neuron migration from neurospheres, which will be beneficial for neural regeneration by restricting the transplanting or endogenous NSPCs in damaged areas. Then, our studies may help to develop novel strategies to enhance neural regeneration by targeting the TN-R protective function.

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