RESEARCH ARTICLE

Spatiotemporal expression of leukemia inhibitory factor receptor protein during neural tube development in embryos with neural tube defects

Dong An[1, 2], Xiao-Wei Wei[1], He-Nan Zhang[1], Dan Liu[1], Wei Ma[1], Zheng-Wei Yuan[1, *]

1 Key Laboratory of Health Ministry for Congenital Malformation, Shengjing Hospital, China Medical University, Shenyang, Liaoning Province, China

2 Department of Pediatrics, The First Hospital of China Medical University, Shenyang, Liaoning Province, China

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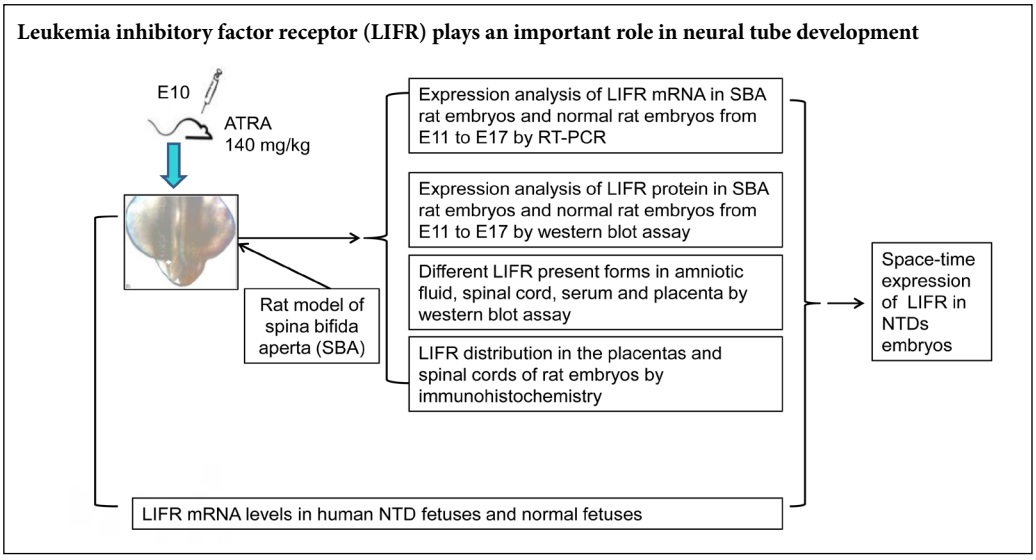
Graphical Abstract



Correspondence to:
Zheng-Wei Yuan, MD, yuanzw@hotmail.com.

orcid:
0000-0002-6223-6612 (Zheng-Wei Yuan)

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Abstract

Leukemia inhibitory factor receptor (LIFR), as a neuroregulatory cytokine receptor, generally shows a neuroprotective effect in central nervous system injuries. In this study, to understand the effect of LIFR on pathogenesis of neural tube defects, we explored spatiotemporal expression of LIFR at different stages of fetal development in normal and neural tube defect embryos. Spina bifida aperta was induced with all-trans retinoic acid on embryonic day 10 in rats, and the spatiotemporal expression of LIFR was investigated in spina bifida aperta rats and healthy rats from embryonic day 11 to 17. Real time-polymerase chain reaction and western blot assay were used to examine mRNA and protein expression of LIFR in healthy control and neural tube defect embryos. Results of the animal experiment demonstrated that expression of LIFR protein and mRNA in the spinal cords of normal rat embryos increased with embryonic development. LIFR was significantly downregulated in the spinal cords of spina bifida aperta rats compared with healthy rats from embryonic days 11 to 17. Immunohistochemical staining showed that the expression of LIFR in placenta and spinal cord in spina bifida aperta rat embryos was decreased compared with that in control embryos at embryonic day 15. Results from human embryo specimens showed that *LIFR* mRNA expression was significantly down-regulated in spinal cords of human fetuses with neural tube defects compared with normal controls at a gestational age of 24 to 33 weeks. The results were consistent with the down-regulation of LIFR in the animal experiments. Our study revealed spatiotemporal changes in expression of LIFR during embryonic neurulation. Thus, LIFR might play a specific role in neural tube development. All animal and human experimental procedures were approved by the Medical Ethics Committee of Shengjing Hospital of China Medical University, China (approval No. 2016PS106K) on February 25, 2016.

Key Words: *amniotic fluid; development; embryogenesis; leukemia inhibitory factor receptor; nerve regeneration; neural tube defect; placenta; spatiotemporal expression; spina bifida aperta; spinal cord; serum*

Chinese Library Classification No. *R446; R364; R772*

Introduction

Neural tube defects (NTDs) are severe congenital malformations in the central nervous system, affecting from 1/1000 to 10/1000 live births (Au et al., 2010; Li et al., 2017). Anencephaly and spina bifida are the two most common categories of NTDs (Avagliano et al., 2018). NTD is generally thought to occur due to failure of complete neurulation during embryogenesis (He et al., 2017). Neurulation is a complicated multi-stage process involving precise space-time regulation of cell development (Tanabe and Jessell, 1996; Smith and Schoenwolf, 1997; Colas and Schoenwolf, 2001). At different developmental stages, disturbances in the control mechanism for neuroregulatory cytokines could contribute to the failure of normal neural tube closure and defecatory dysfunctions. Thus, the study of cytokines involved in regulating the processes of neurulation is needed to help elucidate the mechanisms of NTD development, to prevent NTD pregnancies, and to provide new clues for treatment.

The strategy used to explore the underlying mechanism is to study candidate risk factors identified from NTD mouse models (Harris and Juriloff, 2007). The neurulation processes in humans and rodent models are very similar and rarely involve variation in environmental factors. All-trans retinoic acid (atRA) is necessary for embryonic development (Zhao et al., 2008). However, excess atRA in pregnant mammals can cause many abnormalities of the spinal cord (Lammer et al., 1985). A spina bifida rat model induced by maternal atRA administration has been used to investigate the embryogenesis and pathophysiology of NTDs in fetal rats (Diez-Pardo et al., 1995).

Our previous study (An et al., 2015) investigated differential serum proteome profiles between spina bifida aperta (SBA) fetuses and normal pregnant rats in the early embryonic stage using quantitative proteomics. Among the candidate differentially expressed molecules, leukemia inhibitory factor receptor (LIFR) was chosen for further analysis because it showed significant changes in the serum of pregnant rats with SBA embryos at an early stage of malformation and has been associated with numerous activities within the nervous system (Murphy et al., 1997). Earlier studies on LIFR-mediated signals suggested the involvement of LIFR in promoting the survival of progenitor cells in developing brains (Hatta et al., 2002; Hsu et al., 2007; Bauer et al., 2009). However, LIFR expression has not been reported previously in the embryogenesis of SBA. To explore the specific role of LIFR in neural tube morphogenesis, we investigated the spatiotemporal distribution of LIFR protein in both normal and atRA-induced SBA rat embryos from embryonic day (E)11 to E17. To confirm the presence of consistent trends between the animal model and human, LIFR expression was explored in human fetal samples to further explore the development of therapeutic interventions.

Materials and Methods

Animals and sample preparation

Seventy-eight specific-pathogen-free female Wistar rats aged 10 to 12 weeks and weighing 240 to 300 g were purchased from Liaoning Changsheng Biotechnology Co. Ltd., China [animal license number: SCXK (Liao) 2015-0001]. The procedures for the fetal rat model of SBA have been reported in previous studies (Cai et al., 2007). All experimental procedures were approved by the Animal Ethics Committee of Shengjing Hospital (approval No. 2016PS106K) on February 25, 2016. The experimental procedure followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Seventy-eight pregnant rats were randomly divided into two groups. The SBA group (n = 46) received a gavage feed of atRA (140 mg/kg; 4% wt/vol in olive oil; Sigma, St. Louis, MO, USA) on E10 (Cai et al., 2007). The normal control group (n = 32) received an equal quantity of olive oil at E10. Embryos were harvested by cesarean delivery. The presence of SBA was determined under a stereomicroscope (Nikon, Tokyo, Japan). The spinal cords (from the forelimb buds to the tail bud) were collected for analysis. For immunohistochemical staining, the samples were placed in 4% formaldehyde for fixation and serially sectioned into 4-μm sections. Fresh amniotic fluid and sera samples were obtained immediately after centrifugation (12 000 × g for 15 minutes for amniotic fluid; 3000 × g for 20 minutes for sera). The specimens of each group from at least 3 dams on every embryonic day were placed at –80°C until analysis.

Human NTDs fetuses and controls

This research was approved by the Medical Ethics Committee in Shengjing Hospital (approval No. 2016PS106K) on February 25, 2016. In the NTD group (n = 10), spinal cords were obtained from fetuses with NTDs after labor induction or abortion in Shengjing Hospital in China. In the control group (n = 10), spinal cords were dissected from terminated fetuses with no congenital malformations of the central nervous system. The diagnosis was based on the results of ultrasonography and autopsy results following abortion. The NTD samples were matched to normal controls by gestational week and collection date. The clinical features of the subjects are shown in **Table 1**. Tissue samples from fetuses were stored at –80°C. All participants signed informed consent.

Real-time PCR

After sample collection, total mRNA from the spinal cords of NTD and control rats and from human embryos was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA with an $A_{260\ nm/280\ nm}$ ratio of 1.8 to 2.0 was used. RNA was reverse-transcribed into complementary DNA using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). PCR was accomplished using the SYBR PremixEx Taq II kit (TaKaRa) on an ABI7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). PCR was performed with the following cycling parameters: 95°C for 30 seconds, 45 cycles of denaturation at 95°C for 5 seconds and 60°C for 20 seconds. *GAPDH* was used as the reference gene to normalize expression values. The relative levels of mRNA were

Table 1 Clinical features of the human samples

	Fetuses with NTDs			Normal controls		
ID	Age	Sex	Diagnosis	Age	Sex	Diagnosis
1	GA 26 wks	F	Myelomeningocele	GA 25 wk+6 d	F	Induced labor
2	GA 29 wks+3 d	F	Spina Bifida	GA 28 wk+6 d	F	Induced labor
3	GA 27 wks+6 d	F	Spina Bifida	GA 26 wk+5 d	F	Spontaneous abortion
4	GA 33 wks	M	Spina Bifida	GA 34 wk	M	Fetal intrauterine distress
5	GA 30 wks+6 d	F	Spina Bifida	GA 32 wk+1 d	F	Induced labor
6	GA 24 wks	M	Spina Bifida	GA 24 wk+1 d	M	Unplanned gestation
7	GA 29 wks+6 d	F	Hydrocephalus	GA 31 wk+4 d	F	Induced labor
8	GA 29 wks+4 d	M	Hydrocephalus	GA 29 wk+4 d	M	Threatened premature labor
9	GA 31 wks+1 d	M	Hydrocephalus	GA 30 wk+1 d	M	Induced labor
10	GA 24 wks+1 d	F	Spina Bifida	GA 25 wk+6 d	F	Spontaneous abortion

F: Female; GA: gestational age; M: male; NTDs: neural tube defects.

recorded according to the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). Information on the primers of target genes and *GAPDH* is listed in **Table 2**.

Western blot assay

After sample collection, 50 μg of protein extract from rat samples was denatured, electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline Tween for 2 hours and then incubated with the primary rabbit polyclonal anti-LIFR [1:400 (spinal cord); 1:100 (amniotic fluid); 1:800 (serum); sc-659, Santa Cruz Biotechnology, Santa Cruz, CA, USA] and mouse anti-GAPDH (1:10,000, KC-5G4, KangChen Biotechnology, Shanghai, China) overnight at 4°C. After incubation with a goat anti-mouse horseradish peroxidase-conjugated antibody (1:2000, CW0103; ComWin Biotechnology, Beijing, China) for 2 hours at room temperature, signals were visualized with chemiluminescent agent (Millipore). The relative optical densities of each band were calculated by using ImageJ software (National Institutes of Health, Bethesda, MD, USA), with GAPDH as the loading control.

Immunohistochemical analysis

After the protein expression analysis, immunohistochemical staining for LIFR was performed on the spinal cord sectioned transversely and placenta sectioned sagittally at E15. Sections were subjected to microwave antigen retrieval for 10 minutes. The slides were immersed in 3% H_2O_2 for 20 minutes to block endogenous peroxidase activity and incubated with primary rabbit polyclonal anti-LIFR (1:100, sc-659, Santa Cruz Biotechnology) at 4°C overnight. After washing, sections were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (SP-9001, ZSGB-BIO, Beijing, China) for 20 minutes at room temperature. Sections were stained with diaminobenzidine and counterstained with hematoxylin. For negative controls, the primary antibodies were incubated with non-immune goat antiserum. Cells positive for LIFR protein were a yellow or brownish-yellow color.

Statistical analysis

All data are shown as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS 20.0 software (IBM, Armonk, NY, USA). Comparison analysis was conducted using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

atRA induces NTDs in rats

In the normal control group, 284 embryos were collected from 32 dams and no malformations were discovered. In the SBA group, 363 live embryos were collected from 46 dams at E11, E13, E15, and E17. Forty-five embryos died or were absorbed in utero, and 59.2% (215/363) showed SBA under stereomicroscopy examination (**Figure 1**). Results in embryos of different ages for real-time RT-PCR, western blot assay, and immunohistochemical staining are shown in **Table 3**.

LIFR mRNA levels in rat embryos during development

LIFR mRNA levels were measured in the control and SBA groups (**Figure 2**). Overall, the mRNA levels of *LIFR* in spinal cords of both groups increased with increasing embryonic day from E11 to E17. Specifically, *LIFR* mRNA levels in

Table 2 Sequences of primers of *LIFR* (rat), *LIFR* (human) and *GAPDH* (rat and human)

mRNA	Accession number	Primer sequences (5′–3′)	Annealing temperature (°C)	Product size (bp)
Lifr (rat)	NM_031048.1	Sense: ACT CCG TGG GAT TGA TTA TT Antisense: CTT CTA TTT TAG GGG GTA TTG A	60	270
GAPDH (rat)	NM_017008.4	Sense: GGC ACA GTC AAG GCT GAG AAT G Antisense: ATG GTG GTG AAG ACG CCA GTA	60	143
LIFR (human)	NM_002310.5	Sense: CCT CAG CAC AAA GCA GAG ATA CGA Antisense: GAG AAT CCC CTT TCC CAT CCC AA	60	165
GAPDH (human)	NM_002046.7	Sense: GCA CCG TCA AGG CTG AGA AC Antisense: TGG TGA AGA CGC CAG TGG A	60	138

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; LIFR: leukemia inhibitory factor receptor.

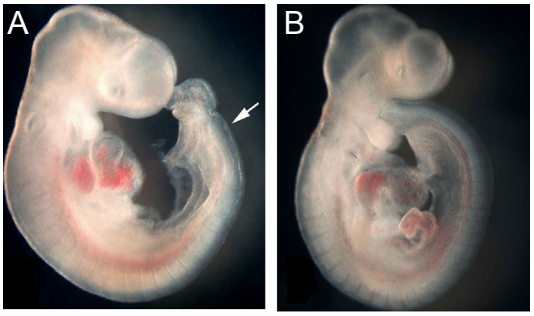

Figure 1 Rat embryos on embryonic day 11.
Rat embryos from (A) atRA-treated pregnancies and (B) normal pregnancies. Arrow shows spina bifida aperta. atRA: All-trans retinoic acid.

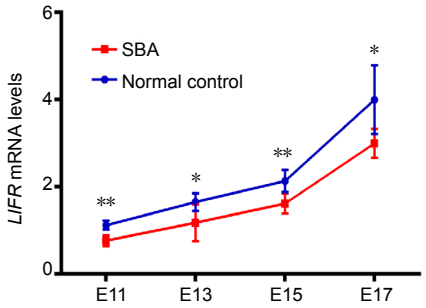

Figure 2 Real time-PCR analysis of *LIFR* mRNA levels in the spinal cord of normal control (blue) and SBA (red) groups in rat embryos.
$*P < 0.05$, $**P < 0.01$, *vs.* normal control group. Data are presented as the mean ± SD ($n = 6$ per group at each time point; Student's *t*-test). E: Embryonic day; LIFR: Leukemia inhibitory factor receptor; SBA: spina bifida aperta.

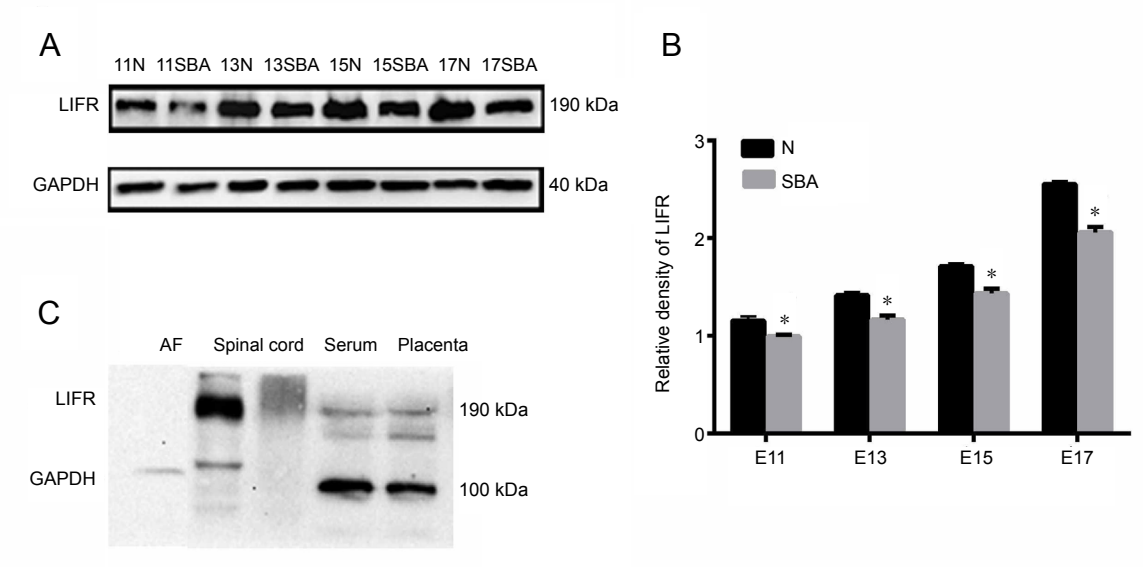

Figure 3 Western blot assay of LIFR in rat embryos.
(A, B) Western blot results of LIFR protein expression in the spinal cords of rat embryos in N and SBA groups on E11, E13, E15, and E17. There was a significant difference in LIFR protein expression in the spinal cords between SBA and N groups at each time point. $*P < 0.01$, *vs.* N group. Data are presented as the mean ± SD ($n = 3$ per group at each time point; Student's *t*-test). (C) LIFR detectable bands in the AF, spinal cord, serum and placenta at E15. LIFR mainly showed the 190-kDa band in spinal cord and the 100-kDa band in AF, serum, and placenta. AF: Amniotic fluid; E: embryonic day; LIFR: leukemia inhibitory factor receptor; N: normal control; SBA: spina bifida aperta.

the control group remained constant initially with significant changes at E17 compared with other observed days (E11, E13, E15). At E17, *LIFR* mRNA expression notably increased (3.59-fold) in the control group compared with the levels observed at E11. In the SBA group, *LIFR* mRNA was lowly expressed at the corresponding time points. *LIFR* gene expression was significantly down-regulated in the SBA rats compared with controls from E11 to E17 ($P < 0.05$).

Western blot assay of LIFR protein expression in rat embryos during development

Consistent with the mRNA expression, in both control and SBA groups, LIFR protein levels in spinal cord gradually increased over embryonic development from E11 to E17, sharply increasing at E17 (**Figure 3A** and **B**). The expression of LIFR protein in spinal cord was lower in SBA rats compared with controls ($P < 0.05$; **Figure 3B**). Furthermore, detectable LIFR bands were present in different forms in amniotic fluid, spinal cord, serum, and placenta, with one main band (190 kDa) observed in spinal cord tissue and one main band (100 kDa) observed in amniotic fluid, serum, and placenta (**Figure 3C**).

Immunohistochemistry results of LIFR distribution in the placenta and spinal cord of rat embryos during development

Based on the results of the western blot assay, we chose E15 as the time point for immunohistochemical staining. In normal rat embryos, cells staining positive for LIFR were widely distributed in the spinal cord as well as on the fetal and maternal sides of placental tissues. Moreover, compared with control rats, we observed decreased expression of LIFR in spinal cords and placentas in SBA rats. Cells positive for LIFR showed brownish-yellow staining, and were localized

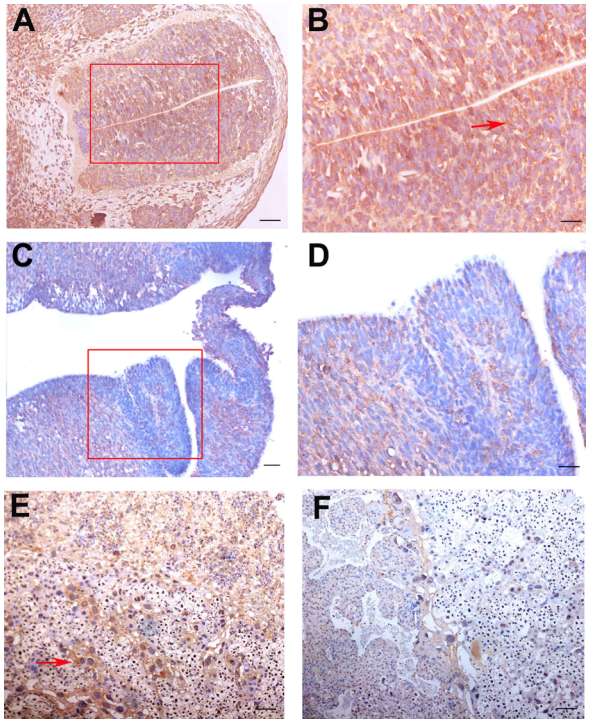

Figure 4 Immunohistochemical distribution of LIFR in rat embryos from SBA and normal control groups at embryonic day 15.
(A, B) Spinal cord in normal controls. In spinal cords, LIFR-positive staining was localized in the cytomembrane and cytoplasm (arrow). (C, D) Spinal cord in SBA rats. (E) Placenta in normal controls. In placenta, LIFR was expressed in the cytoplasm (arrow). (F) Placenta in SBA rats. Red rectangles in A and C are shown at higher magnification in B and D, respectively. (A, C, E, F) Scale bars: 100 μm. (B, D) Scale bars: 50 μm. LIFR: Leukemia inhibitory factor receptor; SBA: spina bifida aperta.

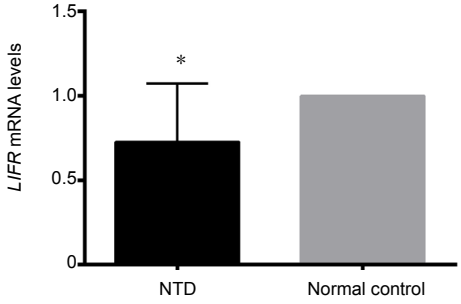

Figure 5 Real time-PCR reaction analysis of LIFR mRNA levels in the spinal cords of human fetuses between the normal control and NTD groups at different gestational ages from 24 to 33 weeks.
*$P < 0.05$, *vs.* normal control group. Data are represented as the mean ± SD ($n = 10$ per group at different gestational ages; Student's *t*-test). LIFR: Leukemia inhibitory factor receptor; NTD: neural tube defect.

in the cytoplasm and cytomembrane in spinal cord. In placental tissues, LIFR immunoreactivity was localized in the cytoplasm (**Figure 4**).

LIFR mRNA levels in the spinal cords of human NTD fetuses

To explore whether there were similarities in LIFR expression during neural development between rat models and human, we measured *LIFR* mRNA levels in spinal cord tissue of human NTD fetuses and controls at gestational ages from 24 to 33 weeks. Consistent with the rat models, levels of *LIFR* mRNA in spinal cord were decreased significantly in human NTD fetuses (0.729-fold) relative to controls (**Figure 5**).

Discussion

Leukemia inhibitory factor (LIF), as a neurotrophic factor, is highly expressed in various central nervous system diseases and related animal models (Liu et al., 2019). Generally, LIF shows a neuroprotective effect in nervous system injuries (Butzkueven et al., 2006; Soilu-Hänninen et al., 2010; Rowe et al., 2014). Low-affinity LIFR bound to gp130 in LIF receptor complex is required for the biological activities of LIF, similar to several other neuroregulatory cytokines, including ciliary neurotrophic factor and oncostatin-M (Gearing, 1993; Gearing et al., 1994). LIFR is present in two forms: membrane-bound and soluble. Soluble receptors are widely distributed in mammalian biological fluids. Previous studies have reported that soluble LIFR could play an antagonistic role in LIF signal transduction (Layton et al., 1992) to block unwanted systemic actions. Previously, we used SBA rat models induced by atRA to study the mechanism of NTDs in our laboratory (Cai et al., 2007; Fan et al., 2011; Wei et al., 2013; Xue et al., 2018). In this study, we observed the spatial and temporal distribution of LIFR in SBA rat embryos during neurodevelopment. Our research suggests that LIFR plays a specific role in neurodevelopment and is associated with the pathogenesis of SBA.

In our research, LIFR expression in spinal cord increased gradually over time from E11 to E17 and then notably increased at E17, further suggesting its specific biological activity in spinal cord development, especially in the development stage of embryos. Other researchers have reported that the LIFR neuroregulatory cytokine-signaling pathway involves the regulation of differentiation of neuronal precursor cells, survival of neurons, development of astrocytes and oligodendrocytes, and trophic effects in mouse (Murphy et al., 1997). As LIFR is necessary for neuroregulatory signaling, LIFR expression during the essential stages of neurodevelopment might have a specific role in differentiation and survival of neuronal cells, thus contributing to neurulation. Our data also demonstrated specific spatial distribution for LIFR in neural development. Our immunohistochemical results showed that LIFR was widely expressed in spinal cord and placental tissue during nervous system development. There was greater LIFR expression on the fetal side than on the maternal side in the placenta, suggesting that LIFR may have specific roles in embryonic growth and neural development. Researchers reported that $LIFR^{-/-}$ mice experience perinatal death due to a combination of pleiotropic defects, including placental architecture disruption and loss of mineralized bone. In addition, histological sections of late-term fetuses show decreased numbers of spinal cord and brainstem astrocytes (Ware et al., 1995). These results suggest that LIFR is required for early embryo development and placen-

Table 3 Distribution of embryos for different assays at each time point

	Normal control			SBA		
	RT-PCR	Western blot assay	Immunohistochemistry	RT-PCR	Western blot assay	Immunohistochemistry
E11	24	24		25	25	
E13	19	19		20	21	
E15	20	20	12	22	22	12
E17	21	21		23	23	
Total	84	84	12	90	91	12

Data are expressed as *n*. E: Embryonic day; RT-PCR: real time-polymerase chain reaction; SBA: spina bifida aperta.

tal function.

Compared with control group results, our results in rat SBA embryos and human NTD fetuses demonstrated that LIFR expression was decreased in the spinal cord, suggesting that down-regulation of LIFR during developmental stages affected neurulation and contributed to the occurrence of NTDs. LIF-LIFR signaling promotes survival for neural progenitor cells *in vitro* by decreasing caspase-dependent apoptosis and enhancing proliferation of neural progenitor cells (Majumder et al., 2012). Our previous study found an increase in apoptosis and a reduction in proliferation in neural progenitor cells of atRA-induced SBA embryos from E11 to E13 (Wei et al., 2012). Fewer sensory neurons were produced from neural crest cells in LIFR-deficient mice (Murphy et al., 1997). Our prior research indicated a decrease in sensory and motor neurons associated with spinal malformation (Yuan et al., 2003; Guan et al., 2009). One study indicated that atRA exposure could promote lipid, protein, enzyme, and DNA peroxidation, leading to increased levels of reactive oxygen species (Tokarz et al., 2016). However, the neuroprotective mechanism of LIF signaling could reduce the amounts of spontaneously produced H_2O_2-induced reactive oxygen species in cultured neural progenitor cells (Majumder et al., 2012). Collectively, these results imply that LIF-LIFR signal is involved in survival, growth, and differentiation of neural progenitor cells. Therefore, a decrease in LIFR expression may contribute to neural development.

We observed that LIFR was present in the spinal cord, placenta, serum, and amniotic fluid in different forms. LIFR existed in a membrane-bound form in the spinal cord, revealing a main band of 190 kDa, and existed in soluble form in serum, amniotic fluid, and placenta, with a main band of 100 kDa. In the current study, we showed that membrane-bound LIFR was down-regulated in the spinal cord and placenta of SBA groups compared with normal control groups. Conversely, in our prior research (An et al., 2015), iTRAQ (isobaric tags for relative and absolute quantitation) results and ELISA validation results consistently indicated that the levels of soluble LIFR in serum increased in SBA rats compared with healthy controls. This opposite expression of different forms (membrane-bound and soluble) in different locations between the SBA and control groups probably relates to antagonistic effect for the two different forms. Thus, either a decrease in the membrane-bound form of LIFR or an increase in the soluble receptor form might be responsible for the abnormal neural development.

In summary, we demonstrated spatiotemporal changes of LIFR expression during embryonic neurulation from E11 to E17, suggesting an important role for LIFR during nervous system development. We detected two different forms with different spatial distributions: a membrane-bound form (190 kDa) in the spinal cord and a soluble form (100 kDa) in the serum, amniotic fluid, and placenta. The two forms of LIFR have inverse biological functions, the membrane-bound form being responsible for local signal transduction and the soluble form antagonizing LIF signal induction. A decrease in LIFR expression in spinal cord and placenta or increase of soluble receptor might be associated with NTD development. Similar trends were observed in human samples: down-regulation of *LIFR* mRNA was observed in spinal cords of NTD fetuses compared with normal fetuses at a gestational age of 24 to 33 weeks. These changes in LIFR expression provide novel insights for understanding the role of LIFR in neural development. Ongoing research on LIFR signaling pathways, target molecules, and interaction partners during embryonic development may reveal further contributions to the multifactorial pathogenesis of NTDs and facilitate development of therapeutic interventions.

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Conflicts of interest: *The authors declare that there are no conflicts of interest associated with this manuscript.*

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