Isl-1 down-regulates DRG cell proliferation during chicken embryo development

Dawei Chen, Guoxin Wang, Haoshu Luo, Jiali Liu, Sheng Cui

State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing People's Republic of China.

Correspondence to: Sheng Cui, PhD.
State Key Laboratory of Agrobiotechnology
College of Biological Sciences, China Agricultural University,
Beijing 100193, P.R. China.
TEL: +86-10-62733443; FAX: +86-10-62733443; E-MAIL: cuisheng@cau.edu.cn

Submitted: 2009-12-04 Accepted: 2010-01-31 Published online: 2010-02-17

Key words: **chicken embryo DRG; cell proliferation; Isl-1; RNAi; over expression**

Neuroendocrinol Lett 2010; **31**(1):67–72 **PMID:** 20150874 NEL310110A12

Abstract

OBJECTIVE: Protein Isl-1 RNA interference and over expression in early chicken embryo dorsal root ganglia (DRG) were used to investigate the function of Isl-1 in DRG cell proliferation.
METHODS: Isl-1 targeted shRNA expression vector and Isl-1 over-expression vector were transfected into chicken embryo DRG by in ovo electroporation. Then, the DRG proliferation rate was detected by BrdU immunohistochemistry.
RESULTS: The rate of DRG cell proliferation increased after Isl-1 knock-down and decreased after Isl-1 over-expression.
CONCLUSIONS: In this study, we found that Isl-1 negatively modulates DRG cell proliferation.

Abbreviations :

DRG	- Dorsal root ganglia
NT	- Neural tube
NC	- Neural crest
CNS	- central nervous system
PNS	- peripheral nervous system
RNAi	- RNA interference
BrdU	- 5-bromo-2-deoxyuridine
IHC	- immunohistochemistry
shRNA	- short hairpin RNA

INTRODUCTION

Isl-1 is a LIM homeodomain contending transcription factor. In chicken embryo DRG, Isl-1 expression was first detected at st18, then increased rapidly. Finally, nearly all of the DRG cells are Isl-1$^+$ by St29 (Cui *et al.* 2000). Furthermore, Isl-1 is predominantly expressed in DRG internal cells and the proliferating DRG cells are primarily present in the periphery of the ganglia (Avivi & Goldstein 1999). This suggests that Isl-1 expression has a distinct spatial relationship with DRG cell proliferation during embryo development. Isl-1 is regarded as one of the earliest differentiation markers for special neural cells in the vertebrate central nervous system (Ericson *et al.* 1992) and peripheral nervous system (Avivi & Goldstein 1999). The results of In vitro and in vivo experiments suggested that Isl-1 does not dual labeled with proliferation marker in DRG cells (Varly *et al.* 1995; Avivi & Goldstein 1999). Thus, Isl-1 has been regard as one of the earliest differentiation marker of embryo DRG cells (Avivi & Goldstein 1999). Since the Isl-1 expression is regarded as a marker for DRG cell-cycle withdrawal, we are interested in whether Isl-1 has a role in the process of DRG cell-cycle withdrawal.

Gene knockout/knockdown is a straightforward method for gene function study, by which we may directly investigate the function of Isl-1 in

ORIGINAL ARTICLE

chicken embryo DRG cell proliferation. However, the primary difficulty in the usage of this technique is that Isl-1 mutation halts embryonic development at the early stage because of this mutation causing impairment in vascular development (Pfaff *et al.* 1996). Isl-1-mutated mouse embryos show developmental anomalies at E9 and die at E11 (Pfaff *et al.* 1996). However, the DRG is not formed until E10 in the mouse embryo (Farinas *et al.* 2002). Therefore, further study regarding the function of Isl-1 expression during DRG development is limited. RNA interference (RNAi) is another remarkable gene knockdown process that has been successfully used to repress target genes in cells and organisms ranging from worms to mammals (Hannon 2002). Vector-based shRNA designed based on target gene sequences is able to induce RNAi (Brummelkamp *et al.* 2002). shRNA expression vectors can be transfected to the chicken embryo by in ovo electroporation method, which is a powerful method that enables the efficient introduction of expression constructs into various tissues in the chicken embryo (Stern 2005). DRG is formed by neural crest cell migration (Teillet & Douarin 1987; Lallier & Bronner 1988), which is located at the top of neural tube. Thus, we may use in ovo electroporation method to transfect shRNA expression plasmid into neural crest (NC) cells, which are located at the dorsal side of the NT at St16 (Hamburger & Hamilton 1951). As a result, the DRG cells will finally inherited the plasmid from their NC cell progenitor. In this study, Isl-1-targeting shRNA expression vector and Isl-1 overexpression vector were introduced into the DRG cells of the chicken embryo, and the relationship between Isl-1 expression and DRG cell proliferation was investigated.

MATERIALS AND METHODS

The pEGFP-H1-shRNA plasmid, which contents a random shRNA sequence, was kindly gifted by Prof. Beate Brand-Saberi (Dai *et al.* 2005). This efficient enhanced green fluorescent protein (EGFP) and shRNA dual expression system was used to perform the vector-based RNAi. In order to construct Isl-1 targeted RNAi vector, we replaced the random shRNA sequence of pEGFP-H1-shRNA plasmid by an isl-1 targeted shRNA sequence, which is referenced by Chesnutt and Niswander (Chesnutt & Niswander 2004). The Isl-1i shRNA expression cassette was generated by overlap extension (SOE)-PCR amplification (Horton *et al.* 1989). Briefly, for the first step, pEGFP-H1-shRNA vector DNA was used as the template; 2 fragments were amplified separately using the following primers (the Isl-1-targeted shRNA sequences and loop were synthesized on the primer Soe1-a and Soe2-s): Soe1-s: 5'-CTGGCACGAC AGGTT-3'; Soe1-a: 5'-TTCAAGAGA TCGGACTGAG GCCAGTCATT TTTTTTTGGA ATTCAAGCTT GGCGTAATC-3'; Soe2-s: 5'-TCTCTTGAAT GGACT-GAGG CCAGTCATTT TGGATCCGAG TGGTCT-3'; and Soe2-a: 5'-CATTCGCCAT TCAGG-3'. These 2 PCR reactions were carried out under similar conditions (20 cycles of 94 °C for 50 seconds, 50 °C for 50 seconds, and 72 °C for 90 seconds). The products were then mixed in a 1:1 molecule ratio and subjected to a second round of PCR where they were used as the templates. The second PCR was carried out using the external primers Soe1-s and Soe2-a at following situations: 20 cycles of 94 °C for 50 seconds, 50 °C for 50 seconds, and 72 °C for 1 minute. The splicing segment containing the Isl-1 shRNA expression cassette was digested using BamHI and HindIII and joint into a linerized pEGFP-H1-shRNA vector. The original pEGFP-H1-shRNA vector that content random shRNA sequence was used as negative control in this study.

The Isl-1 overexpression vector (pXJ40-Myc-tagged-isl-1) was kindly gifted by Prof. Xinmin Cao (Hao *et al.* 2005). It contains the complete Isl-1 sequence fused with a myc-tag reporter gene sequence. Before injection and in ovo electroporation, the pXJ40-Myc-tagged-isl-1 vector was mixed with the pEGFP-N1 vector in a 5:1 molecule ratio, which was used as an independent marker of electroporation efficiency.

White Leghorn eggs were obtained from China Agricultural University and horizontally incubated at 38.5 °C under 50% humidity conditions for 52 hours (St16). The in ovo electroporation was applied as described (Kos *et al.* 2001). In brief, a 1 cm × 1 cm double-sided tape was pasted on the operating area to prevent the shell from cracking. The eggs were then windowed to visualize the embryos. An aliquot of plasmid solutions containing 1 mM $MgCl_2$, 0.01 M phosphate-buffered saline (PBS), 0.05% fast green, and 1 μg/μL plasmid was later injected into the lumen of the neural tube. Electroporation was applied using a square-wave generator (RM6240B; Chengdu Instrument Factory) (40 V, 20 ms pulse width, 5 pulses with 1 second intervals between each pulse). The window on egg was covered by cover-glass and sealed by paraffin. The eggs continued to incubate at 38.5 °C for an additional 24 hours. Following this, 10 μL of 10 mM 5-bromo-2-deoxyuridine (BrdU) solution was injected into the vitelline vein an hour before embryo harvest.

24h after in vivo electroporation, chicken embryos were sacrificed by decapitation. Then, the embryos were fixed in 4% paraformaldehyde at 4 °C over night followed by treatment with 20% sucrose PBS for another 4–8 hours. The fixed embryo trunk was then embedded in Jung tissue freezing medium (Leica, Germany). After that, 10-μm-thick serial frozen sections were cut at –22 °C. GFP expression on the sections were observed and photographed under a fluorescence microscope (Leica LB30T; Heidelberg, Germany). Then the sections were used for further immunohistochemistry (IHC) staining.

Isl-1 Immounohistochemistry stain was applied as described before (Cui & Goldstein 2000). In brief, antigen fix applied by water bath at 95 °C in 0.01 M TCA solution for 20 min. mouse monoclonal anti-

body against Islet-1 (40.2D6, Developmental Studies Hybridoma Bank, Iowa City, IA, USA, 1:50) was added to the sections and incubated overnight at 4 °C. Sections were gently washed with PBS and then incubated in biotin labeled house anti-mouse IgG (HAMB 1:150) 2h at room temperature. After rinsed by PBS, sections were incubated in horseradish peroxidase streptavidin (HRP-SP, 1:150) for 2 h at room temperature. The Immounolabeling was visualized using diaminobenzidine (DAB). Finally, sections were photographed under fluorescence microscope photograph system (Leica). The GFP and Isl-1 picture of same section was merged by photoshop software. Positive cells number divided by total cells number within the GFP expression area of DRG was calculated as the positive cells rate.

The Myc-tag which was fused with the exogenous Isl-1 was used to determine the effect of Isl-1 overexpression. Myc-tag was labeled by mouse anti-myc-tag (1:100; C1302; Cell Signaling) antibody. The following method is the same to Isl-1 IHC staining ing.

In order to identify the proliferation cell rate, BrdU immouno-stain applied as described before (Liu *et al.* 2005). In brief, DNA was denatured in 2N HCl at 37 °C for 30min, BrdU sties were exposed by 0.01% trypsin (sigma) at 37 °C for 10 min and nonspecific staining was blocked with 1% bovine serum albumin (BSA) for an hour. Primary antibody (1:50, G3G4, Developmental Studies Hybridoma Bank, University of Iowa) was added to the sections and incubated over night at 4°C. After that, sections were washed by PBS and incubated with second antibody horse anti mice IgG (HAMB, 1:150) for 2 h at room temperature and followed by incubating with HRP-SP (1:150) for 2 h at room temperature. The Immounolabeling was then visualized using diaminobenzidine (DAB). The bounder of DRG was marked by dual label HNK-1 (Sanders & Cheung 1985), sections treated in 2% Triton-X100 in PBS for 15min and incubateed with HNK-1 antibody (sigma c6680 1:70) over night at 4 °C. Sections were washed in PBS and incubated with biotin labeled goat anti-mouse IgM (GAMB, 1:150) for 2h at room temperature. After that, sections were incubated with avidin conjugated Texas red labeled avidin (1:10 vector) for 2h at room temperature. Finally, sections were photographed under microscope photograph system (Leica). After the merge of the BrdU picture with HNK and GFP picture of the same section, DRG cells proliferation cell rates were calculated by counting the percentage of BrdU immunopositive cells number to the total cells number. The numbers of Isl-1$^+$ and BrdU$^+$ cells in DRG were detected by counting the number of immunopositive nuclei within the boundary of the DRG in each section, and all positive cell numbers on the serial sections of the same DRG were summed up together. Total number of DRG cells on the sections was calculated by counting both the negative nuclei and immunopositive nuclei within the DRG boundary and summed up by the same way as the positive cell number. Cell number is counted from the images, which were photographed under a microscope using a 40× objective (Leica LB30T; Heidelberg, Germany), by the image analysis software (AlphaImager 2200; Alpha Innotech, San Leandro, CA, USA). DRG volume was calculated as described in the previous study (Liu *et al.* 2005).

In this study, data were presented as mean ± SEM. The number of embryos in each treatment was represented as "n." Differences between the means of each repeated measure group of the embryos (n≥4) were tested for statistical significance using one-way ANOVA, followed by Student's t test. $p<0.05$ was considered as statistically significant.

RESULTS

Isl-1 targeted RNAi and Isl-1 over expression efficiency

In order to knockdown Isl-1 in the developing chicken embryo DRG, the pEGFP-H1-shRNA vector was transfected into st16 chicken embryo right side neural crest by in ovo electroporation. After 24h post transfection, Isl-1 was stained by IHC. The efficacy of Isl-1 knockdown was determined by counting the Isl-1$^+$ cell rate in the pEGFP-H1-shRNA-Isl-1i transfected DRG (Isl-1 knockdown arm). The counter side DRG was not transfected, which was used as a negative control (wild type arm). The Isl-1$^+$ cells rate in wild type arm was significantly higher than that of the Isl-1 knockdown arm ($p<0.05$, Figure 2). In order to determine whether the changes of Isl-1$^+$ cells rate in DRG is a side effect of GFP expression, non-specific effect of shRNA expression or Isl-1 targeted RNAi effect, pEGFP-H1-shRNA vector contenting a random shRNA sequence was transfected to DRG using the same method (random shRNA arm). Random shRNA arm had the same Isl-1$^+$ cells rate to wild type arm, which was significantly higher than Isl-1 knockdown arm ($p<0.05$, Figure 2). These results showed that the Isl-1 was successfully knock-downed by Isl-1 targeted RNAi.

Isl-1 over-expression in chicken embryo DRG was also determined by Isl-1$^+$ DRG cells rate and double checked by Myc-tag fused to exogenous Isl-1. Isl-1$^+$ DRG cells rate in the Isl-1 over-expression arm is significantly higher than that in the wild type arm ($p<0.05$, Figure 2). What's more, Myc-tag IHC staining consistent with Isl-1 over-expression DRG (Figure 1) indicated that the Isl-1 was successfully over-expressed by pXJ40-Myc-tagged-isl-1 vector.

In order to investigate if Isl-1 has a role in DRG cell proliferation, we estimated the DRG cell proliferation rate by calculating the BrdU positive DRG cells present to the total number of DRG cells after Isl-1 knockdown or over-expression. The results (Figure 4A) showed that the DRG cell proliferation rate of Isl-1 knockdown arm (Figure 3, A–C) was significantly higher than that in the random shRNA arm ($p<0.001$, Figure 3G–I) and the wild type arm ($p<0.001$).

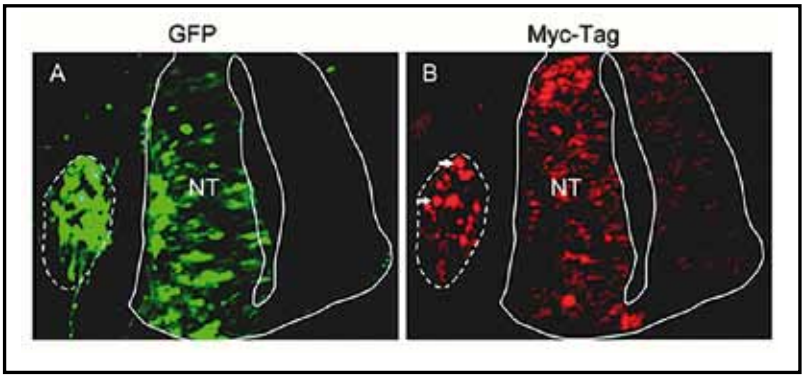

Fig1. Isl-1 over-expression in the E3.5 chicken embryo DRG. GFP expression showed the transfect efficiency (Green; A) and the Myc-tag IHC stain (Red; B) showed Isl-1 expression. The broken lines indicated DRG outer boundary and red lines indicated neural tube (NT) outer boundary. Bar = 60 μm.

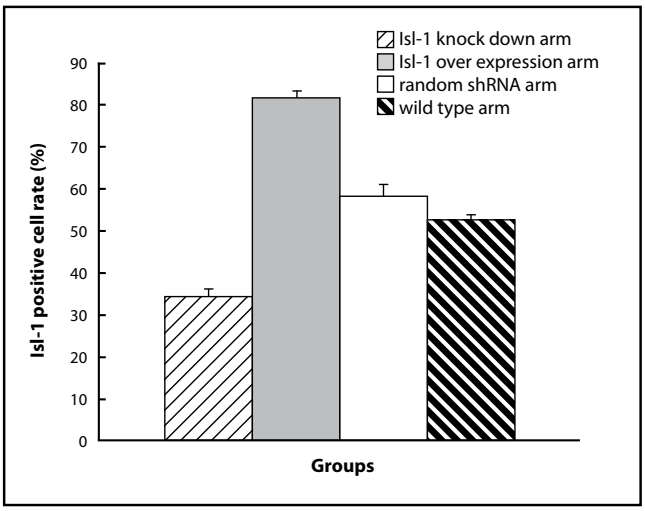

Fig. 2. The percentages of Isl-1+ cells to the total DRG cells in the Isl-1 targeted pEGFP-H1-shRNA vector transfected chicken embryo (Isl-1 knock-down arm), the random shRNA content pEGFP-H1-shRNAI vector transfected chicken embryo (random shRNA arm), the none treatment chicken embryo (wild type arm) and the Isl-1 over-expression vector-transfected DRG (Isl-1 over-expression arm). There is a significant difference between Isl-1 knock-down arm, Isl-1 over-expression arm and control groups (wild type arm and random shRNA arm, $p<0.001$) and no significant difference between each control group ($p>0.05$).

In order to further confirm the above results, we subsequently examined the influence of Isl-1 over-expression on the DRG cell proliferation rate. The proliferation rate in Isl-1 over-expression arm (Figure 3D–F) was significantly lower than that in the random shRNA arm ($p<0.001$) and wild type arm ($p<0.001$).

Since the right side neural tube had also been transfected, it is possible that the changes of Isl-1 expression in neural tube influence the DRG cell proliferation. Thus, we established a group of chicken embryos, which we transfected the Isl-1 targeted pEGFP-H1-shRNA vector into the neural tube but not the DRG (transfect NT only arm, Figure 3J–L). The proliferation rate of this group had no significant difference to the random shRNA arm ($p>0.05$) and wild type arm ($p>0.05$), which was significantly lower than the Isl-1 knockdown arm. This result indicated that only the Isl-1 expression alter in DRG respond to the changes of DRG cell proliferation rate.

The possible reasons for the promotion of DRG cell proliferation rate is the increase of the proliferation cells number or the decrease the number of total cells. However, the change patterns of DRG volume and total DRG cell number were similar to the DRG cell proliferation rate after Isl-1 RNAi and over-expression (Figure 4B,C). The DRG size and total cell number of Isl-1 knockdown arm were significantly higher than those of the random shRNA arm ($p<0.001$), wild type arm ($p<0.001$ Figure 4B,C) and NT only transfected arm. Meanwhile, the DRG size and total cell number of Isl-1 over-expression arm were significantly lower than other groups ($p<0.001$). These results indicated that the change of DRG cell proliferation rate is due to the change of proliferating cells number. Thus, we can conclude that Isl-1 expression in developing chicken embryo DRG negatively modulates the DRG cell proliferation.

DISCUSSION

In the chicken embryo DRG, Isl-1 is first detected at Stage (St) 18, then the Isl-1 expression level continues to increase rapidly and nearly all the cells are Isl-1$^+$ by St29 (Cui & Goldstein 2000). Furthermore, Isl-1 is predominantly expressed in DRG internal cells and proliferating DRG cells are primarily present in the periphery of the ganglia (Avivi & Goldstein 1999). This suggests that Isl-1 expression has a distinct spatial relationship with DRG cell proliferation in the embryo.

In our study, we found significantly increased proliferation rate in chicken embryo DRG after Isl-1 RNAi. In addition, the Isl-1 over-expression experiment has confirmed the results of Isl-1 RNAi. DRG cell proliferation rate in Isl-1 over-expression embryos was significantly lower than those of control and RNAi groups. These results revealed that the variation of the DRG cell proliferation rate is negatively correlated with the Isl-1 expression in chicken embryo DRG. Altogether, our results suggested that Isl-1 negatively regulates the DRG cell proliferation in the early chicken embryo DRG.

This result is supported by the study of Froriep's DRG, a transient DRG in the occipital (cranial) sclerotomes (reviewed in Lim *et al.* 1987). Froriep's DRG reduce growth and eventually degenerate during the embryo development at early stage of the embryo devel-

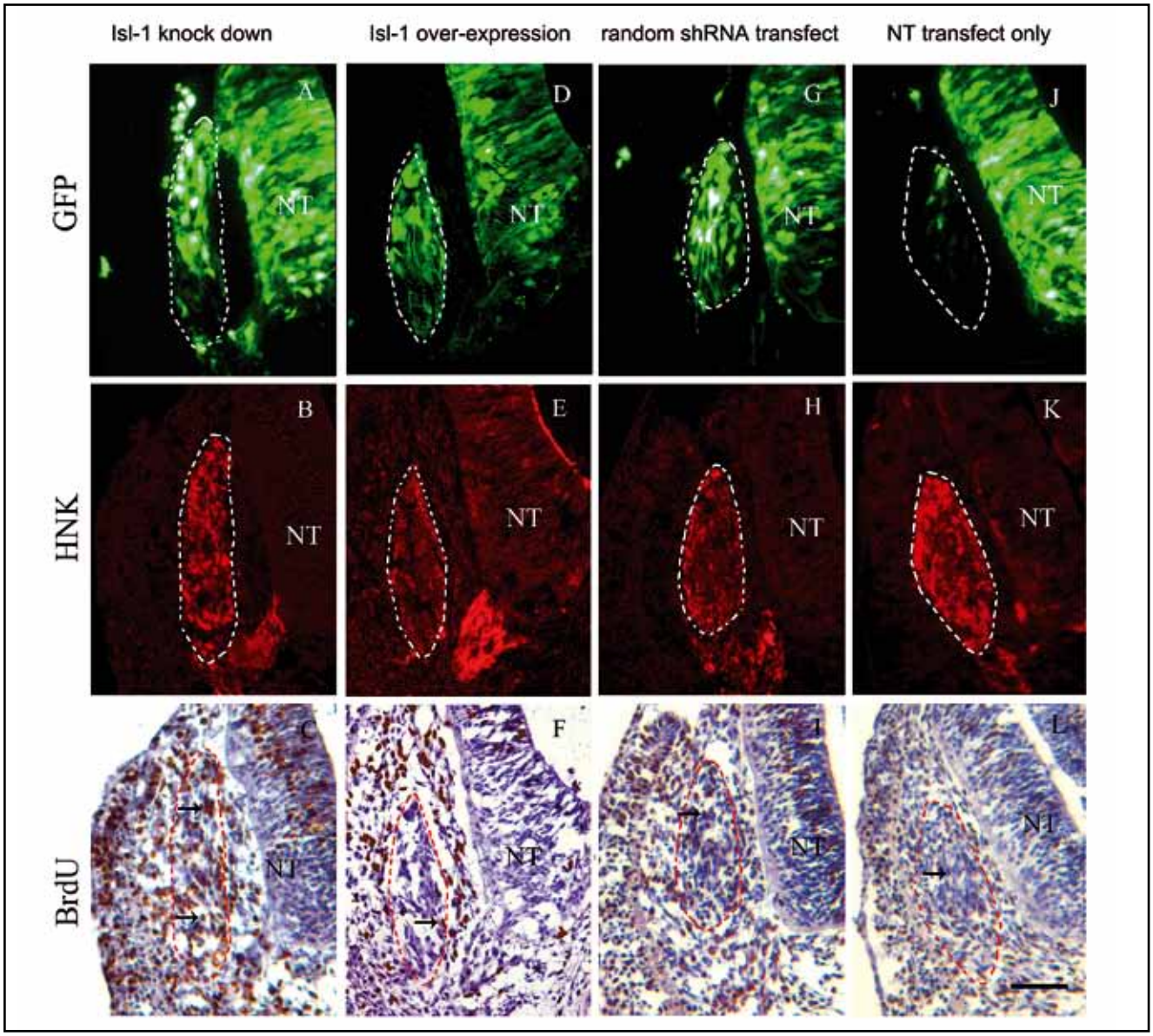

Fig. 3. Isl-1 RNAi in DRG significantly increased the rate of cell proliferation in the E3.5 chicken embryo DRG, while Isl-1 over-expression had the contrary effect. On the same section, cells transfected with vector were labeled by GFP (green; A, D, G, and J); the edge of DRG were labeled by HNK-1 (red; B, E, H, and K); and the S phase cells were labeled by BrdU IHC stain (brown; C, F, I, and L). Fig A-C showed the Isl-1 targeted RNAi vector transfected DRG (Isl-1 over-expression arm); Fig D-F showed the Isl-1 over-expression vector transfected DRG (Isl-1 knockdown arm); Fig G-I showed the control vector-transfected DRG (random shRNA arm), Fig J-L showed the embryo in which the Isl-1 RNAi vector was transfected into neural tube but not in DRG (transfect NT only arm). Black arrows indicated the BrdU+ cells. The broken lines indicated DRG outer boundaries. Bar = 30 μm.

opment, which is caused by an increasing apoptosis and a decreasing proliferation compare to the normal DRG (Rosen et. al. 1996). However, the Isl-1 expression in Froriep's DRG is significant higher than that in normal DRG (Avivi *et al.* 2002). This result also suggests that Isl-1 expression may decrease the DRG cell proliferation. This phenomenon is conformed to our result and indicates that Froriep's DRG abnormal development may be due to the high expression of Isl-1.

In this study, we found that Isl-1 negatively modulates DRG cell proliferation. However, the results of the experiments in vivo and in vitro showed that there was no S-phase cell co-expressed Isl-1 in chicken embryo DRG (Varly *et al.* 1995; Avivi & Goldstein 1999), which leads us to wonder how Isl-1 modulates DRG cell proliferation? The detailed mechanism underlying the DRG cell proliferation modulation is still unclear and further investigation is necessary in this regard.

ACKNOWLEDGEMENTS

This study was supported by grants to Jiali Liu and Sheng Cui from the Natural Science Foundation of China (30671515 and 30630011) and Transgenic organisms safety assessment program (2008ZX08011-006). We thank Prof. Beate Brand-Saberi, Department of

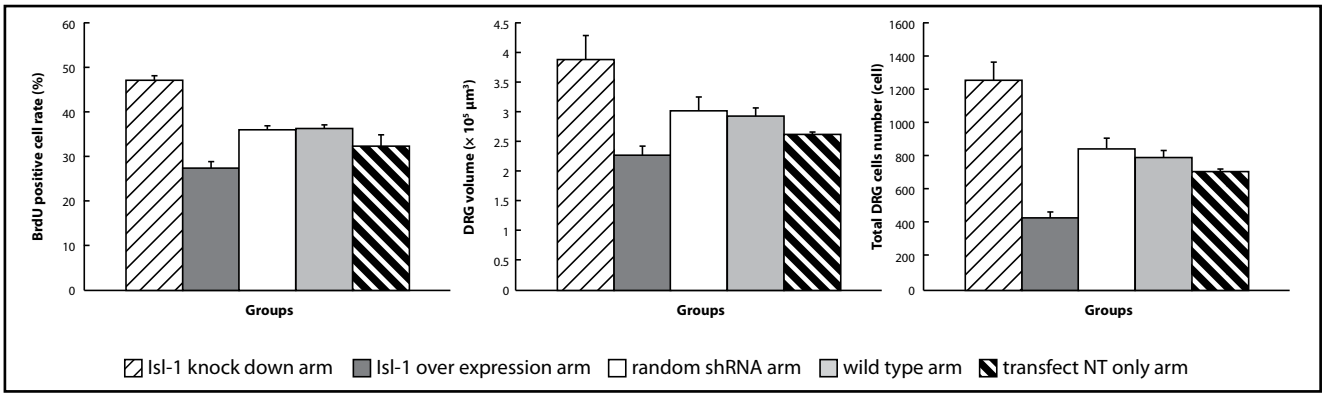

Fig. 4. The rates of DRG cell proliferation, total DRG cells number and DRG volume in the Isl-1 targeted pEGFP-H1-shRNA vector transfected chicken embryo (Isl-1 knock-down arm), the Isl-1 over-expression vector transfected DRG (Isl-1 over-expression arm), the random shRNA content pEGFP-H1-shRNAl vector transfected chicken embryo (random shRNA arm), the none treatment chicken embryo (wild type arm) and the embryos transfected with the Isl-1 targeted pEGFP-H1-shRNA vector into the neural tube without DRG (transfect NT only arm). The Isl-1 knock-down arms were significantly higher than control groups ($p<0.001$). The Isl-1 over-expression arm was significantly lower than the other groups ($p<0.001$). No significant difference was observed in the rate of cell proliferation, total DRG cells number and DRG volume of NT only arm ($p>0.05$).

Molecular Embryology, Institute of Anatomy and Cell Biology, Freiburg University, Germany, for the generous gifts of the peGFP-H1-shRNA vector. The pXJ40-Myc-tagged-isl-1 vector was a generous gift from Prof Xinmin Cao, Signal Transduction Laboratory, Institute of Molecular and Cell Biology, Republic of Singapore. The 40.2D6 Isl-1 and G3G4 BrdU antibody were developed by Thomas Jessel and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA.

REFERENCES

1 Avivi C and Goldstein RS. (1999). Differential expression of Isl-1 in neural crest-derived ganglia: Isl-1+ dorsal root ganglion cells are post-mitotic and Isl-1$^+$ sympathetic ganglion cells are still cycling. Brain Res. Dev. Brain Res. **115**: 89–92.
2 Avivi C, Cui S, Goren S, Goldstein RS. (2002). Differences in neuronal differentiation between the transient cranial (Froriep's) and normal dorsal root ganglia Brain Res. Dev. Brain Res. **135**: 19–28.
3 Brummelkamp TR, Bernards R, Agami R. (2002) A system for stable expression of short interfering RNAs in mammalinan cells. Science. **296**: 550–553.
4 Cui S and Goldstein RS. (2000). Early markers of neuronal differentiation in DRG: Isl-1 expression precedes that of Hu. Brain Res. Dev. Brain Res. **121**: 209–212.
5 Chesnutt C and Niswander L. (2004). Plasmid-based short-hairpin RNA interference in the chicken embryo. Genesis. **39**: 73–78.
6 Dai F, Yusuf F, Farjah GH, Brand-Saberi B. (2005) RNAi-induced targeted silencing of developmental control genes during chicken embryogenesis. Dev. Biol. **285**: 80–90.
7 Ericson J, Thor S, Edlund T, Jessell TM, Yamada T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Isl-1. Science. **256**: 1555–1560.
8 Farinas I, Cano-Jaimez M, Bellmunt E, Soriano M. (2002). Regulation of neurogenesis by neurotrophins in developing spinal sensory ganglia. Brain Res. Bull. **57**: 809–816.
9 Hannon GJ. (2002). RNA interference. Nature. **418**: 244–251.
10 Hamburger V and Hamilton HL. (1951) A series of normal stages in the development of the chick embryo. J Morphol. **88**: 49–92.
11 Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77**: 61–68
12 Hao A, Novotny-Diermayr V, Bian W, Lin B, Lim CP, Jing N, Cao X. (2005). The LIM/homeodomain protein Islet1 recruits Janus tyrosine kinases and signal transducer and activator of transcription 3 and stimulates their activities. Mol. Biol. Cell. **16**: 1569–1583.
13 Kos R, Reedy MV, Johnson RL, Erickson CA. (2001). The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. Development. **128**: 1467–1478
14 Lallier TE and Bronner-Fraser M. (1988). A spatial and temporal analysis of dorsal root and sympathetic ganglion formation in the avian embryo. Dev. Biol. **127**: 99–112.
15 Lim TM, Lunn ER, Keynes RJ, Stern CD. (1987). The differing effects of occipital and trunk somites on neural development in the chick embryo. Development. **100**: 525– 533.
16 Liu J, Chen D, Goldstein RS, Cui S. (2005). Effects of male and female sex steroids on the development of normal and the transient Froriep's dorsal root ganglia of the chick embryo. Brain Res. Dev. Brain Res. **155**: 14–25.
17 Pfaff SL, Mendelsohn M, Stewart CL, Edlund T, Jessell TM. (1996). Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell. **84**: 309–320.
18 Rosen O, Geffen R, Avivi C, Goldstein RS. (1996). Growth, proliferation, and cell death in the ontogeny of transient DRG (Froriep's ganglia) of chick embryos. J. Neurobiol. **30**(2): 219–30.
19 Stern CD. (2005). The chick; a great model system becomes even greater. Dev. Cell. **8**: 9–17.
20 Sanders EJ and Cheung E. (1988). Effects of HNK-1 monoclonal antibody on the substratum attachment and survival of neural crest and sclerotome cells in culture. J. Cell. Sci. **90**: 115–122
21 Teillet MA, Kalcheim C, Le Douarin NM. (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. Dev. Biol. **120**: 329–47.
22 Varley JE, Wehby RG, Rueger DC, Maxwell GD. (1995). Number of adrenergic and Isl-1 immunoreactive cells is increased in avian trunk neural crest cultures in the presence of human recombinant osteogenic protein-1. Dev. Dyn. **203**: 434–447.