약학석사 학위논문

EsrrG: 운동신경억제와 V2b IN 분화에의 영향

EsrrG Plays a Role in Suppressing Motor Neurons and Inducing V2b Interneurons

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이지연

Abstract

EsrrG Plays a Role in Suppressing Motor Neurons and Inducing V2b interneurons

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One of the major challenges in neuroscience is understanding how diverse neuronal subtypes are generated from a relatively uniform neural precursor. Elucidation of molecular mechanism regarding neuronal development will be of much value to our society as it would provide crucial basis for developing new drugs or cell therapy for treating neurodegenerative diseases. The goal of my research project was to gain further insight into genes that are involved in neuronal development based on the ChIP-seq and RNA-seq data on Motor neuron hexamer by Professor Seunghee Lee (MN-hexamer). Among the 500 genes identified as potential targets of motor neuron hexamer (MN-hexamer), 52 also possessed DNA binding response elements of Estrogen Related Receptor (ERRE). The research began with hypothesis that Estrogen Related Receptor could, independently or in cooperation with the MN-hexamer, direct motor neuron development. To see if the putative genes were expressed at motor neuron domain in the

spinal cord, *in situ* hybridization (ISH) was performed with the mouse and chick spinal cord tissue transverse sections using the probes that I cloned. After screening for mRNA localization patterns of numerous genes, a few of them were chosen for further study. Interestingly, Estrogen Related Receptor Gamma (EsrrG) displayed migratory expression pattern depending on developmental stages. Luciferase reporter assay and GFP reporter assay were performed with the constructs that I cloned. Reporter assays confirmed that the target gene promoters were indeed activated by EsrrG. This was further validated by Chromatin Immunoprecipitation (ChIP) which showed that EsrrG physically bound to ERRE located upstream of Notch4. In order to gain insight into the function of EsrrG during spinal cord development, I performed the function analysis: For the gain of function analysis, chick embryo spinal cords were electroporated with the expression plasmids, either full length construct or the construct bearing transcription activating domain VP16. For the loss of function analysis, both chick and mice were analyzed. Chick embryos had the spinal cords electroporated with shRNA knockdown constructs or constructs bearing engrailed repressor domain, which acts as a dominant-negative form. Mutant mice embryos were harvested at various developmental stages by breeding EsrrG-floxed mice with Nestin-Cre mice, and obtaining knockout embryos which have EsrrG deleted at early neural progenitor stage. Surprisingly, I was able to conclude that EsrrG favored V2b inhibitory interneuron and played a certain role in suppressing motor neuron formation. I obtained consistent results during embryonic stem cell differentiation to motor neuron using the doxycycline inducible EsrrG stem cell line (iEsrrG) that I established. During differentiation of iEsrrG-ESC to motor neurons by treatment with

retinoic acid and Sonic hedgehog agonist, doxycycline treatment led to very significant decreases in motor neuron markers Hb9 and Isl1 both in RT-PCR and IHC.

My research has identified, for the first time, a novel role EsrrG plays in spinal cord neural development by establishing V2b interneuron and motor neuron cell fate decision. Further molecular mechanistic study and mutant mice study are in progress and closer examination of EsrrG at various embryonic stages and elucidation of its downstream signaling network will be the subjects for immediate future studies.

Key words: EsrrG, V2b IN, GATA3, neurogenesis, motor neuron, Notch4

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List of Abbreviations

ChIP	Chromatin Immunoprecipitation
Dox	Doxycycline
dpi	Days post injection
ERRE	Estrogen Related Receptor Response Elements
EsrrG	Estrogen Related Receptor Gamma
HH	Hamburger-Hamilton stage
IHC	Immunohistochemistry
ISH	*In situ* hybridization
Purm	Purmorphamine
RA	Retinoic Acid
RT-PCR	Reverse transcription polymerase chain reaction
SC	Spinal cord
Shh	Sonic Hedgehog

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I. Introduction

Despite high societal pressure to elucidate neuronal network circuitry due to increasing number of neurodegenerative diseases and spinal cord injury cases, relatively little is understood regarding molecular mechanism involving neurogenesis. Spinal cord develops from ectoderm guided by external factors along the dorsoventral and anterior posterior axes, and consists of sensory neurons, interneurons, and motor neurons (Dasen et al., 2009). In ventral part of the spinal cord, Sonic hedgehog (Shh) morphogen is secreted from the notochord, creating a concentration gradient from ventral Shh high, to dorsal Shh low (Ulloa et al., 2007). In addition to Shh, retinoic acid (RA) and fibroblast growth factors (FGFs) are secreted from mesoderm to help guide neuronal patterning of the spinal cord (Jessell et al., 2000). These external factors in turn give rise to combination of diverse sets of transcription factors, which serve to further define neuronal subsets in different domains of the spinal cord across development (Ulloa et al., 2007). The ventral most part of the spinal cord, which is exposed to highest concentration of Shh, becomes V3 interneurons, and the region dorsal to V3 IN differentiate into MN, V2, V1, and V0 interneurons (Dessaud et al., 2008). In the ventral side of the spinal cord critical for MN differentiation, Shh induces class II transcription factor Olig2 (Novitch et al., 2003). Similarly, the region dorsal to where Olig2 is expressed is Irx3, which in turn exhibits cross inhibitory action with Olig2 to establish V2 and MN neuronal boundary (Novitch et al., 2001). In postmitotic stages, Hb9 and Isl1/2 mark motor neuron identity (Lee et al., 2012).

In this study, we originally intended to elucidate molecular mechanism downstream of MN-hexamer, which consists of Isl1, Lhx3, and NLI, and is known to play a key role in specifying motor neuron fate (Lee et al., 2012). MN hexamer complex turns on Hb9, a well-known homeobox gene and a motor neuron marker, and suppress V2a excitatory interneurons, marked by Chx10 (Lee et al., 2012). From the ChIP-seq data of inducible Isl1-Lhx3 cell line, about 500 putative target genes of MN-hexamer were identified. Interestingly, among these targets, 52 also had Estrogen related receptor binding sites, or ERRE. From this observation, we hypothesized that Estrogen related receptor could, independently or downstream of MN-hexamer complex, play a role in MN fate specification. Estrogen related receptor family, otherwise referred to as NR3B, are orphan nuclear receptor superfamily and consists of EsrrA, EsrrB, and EsrrG (Tremblay et al., 2007). They are known to be constitutively active transcription factors and their ligands remain unknown, hence the word orphan nuclear receptor (Tremblay et al., 2007). All three ERR isoforms share sequence similarity in its DNA binding domain (DBD) (Tremblay et al., 2007). EsrrB is well known to play crucial role in ESC self-renewal by acting as a direct downstream target of Nanog and Gsk3/Tcf3 axis (Festuccia et al., 2012; Martello et al., 2012). EsrrG is important in normal kidney renal papilla development and oxidative metabolism transition in heart (Berry et al., 2010; Alaynick et al., 2007).

II. Material and Methods

1. DNA Constructs

Mouse EsrrG was cloned to pCS2 vector containing a HA-epitope tag. Constitutively active EsrrG was cloned by cloning DNA Binding Domain (DBD) of EsrrG into myc-tagged pCS2 vector containing a VP16 transcriptional activation domain. EsrrG-DBD was also cloned into myc-tagged pCS2 containing an Engrailed repressor domain, which acts as a dominant negative form.

In situ hybridization probes for mouse EsrrG and chick EsrrG were generated by cloning 400-500bp fragments into pBluescript vector. The following oligonucleotides were used for the cloning:

msEsrrG forward : 5'- CGGAATTCGCAAGGCATTCTTCAAGAGG-3' and reverse: 5'- CCGCTCGAGGAGGAGGCTCATCTGGTCTG-3'

ckEsrrG forward 5'-CGGAATTCGACTCACCTCCCCTCTACCC-3' and reverse 5'-CCGCTCGAGCTGCATCTATTCTGCGCTTG-3',

msNotch4 forward: 5'-CGGAATTCCAGGGGCTCTCTGTGACTTC-3' and reverse 5'-CCGCTCGAGGGCTAAAGAGTGGCAAGCTG-3'.

2. Generation and Differentiation of iEsrrG-ESCs

A gene encoding 3xFlag-tagged EsrrG construct was inserted into Tet-inducible p2Lox plasmid and transfected with pSALK-Cre into ESC lines. The primers used for

the cloning are: forward 5'-CCGCAATTGATGGATTCGGTAGAA-3' and reverse 5'-ATAAGAATGCGGCCGCTCAGACCTTGGCCTC-3'.

The A172LoxP ESC line was maintained in an undifferentiated state on 0.1% gelatin-coated dishes in the ESC growth media that consisted of Knockout DMEM, 10% (vol/vol) FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and recombinant leukemia inhibitory factor (LIF, 1000 units/mL, Chemicon). A gene encoding FLAG-tagged Isl1-Lhx3 fusion construct was inserted into Tetinducible plasmid p2Lox and were cotransfected with pSALKCre into ESC lines by electroporation. Stable transfectants were isolated by selection with neomycin (G418, 400-600 μg/mL) for 5-7 d. Doxycycline (Dox)-dependent induction of Flag-EsrrG was confirmed by RT-PCR, Western blotting, and immunohistochemical analyses using mouse a-FLAG antibody (sigma).

iEsrrG ESC was differentiated into neurons and motor neurons (MNs) in 6 d period by treatment with 0.5uM RA alone or with 0.5uM RA and 1uM Purmorphamine respectively, starting at 2nd day of cell differentiation until the 5th day, the day before harvest. 20ug of Doxycycline was treated 4th day and 5th day of differentiation to induce EsrrG.

3. In ovo Electroporation and Immunohistochemistry

Neural tubes of Chick embryos were electroporated in Hamburger-Hamilton stage 13, harvested 2~4 d post-electroporation and fixed in 4% paraformaldehyde for two

hours on a rotator in cold room, then left in 1X PBS overnight. They were then cryoprotected in 30% sucrose for two hours on a rotator in cold room and mounted and embedded in Optimal Cutting Temperature (OCT), which was cryosectioned on Leica cryostat at -20℃ for immunohistochemistry or in situ hybridization. For immunohistochemical analyses, tissues were cut at 12um and for in situ hybridization, tissues were cut at 18um. Representative sets of images from reproducible results were presented. Mouse embryos were collected at indicated developmental stages and processed similarly to chicken embryos as described previously. For immunohistochemistry assays, the following antibodies were used; mouse anti-HA (Covance), mouse anti-flag (sigma), mouse anti-Mnr2/Hb9 (5C10, DSHB), rabbit anti-Isl1/2 (19), rabbit anti-GATA3, mouse anti-EsrrG (Perseus Proteomics), rabbit anti-GFP (A6455, Molecular Probes).

4. ChIP Assays

3xflag-iEsrrG ESC line described above was used for ChIP assay. For ChIP assay performed in self-renewal ESC state, iEsrrG-ESCs were cultured on 0.1% gelatin-coated dishes in the ESC grwoth media with LIF in the presence or absence of Dox (2ug/ml), which induces the expression of FLAG-tagged EsrrG, for 1 d. The cells were washed with PBS buffer, fixed by 1% formaldehyde for 10 min at room temperature and quenched by 125mM glycine. Cells were washed with Buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA,10 mM Hepes, pH 6.5) and

Buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) sequentially. Then, cells were lysed with lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris·HCl, pH 8.0, protease inhibitor mixture) and were subjected to sonication for DNA shearing. Next, cell lysates were diluted 1:10 in ChIP buffer (0.5% Triton X-100, 2 mM EDTA, 100 mM NaCl, 50 mM Tris·HCl, pH 8.0, protease inhibitor mixture) and, for immunoclearing, were incubated with IgG and protein A agarose beads for 2 h at 4 ℃. Supernatant was collected after quick spin and incubated with α-FLAG antibody (Sigma) and protein A agarose beads to precipitate FLAG-EsrrG/chromatin complex overnight at 4 ℃. After pull-down of FLAG-EsrrG/chromatin/antibody complex with protein A agarose beads, the beads were washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl, pH 8.0, 150 mM NaCl), TSE II (same components as in TSE I except 500 mM NaCl), and Buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris·HCl, pH 8.0) sequentially for 7 min at each step. Then the beads were washed with TrisEDTA (TE) buffer two times. FLAG-Isl1-Lhx3/chromatin complexes were eluted in elution buffer (1% SDS, 1 mM EDTA, 0.1 M NaHCO3, 50 mM Tris·HCl, pH 8.0) and decross-linked by incubating at 65 ℃ overnight. Eluate was incubated at 50 ℃ for more than 2 h with Proteinase K. Next, DNA was purified with Phenol/chloroform and DNA pellet was precipitated by ethanol and resolved in water overnight at room temperature. PCR was done with enhNotch4 and msCDH1-ERRE using the primers mCDH1 forward: 5’-GGGGACACTCTTTCGACCTT-3’

and reverse: 5'-GTGGAGGGGTTTGGGTTTTC-3', which include ERRE: TGACCTTGG. Enh-Notch4 forward: 5'-GAAGATTCTATGAAGCTCAGGGTTTGATTTCCA-3' and reverse: 5'-CGGGATCCTCAGCCTCGAGCAGTCCTGGAAGC-3'.

5. Mouse Genetics

Mouse strains used in this study are $Nestin^{Cre}$, $EsrrB^{flox/flox}$ and $EsrrG^{flox/flox}$ mice were kind gifts from Dr. Kralli, at the Scrips Research Institute (TSRI), and were originally created by Professor Chambon's lab at IGBMC. Seoul National University Institutional Animal Care and Use Committes approved all the maintenance and experimental procedures. The primers used for genotyping are: EsrrG flox primer forward: 5'-GTTTTAAAGGCCCTTGGTGATCTCGC-3' and reverse: 5'-CTGCAACCCTTGGACTGCCAGAAC-3'. EsrrG KO primer forward: 5'-CCCTTATGCTGATTACCTTCTTGTA-3' and reverse: 5'-CTGCAACCCTTGGACTGCCAGAAC-3'.

6. RT-PCR

Total RNA was extracted using Trizol (Invitrogen) and reverse transcription (RT) was performed using SuperScript III (Invitrogen). The following primers were used for RT-PCR:

Cyclophilin A, forward 5′-GTCTCCTTCGAGCTGTTTGC-3’ and reverse 5′-GATGCCAGGACCTGTATGCT;

msEsrrG, forward 5’-TGAAGTGTCTCAAAGTGGGC-3’ and reverse 5’-GTTCAGCCACCAACAAATGC-3’;

msHb9 forward 5’-CGGCGCTTTCCTACTCATAC-3’ and reverse 5’-ACTTCCCCAAGAGGTTCGAC-3’;

msSox1 forward 5’-AGACTTCGAGCCGACAAGAG-3’ and reverse 5’-AACTGTGCAAACAGGTGCAG-3’;

msNotch4 forward: 5’-CAGCTTGCCACTCTTTAGCC-3’ and reverse 5’-CAGCTTGCCACTCTTTAGCC-3’; and

Olig2 forward 5’-GGATGCTTATTACAGACCGA-3’ and reverse 5’-AAAAGATCATCGGGTTCTGG-3’.

7. Statistical Analysis of the Data

Data are presented as mean ± SD or mean ± SEM as indicated. Statistical comparisons were conducted by two-tailed Student t test.

III. Results

1. EsrrG is expressed in ventral area of the spinal cord during development and exhibits dorsal migratory pattern

Previous studies have shown that MN-hexamer consisting of Isl1, Lhx3, and NLI specifies motor neuron fate while suppressing V2a interneuron fate (Lee et al 2012). To see if any of the Estrogen related receptor family is indeed a target of MN-hexamer, mRNA expression patterns in transverse spinal cord sections of mice were analyzed. As hypothesized, if any of the Estrogen related receptor family indeed plays a role in motor neuron specification, its expression pattern would most likely be localized at ventral motor neuron area of the spinal cord. However, EsrrA and EsrrB were expressed at low levels in the spinal cord (data not shown). Interestingly, *in situ* hybridization of EsrrG on mice tissues indicated that EsrrG is expressed at a rather unique pattern, as EsrrG dorsally migrates out of ventral area across developmental stages (Figure 1A). Note that EsrrG mRNA expression peaks at e12.5. In chick spinal cord, EsrrG also exhibits unique expression pattern and was not found to be ectopically turned on upon Isl1+Lhx3 injection, suggesting that it may not act as a downstream target of MN-hexamer (Figure 1B). Next, we focused on EsrrG among Estrogen related receptor family and sought to further analyze the functional identity of EsrrG using immunohistochemistry of EsrrG with diverse neuronal markers.

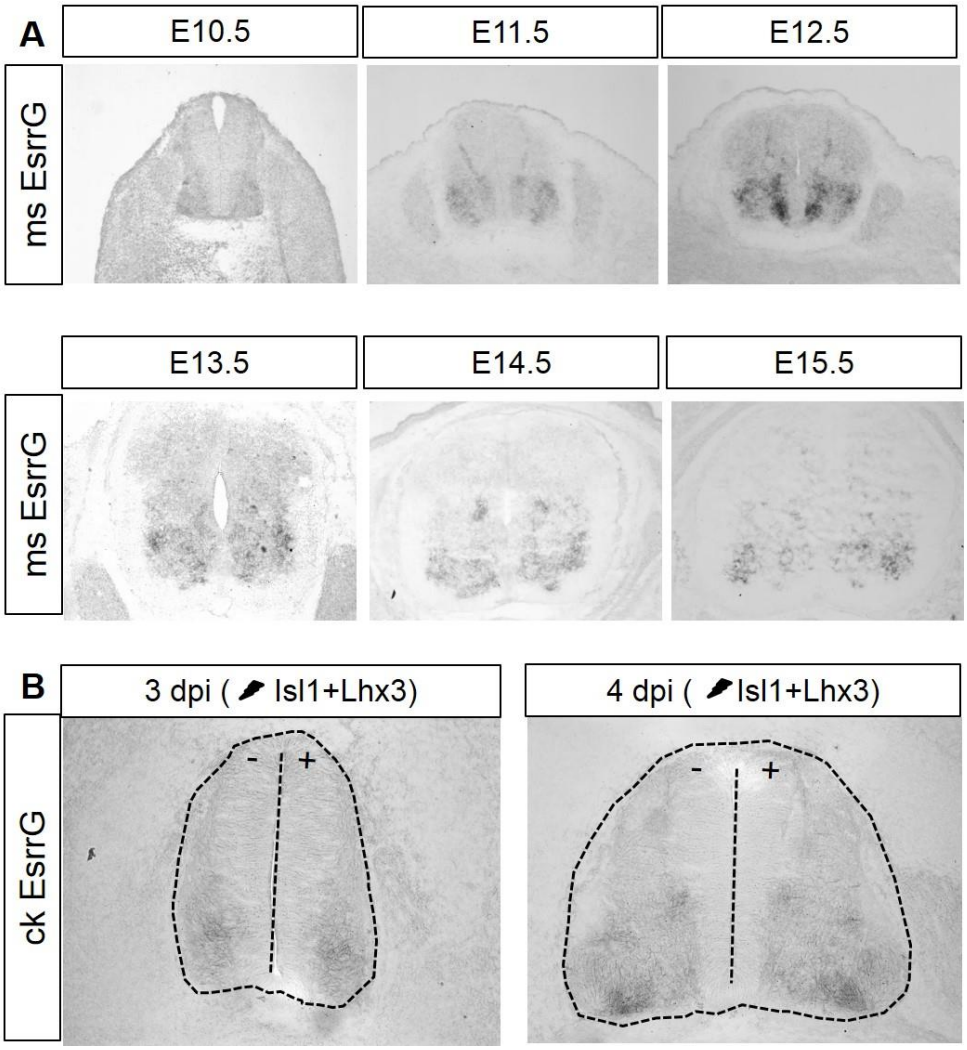


Figure 1. EsrrG exhibits migratory expression pattern during SC development.

(A) ISH of mouse embryonic spinal cord from e10.5 to e15.5 using EsrrG mouse antisense probe.

(B) ISH of chick EsrrG in chick spinal electroporated with ISl1

2. EsrrG co-localizes mostly with GATA3, a V2b inhibitory interneuron marker

EsrrG was co-stained with various neuronal markers during mice embryonic development, including motor neuron markers Hb9, Isl1, and Lhx3, V2a excitatory interneuron marker Chx10, V2b inhibitory interneuron marker GATA3, and V2c marker Sim1. In contrast to the initial hypothesis which expected that EsrrG would co-localize mostly with MN marker Hb9, EsrrG was found to have most significant overlap with V2b interneuron marker GATA3 across developmental stages e10.5 to e14.5 (Figure 2A). At mouse embryonic stages e12.5 and e13.5, 53.6% and 50.4% of GATA3 population were found to co-localize with EsrrG, respectively (Figure 2B). Surprisingly, at e12.5, EsrrG showed mutually exclusive pattern with MN marker Hb9, with Hb9 population appearing to be surrounded by EsrrG (Figure 2C). GATA3 has been well studied in nervous system development, as loss of GATA3 has been found to impair facial branchiomotor neuron migration in the hindbrain and survival of sympathetic neurons (Pata et al., 1999; Tsarovina et al., 2010). From the series of co-localization studies, EsrrG emerged to be potentially important in V2b interneuron specification. To further confirm the functional identity of EsrrG in determining neuronal fate, gain of function and loss of function phenotype studies were performed in mice and chick tissues.

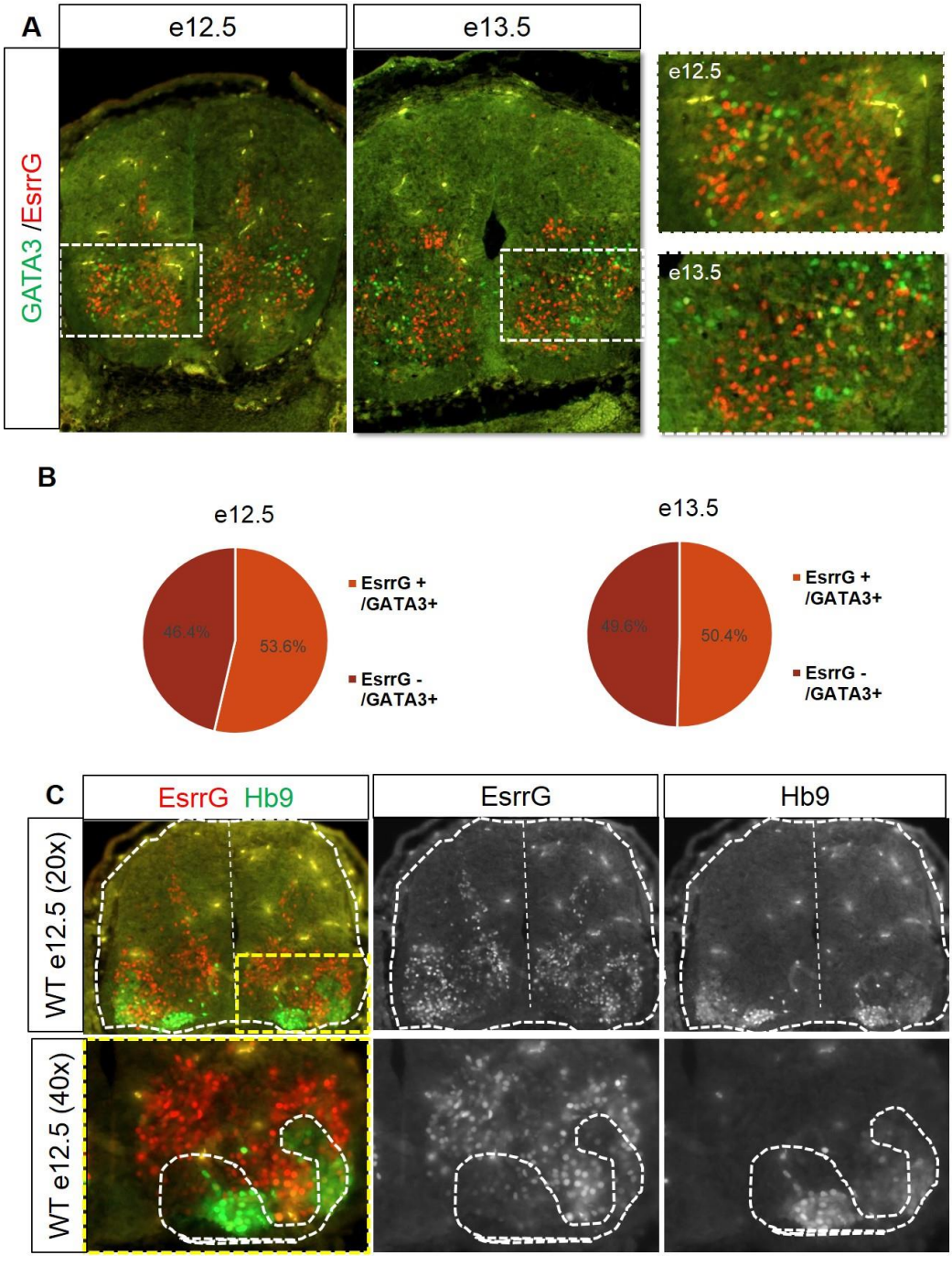

A
e12.5
e13.5
GATA3 /EsrrG
e12.5
e13.5
B
e12.5
46.4%
53.6%
EsrrG +
/GATA3+
EsrrG -
/GATA3+
e13.5
49.6%
50.4%
EsrrG +
/GATA3+
EsrrG -
/GATA3+
C
EsrrG Hb9
EsrrG
Hb9
WT e12.5 (20x)
WT e12.5 (40x)

Figure 2. EsrrG co-localizes mostly with V2b inhibitory neuronal marker GATA3.

(A) Co-staining of V2b inhibitory neuronal marker GATA3 (green) with EsrrG (red) shows colocalized population at mouse embryonic days e12.5 and e13.5.

(B) Quantification analysis of figure 2A showing percentages of GATA3 that also expresses EsrrG at e12.5 and e13.5 (orange).

(C) Co-staining of MN marker Hb9 (green) with EsrrG (red) shows virtually no co-localized cells and non-overlapping patterns at mouse embryonic day e12.5.

3. Gain of function and loss of function studies of EsrrG in spinal cords of chick embryos

Chick embryos are ideal model organisms in developmental science due to their availability and easiness in surgical manipulations (Nakamura et al., 2004). In this study, chick *in ovo* electroporation technique was used, where the plasmid of interest was injected into the spinal cord of developing chick embryos at Hamburger Hamilton stages 12-13. Then the transient, square pulse was applied so that negatively charged DNA is moved to the side of spinal cord adjacent to a positively charged electrode (Nakamura et al., 2004). The embryos were harvested 3 days post injection (Figure 3A, 3B). This allows for gene of interest to be expressed in one side of the spinal cord, and the gene's effect on neuronal distribution or morphology can be studied in comparison to the other half side, or the unelectroporated control side, all in one embryo.

Consistent with previous observations that EsrrG appears be important in V2b interneuron specification, gain of function study in chick embryos electroporated with HA tagged full length EsrrG led to ectopic expression of GATA3 (Figure 3A). In addition, a slight decrease in MN marker Isl1 was observable in chick spinal cord side electroporated with EsrrG (Figure 3A). At cervical level, EsrrG-HA injection led to statistically significant increase of Gata3 at P=0.0382 (Figure 3B). Similarly, at thoracic level, EsrrG-HA injection led to statistically significant decrease of Hb9 at P=0.045. Next, loss of function study was conducted using plasmid encoding EsrrG DNA binding domain (DBD)

fused to Engrailed Repressor (EnR) domain, which acts as a dominant negative form to inhibit endogenous EsrrG from binding to its downstream targets (Markel et al., 2002). When myc-tagged EsrrG-DBD_EnR was injected into chick spinal cord, notable decrease in GATA3, as predicted, was observed and marked expansion of MN marker Isl1 was observed (Figure 3D). The following observations were confirmed in chick embryos harvested 4 days post injection (data not shown). Similarly, electroporation of myc-tagged plasmid encoding EsrrG DNA binding domain fused to constitutively active VP16 domain led to increased GATA3 and decreased Isl1 (Figure 3E. The gain of function and loss of function analysis in chick spinal cord consistently indicated that EsrrG plays a role in activating V2b interneuron marker GATA3 while repressing MN marker Isl1. Next, we sought to determine the effect of neural specific deletion of EsrrG in mice embryos using Nestin-cre transgenic mice, as homozygous deletion of EsrrG was found to result in deaths postnatal day 1 due to severe cardiac malfunction (Alaynick et al., 2007).

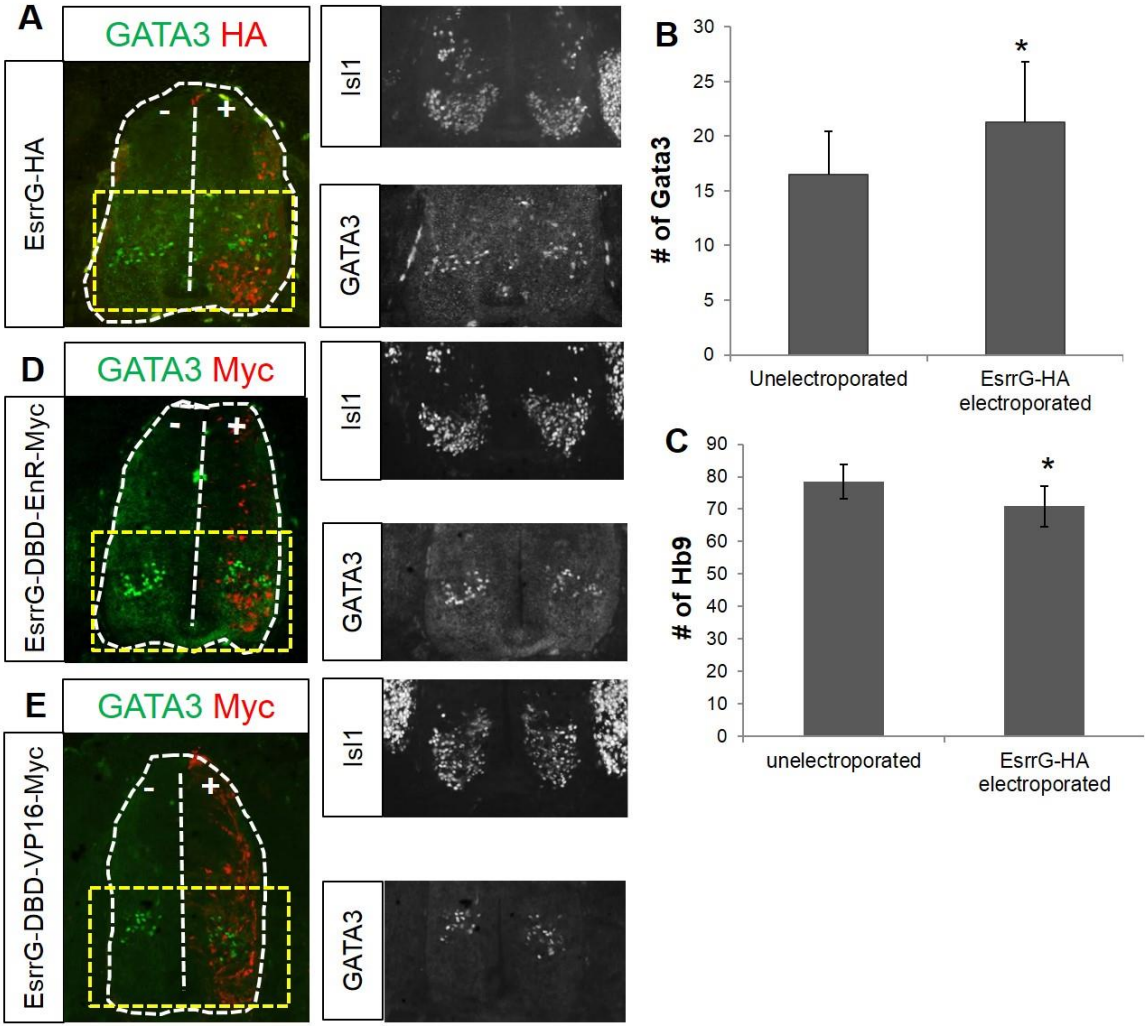

A
GATA3 HA
EsrrG-HA
Isl1
GATA3
D
GATA3 Myc
EsrrG-DBD-EnR-Myc
Isl1
GATA3
E
GATA3 Myc
EsrrG-DBD-VP16-Myc
Isl1
GATA3
B
# of Gata3
30
25
20
15
10
5
0
*
Unelectroporated
EsrrG-HA electroporated
C
# of Hb9
90
80
70
60
50
40
30
20
10
0
*
unelectroporated
EsrrG-HA electroporated

Figure 3. EsrrG plays a role in specifying V2b interneuron and repressing motor neuron (MN) in chick embryonic spinal cord.

(A) Chick spinal cord electroporated with HA tagged full length EsrrG leads to increased GATA3 formation and slightly decreased MN marker Isl1.

(B) At cervical level, EsrrG-HA injection at 3dpi leads to statistically significant increase of Gata3 ($P=0.0382$,$^*P<0.05$).

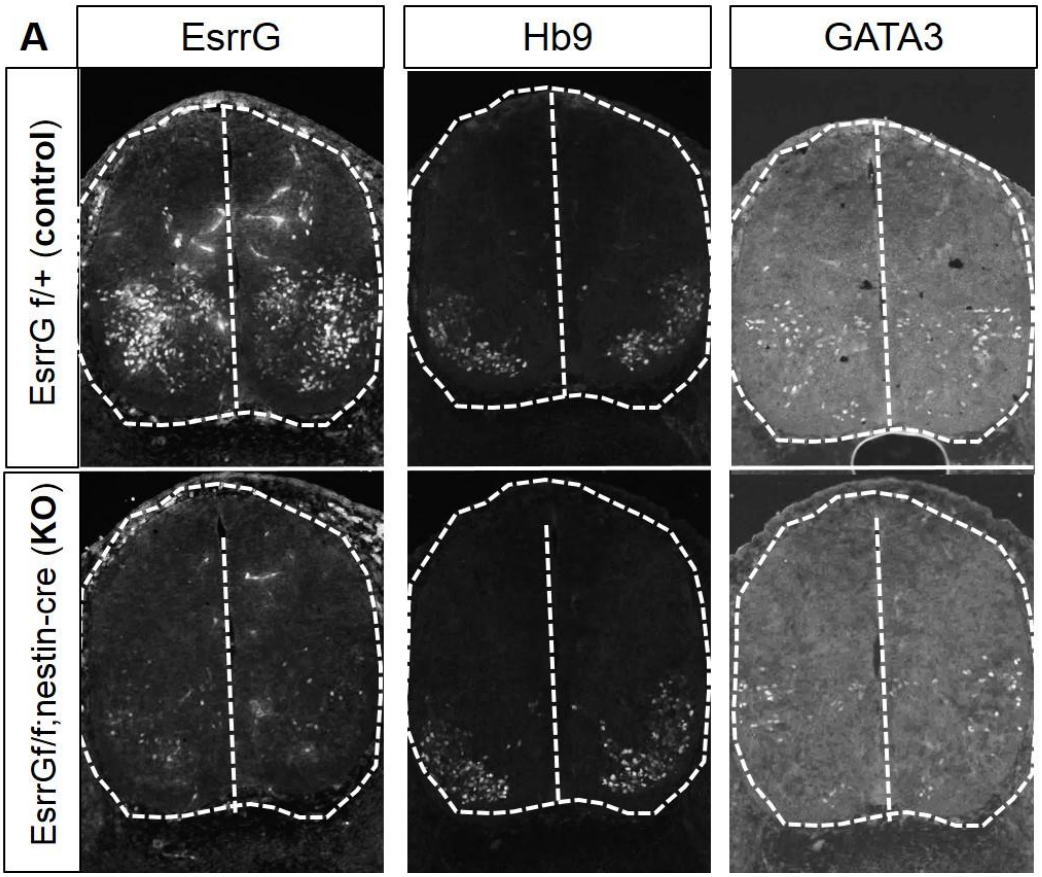
(C) At thoracic level, EsrrG-HA injection at 3dpi leads to statistically significant decrease of Hb9 ($P=0.045$, $^*P<0.05$).

(D) Chick spinal cord electroporated with dominant negative form of EsrrG (EsrrG-DBD-EnR-Myc) leads to reduced GATA3 formation and increased Isl1 formation.

(E) Chick spinal cord electroporated with activated form of EsrrG (EsrrG-DBD-VP16-Myc) leads to slightly increased GATA3 formation.

4. Neural specific deletion of EsrrG leads to similar results

Nestin-cre mice enables for highly efficient recombination of floxed alleles in the nervous system starting at mouse embryonic day 10.5 (Dubois et al., 2006). Timed mating of male EsrrG fl/+;Nestin-Cre mice with female EsrrG fl/fl enabled for embryos with wild type (EsrrG fl/+), heterozygous (EsrrGfl/+; Nestin-Cre), and knockout (EsrrG fl/fl;Nestin-Cre) genotypes. Pregnant female mice were harvested at desired developmental stages. Resulting wild type and mutant embryos at developmental stages e10.5, e11.5, e12.5, and e15.5 were analyzed. Marked and efficient deletion of EsrrG was confirmed in all stages through immunohistochemistry (Figure 4A). Similar to chick functional studies, neural specific EsrrG deletion using Nestin-cre mice led to significant decrease in GATA3 (22.8%) at e12.5 thoracic levels (Figure 4A, 4B). Consistently, there was a slight expansion in MN marker Hb9 when EsrrG was deleted (Figure 4A). Other neural markers such as Isl1, Lhx3, Olig2, Sim1, Tuj1, and proliferation marker BrdU were analyzed. The results in mice conditional knockout study further validated previous results and pointed that EsrrG is an important factor in promoting V2b interneuron specification. Next, EsrrG's effect on differentiation of embryonic stem cell (ESC) to motor neuron (MN) was assessed using inducible EsrrG cell line construct, to further validate the results in cell line *in vitro* context.


A
EsrrG
Hb9
GATA3
EsrrG f/+ (control)
EsrrGf/f;nestin-cre (KO)

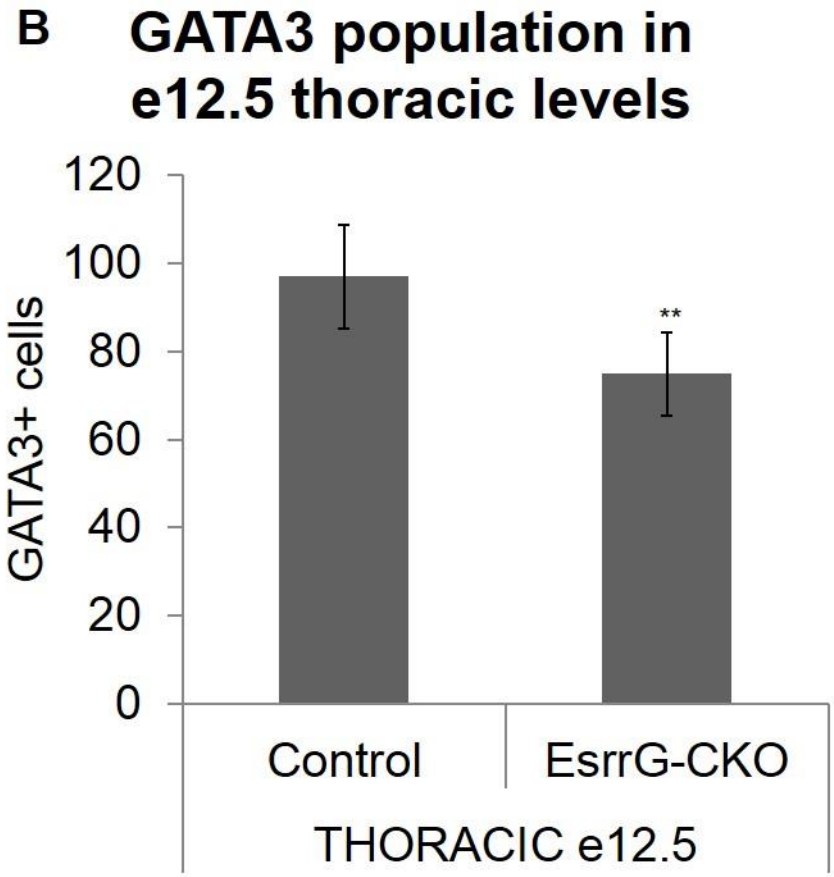


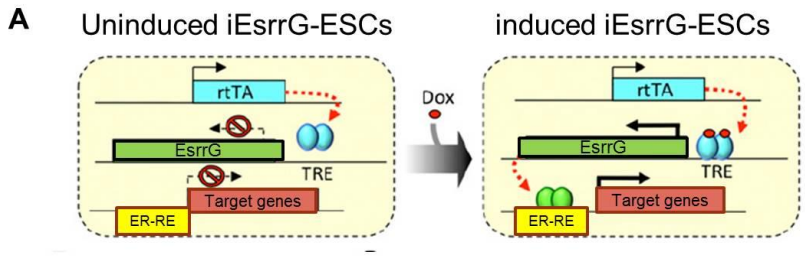
Figure 4. Loss of EsrrG in neural progenitor stage results in reduced GATA3 formation and slightly increased MN formation.

(A) Neural specific deletion of EsrrG at e12.5 leads to slightly increased Hb9 and decreased GATA3.

(B) Quantitative analysis of GATA3 cell populations indicates statistically significant decrease of GATA3 upon loss of EsrrG ($p=0.0011$).

5. Induction of EsrrG during MN differentiation leads to MN suppression

Doxycycline inducible EsrrG ESC line (iEsrrG) was treated with retinoic acid (RA) and Sonic hedgehog agonist purmorphamine (Purm), to differentiate ESC to motor neuron (Figure 5A, Figure 5B). As expected, EsrrG induction significantly impaired differentiation of ESC to MN, as can be seen by visibly reduced formation of Hb9 (Figure 5C). MN marker Isl1 formation was also reduced upon dox treatment (data not shown). Quantitative analysis was performed by counting the number of Hb9 positive cells and dividing it by the number of nuclei (DAPI) in each cell aggregate, to assess MN differentiation efficiency. This showed very significant 49% decrease in Hb9 formation when EsrrG was induced during MN differentiation (Figure 5D). Similarly, reduction of other MN marker Isl1 upon EsrrG induction was also observed (data not shown). Thus, EsrrG was found to play a role in suppressing motor neuron formation in ESC context, further validating previous results. We next sought to examine the molecular mechanism of how EsrrG exerts its effect on specifying V2b interneuron and repressing motor neuron through reporter assays and Chromatin Immunoprecipitation (ChIP) assays.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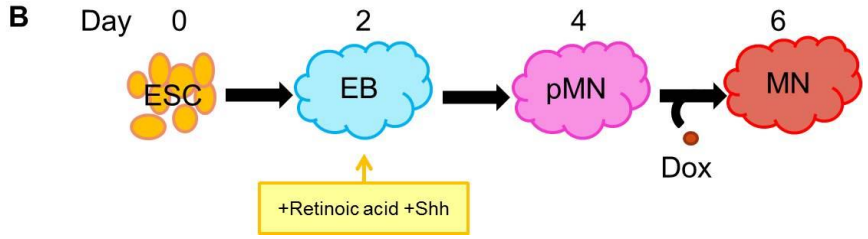
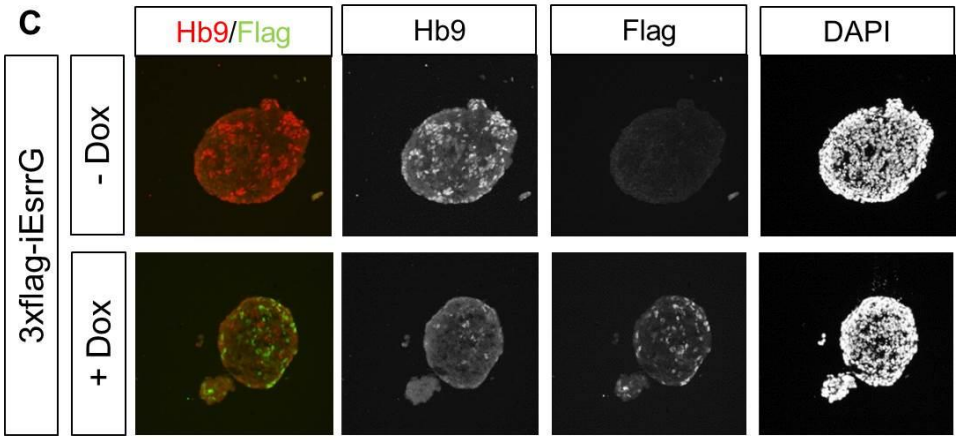
rtTA
EsrrG
TRE
Target genes
ER-RE
Dox


B
Day 0
2
4
6
ESC
EB
pMN
MN
Dox
+Retinoic acid +Shh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Hb9/Flag
Hb9
Flag
DAPI
3xflag-iEsrrG
- Dox
+ Dox

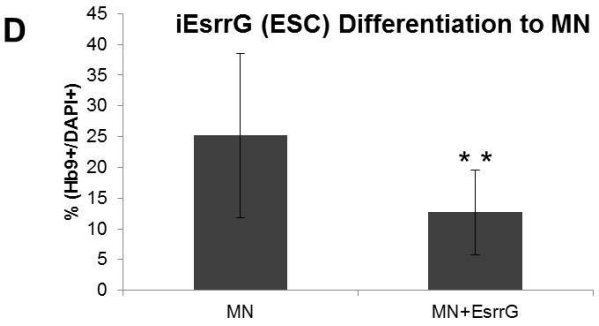
D
iEsrrG (ESC) Differentiation to MN
% (Hb9+/DAPI+)
45
40
35
30
25
20
15
10
5
0
* *
MN
MN+EsrrG

Figure 5. EsrrG induction impairs proper motor neuron formation during ESC differentiation to MN

(A) Doxycycline inducible EsrrG ESC (iEsrrG) line construct.

(B) Scheme of differentiation of ESC to MN using RA and Shh agonist Purmorphamine.

(C) Upon dox treatment, EsrrG is detected using flag antibody and marked reduction in MN marker Hb9 (bottom) is observable compared to Dox-negative sample (top).

(D) Quantitative analysis shows that EsrrG induction leads to very significant decrease in Hb9 ($P=0.00134$, $^{**}P<0.005$).

6. Notch4 as a potential target of EsrrG

From the previous ChIP-seq result of MN-hexamer which identified genes containing MN-hexamer binding site (HxRE) and Estrogen Related Receptor binding site (ERRE), Notch4, Mib2, and EsrrB were chosen (Lee et al 2012). Notch signaling has been studied extensively in many model organisms such as mice, drosophila, and zebrafish; and is known to play key roles in cell fate diversification, cell fate decision, and neurogenesis (Cau et al., 2009). In zebrafish, existence of Notch signaling has been shown to suppress pMN formation and induce KA interneuron formation, but relatively little is known about Notch4 signaling (Shin et al., 2007). RT-PCR was conducted using inducible 3xflag-EsrrG ESC line that was differentiated into neurons, by treatment of RA only, or motor neurons, by treatment of RA and Purm, as described previously. Interestingly, in neuronal context, dox treatment resulted in increased Notch4 gene expression (Figure 6A). Also, consistent with previous immunohistochemistry results in Figure 5, RT-PCR also showed decreased MN markers Olig2 and Hb9 when EsrrG is induced during MN differentiation (Figure 6A). Induction of GATA3 during neurogenesis by RT-PCR was not observed, however. Additionally, V2c interneuon marker Sox1 was also upregulated in neurogenesis upon EsrrG induction (Figure 6A). We next strived to better understand molecular mechanistic interaction through Chromatin Immunoprecipitation and GFP reporter assay.

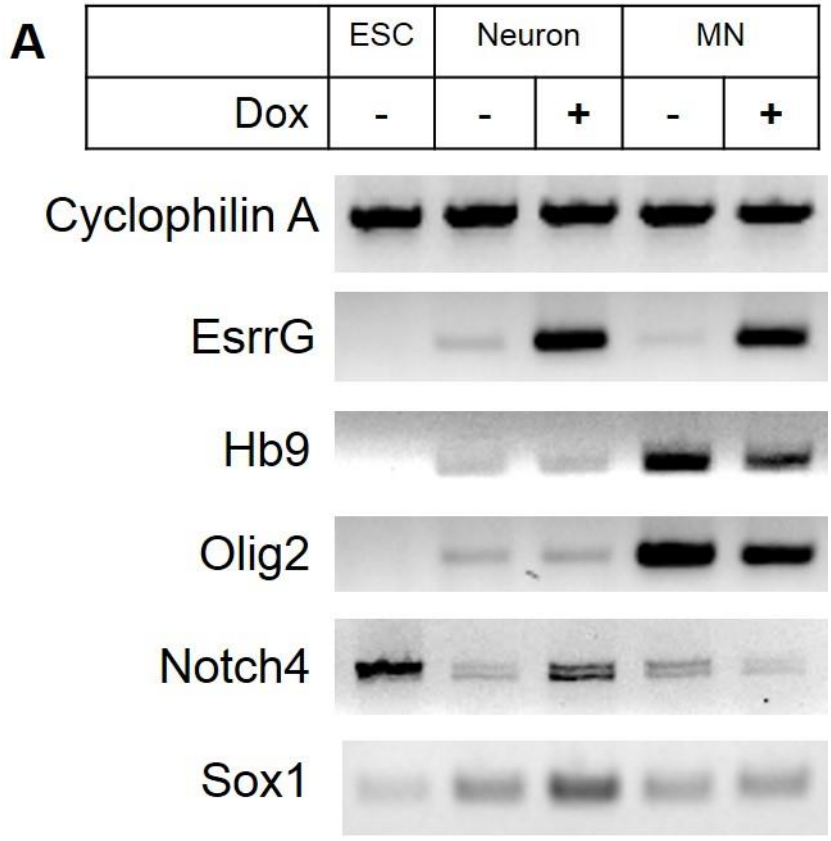

Figure 6. Notch4 as a potential downstream target of EsrrG

(A) RT-PCR of key neuronal markers in ESC state, neuronal state and MN state without or with EsrrG induction.

7. Molecular mechanistic study of EsrrG reveals that it binds to enh-Notch4 ERRE during MN differentiation

Chromatin Immunoprecipitation (ChIP) is a technique that allows one to study physical interaction between a protein of interest, usually a transcription factor, and the DNA elements which it binds *in vivo* (Kuo et al, 1999). ChIP was performed using doxycycline inducible 3xflag-iEsrrG ESC line in MN context. EsrrG binding to ERRE of Notch4-enh was confirmed by PCR in MN context (Figure 7A). msCDH1-enh including ERRE region was used as a positive control, and EsrrG's binding to ERRE of msCDH1-enh was best observable in ESC context (Tiraby et al., 2011; data not shown). Interestingly, PCR of Hb9-enhancer m250-luc showed minimal binding of EsrrG during MN differentiation (Figure 7C). Quantitative analysis using qPCR showed that EsrrG was enriched 4.84 times more at enh-Notch4 during MN differentiation in dox treated sample compared to dox negative sample (Figure 7D). Thus, we validated that EsrrG physically binds to ERRE of enh-Notch4 during MN differentiation.

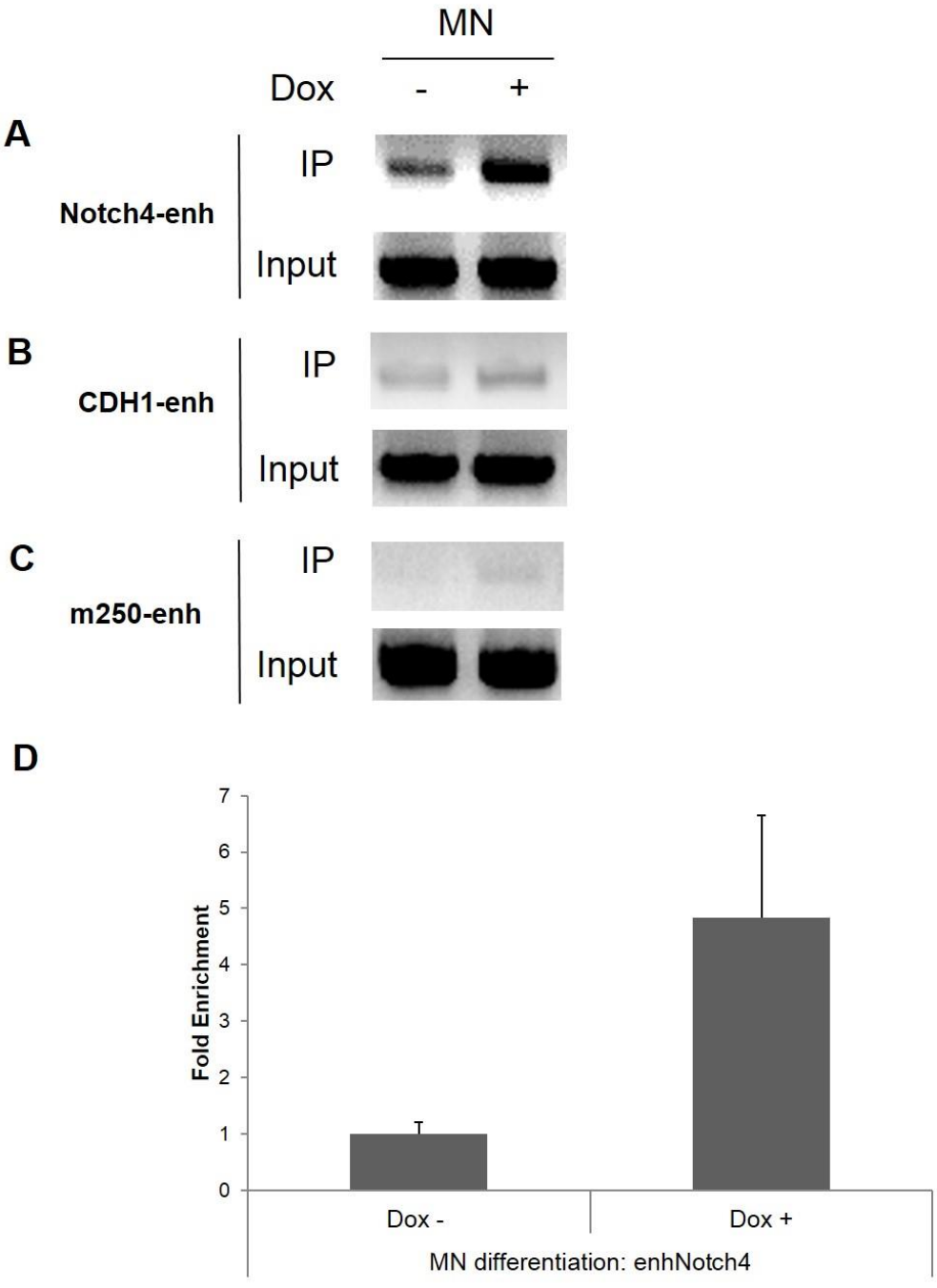
MN
Dox
-
+
A
Notch4-enh
IP
Input
B
CDH1-enh
IP
Input
C
m250-enh
IP
Input
D
Fold Enrichment
0
1
2
3
4
5
6
7
Dox -
Dox +
MN differentiation: enhNotch4

Figure 7. Molecular mechanistic study of EsrrG reveals that EsrrG physically binds to enh-Notch4 ERRE elements.

(A) EsrrG binds to ERRE of enh-Notch4 during MN differentiation.

(B) As control, CDH1 intron including ERRE region was selected.

(C) m250-enh during MN differentiation shows minimal binding of EsrrG.

(D) Quantitative analysis of enrichment of EsrrG on enh-Notch4 ERRE during motor neuron differentiation.

8. EsrrG activates promoter strength of enh-Notch4

Next we assessed whether EsrrG was capable of turning on promoter strength of enh-Notch4. From the ChIP-seq of MN hexamer, putative MN-hexamer binding site (HxRE-short) and EsrrG binding site (ERRE) were identified upstream of Notch4 coding region (Lee et al 2014). Previously in figure 7, EsrrG's binding on ERRE of enh-Notch4 was observed. To further test whether binding of EsrrG on ERRE of enh-Notch4 leads to promoter activation, GFP reporter assay was performed. Since MN-hexamer is a dimeric protein, at least two copies of HxRE sites are needed to see if the site is functionally active (Lee et al 2012). Thus, two copies of the enhancer region were cloned into GFP reporter vector (Figure 8A). Injection of enh-Notch4 (2x)-GFP alone led to minimal GFP detection, and addition of EsrrG led to strong expression of GFP where EsrrG is expressed (Figure 8B). Interestingly, injection of Isl1 and Lhx3 with enh-Notch4 (2x)-GFP did not lead to GFP activation (data not shown). In addition, injection of enh-Notch4 (2x)-GFP with Isl1, Lhx3, and EsrrG did not lead to further enhancement of GFP compared to addition of EsrrG only (data not shown). These results were consistent with previous data and further supported that Notch4 has a potential as a direct downstream target of EsrrG.

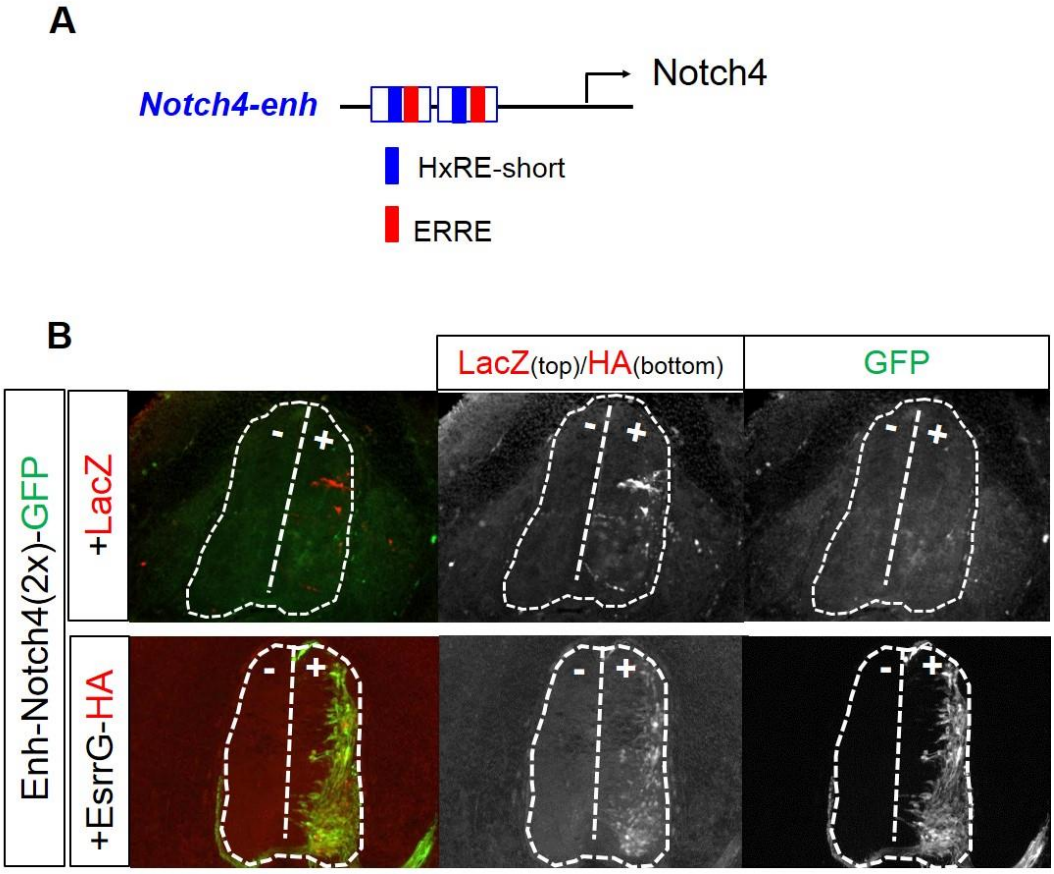

Figure 8. EsrrG activates promoter strength of enh-Notch4.

(A) Two copies of upstream region of Notch4 containing MN hexamer binding site (HxRE) and Estrogen Related Receptor binding site (ERRE) were cloned into GFP reporter vector.

(B) GFP reporter assay shows significant activation of GFP in region injected with EsrrG-HA, suggesting that EsrrG turns on promoter strength of enh-Notch4.

IV. Discussion

Due to the aging society and increased frequency of neurodegenerative diseases, a better understanding of how diverse neuronal subtypes are generated from relatively uniform neural precursor cells will be instrumental in developing treatments for nervous system diseases. This study identified, for the first time, a novel role EsrrG plays in promoting GATA3 fate specification and suppressing MN formation. In addition, this study also hints at Notch4 as a potential mediator acting downstream of EsrrG for the first time. As can be seen in figure 2, although significant proportion of GATA3 population co-localizes with EsrrG cell population, almost half of GATA3 remain EsrrG negative. Similarly, since EsrrG exists in broader domain than GATA3 population, EsrrG may play additional roles concerning neuronal development other than V2b IN specification. These additional roles of EsrrG across developmental stages are yet to be discovered and will be the subject for future studies. Also, since loss of EsrrG in EsrrG-CKO mice did not lead to complete loss of GATA3, there may be other compensatory factors that could substitute for the role of EsrrG. For example, EsrrB, which shares sequence similarity in the DNA binding domain to that of EsrrG, could have dual role with EsrG in establishing neuronal fate specification. Although not shown in this dissertation, generation of EsrrB and EsrrG conditional KO mice are in progress. Next, we aim to assess effect of deletion of both EsrrB and EsrrG at neual progenitor stage during mice spinal cord development. This study was able to differentiate iEsrrG ESC to motor neurons, since its differentiation context has been well studied. If conditions for

differentiating ESC to V2b interneurons are discovered, it would be very interesting to see the effect of turning on EsrrG during differentiation of ESC to V2b IN. We were able to identify only Notch4 as a putative target of EsrrG, but we cannot exclude the possibility of other factors that are regulated by EsrrG, which could, cooperatively or independently with Notch4, help EsrrG exert its effect in spinal cord neuronal development. Since Notch4 is not expressed in chick, we could not show that overexpression of EsrrG leads to ectopic expression of Notch4. Further molecular mechanistic study involving whether or not Notch4 is indeed a chief factor responsible for binary cell fate decision inducing GATA3 and suppressing Hb9 are necessary to complete the story.

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요약 (국문초록)

EsrrG: 운동신경억제와 V2b IN 분화에의 영향

중추신경계는 뇌와 척수로 구성되어 있고 몸으로 전달되는 감각세포들의 시그널은 중간 신경세포로 전달되어 시냅스를 형성하고 다양한 정보들을 모아 운동신경에 전달함으로써 운동을 조절한다. 많은 퇴행성신경질환들은 치료방법이 밝혀지지 않은 것들이 많은데 이는 정확한 기전이 밝혀지지 않은 질병들이 많기 때문이다. 세포치료방법이나 약물을 연구하고 개발하는 데에는 이처럼 기초과학 연구가 필수적이다. Motor neuron hexamer 는 세포의 운명을 운동신경세포로 결정 하는데 중요한 전사인자로 밝혀졌다. 본 연구에서는 ligand 가 밝혀지지 않으며 상시 활성화되어 있는 전사인자인 Estrogen Related Receptor Gamma (EsrrG)가 Motor neuron hexamer 의 타겟인지, 그리고 척수내에서 운동신경세포와 중간신경세포의 바운더리를 결정하는데 어떤 역할을 하는지 밝히고자 하였다. Chick embryo에서 EsrrG 가 과발현 되었을때와 mouse embryo에서 EsrrG 가 early neural progenitor 단계에서 제거되었을 때의 양상을 통해 EsrrG 는 운동신경세포의 마커인 Hb9 이나 Isl1을 억제하고 V2b interneuron 마커인 GATA3를 활성화시킨다는 것을 알 수 있었다. 이 결과는 줄기세포에서 EsrrG 가 doxycycline 에 의해 운동신경세포 분화전 발현되게 된 경우 운동신경 형성을 방해한다는 것과 일치하였다. Chromatin Immunoprecipitation을 통해 EsrrG response elements (ERRE) 가 있는 putative genes 중에서 EsrrG 가 실제로 운동신경세포 분화도중 Notch4 의

ERRE 에 binding 하여 Notch4 의 promoter를 활성화시킨다는 것을 밝혀내었다.

주요어 : EsrrG, motor neuron, V2b interneuron, GATA3, Notch4, neurogenesis

학번 : 2013-21608 이름 : 이지연

약학석사 학위논문

EsrrG: 운동신경억제와 V2b IN 분화에의 영향

EsrrG Plays a Role in Suppressing Motor Neurons and Inducing V2b Interneurons

2015 년 2 월

서울대학교 대학원

약학과 약물학 전공

이지연

Abstract

EsrrG Plays a Role in Suppressing Motor Neurons and Inducing V2b interneurons

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One of the major challenges in neuroscience is understanding how diverse neuronal subtypes are generated from a relatively uniform neural precursor. Elucidation of molecular mechanism regarding neuronal development will be of much value to our society as it would provide crucial basis for developing new drugs or cell therapy for treating neurodegenerative diseases. The goal of my research project was to gain further insight into genes that are involved in neuronal development based on the ChIP-seq and RNA-seq data on Motor neuron hexamer by Professor Seunghee Lee (MN-hexamer). Among the 500 genes identified as potential targets of motor neuron hexamer (MN-hexamer), 52 also possessed DNA binding response elements of Estrogen Related Receptor (ERRE). The research began with hypothesis that Estrogen Related Receptor could, independently or in cooperation with the MN-hexamer, direct motor neuron development. To see if the putative genes were expressed at motor neuron domain in the

spinal cord, *in situ* hybridization (ISH) was performed with the mouse and chick spinal cord tissue transverse sections using the probes that I cloned. After screening for mRNA localization patterns of numerous genes, a few of them were chosen for further study. Interestingly, Estrogen Related Receptor Gamma (EsrrG) displayed migratory expression pattern depending on developmental stages. Luciferase reporter assay and GFP reporter assay were performed with the constructs that I cloned. Reporter assays confirmed that the target gene promoters were indeed activated by EsrrG. This was further validated by Chromatin Immunoprecipitation (ChIP) which showed that EsrrG physically bound to ERRE located upstream of Notch4. In order to gain insight into the function of EsrrG during spinal cord development, I performed the function analysis: For the gain of function analysis, chick embryo spinal cords were electroporated with the expression plasmids, either full length construct or the construct bearing transcription activating domain VP16. For the loss of function analysis, both chick and mice were analyzed. Chick embryos had the spinal cords electroporated with shRNA knockdown constructs or constructs bearing engrailed repressor domain, which acts as a dominant-negative form. Mutant mice embryos were harvested at various developmental stages by breeding EsrrG-floxed mice with Nestin-Cre mice, and obtaining knockout embryos which have EsrrG deleted at early neural progenitor stage. Surprisingly, I was able to conclude that EsrrG favored V2b inhibitory interneuron and played a certain role in suppressing motor neuron formation. I obtained consistent results during embryonic stem cell differentiation to motor neuron using the doxycycline inducible EsrrG stem cell line (iEsrrG) that I established. During differentiation of iEsrrG-ESC to motor neurons by treatment with

retinoic acid and Sonic hedgehog agonist, doxycycline treatment led to very significant decreases in motor neuron markers Hb9 and Isl1 both in RT-PCR and IHC.

My research has identified, for the first time, a novel role EsrrG plays in spinal cord neural development by establishing V2b interneuron and motor neuron cell fate decision. Further molecular mechanistic study and mutant mice study are in progress and closer examination of EsrrG at various embryonic stages and elucidation of its downstream signaling network will be the subjects for immediate future studies.

Key words: EsrrG, V2b IN, GATA3, neurogenesis, motor neuron, Notch4

Student number: 2013-21608

Table of Contents

List of Abbreviations

ChIP	Chromatin Immunoprecipitation
Dox	Doxycycline
dpi	Days post injection
ERRE	Estrogen Related Receptor Response Elements
EsrrG	Estrogen Related Receptor Gamma
HH	Hamburger-Hamilton stage
IHC	Immunohistochemistry
ISH	*In situ* hybridization
Purm	Purmorphamine
RA	Retinoic Acid
RT-PCR	Reverse transcription polymerase chain reaction
SC	Spinal cord
Shh	Sonic Hedgehog

List of Figures

I. Introduction

Despite high societal pressure to elucidate neuronal network circuitry due to increasing number of neurodegenerative diseases and spinal cord injury cases, relatively little is understood regarding molecular mechanism involving neurogenesis. Spinal cord develops from ectoderm guided by external factors along the dorsoventral and anterior posterior axes, and consists of sensory neurons, interneurons, and motor neurons (Dasen et al., 2009). In ventral part of the spinal cord, Sonic hedgehog (Shh) morphogen is secreted from the notochord, creating a concentration gradient from ventral Shh high, to dorsal Shh low (Ulloa et al., 2007). In addition to Shh, retinoic acid (RA) and fibroblast growth factors (FGFs) are secreted from mesoderm to help guide neuronal patterning of the spinal cord (Jessell et al., 2000). These external factors in turn give rise to combination of diverse sets of transcription factors, which serve to further define neuronal subsets in different domains of the spinal cord across development (Ulloa et al., 2007). The ventral most part of the spinal cord, which is exposed to highest concentration of Shh, becomes V3 interneurons, and the region dorsal to V3 IN differentiate into MN, V2, V1, and V0 interneurons (Dessaud et al., 2008). In the ventral side of the spinal cord critical for MN differentiation, Shh induces class II transcription factor Olig2 (Novitch et al., 2003). Similarly, the region dorsal to where Olig2 is expressed is Irx3, which in turn exhibits cross inhibitory action with Olig2 to establish V2 and MN neuronal boundary (Novitch et al., 2001). In postmitotic stages, Hb9 and Isl1/2 mark motor neuron identity (Lee et al., 2012).

In this study, we originally intended to elucidate molecular mechanism downstream of MN-hexamer, which consists of Isl1, Lhx3, and NLI, and is known to play a key role in specifying motor neuron fate (Lee et al., 2012). MN hexamer complex turns on Hb9, a well-known homeobox gene and a motor neuron marker, and suppress V2a excitatory interneurons, marked by Chx10 (Lee et al., 2012). From the ChIP-seq data of inducible Isl1-Lhx3 cell line, about 500 putative target genes of MN-hexamer were identified. Interestingly, among these targets, 52 also had Estrogen related receptor binding sites, or ERRE. From this observation, we hypothesized that Estrogen related receptor could, independently or downstream of MN-hexamer complex, play a role in MN fate specification. Estrogen related receptor family, otherwise referred to as NR3B, are orphan nuclear receptor superfamily and consists of EsrrA, EsrrB, and EsrrG (Tremblay et al., 2007). They are known to be constitutively active transcription factors and their ligands remain unknown, hence the word orphan nuclear receptor (Tremblay et al., 2007). All three ERR isoforms share sequence similarity in its DNA binding domain (DBD) (Tremblay et al., 2007). EsrrB is well known to play crucial role in ESC self-renewal by acting as a direct downstream target of Nanog and Gsk3/Tcf3 axis (Festuccia et al., 2012; Martello et al., 2012). EsrrG is important in normal kidney renal papilla development and oxidative metabolism transition in heart (Berry et al., 2010; Alaynick et al., 2007).

II. Material and Methods

1. DNA Constructs

Mouse EsrrG was cloned to pCS2 vector containing a HA-epitope tag. Constitutively active EsrrG was cloned by cloning DNA Binding Domain (DBD) of EsrrG into myc-tagged pCS2 vector containing a VP16 transcriptional activation domain. EsrrG-DBD was also cloned into myc-tagged pCS2 containing an Engrailed repressor domain, which acts as a dominant negative form.

In situ hybridization probes for mouse EsrrG and chick EsrrG were generated by cloning 400-500bp fragments into pBluescript vector. The following oligonucleotides were used for the cloning:

msEsrrG forward : 5'- CGGAATTCGCAAGGCATTCTTCAAGAGG-3' and reverse: 5'- CCGCTCGAGGAGGAGGCTCATCTGGTCTG-3'

ckEsrrG forward 5'-CGGAATTCGACTCACCTCCCCTCTACCC-3' and reverse 5'-CCGCTCGAGCTGCATCTATTCTGCGCTTG-3',

msNotch4 forward: 5'-CGGAATTCCAGGGGCTCTCTGTGACTTC-3' and reverse 5'-CCGCTCGAGGGCTAAAGAGTGGCAAGCTG-3'.

2. Generation and Differentiation of iEsrrG-ESCs

A gene encoding 3xFlag-tagged EsrrG construct was inserted into Tet-inducible p2Lox plasmid and transfected with pSALK-Cre into ESC lines. The primers used for

the cloning are: forward 5'-CCGCAATTGATGGATTCGGTAGAA-3' and reverse 5'-ATAAGAATGCGGCCGCTCAGACCTTGGCCTC-3'.

The A172LoxP ESC line was maintained in an undifferentiated state on 0.1% gelatin-coated dishes in the ESC growth media that consisted of Knockout DMEM, 10% (vol/vol) FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and recombinant leukemia inhibitory factor (LIF, 1000 units/mL, Chemicon). A gene encoding FLAG-tagged Isl1-Lhx3 fusion construct was inserted into Tetinducible plasmid p2Lox and were cotransfected with pSALKCre into ESC lines by electroporation. Stable transfectants were isolated by selection with neomycin (G418, 400-600 μg/mL) for 5-7 d. Doxycycline (Dox)-dependent induction of Flag-EsrrG was confirmed by RT-PCR, Western blotting, and immunohistochemical analyses using mouse a-FLAG antibody (sigma).

iEsrrG ESC was differentiated into neurons and motor neurons (MNs) in 6 d period by treatment with 0.5uM RA alone or with 0.5uM RA and 1uM Purmorphamine respectively, starting at 2nd day of cell differentiation until the 5th day, the day before harvest. 20ug of Doxycycline was treated 4th day and 5th day of differentiation to induce EsrrG.

3. In ovo Electroporation and Immunohistochemistry

Neural tubes of Chick embryos were electroporated in Hamburger-Hamilton stage 13, harvested 2~4 d post-electroporation and fixed in 4% paraformaldehyde for two

hours on a rotator in cold room, then left in 1X PBS overnight. They were then cryoprotected in 30% sucrose for two hours on a rotator in cold room and mounted and embedded in Optimal Cutting Temperature (OCT), which was cryosectioned on Leica cryostat at -20℃ for immunohistochemistry or in situ hybridization. For immunohistochemical analyses, tissues were cut at 12um and for in situ hybridization, tissues were cut at 18um. Representative sets of images from reproducible results were presented. Mouse embryos were collected at indicated developmental stages and processed similarly to chicken embryos as described previously. For immunohistochemistry assays, the following antibodies were used; mouse anti-HA (Covance), mouse anti-flag (sigma), mouse anti-Mnr2/Hb9 (5C10, DSHB), rabbit anti-Isl1/2 (19), rabbit anti-GATA3, mouse anti-EsrrG (Perseus Proteomics), rabbit anti-GFP (A6455, Molecular Probes).

4. ChIP Assays

3xflag-iEsrrG ESC line described above was used for ChIP assay. For ChIP assay performed in self-renewal ESC state, iEsrrG-ESCs were cultured on 0.1% gelatin-coated dishes in the ESC grwoth media with LIF in the presence or absence of Dox (2ug/ml), which induces the expression of FLAG-tagged EsrrG, for 1 d. The cells were washed with PBS buffer, fixed by 1% formaldehyde for 10 min at room temperature and quenched by 125mM glycine. Cells were washed with Buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA,10 mM Hepes, pH 6.5) and

Buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) sequentially. Then, cells were lysed with lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris·HCl, pH 8.0, protease inhibitor mixture) and were subjected to sonication for DNA shearing. Next, cell lysates were diluted 1:10 in ChIP buffer (0.5% Triton X-100, 2 mM EDTA, 100 mM NaCl, 50 mM Tris·HCl, pH 8.0, protease inhibitor mixture) and, for immunoclearing, were incubated with IgG and protein A agarose beads for 2 h at 4 ℃. Supernatant was collected after quick spin and incubated with α-FLAG antibody (Sigma) and protein A agarose beads to precipitate FLAG-EsrrG/chromatin complex overnight at 4 ℃. After pull-down of FLAG-EsrrG/chromatin/antibody complex with protein A agarose beads, the beads were washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl, pH 8.0, 150 mM NaCl), TSE II (same components as in TSE I except 500 mM NaCl), and Buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris·HCl, pH 8.0) sequentially for 7 min at each step. Then the beads were washed with TrisEDTA (TE) buffer two times. FLAG-Isl1-Lhx3/chromatin complexes were eluted in elution buffer (1% SDS, 1 mM EDTA, 0.1 M NaHCO3, 50 mM Tris·HCl, pH 8.0) and decross-linked by incubating at 65 ℃ overnight. Eluate was incubated at 50 ℃ for more than 2 h with Proteinase K. Next, DNA was purified with Phenol/chloroform and DNA pellet was precipitated by ethanol and resolved in water overnight at room temperature. PCR was done with enhNotch4 and msCDH1-ERRE using the primers mCDH1 forward: 5’-GGGGACACTCTTTCGACCTT-3’

and reverse: 5'-GTGGAGGGGTTTGGGTTTTC-3', which include ERRE: TGACCTTGG. Enh-Notch4 forward: 5'-GAAGATTCTATGAAGCTCAGGGTTTGATTTCCA-3' and reverse: 5'-CGGGATCCTCAGCCTCGAGCAGTCCTGGAAGC-3'.

5. Mouse Genetics

Mouse strains used in this study are $Nestin^{Cre}$, $EsrrB^{flox/flox}$ and $EsrrG^{flox/flox}$ mice were kind gifts from Dr. Kralli, at the Scrips Research Institute (TSRI), and were originally created by Professor Chambon's lab at IGBMC. Seoul National University Institutional Animal Care and Use Committes approved all the maintenance and experimental procedures. The primers used for genotyping are: EsrrG flox primer forward: 5'-GTTTTAAAGGCCCTTGGTGATCTCGC-3' and reverse: 5'-CTGCAACCCTTGGACTGCCAGAAC-3'. EsrrG KO primer forward: 5'-CCCTTATGCTGATTACCTTCTTGTA-3' and reverse: 5'-CTGCAACCCTTGGACTGCCAGAAC-3'.

6. RT-PCR

Total RNA was extracted using Trizol (Invitrogen) and reverse transcription (RT) was performed using SuperScript III (Invitrogen). The following primers were used for RT-PCR:

Cyclophilin A, forward 5′-GTCTCCTTCGAGCTGTTTGC-3’ and reverse 5′-GATGCCAGGACCTGTATGCT;

msEsrrG, forward 5’-TGAAGTGTCTCAAAGTGGGC-3’ and reverse 5’-GTTCAGCCACCAACAAATGC-3’;

msHb9 forward 5’-CGGCGCTTTCCTACTCATAC-3’ and reverse 5’-ACTTCCCCAAGAGGTTCGAC-3’;

msSox1 forward 5’-AGACTTCGAGCCGACAAGAG-3’ and reverse 5’-AACTGTGCAAACAGGTGCAG-3’;

msNotch4 forward: 5’-CAGCTTGCCACTCTTTAGCC-3’ and reverse 5’-CAGCTTGCCACTCTTTAGCC-3’; and

Olig2 forward 5’-GGATGCTTATTACAGACCGA-3’ and reverse 5’-AAAAGATCATCGGGTTCTGG-3’.

7. Statistical Analysis of the Data

Data are presented as mean ± SD or mean ± SEM as indicated. Statistical comparisons were conducted by two-tailed Student t test.

III. Results

1. EsrrG is expressed in ventral area of the spinal cord during development and exhibits dorsal migratory pattern

Previous studies have shown that MN-hexamer consisting of Isl1, Lhx3, and NLI specifies motor neuron fate while suppressing V2a interneuron fate (Lee et al 2012). To see if any of the Estrogen related receptor family is indeed a target of MN-hexamer, mRNA expression patterns in transverse spinal cord sections of mice were analyzed. As hypothesized, if any of the Estrogen related receptor family indeed plays a role in motor neuron specification, its expression pattern would most likely be localized at ventral motor neuron area of the spinal cord. However, EsrrA and EsrrB were expressed at low levels in the spinal cord (data not shown). Interestingly, *in situ* hybridization of EsrrG on mice tissues indicated that EsrrG is expressed at a rather unique pattern, as EsrrG dorsally migrates out of ventral area across developmental stages (Figure 1A). Note that EsrrG mRNA expression peaks at e12.5. In chick spinal cord, EsrrG also exhibits unique expression pattern and was not found to be ectopically turned on upon Isl1+Lhx3 injection, suggesting that it may not act as a downstream target of MN-hexamer (Figure 1B). Next, we focused on EsrrG among Estrogen related receptor family and sought to further analyze the functional identity of EsrrG using immunohistochemistry of EsrrG with diverse neuronal markers.

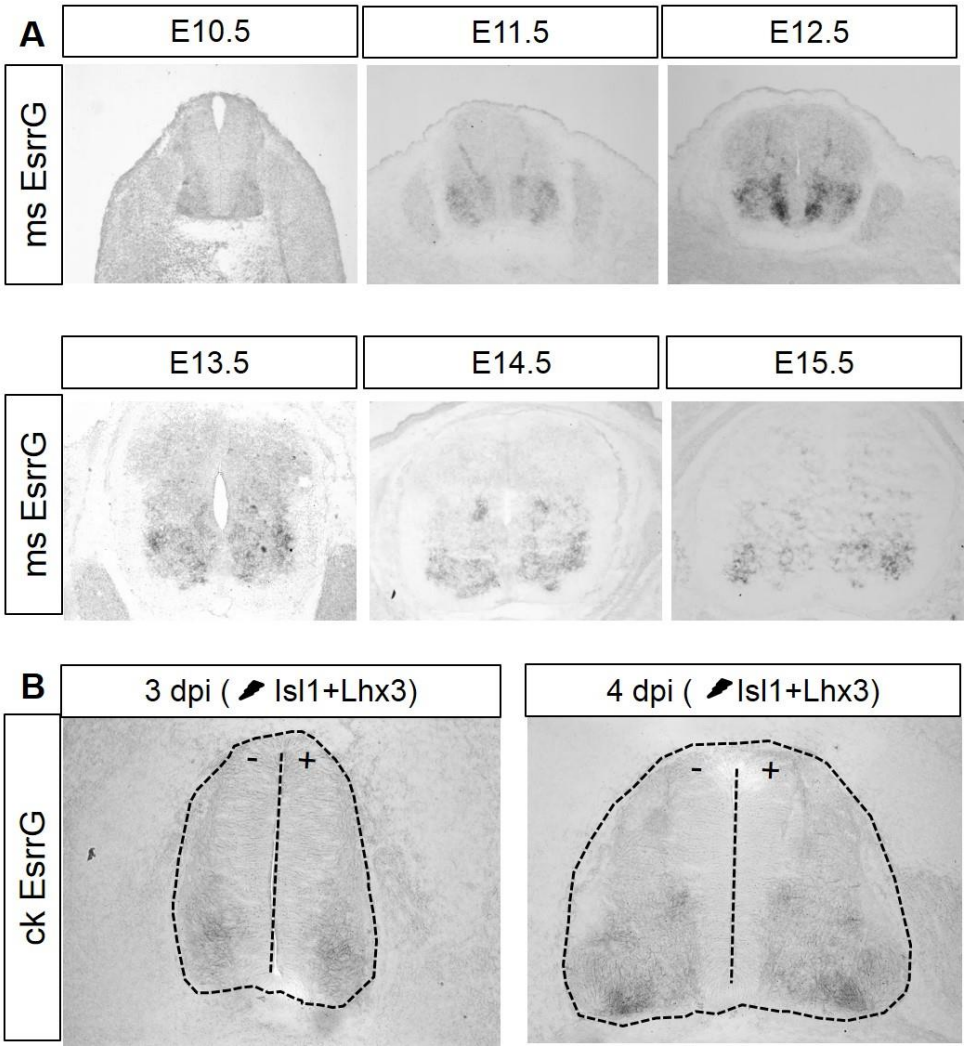


Figure 1. EsrrG exhibits migratory expression pattern during SC development.

(A) ISH of mouse embryonic spinal cord from e10.5 to e15.5 using EsrrG mouse antisense probe.

(B) ISH of chick EsrrG in chick spinal electroporated with ISl1

2. EsrrG co-localizes mostly with GATA3, a V2b inhibitory interneuron marker

EsrrG was co-stained with various neuronal markers during mice embryonic development, including motor neuron markers Hb9, Isl1, and Lhx3, V2a excitatory interneuron marker Chx10, V2b inhibitory interneuron marker GATA3, and V2c marker Sim1. In contrast to the initial hypothesis which expected that EsrrG would co-localize mostly with MN marker Hb9, EsrrG was found to have most significant overlap with V2b interneuron marker GATA3 across developmental stages e10.5 to e14.5 (Figure 2A). At mouse embryonic stages e12.5 and e13.5, 53.6% and 50.4% of GATA3 population were found to co-localize with EsrrG, respectively (Figure 2B). Surprisingly, at e12.5, EsrrG showed mutually exclusive pattern with MN marker Hb9, with Hb9 population appearing to be surrounded by EsrrG (Figure 2C). GATA3 has been well studied in nervous system development, as loss of GATA3 has been found to impair facial branchiomotor neuron migration in the hindbrain and survival of sympathetic neurons (Pata et al., 1999; Tsarovina et al., 2010). From the series of co-localization studies, EsrrG emerged to be potentially important in V2b interneuron specification. To further confirm the functional identity of EsrrG in determining neuronal fate, gain of function and loss of function phenotype studies were performed in mice and chick tissues.

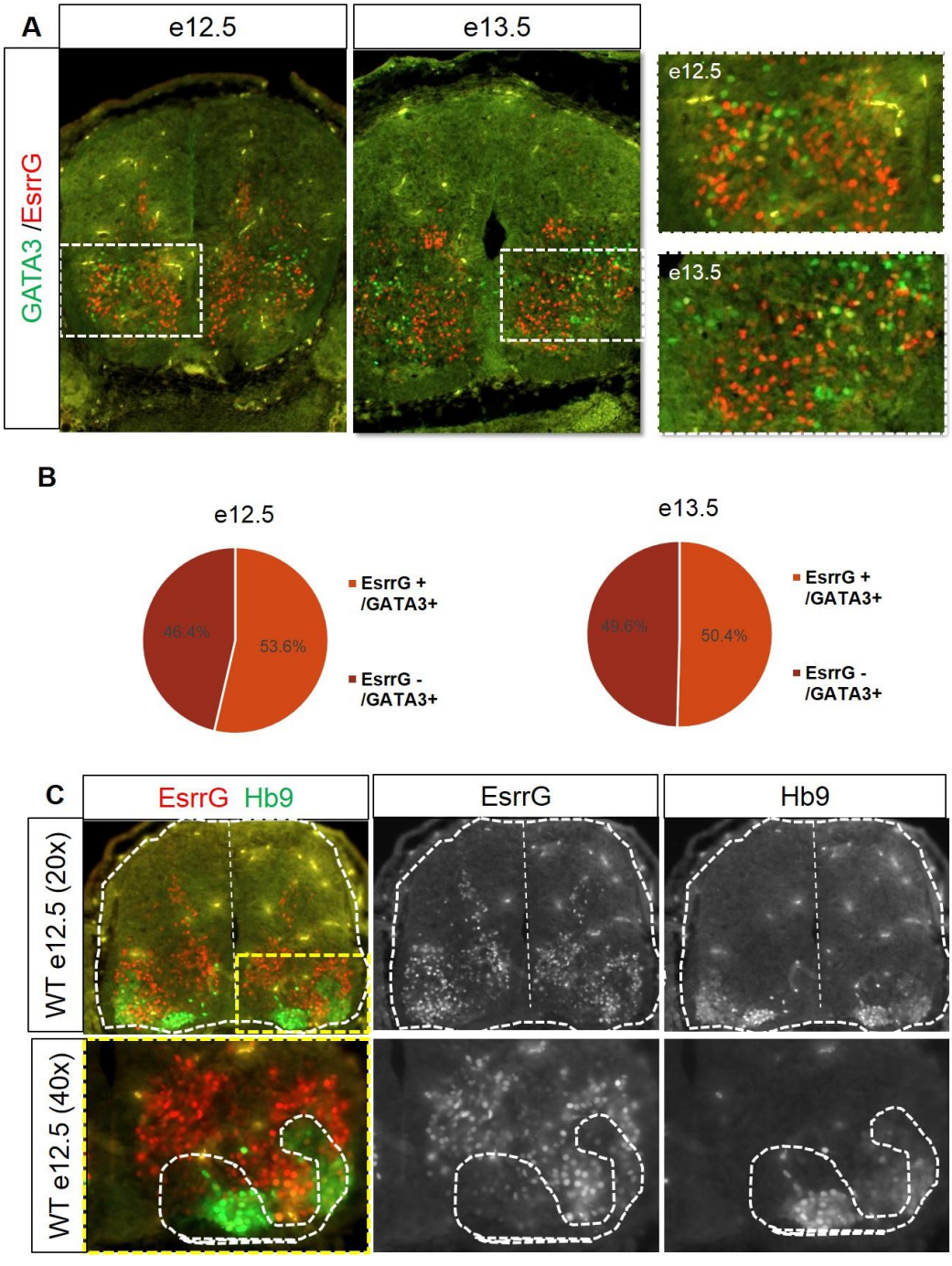

A
e12.5
e13.5
GATA3 /EsrrG
e12.5
e13.5
B
e12.5
46.4%
53.6%
EsrrG +
/GATA3+
EsrrG -
/GATA3+
e13.5
49.6%
50.4%
EsrrG +
/GATA3+
EsrrG -
/GATA3+
C
EsrrG Hb9
EsrrG
Hb9
WT e12.5 (20x)
WT e12.5 (40x)

Figure 2. EsrrG co-localizes mostly with V2b inhibitory neuronal marker GATA3.

(A) Co-staining of V2b inhibitory neuronal marker GATA3 (green) with EsrrG (red) shows colocalized population at mouse embryonic days e12.5 and e13.5.

(B) Quantification analysis of figure 2A showing percentages of GATA3 that also expresses EsrrG at e12.5 and e13.5 (orange).

(C) Co-staining of MN marker Hb9 (green) with EsrrG (red) shows virtually no co-localized cells and non-overlapping patterns at mouse embryonic day e12.5.

3. Gain of function and loss of function studies of EsrrG in spinal cords of chick embryos

Chick embryos are ideal model organisms in developmental science due to their availability and easiness in surgical manipulations (Nakamura et al., 2004). In this study, chick *in ovo* electroporation technique was used, where the plasmid of interest was injected into the spinal cord of developing chick embryos at Hamburger Hamilton stages 12-13. Then the transient, square pulse was applied so that negatively charged DNA is moved to the side of spinal cord adjacent to a positively charged electrode (Nakamura et al., 2004). The embryos were harvested 3 days post injection (Figure 3A, 3B). This allows for gene of interest to be expressed in one side of the spinal cord, and the gene's effect on neuronal distribution or morphology can be studied in comparison to the other half side, or the unelectroporated control side, all in one embryo.

Consistent with previous observations that EsrrG appears be important in V2b interneuron specification, gain of function study in chick embryos electroporated with HA tagged full length EsrrG led to ectopic expression of GATA3 (Figure 3A). In addition, a slight decrease in MN marker Isl1 was observable in chick spinal cord side electroporated with EsrrG (Figure 3A). At cervical level, EsrrG-HA injection led to statistically significant increase of Gata3 at P=0.0382 (Figure 3B). Similarly, at thoracic level, EsrrG-HA injection led to statistically significant decrease of Hb9 at P=0.045. Next, loss of function study was conducted using plasmid encoding EsrrG DNA binding domain (DBD)

fused to Engrailed Repressor (EnR) domain, which acts as a dominant negative form to inhibit endogenous EsrrG from binding to its downstream targets (Markel et al., 2002). When myc-tagged EsrrG-DBD_EnR was injected into chick spinal cord, notable decrease in GATA3, as predicted, was observed and marked expansion of MN marker Isl1 was observed (Figure 3D). The following observations were confirmed in chick embryos harvested 4 days post injection (data not shown). Similarly, electroporation of myc-tagged plasmid encoding EsrrG DNA binding domain fused to constitutively active VP16 domain led to increased GATA3 and decreased Isl1 (Figure 3E. The gain of function and loss of function analysis in chick spinal cord consistently indicated that EsrrG plays a role in activating V2b interneuron marker GATA3 while repressing MN marker Isl1. Next, we sought to determine the effect of neural specific deletion of EsrrG in mice embryos using Nestin-cre transgenic mice, as homozygous deletion of EsrrG was found to result in deaths postnatal day 1 due to severe cardiac malfunction (Alaynick et al., 2007).

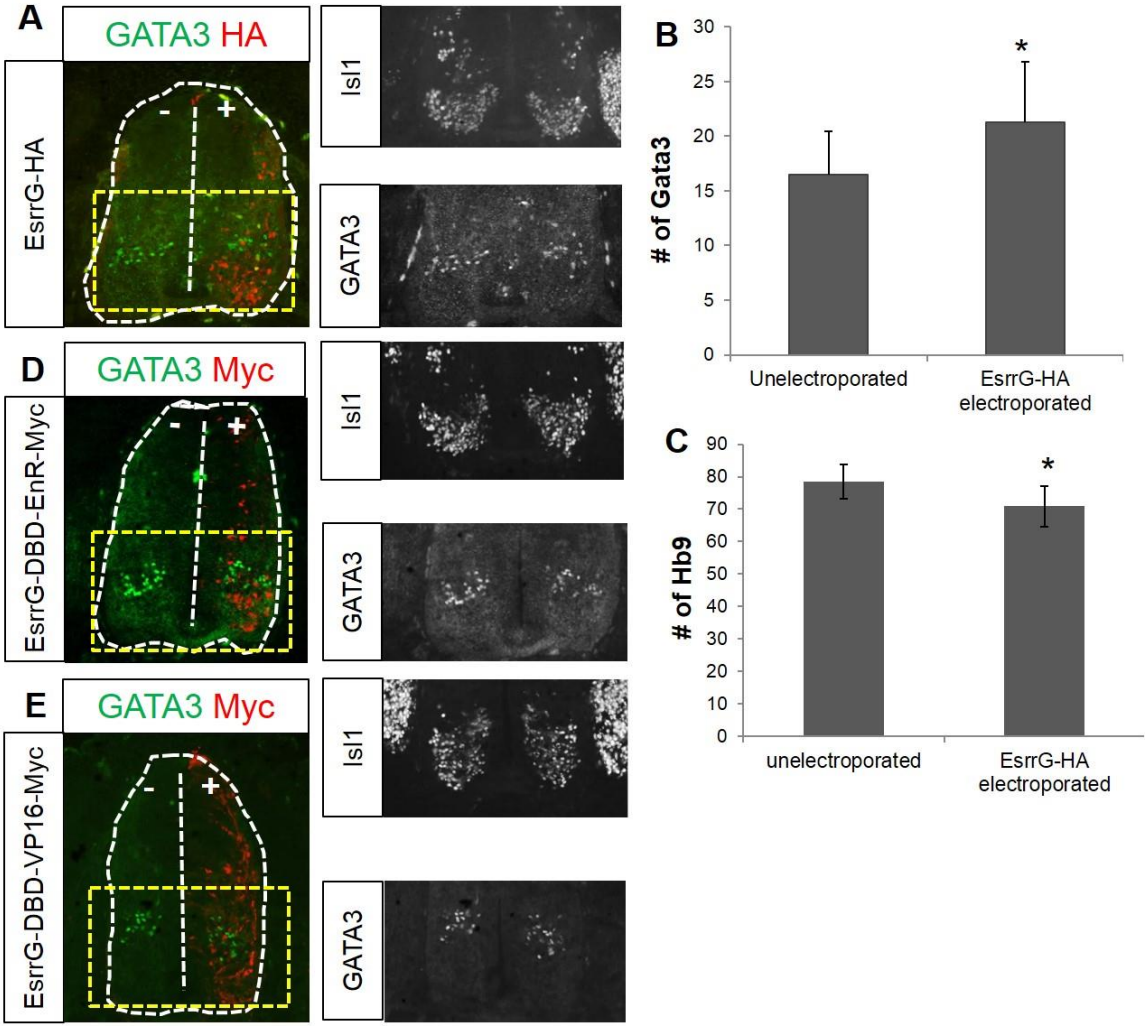

A
GATA3 HA
EsrrG-HA
Isl1
GATA3
D
GATA3 Myc
EsrrG-DBD-EnR-Myc
Isl1
GATA3
E
GATA3 Myc
EsrrG-DBD-VP16-Myc
Isl1
GATA3
B
# of Gata3
30
25
20
15
10
5
0
*
Unelectroporated
EsrrG-HA electroporated
C
# of Hb9
90
80
70
60
50
40
30
20
10
0
*
unelectroporated
EsrrG-HA electroporated

Figure 3. EsrrG plays a role in specifying V2b interneuron and repressing motor neuron (MN) in chick embryonic spinal cord.

(A) Chick spinal cord electroporated with HA tagged full length EsrrG leads to increased GATA3 formation and slightly decreased MN marker Isl1.

(B) At cervical level, EsrrG-HA injection at 3dpi leads to statistically significant increase of Gata3 ($P=0.0382$,$^*P<0.05$).

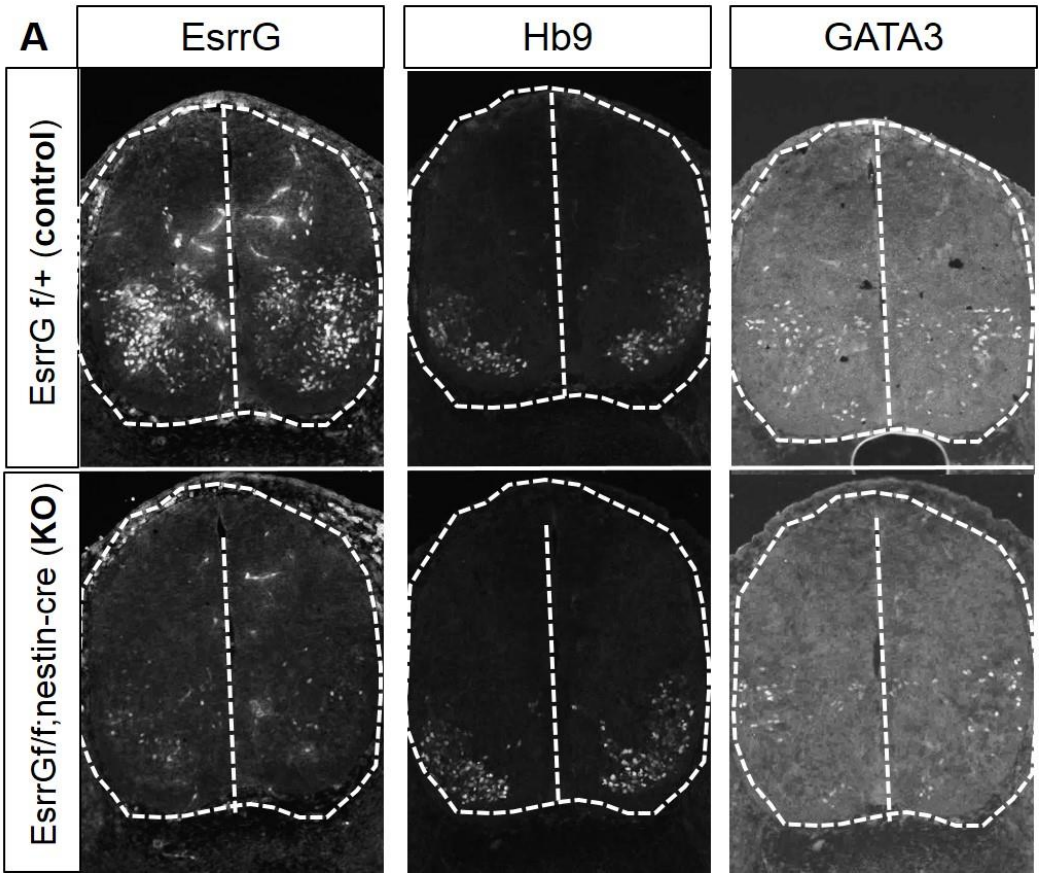
(C) At thoracic level, EsrrG-HA injection at 3dpi leads to statistically significant decrease of Hb9 ($P=0.045$, $^*P<0.05$).

(D) Chick spinal cord electroporated with dominant negative form of EsrrG (EsrrG-DBD-EnR-Myc) leads to reduced GATA3 formation and increased Isl1 formation.

(E) Chick spinal cord electroporated with activated form of EsrrG (EsrrG-DBD-VP16-Myc) leads to slightly increased GATA3 formation.

4. Neural specific deletion of EsrrG leads to similar results

Nestin-cre mice enables for highly efficient recombination of floxed alleles in the nervous system starting at mouse embryonic day 10.5 (Dubois et al., 2006). Timed mating of male EsrrG fl/+;Nestin-Cre mice with female EsrrG fl/fl enabled for embryos with wild type (EsrrG fl/+), heterozygous (EsrrGfl/+; Nestin-Cre), and knockout (EsrrG fl/fl;Nestin-Cre) genotypes. Pregnant female mice were harvested at desired developmental stages. Resulting wild type and mutant embryos at developmental stages e10.5, e11.5, e12.5, and e15.5 were analyzed. Marked and efficient deletion of EsrrG was confirmed in all stages through immunohistochemistry (Figure 4A). Similar to chick functional studies, neural specific EsrrG deletion using Nestin-cre mice led to significant decrease in GATA3 (22.8%) at e12.5 thoracic levels (Figure 4A, 4B). Consistently, there was a slight expansion in MN marker Hb9 when EsrrG was deleted (Figure 4A). Other neural markers such as Isl1, Lhx3, Olig2, Sim1, Tuj1, and proliferation marker BrdU were analyzed. The results in mice conditional knockout study further validated previous results and pointed that EsrrG is an important factor in promoting V2b interneuron specification. Next, EsrrG's effect on differentiation of embryonic stem cell (ESC) to motor neuron (MN) was assessed using inducible EsrrG cell line construct, to further validate the results in cell line *in vitro* context.


A
EsrrG
Hb9
GATA3
EsrrG f/+ (control)
EsrrGf/f;nestin-cre (KO)

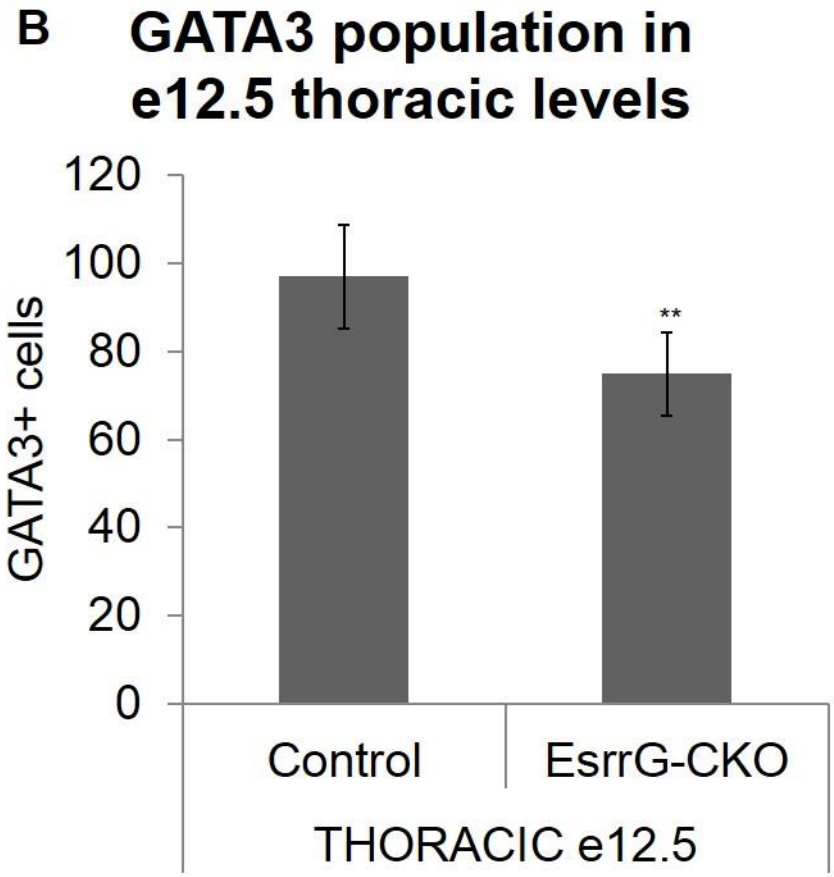


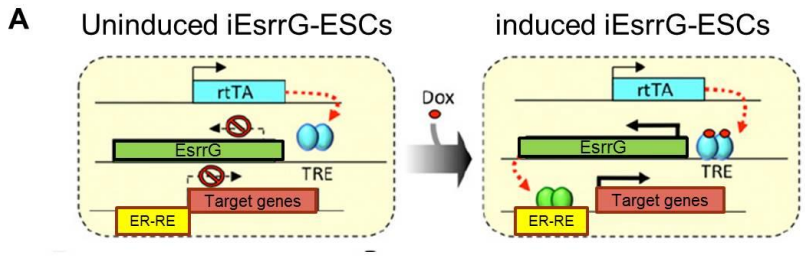
Figure 4. Loss of EsrrG in neural progenitor stage results in reduced GATA3 formation and slightly increased MN formation.

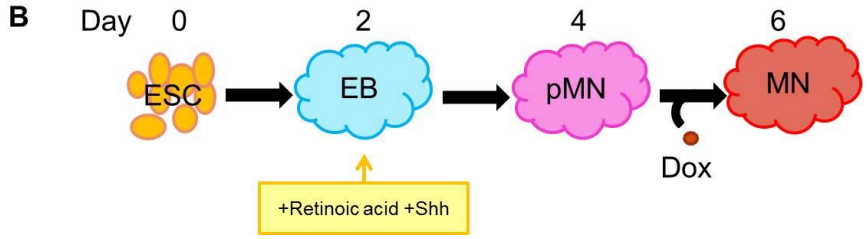
(A) Neural specific deletion of EsrrG at e12.5 leads to slightly increased Hb9 and decreased GATA3.

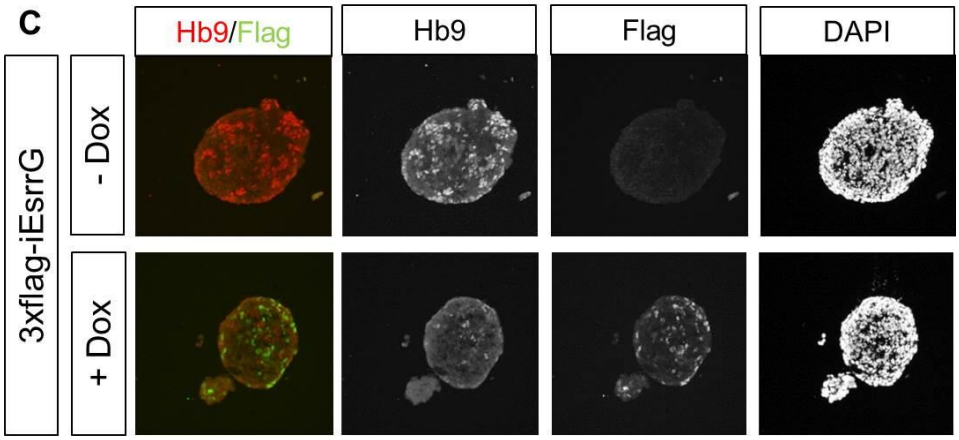
(B) Quantitative analysis of GATA3 cell populations indicates statistically significant decrease of GATA3 upon loss of EsrrG ($p=0.0011$).

5. Induction of EsrrG during MN differentiation leads to MN suppression

Doxycycline inducible EsrrG ESC line (iEsrrG) was treated with retinoic acid (RA) and Sonic hedgehog agonist purmorphamine (Purm), to differentiate ESC to motor neuron (Figure 5A, Figure 5B). As expected, EsrrG induction significantly impaired differentiation of ESC to MN, as can be seen by visibly reduced formation of Hb9 (Figure 5C). MN marker Isl1 formation was also reduced upon dox treatment (data not shown). Quantitative analysis was performed by counting the number of Hb9 positive cells and dividing it by the number of nuclei (DAPI) in each cell aggregate, to assess MN differentiation efficiency. This showed very significant 49% decrease in Hb9 formation when EsrrG was induced during MN differentiation (Figure 5D). Similarly, reduction of other MN marker Isl1 upon EsrrG induction was also observed (data not shown). Thus, EsrrG was found to play a role in suppressing motor neuron formation in ESC context, further validating previous results. We next sought to examine the molecular mechanism of how EsrrG exerts its effect on specifying V2b interneuron and repressing motor neuron through reporter assays and Chromatin Immunoprecipitation (ChIP) assays.


A
Uninduced iEsrrG-ESCs
induced iEsrrG-ESCs
rtTA
EsrrG
TRE
Target genes
ER-RE
Dox


B
Day 0
2
4
6
ESC
EB
pMN
MN
Dox
+Retinoic acid +Shh


C
Hb9/Flag
Hb9
Flag
DAPI
3xflag-iEsrrG
- Dox
+ Dox

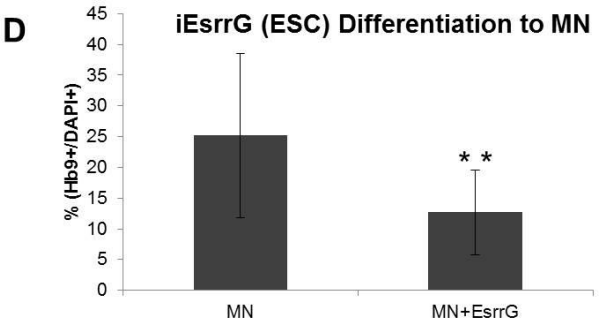

D
iEsrrG (ESC) Differentiation to MN
% (Hb9+/DAPI+)
45
40
35
30
25
20
15
10
5
0
* *
MN
MN+EsrrG

Figure 5. EsrrG induction impairs proper motor neuron formation during ESC differentiation to MN

(A) Doxycycline inducible EsrrG ESC (iEsrrG) line construct.

(B) Scheme of differentiation of ESC to MN using RA and Shh agonist Purmorphamine.

(C) Upon dox treatment, EsrrG is detected using flag antibody and marked reduction in MN marker Hb9 (bottom) is observable compared to Dox-negative sample (top).

(D) Quantitative analysis shows that EsrrG induction leads to very significant decrease in Hb9 ($P=0.00134$, $^{**}P<0.005$).

6. Notch4 as a potential target of EsrrG

From the previous ChIP-seq result of MN-hexamer which identified genes containing MN-hexamer binding site (HxRE) and Estrogen Related Receptor binding site (ERRE), Notch4, Mib2, and EsrrB were chosen (Lee et al 2012). Notch signaling has been studied extensively in many model organisms such as mice, drosophila, and zebrafish; and is known to play key roles in cell fate diversification, cell fate decision, and neurogenesis (Cau et al., 2009). In zebrafish, existence of Notch signaling has been shown to suppress pMN formation and induce KA interneuron formation, but relatively little is known about Notch4 signaling (Shin et al., 2007). RT-PCR was conducted using inducible 3xflag-EsrrG ESC line that was differentiated into neurons, by treatment of RA only, or motor neurons, by treatment of RA and Purm, as described previously. Interestingly, in neuronal context, dox treatment resulted in increased Notch4 gene expression (Figure 6A). Also, consistent with previous immunohistochemistry results in Figure 5, RT-PCR also showed decreased MN markers Olig2 and Hb9 when EsrrG is induced during MN differentiation (Figure 6A). Induction of GATA3 during neurogenesis by RT-PCR was not observed, however. Additionally, V2c interneuon marker Sox1 was also upregulated in neurogenesis upon EsrrG induction (Figure 6A). We next strived to better understand molecular mechanistic interaction through Chromatin Immunoprecipitation and GFP reporter assay.

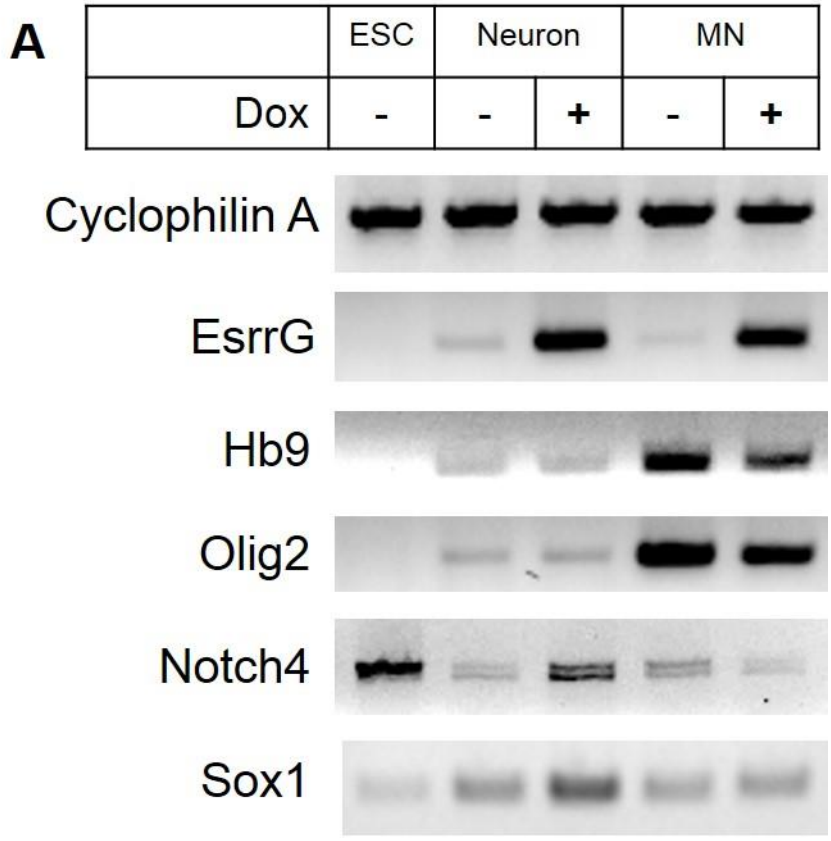


Figure 6. Notch4 as a potential downstream target of EsrrG

(A) RT-PCR of key neuronal markers in ESC state, neuronal state and MN state without or with EsrrG induction.

7. Molecular mechanistic study of EsrrG reveals that it binds to enh-Notch4 ERRE during MN differentiation

Chromatin Immunoprecipitation (ChIP) is a technique that allows one to study physical interaction between a protein of interest, usually a transcription factor, and the DNA elements which it binds *in vivo* (Kuo et al, 1999). ChIP was performed using doxycycline inducible 3xflag-iEsrrG ESC line in MN context. EsrrG binding to ERRE of Notch4-enh was confirmed by PCR in MN context (Figure 7A). msCDH1-enh including ERRE region was used as a positive control, and EsrrG's binding to ERRE of msCDH1-enh was best observable in ESC context (Tiraby et al., 2011; data not shown). Interestingly, PCR of Hb9-enhancer m250-luc showed minimal binding of EsrrG during MN differentiation (Figure 7C). Quantitative analysis using qPCR showed that EsrrG was enriched 4.84 times more at enh-Notch4 during MN differentiation in dox treated sample compared to dox negative sample (Figure 7D). Thus, we validated that EsrrG physically binds to ERRE of enh-Notch4 during MN differentiation.

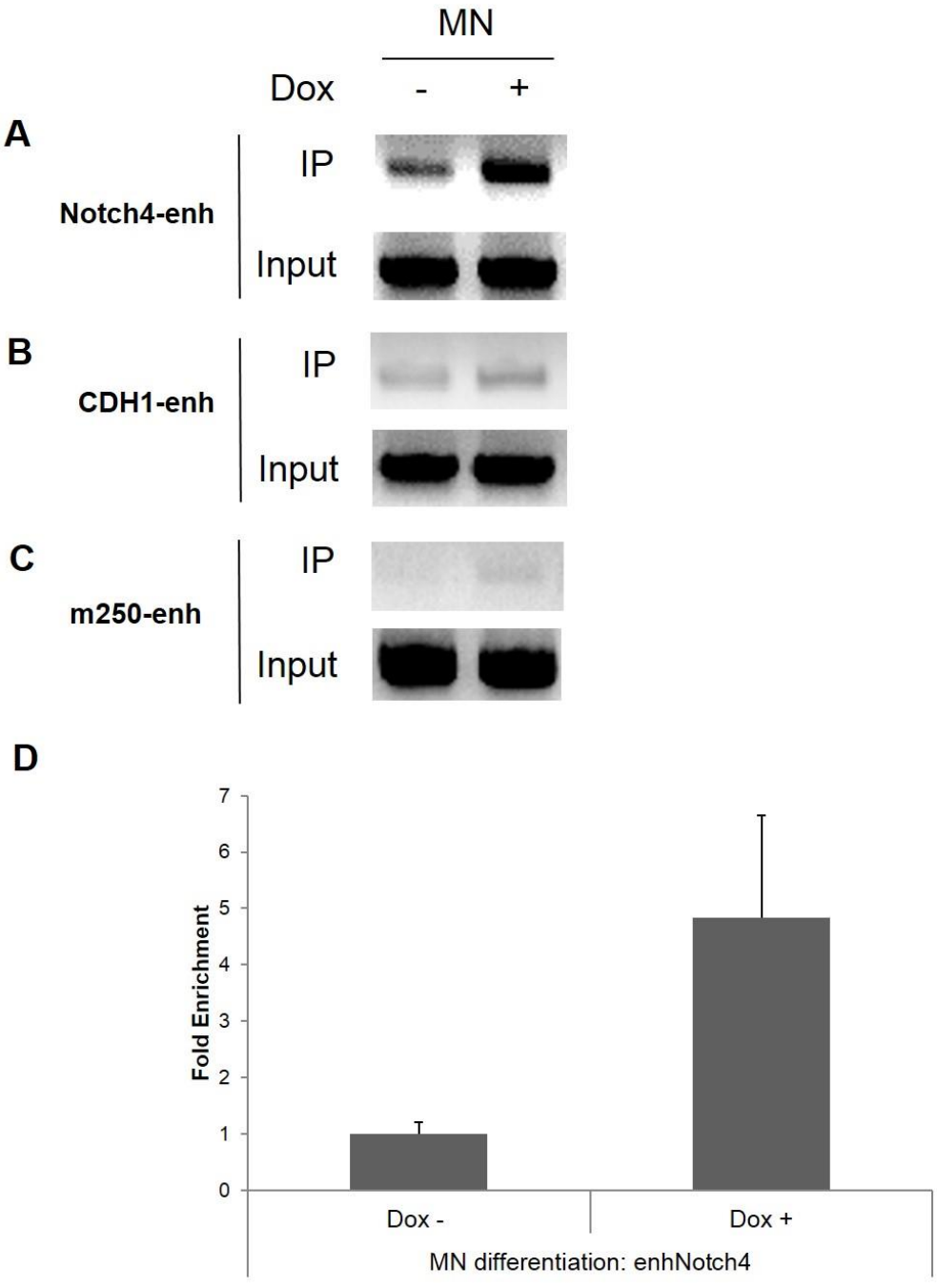

MN
Dox
-
+
A
Notch4-enh
IP
Input
B
CDH1-enh
IP
Input
C
m250-enh
IP
Input
D
Fold Enrichment
0
1
2
3
4
5
6
7
Dox -
Dox +
MN differentiation: enhNotch4

Figure 7. Molecular mechanistic study of EsrrG reveals that EsrrG physically binds to enh-Notch4 ERRE elements.

(A) EsrrG binds to ERRE of enh-Notch4 during MN differentiation.

(B) As control, CDH1 intron including ERRE region was selected.

(C) m250-enh during MN differentiation shows minimal binding of EsrrG.

(D) Quantitative analysis of enrichment of EsrrG on enh-Notch4 ERRE during motor neuron differentiation.

8. EsrrG activates promoter strength of enh-Notch4

Next we assessed whether EsrrG was capable of turning on promoter strength of enh-Notch4. From the ChIP-seq of MN hexamer, putative MN-hexamer binding site (HxRE-short) and EsrrG binding site (ERRE) were identified upstream of Notch4 coding region (Lee et al 2014). Previously in figure 7, EsrrG's binding on ERRE of enh-Notch4 was observed. To further test whether binding of EsrrG on ERRE of enh-Notch4 leads to promoter activation, GFP reporter assay was performed. Since MN-hexamer is a dimeric protein, at least two copies of HxRE sites are needed to see if the site is functionally active (Lee et al 2012). Thus, two copies of the enhancer region were cloned into GFP reporter vector (Figure 8A). Injection of enh-Notch4 (2x)-GFP alone led to minimal GFP detection, and addition of EsrrG led to strong expression of GFP where EsrrG is expressed (Figure 8B). Interestingly, injection of Isl1 and Lhx3 with enh-Notch4 (2x)-GFP did not lead to GFP activation (data not shown). In addition, injection of enh-Notch4 (2x)-GFP with Isl1, Lhx3, and EsrrG did not lead to further enhancement of GFP compared to addition of EsrrG only (data not shown). These results were consistent with previous data and further supported that Notch4 has a potential as a direct downstream target of EsrrG.

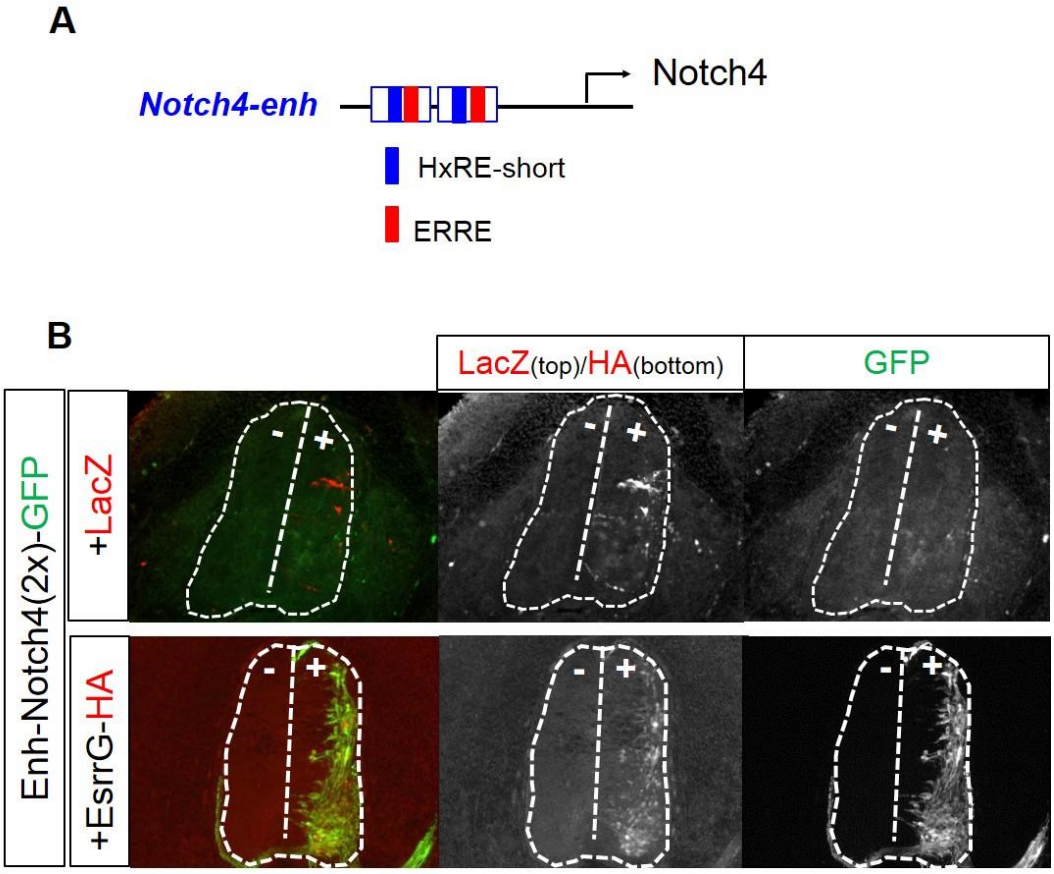


Figure 8. EsrrG activates promoter strength of enh-Notch4.

(A) Two copies of upstream region of Notch4 containing MN hexamer binding site (HxRE) and Estrogen Related Receptor binding site (ERRE) were cloned into GFP reporter vector.

(B) GFP reporter assay shows significant activation of GFP in region injected with EsrrG-HA, suggesting that EsrrG turns on promoter strength of enh-Notch4.

IV. Discussion

Due to the aging society and increased frequency of neurodegenerative diseases, a better understanding of how diverse neuronal subtypes are generated from relatively uniform neural precursor cells will be instrumental in developing treatments for nervous system diseases. This study identified, for the first time, a novel role EsrrG plays in promoting GATA3 fate specification and suppressing MN formation. In addition, this study also hints at Notch4 as a potential mediator acting downstream of EsrrG for the first time. As can be seen in figure 2, although significant proportion of GATA3 population co-localizes with EsrrG cell population, almost half of GATA3 remain EsrrG negative. Similarly, since EsrrG exists in broader domain than GATA3 population, EsrrG may play additional roles concerning neuronal development other than V2b IN specification. These additional roles of EsrrG across developmental stages are yet to be discovered and will be the subject for future studies. Also, since loss of EsrrG in EsrrG-CKO mice did not lead to complete loss of GATA3, there may be other compensatory factors that could substitute for the role of EsrrG. For example, EsrrB, which shares sequence similarity in the DNA binding domain to that of EsrrG, could have dual role with EsrG in establishing neuronal fate specification. Although not shown in this dissertation, generation of EsrrB and EsrrG conditional KO mice are in progress. Next, we aim to assess effect of deletion of both EsrrB and EsrrG at neual progenitor stage during mice spinal cord development. This study was able to differentiate iEsrrG ESC to motor neurons, since its differentiation context has been well studied. If conditions for

differentiating ESC to V2b interneurons are discovered, it would be very interesting to see the effect of turning on EsrrG during differentiation of ESC to V2b IN. We were able to identify only Notch4 as a putative target of EsrrG, but we cannot exclude the possibility of other factors that are regulated by EsrrG, which could, cooperatively or independently with Notch4, help EsrrG exert its effect in spinal cord neuronal development. Since Notch4 is not expressed in chick, we could not show that overexpression of EsrrG leads to ectopic expression of Notch4. Further molecular mechanistic study involving whether or not Notch4 is indeed a chief factor responsible for binary cell fate decision inducing GATA3 and suppressing Hb9 are necessary to complete the story.

V. References

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요약 (국문초록)

EsrrG: 운동신경억제와 V2b IN 분화에의 영향

중추신경계는 뇌와 척수로 구성되어 있고 몸으로 전달되는 감각세포들의 시그널은 중간 신경세포로 전달되어 시냅스를 형성하고 다양한 정보들을 모아 운동신경에 전달함으로써 운동을 조절한다. 많은 퇴행성신경질환들은 치료방법이 밝혀지지 않은 것들이 많은데 이는 정확한 기전이 밝혀지지 않은 질병들이 많기 때문이다. 세포치료방법이나 약물을 연구하고 개발하는 데에는 이처럼 기초과학 연구가 필수적이다. Motor neuron hexamer 는 세포의 운명을 운동신경세포로 결정 하는데 중요한 전사인자로 밝혀졌다. 본 연구에서는 ligand 가 밝혀지지 않으며 상시 활성화되어 있는 전사인자인 Estrogen Related Receptor Gamma (EsrrG)가 Motor neuron hexamer 의 타겟인지, 그리고 척수내에서 운동신경세포와 중간신경세포의 바운더리를 결정하는데 어떤 역할을 하는지 밝히고자 하였다. Chick embryo에서 EsrrG 가 과발현 되었을때와 mouse embryo에서 EsrrG 가 early neural progenitor 단계에서 제거되었을 때의 양상을 통해 EsrrG 는 운동신경세포의 마커인 Hb9 이나 Isl1을 억제하고 V2b interneuron 마커인 GATA3를 활성화시킨다는 것을 알 수 있었다. 이 결과는 줄기세포에서 EsrrG 가 doxycycline 에 의해 운동신경세포 분화전 발현되게 된 경우 운동신경 형성을 방해한다는 것과 일치하였다. Chromatin Immunoprecipitation을 통해 EsrrG response elements (ERRE) 가 있는 putative genes 중에서 EsrrG 가 실제로 운동신경세포 분화도중 Notch4 의

ERRE 에 binding 하여 Notch4 의 promoter를 활성화시킨다는 것을 밝혀내었다.

주요어 : EsrrG, motor neuron, V2b interneuron, GATA3, Notch4, neurogenesis

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