Molecules and Cells

Goosecoid Controls Neuroectoderm Specification via Dual Circuits of Direct Repression and Indirect Stimulation in *Xenopus* Embryos

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Spemann organizer is a center of dorsal mesoderm and itself retains the mesoderm character, but it has a stimulatory role for neighboring ectoderm cells in becoming neuroectoderm in gastrula embryos. *Goosecoid* (*Gsc*) overexpression in ventral region promotes secondary axis formation including neural tissues, but the role of *gsc* in neural specification could be indirect. We examined the neural inhibitory and stimulatory roles of *gsc* in the same cell and neighboring cells contexts. In the animal cap explant system, *Gsc* overexpression inhibited expression of neural specific genes including *foxd4l1.1*, *zic3*, *ncam*, and *neurod*. Genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) and promoter analysis of early neural genes of *foxd4l1.1* and *zic3* were performed to show that the neural inhibitory mode of *gsc* was direct. Site-directed mutagenesis and serially deleted construct studies of *foxd4l1.1* promoter revealed that Gsc directly binds within the *foxd4l1.1* promoter to repress its expression. Conjugation assay of animal cap explants was also performed to demonstrate an indirect neural stimulatory role for *gsc*. The genes for secretory molecules, Chordin and Noggin, were up-regulated in *gsc* injected cells with the neural fate only achieved in *gsc* uninjected neighboring cells. These experiments suggested that *gsc* regulates neuroectoderm formation negatively when expressed in the same cell and positively in neighboring cells via soluble factors. One is a direct suppressive circuit of neural genes in *gsc* expressing mesoderm cells and the other is an indirect stimulatory circuit for neurogenesis in neighboring ectoderm cells via secreted BMP antagonizers.

Keywords: chordin, dorsal organizer, Gsc, Gsc response element, neuroectoderm, Noggin, transcriptional regulation, *Xenopus*

INTRODUCTION

Spemann organizer has been established as a center of dorsal mesoderm in the early embryo; this is demonstrated by the organizer transplantation into the ventral side of another embryo, resulting in duplication of the body axis including the head and trunk (Cho et al., 1991; De Robertis et al., 2000; Nieto, 1999). In the same experiment, the duplicated complete axis contained well-organized body patterning of all three germ layers. This structural patterning of Spemann organizer was considered to be due to instructive signaling to induce neuroectoderm formation from the ectoderm. However, search for such instructive signal(s) led to the unexpected finding that instead of providing instructive signals, inhibitory signals emanate from the organizer. The

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main organizer genes including *chordin*, *noggin*, *dkk*, and *cerberus* have shown to be inhibitory for instructive signals such as Bmps, Wnts and Nodals (De Robertis et al., 2000; Nieto, 1999; Roskoski, 2020). Also for the organizer in frog, chick, mouse and fish, the organizer is dually active in neural and mesodermal specification. This is demonstrated with the transplanted organizer altering the fate of adjacent tissue/cells and modifying the dorsoventral and anteroposterior pattern of the developing embryos while the organizer maintains its own dorsal mesoderm property as the transplanted tissues always differentiate into chordamesoderm, lastly the notochord (Beddington and Robertson, 1999; De Robertis et al., 2000; Harland and Gerhart, 1997; Lemaire and Kodjabachian, 1996; Nieto, 1999; Yao and Kessler, 2001). Thus far, the mechanism(s) behind the aforementioned dual characteristics of the organizer have remained largely undefined.

Consistent with the conserved natured of the organizer across species, a conserved group of genes have been identified associated with the organizer. One of the first genes identified in the organizer was the homeobox transcription factor (TF) *goosecoid* (Cho et al., 1991). *Gsc* is one of the earliest genes expressed in the organizer and it replicates the inductive activities of the Spemann organizer found across the animal phyla, from hydra to human (Cho et al., 1991; Yao and Kessler, 2001), and a gain-of-function experiment by *gsc* injection to the ventral side remarkably mimics organizer function with complete axis duplication at the later stage (Cho et al., 1991). *Gsc* transcriptionally represses target genes including *wnt8a* and BMP signaling components identified in mouse, zebrafish and *Xenopus* (Artinger et al., 1997; Christian and Moon, 1993; Dixon Fox and Bruce, 2009; Fainsod et al., 1994; Latinkic and Smith, 1999; Latinkic et al., 1997; Seiliez et al., 2006; Steinbeisser et al., 1995; Yasuo and Lemaire, 2001). Binding of Gsc to DNA is via a paired-type homeodomain and it contains a repressor domain composed of a conserved N-terminal heptapeptide, also known as the engrailed homology domain of Gsc. As such, Gsc functions as a transcriptional repressor with several studies demonstrating a repressor activity for Gsc in cell culture and also in developing embryos (Danilov et al., 1998; Ferreiro et al., 1998; Latinkic and Smith, 1999; Mailhos et al., 1998; Smith and Jaynes, 1996).

Gsc is also one of the organizer genes responsible for dorso-ventral patterning (Niehrs et al., 1993). Introduction of *gsc* leads to dorsalization of embryos, while knock-down of *gsc* prevents head formation in *Xenopus* (Steinbeisser et al., 1995) and zebrafish embryos (Rivera-Perez et al., 1995). The expression pattern and repressor function of *gsc* suggest that *gsc* represses the genes interfering in the function of organizer or/or dorsal mesoderm differentiation (such as formation of chordamesoderm) (Christian and Moon, 1993; Fainsod et al., 1994; Steinbeisser et al., 1995). However, the direct target gene(s) of Gsc are still largely unexplored, especially for neuro-ectoderm specification. Although Gsc is a repressor TF, it also promotes neural fate of the neighboring dorsal ectodermal cells, leading to neuro-ectoderm in a "noncell-autonomous" manner (a small number of injected cells orchestrating additional uninjected neighboring cells). It is known that Gsc induces ectopic expression of *chordin* but not that of *noggin* (Sasai et al., 1994). *Chordin* and *noggin* are Bmp antagonizers which promote ectoderm to neuroectoderm formation (Kuroda et al., 2004; Sasai et al., 1994). With the cell-autonomous and noncell-autonomous regulation of the organizer genes in neuroectoderm and mesoderm specification requiring further study, *gsc* is a good candidate gene for such studies.

Foxd4l1.1 is a forkhead/winged helix TF that functions in a variety of differentiation processes (Jackson et al., 2010; Katoh et al., 2013; Katoh and Katoh, 2004; Pohl and Knochel, 2005). *Foxd4l1.1* is one of the earliest genes expressed in the neuro-ectoderm as an upstream gene for various neural regulatory networks and it maintains the neural fate during gastrulation (Yan et al., 2009). *Foxd4l1.1* functions to maintain undifferentiated neuro-ectoderm through suppression of neural inhibitory Bmp/Ventx1.1 axis and neural differentiation towards the neural plate (Fetka et al., 2000; Lee et al., 2009; Sullivan et al., 2001; Yan et al., 2009; Yu et al., 2002). Inhibition of Bmp also induces neuro-ectoderm specific earliest genes, *foxd4l1.1* and *zic3*, in animal cap (AC) explants (Shim et al., 2005; Umair et al., 2018; Yoon et al., 2013; Yu et al., 2016). *Foxd4l1.1* transcription is necessary to maintain the neuro-ectoderm. In addition, *Foxd4l1.1* expression is up-regulated by over-expression of *siamois* and *noggin* (Sullivan et al., 2001) and down-regulated by neural inhibitory axis of Bmp-Ventx1.1 axis in ventral mesoderm and ectoderm (Yoon et al., 2014). Study of the transcriptional repression of *foxd4l1.1* in the organizer (dorsal mesoderm) is still pending for identifying a role in proper patterning and maintenance of dorsal mesoderm (chordamesoderm) in normal embryogenesis.

In the present study, we hypothesized that organizer gene *gsc* displays the dual activity of being neural inhibitory as to maintain dorsal mesoderm in a cell-autonomous manner and being neural stimulatory to convert neighboring ectoderm to neuroectoderm in a noncell-autonomous manner. As expected, *Gsc* co-expression inhibited dominant negative BMP receptor (DNBR) and *chordin/noggin* mediated early and late neural-specific genes including *foxd4l1.1*, *zic3*, *ncam*, *neurod*, *ngnr*, and *otx2* in AC explants. A genome-wide *Xenopus* chromatin immunoprecipitation sequencing (ChIP-seq) analysis of Gsc and promoter analysis of *foxd4l1.1* showed that Gsc directly binds to the Gsc response cis-acting element (GRE) within the 5'-flanking upstream region of *foxd4l1.1* and represses *foxd4l1.1* transcription. Reporter gene assay and site-directed mutagenesis of *foxd4l1.1* promoter constructs defined the GREs as directly inhibiting *foxd4l1.1* transcription in *Xenopus* embryos. Noncell-autonomous and stimulatory roles of *gsc* in neuroectoderm formation were confirmed with conjugation assays of AC explants of *gsc*-injected and non-injected ACs. Collectively, these results suggest that Gsc is a direct repressor of *foxd4l1.1* transcription in order to inhibit neurogenesis in the organizer/dorsal mesoderm while to promote neuroectoderm formation of dorsal ectoderm cells via *gsc*-stimulated positive regulation of *chordin* and *noggin*.

MATERIALS AND METHODS

Ethics statement

This animal study was conducted in accordance with the reg-

ulations of the Institutional Animal Care and Use Committee (IACUC) of Hallym University (Hallym 2019-79, 2019-80). All the research members attended both the educational and training courses for the appropriate care and use of experimental animals at our institution in order to receive an animal use permit. Adult *Xenopus laevis* were grown and tended in approved containers, maintained at a 12-h light/dark (LD 12:12 h) cycle and 18°C ambient temperature, by authorized personnel and according to the guidelines of the Institute of Laboratory Animal Resources of Hallym University for laboratory animal maintenance.

DNA and RNA preparation

All mRNA used for this study were synthesized by linearizing the target vectors with the appropriate restriction enzymes, including Sp6/SacII *for flag-gsc*, Sp6/Acc651 for *flag-chordin*, Sp6/Not1 for *noggin* and Sp6/EcoR1 for DNBR constructs. Each vector was linearized with the appropriate restriction enzyme and used for *in-vitro* transcription using the MEGA script kit according to the manufacturer's instructions (Ambion, USA). Synthetic mRNAs were quantified by a spectrophotometer at 260/280 nm wavelengths (SpectraMax; Molecular Devices, USA).

Cloning of genomic DNA

Cloning of *Foxd4l1.1* and *zic3* genomic DNA (gDNA) into the pGL3-Basic plasmid (Promega, USA) was performed as described previously (Lee et al., 2004).

Promoter constructs

The 5'-flanking region of reporter construct of *foxd4l1.1* (1.5 kb) and *zic3* (1.8 kb) in the pGL3-basic plasmid (Promega) were made by using specific restriction sites, *Kpn*I/*Xho*I and *Kpn*I/*Hin*dIII, respectively. Serially-deleted promoter constructs of *foxd4l1.1* were generated and sub-cloned into a pGL3-basic plasmid by polymerase chain reaction (PCR) amplification (Table 1).

Embryo injection and explants culture

X. laevis were obtained from the Korean *Xenopus* Resource Center for Research (Korea). *Xenopus* embryos were injected after *in vitro* fertilization of oocytes, induced by injection of 500 units of human chorionic gonadotropin (Sigma, USA). RNAs were injected into the animal pole at the one or 2-cell stage embryos and cultured in 30% Marc's Modified Ringer's (MMR) solution. The ACs were then dissected from injected and un-injected embryos at stage 8.0-8.5 and incubated in 1X L-15 growth medium (Gibco/Thermo Fisher Scientific, USA) until stage 11 and 24 in preparation for reverse transcription PCR (RT-PCR).

RNA isolation and RT-PCR

DNBR mRNA (0.5 ng/embryo), *chordin* mRNA (0.5 ng/embryo), *noggin* (0.5 ng/embryo), and *gsc* (1 ng/embryo) were injected into the animal pole at the one or 2-cell stage of *Xenopus* embryos and cultured in 30% MMR solution. ACs were then dissected from the injected and un-injected embryos and incubated until stage 11 and 24 in 1X L-15 growth medium as described previously (Kumar et al., 2019). Total RNA was isolated from whole embryos and AC explants using RNA-Bee reagent, following the manufacturer's instructions (Tel-Test, USA), and was then treated with DNase I to remove genomic DNA contamination. RT-PCR was performed with Superscript II (Invitrogen, USA) as described by the manufacturer and with 2 μg total RNA per reaction. PCR was performed according to the following conditions: 30 s at 94 °C, 30 s at each annealing temperature and 30 s at 72°C, all for with 20-30 cycles of amplification (Table 2).

Morpholino oligomers and activin treatment

Antisense morpholinos (MOs) for *Chordin* and *Gsc* were obtained having similar MOs sequences as previously reported (Oelgeschlager et al., 2003; Sander et al., 2007). Nog-MO1 had the sequence (5'-*ATTTTGTGCAGCTGTGTGCAGCATG*) and Nog-MO2 had the sequence (5'-*TGAAAGTGAAGAATATTTAAGAGA*). All MOs were obtained from Gene Tools (USA). For use, the MOs were diluted in sterile water and then heated at 65°C for 10 min before microinjections as described previously (Ryu et al., 2021). The harvested ACs were treated with activin (25 ng/ml) as described previously (Umair et al., 2020).

Whole mount *in situ* hybridization

Embryos were injected with mRNAs as indicated and subsequently processed for whole-mount *in situ* hybridization using standard methods with anti-sense probes for Foxd4l1.1 (Moore et al., 2004).

Luciferase assays

Relative promoter activities were measured using a luciferase assay system according to the manufacturer's instructions (Promega) and were performed as previously described

Table 1. Primers used for serially-deleted FoxD5b reporter gene constructs

Primer	Primer name	Sequence
Upstream primer	Foxd4l1.1(-1551)	5'-CCGGTACCTAGAGGTTGGATAAAGTCAATTGC-3'
	Foxd4l1.1(-1316)	5'-CCGGTACCTATATGCAGAGCTGCTAATAGTC-3'
	Foxd4l1.1(-1016)	5'-CCGGTACCTATATGCAGAGCTGCTAATAGTC-3'
	Foxd4l1.1(-816)	5'-CCGGTACCTAGAATTCCAGTTCCCATAATC-3'
	Foxd4l1.1(-301)	5'-CCGGTACCTTGGATTGCAAGTTAGTGGCTC-3'
	Foxd4l1.1(-186)	5'-GGGGTACCTTCATTCAGCAAAAGCACAGCC-3'
	Foxd4l1.1(-78)	5'-GGGGTACCAATTCAAGTGCAGATGACTGCC-3'
Downstream primer	Foxd4l1.1-R	5'-ATCTCGAGGCTTGGTTGGCAGTAAGTAG-3'

Table 2. Primers used for RT-PCR amplification

Gene name	Sequence	Annealing temperature (°C)	Cycle
zic3	F5'-TCTCAGGATCTGAACACCT-3' R5'-CCCTATAAGACAAGGAATAC-3'	45	28
Foxd4l1.1	F5'-ACTCTATCAGGCACAACCTGTC-3' R5'-GGTCTGTAGTAAGGCAGAGAGT-3'	50	30
xbra	F5'-GGATCGTTATCACCTCTG-3' R5'-GTGTAGTCTGTAGCAGCA-3'	57	25
Fgf8a	F5'-CTGGTGACCGACCAACTAAG-3' R5'-TGCGAACTCTGCTTCCAAAC-3'	55	28
chch	F5'-ATGTGCGGAGGCTGCGTC-3' R5'-CGTGGGTCATCGGGTAGAAC-3'	60	27
ascl1	F5'-GAGCTGATGAGGTGCAAGAG-3' R5'-TTTGCTCATCTTCTTGTTGG-3'	60	27
ncam	F5'-CACAGTTCCACCAAATGC-3' R5'-GGAATCAAGCGGTACAGA-3'	57	29
xngnr1	F5'-GGATGGTGCTGCTACCGTGCGAGTACC-3' R5'-CAAGCGCAGAGTTCAGGTTGTGCATGC-3'	65	30
otx2	F5'-GGATGGATTTGTTGCACCAGTC-3' R5'-CACTCTCCCAGCTCACTTCTC-3'	57	27
xk81	F5'-TGGTGTTGAACAAGTGCAGG-3' R5'-ACCTCCTCGACAATGGTCTT-3'	57	25
krox20	F5'-AACCGCCCCAGTAAGACC-3' R5'-GTGTCAGCCTGTCCTGTTAG-3'	57	28
neurod	F5'-GTGAAATCCCAATAGACACC-3' R5'-TTCCCCATATCTAAAGGCAG-3'	57	28
HoxB9	F5'-TACTTACGGGCTTGGCTGGA-3' R5'-AGCGTGTAACCAGTTGGCTG-3'	68	26
gsc	F5'-GCTGATTCCACCAGTGCCTCACCAG-3' R5'-GGTCCTGTGCCTCCTCCTCCTCCTG-3'	60	30
noggin	F5'-AGTTGCAGATGTGGCTCT-3' R5'-AGTCCAAGAGTCTGAGCA-3'	57	27
chordin	F5'-TTAGAGAGGAGAGCAACTCGGGCAAT-3' R5'-GTGCTCCTGTTGCGAAACTCTACAGA-3'	57	25
bmp4	F5'-CATCATGATTCCTGGTAACCGA-3' R5'-CTCCATGCTGATATCGTGCAG-3'	57	25
ventx1.1	F5'-CCTTCAGCATGGTTCAACAG-3' R5'-CATCCTTCTTCCTTGGCATC-3'	57	26
ef1α	F5'-CCTGAATCACCCAGGCCAGATTGTG-3' R5'-GAGGGTACTCTGAGAAAGCTCTCCACG-3'	57	19
odc	F5'-GTCAATGATGGAGTGTATGGATC-3' R5'-TCCATTCCGCTCTCCTGAGCAC-3'	55	25

Table 3. Primers used for site-directed mutagenesis

Mutated site	Primer name	Sequence
GRE	Foxd4l1.1mGRE	F5'-GGACCCTCTCACGTGGGAGCTTATCTGATAR-3' R5'-TATCAGATAAGCTCCCACGTGAGAGGGTCC-3'

(Yoon et al., 2014). Five different groups of embryos (3 embryos per group) were harvested and homogenized in 10 μl lysis buffer per embryo. Embryo homogenates at 10 μl each were assayed with 40 μl luciferase substrate and the reporter gene activity was read by an illuminometer (Berthold Technologies, Germany). All experiments were repeated at least three times for independently derived sample sets.

Site-directed mutagenesis

Mutagenesis was performed by a site-directed mutagenesis kit (Muta-Direct; iNtRON Biotechnology, Korea) using primer oligonucleotides in accordance with the manufacturer's instructions (Table 3).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation assay was performed as described previously (Blythe et al., 2009). Embryos were injected at one-cell stage with mRNA encoding 3Flag-Gsc (1 ng/embryo). Injected embryos were collected at stage 11 (100 embryos/sample) and processed according to protocol. Anti-Flag monoclonal antibodies (F-1804; Sigma) or normal mouse IgG (SC-2025; Santa Cruz Biotechnology, USA) were then added to the cell lysates to immune-precipitate the chromatin. ChIP-PCR was performed with the immune-precipitated chromatin using *foxd4l1.1,* promoter region primers for GRE. These primers are shown in (Table 4).

ChIP-sequencing analysis

The Gsc mRNA (0.5 ng/embryo) was injected at the one-cell stage. Approximately 1,000 embryos were harvested at stage 11. The ChIP assay was performed accordingly to a previously described method (Blythe et al., 2009). Total immunoprecipitated chromatin was sequenced by Macrogen (Korea), and raw data with short reads were received in FASTA format. Galaxy (https://usegalaxy.org), an online tool, was used for data analysis as described previously (Zhang et al., 2008). MACS call peak data were used for visualization, and Gsc coverage within the *foxd4l1.1* and *zic3* promoter region was plotted.

Conjugation assay

AC explants were dissected at stages 8 and conjugation of the two required AC explants together was in 30% MMR containing 50 mg/ml gentamycin. The recombinants were cultured for 40 min at 16°C to heal the torn ends of the tissue explants before being transferred to fresh L-15 media.

Statistical analysis

Data were analyzed by unpaired two-tailed Student's *t*-test using GraphPad Prism9 (GraphPad Software, USA). Asterisks designations are ** for $P \leq 0.01$ and *** for $P \leq 0.001$. Non-significance was denoted as "ns".

RESULTS

Gsc suppresses transcription of early and late neural genes expressed by *dnbr*, *chordin*, and *noggin*

Previous reports demonstrate that *gsc* is expressed in the dorsal organizer and involving dorsal mesoderm cells. The mesoderm cells expressing *gsc* sustain their mesodermal identity; however, they greatly influence the interacting neighbor cells (ectoderm) to adopt the neural fate, (Cho et al., 1991; De Robertis et al., 1992). These results indicate that *gsc* has dual roles of directly inhibiting neural gene expression in mesoderm territory and indirectly stimulating neural fate in neighboring ectoderm cells. To examine the role of Gsc on neural specific gene expression, *gsc* mRNA was co-injected with or without *dnbr*, *chordin*, and *noggin*. RT-PCR results showed that *dnbr*, which is known to inhibit BMP signaling and elicit neural genes, prompted expression of early neural marker genes including *foxd4l1.1* and *zic3,* but did not increase those of organizer genes including *chordin* and *noggin* (Fig. 1A, lane 1 vs 4). While *gsc* (with or without introduced DNBR) strongly reduced the expression of early neural genes *foxd4l1.1* and *zic3* (Fig. 1A, lane 1 vs 2) as well as the neural repressor gene ventx1.1, ectopic *gsc* expression strongly induced *chordin* and *noggin* expression (Fig. 1A, lane 2 and 4). Similar results for neural gene expression were obtained when *chordin*/*noggin* combination, instead of DNBR, was injected (Fig. 1B). The ACs showed an elevated expression of *foxd4l1.1* and *zic3*. However, ACs co-injected with *gsc* abolished neural gene expression (Fig. 1B, lane 1, 2, and 4). As expected, all samples injected with *gsc, chordin*, and *noggin* showed no expression of *xbra*, *bmp4*, and *ventx1.1* (Fig. 1B, lane 1, 2, 3, and 4). These results suggest that *gsc* plays an inhibitory role in transcription of neural specific genes, which is consistent with previously known role of Gsc as a transcriptional repressor (Yao and Kessler, 2001). To investigate the consequences of *gsc* overexpression at later stage embryos, the late neural specific markers were also examined. Co-expression of *gsc* inhibited the expression of later neural genes including *ncam, neurod, xngnr1, otx2*, and *krox2* expressed by *dnbr* injection (Fig. 1C, lane 1, 2, 3, and 4) and *chordin* and *noggin* (Fig. 1D, lane 1, 2, 3, and 4). In co-injected embryos, Gsc diminished *ncam* and *neurod* expression, whereas those for *xngnr1* and *otx2* were not much affected (Fig. 1D, lane 1, 2, 3, and 4). These suggest that *xngnr1* and *otx2* may not be direct targets of Gsc. In addition, whole mount *in situ* hybridization (WISH) analysis was performed in whole embryos to examine *foxd4l1.1* expression with or without expressed *gsc*. The WISH results demonstrate that ectopic expression of *gsc* completely diminishes *foxd4l1.1* expression in the injected embryos (Fig. 1E). Taken together, these results indicate *gsc* inhibits neural gene expression while inducing *chordin* and *noggin* expression in AC explants.

Multiple Gsc response elements within *foxd4l1.1* promoter are detected using genome-wide ChIP-seq

Several independent studies demonstrate that Gsc has strong repressor activity through direct binding on cis-acting response element within target genes to block their transcription (Cho et al., 1991; Christian and Moon, 1993; Latinkic and Smith, 1999; Yasuo and Lemaire, 2001). To identify the neural specific gene(s) (*foxd4l1.1* and *zic3*) as direct target(s) of *gsc*, genome wide ChIP-seq analysis was performed using *gsc* injected gastrula embryos (stage 11). Our ChIP-seq analysis showed that Gsc bound within upstream promoter region of early neural genes (*foxd4l1.1* and *zic3*). Peak calling followed by coverage plot analysis done independently for *foxd4l1.1* and *zic3* genome sequences led to mapping of the

Table 4. Primers used for ChIP-PCR assay

Prime name	Sequence	Annealing temperature (°C)	Cycle
Foxd4l1.1ChIP-GRE	F5'-ACCTTGTTGGACTACAGATTC-3' R5'-CAGTCATCTGCACTTGAATTGG-3'	52	30

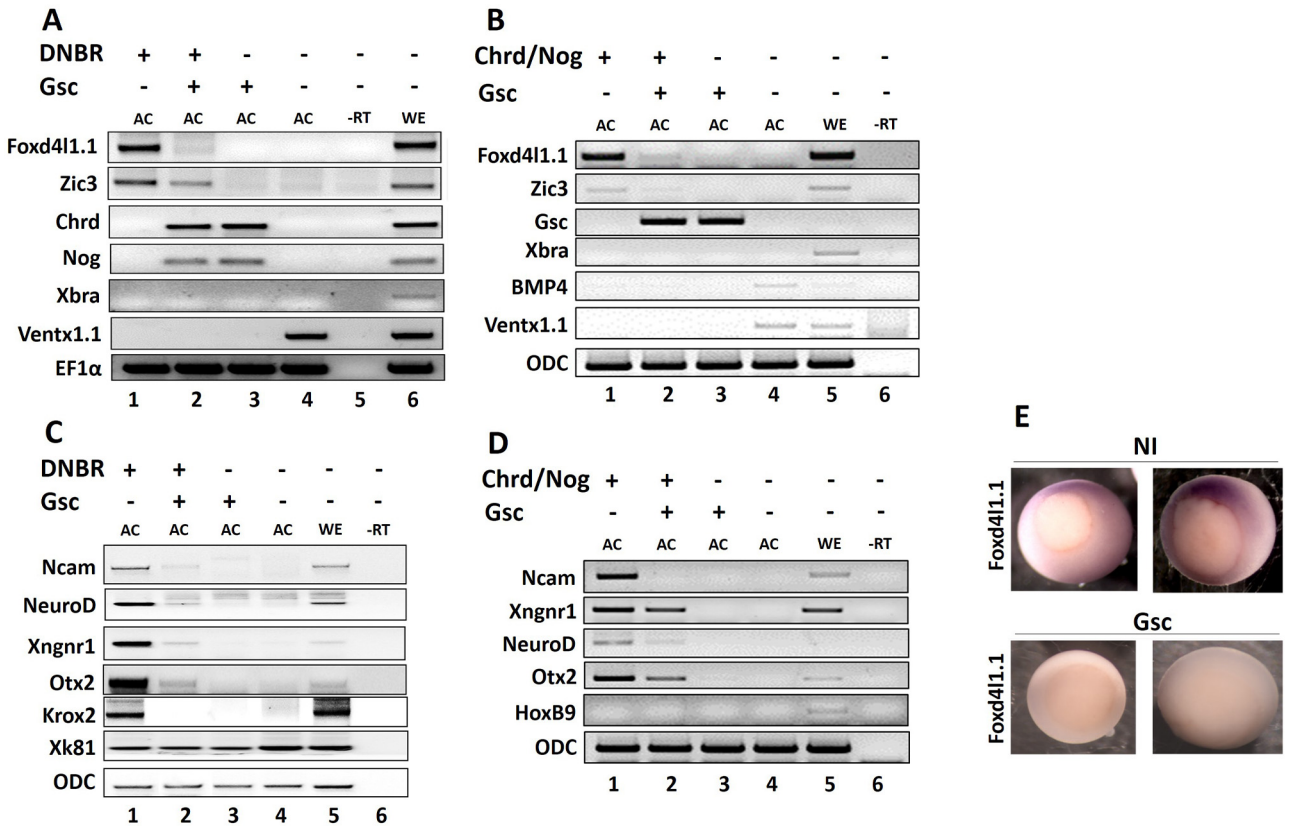

Fig. 1. Inhibition of neural gene expression either mediated by *dnbr* or *chordin* and *noggin* (Chrd/Nog) in AC explants of *Xenopus*. *3Flag-gsc* (1 ng/embryo), *dnbr* (0.5 ng/embryo), *chordin* (0.5 ng/embryo) and *noggin* (0.5 ng/embryo) (Chrd/Nog) were injected at one-cell stage and the AC explants were dissected at stage 8 to grow until (A and B) stage 11 and (C and D) stage 24. The expression profiles of different germ layer specific marker genes were analyzed by RT-PCR. No RT (no reverse transcriptase added) served as a negative control while WE (whole embryos) served a positive control. (E) *3Flag-gsc* (1 ng/embryo) mRNA was injected into one cell embryos, and the injected embryos and non-injected ones (control) were processed for whole mount *in situ* hybridization (WISH) with anti-sense *foxd4l1.1* probe at stage 11.

putative binding sites in promoter regions of *foxd4l1.1* and *zic3* genome (Figs. 2A and 2B). We then selected *foxd4l1.1* for a more detailed analysis of the repressive role of Gsc. *Foxd4l1.1* is one of the earliest neural TFs that is highly expressed in neuroectoderm to maintain neural identity (Kumar et al., 2020; Neilson et al., 2012; Sherman et al., 2017; Yan et al., 2009). *Foxd4l1.1* is one of the most critical genes responsible for neuroectoderm and ectoderm specification (Lee et al., 2014). *Foxd4l1.1* is expressed in neuroectoderm cells of BMP inhibited AC explants and overexpression of *foxd4l1.1* is sufficient to induce neurogenesis in the ectodermal explants of *Xenopus* embryos (Yoon et al., 2013). The analysis of ChIP-coverage region of Gsc revealed four putative GRE within upstream region of *foxd4l1.1* coding sequences (Figs. 2D and 2E). These results indicate that *foxd4l1.1* 5'-flanking region may contain more than one GRE to exert repressor activity. Since *X. laevis* is an allotetraploid that contains two copies of *foxd4l1.1* genome on chromosome (chr) 1l and 1s separately, the conserved regions between chr1l and chr1s were analyzed by homology search/alignment (BLAST) of both DNA sequences. Alignment results indicate conserved GRE containing regions (Fig. 2C) in both copies of *foxd4l1.1* in chr1l and 1s. The results suggest that *foxd4l1.1* and *zic3* would be the direct target(s) of Gsc, where Gsc directly binds and negatively regulates the transcription of neural genes in gastrula embryos.

Site-direct mutagenesis of GRE within *foxd4l1.1* promoter abolishes the repression activity of Gsc

As shown above, ChIP-seq results indicated putative GREs within *foxd4l1.1* and *zic3* promoter regions. To identify this functionally active GRE, 1551 bps long 5'-flanking region of *foxd4l1.1* respect to putative transcription start site (TSS) was cloned into a reporter vector named *foxd4l1.1(–1551)*. We then examined whether *gsc* indeed inhibited the relative reporter activity of *foxd4l1.1(–1551)*. *Foxd4l1.1(–1551)* was injected at one-cell stage with or without *dnbr* and *gsc*. The reporter gene assay was performed at the embryonic stage 11. As expected, *foxd4l1.1(–1551)* relative reporter activity was significantly increased by *dnbr* (2.5 fold) while co-injection with *gsc* decreased the reporter activity down to 9 fold (Fig. 3A, bar 1 to 4). To map out the functionally active GRE within the *foxd4l1.1(–1551)* promoter, serially-deleted promoter constructs were created and sub-cloned into the reporter

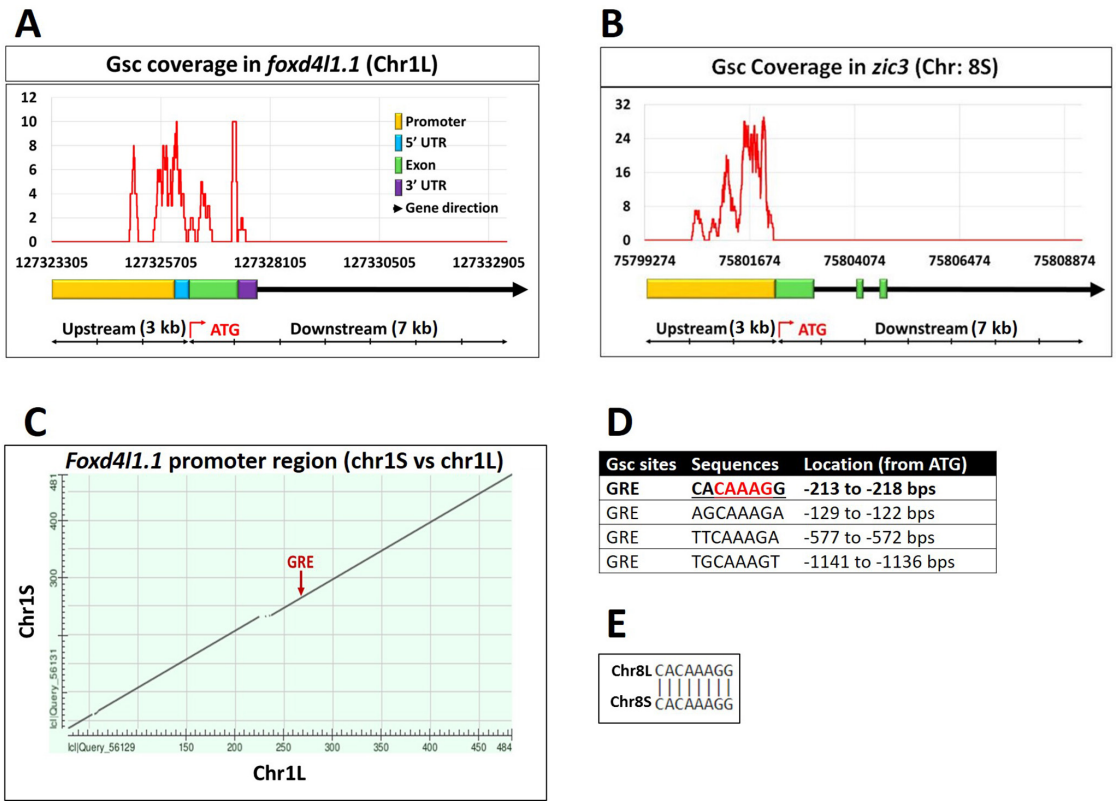

Fig. 2. Identification of GREs within the neural target genes using ChIP-seq of *3Flag-gsc* injected *Xenopus* embryos. (A and B) Coverage plot of *3Flag-gsc* within the *foxd4l1.1* and *zic3* promoter regions. (C) Similarity within both (chr1s and chr1l) copies of *foxd4l1.1* promoter region are shown. The active GRE (-213 to -218 bps) location is highlighted by an arrow. (D) Putative GREs revealed within the *foxd4l1.1* promoter region by ChIP-sequencing. (E) Consensus binding motifs of Gsc within both (chr1s and chr1l) copies of *foxd4l1.1* promoter region.

vector (Fig. 3B). All deleted constructs were then co-injected with or without *gsc* mRNA for reporter activity assays. Co-injection of *gsc* mRNA strongly reduced the relative reporter activities of all constructs from −1551 to −301 bps (by approximately 7 to 9 fold), while the reporter constructs containing −186 bps and −78 bps (*foxd4l1.1(−186)* and *foxd4l1.1(−78)*) showed no significant changes in the reporter activity assay (Fig. 3C, bar 1 to 14). The results indicate that the functional putative GRE would be between −301 to −186 bps (Fig. 3C, bar 9 to 12). Scrutinizing the sequences of *foxd4l1.1(−1551)* promoter region between −301 to −186 bps revealed one putative GRE at −218 to −213 bps (ACAAAG) from the putative TSS (Figs. 2D and 2E). To verify the GRE as an active cis-acting element for Gsc, we performed site-directed mutagenesis of GRE (*AC<u>AAA</u>G* to *AC<u>GGG</u>G*) in *foxd4l1.1(−1551)* and *foxd4l1.1(−301)* reporter constructs separately (Fig. 3D). The mutated *foxd4l1.1(−1551)mGRE*, *foxd4l1.1(−301)mGRE* and those wild type promoter constructs were then injected with or without *gsc* to evaluate the reporter activities. Results show that *gsc* mediated reduction in *foxd4l1.1(−1551)* and *foxd4l1.1(−301)* promoter activities were completely abolished in the mutated *foxd4l1.1(−1551)mGRE* and *foxd4l1.1(−301)mGRE*) constructs (Figs. 3E and 3F, lane 1 to 4). These results strongly indicate that functionally active consensus sequences for *gsc* (its GRE) was located in −218 to −213 bps region within *foxd4l1.1(−1551)* promoter where Gsc binds to inhibit transcription. We next examined whether Gsc directly bound within the proximal promoter region (−218 to −213 bps) of *foxd4l1.1*. To determine Gsc binding within *foxd4l1.1* promoter, Flag-tagged Gsc construct (*3Flag-gsc*) was injected at one-cell stage and ChIP-PCR was performed at stage 11 in whole embryos. The results confirm that Gsc directly binds within the proximal region of endogenous *foxd4l1.1* promoter (Fig. 3G). In addition, *zic3(−1805)* promoter was injected with or without *gsc* mRNA and the reporter assay was performed. As expected, the *zic3(−1805)* relative reporter activity was markedly reduced in *gsc* co-injected samples (Fig. 3H, bar 1 and 2). Taken together, these results collectively confirm that *foxd4l1.1* promoter contains a cis-acting GRE (−218 to −213 bps) where Gsc directly binds and negatively regulates *foxd4l1.1* transcription.

Gsc has dual roles of neural inhibition and stimulation in cell-autonomous and noncell-autonomous fashions, respectively

During early development, Gsc is known as a neural stimulator, probably through induction of *chordin* in a noncell-autonomous manner (Sander et al., 2007). Ectopic expression of *gsc* is known to induce a complete secondary axis including head (neural tissue) in ventral region of *Xenopus*

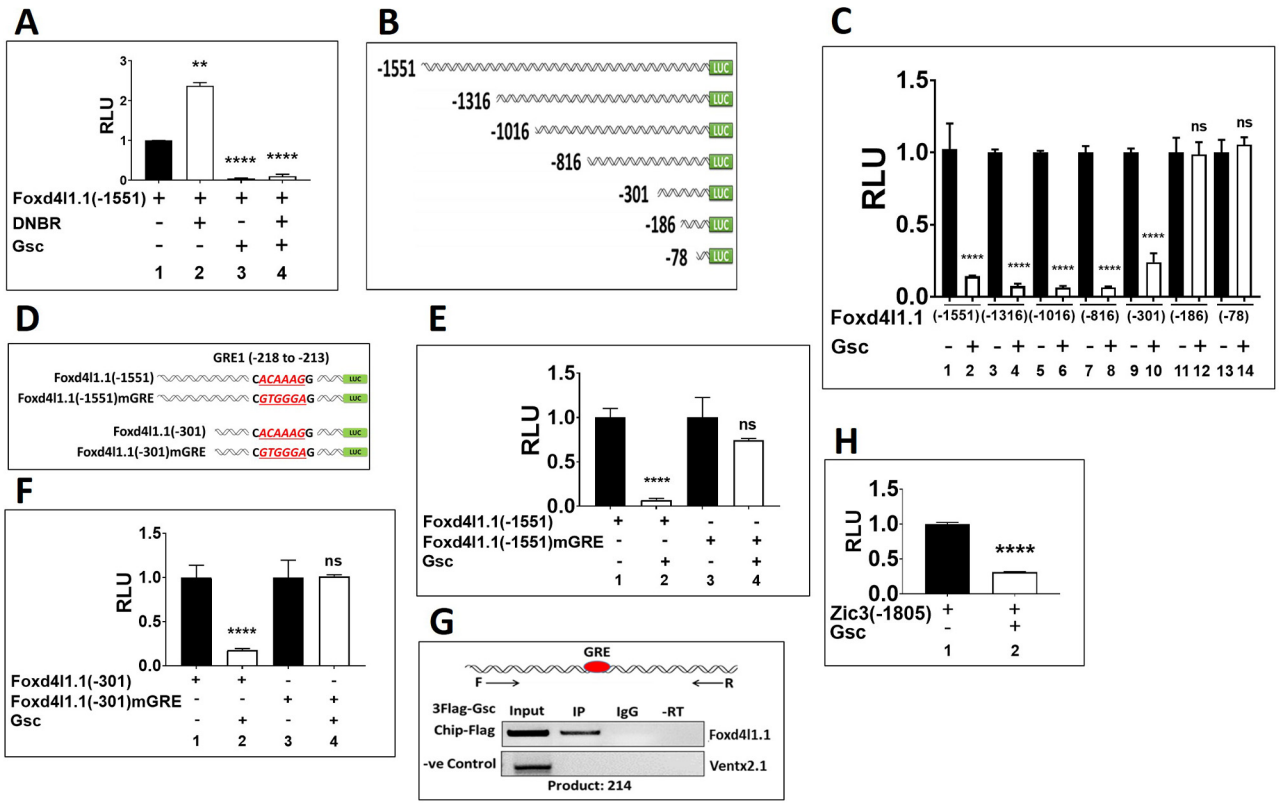

Fig. 3. Abolishment of the repressional activity of Gsc by site-directed mutagenesis of GRE within the *foxd4l1.1(-1551)* reporter construct. (A) *foxd4l1.1(-1551)* (40 pg/embryo), *dnbr* (1 ng/embryo), and *3Flag-gsc* (1 ng/embryo) were injected at one-cell stage and reporter assay was performed at stage 11. RLU, relative reporter activity. (B) Schematic representation of serially-deleted *foxd4l1.1(-1551)* promoter constructs. (C) Serially-deleted *foxd4l1.1* (40 pg/embryo) promoter constructs were injected with and without *3Flag-gsc* (1 ng/embryo) at one-cell stage and relative promoter activity were measured at stage 11. (D) Site-directed mutagenesis scheme for *foxd4l1.1(-1551)* and *foxd4l1.1(-301)* promoter constructs, target sequences highlighted (*red color and italic*). (E and F) Relative reporter assay of *foxd4l1.1(-1551)*, *foxd4l1.1(-1551)mGRE, foxd4l1.1(-301)*, and *foxd4l1.1(-301)mGRE* with or with *gsc* were performed at stage 11. (G) *3Flag-gsc* was injected at one-cell stage and ChIP-PCR was performed at stage 11. Specific primers of *Foxd4l1.1* promoter region (containing GRE) were used for amplification, while ventx2.1 served as negative control. (H) *zic3(-1805)* reporter construct was injected (40 pg/embryo) with or without *3Flag-gsc* (1 ng/embryo) and reporter assay was performed at stage 11. $^{**}P \leq 0.01$; $^{****}P \leq 0.0001$; ns, non-significance.

embryos (De Robertis et al., 1992; Latinkic and Smith, 1999; Steinbeisser et al., 1995; Ulmer et al., 2017). However, Gsc also directly binds to within the proximal promoter region (−218 to −213 bps) of the early neural gene *foxd4l1.1* to inhibit its transcription. To delineate these contradictory dual regulatory roles for Gsc in neural specification, we designed and performed a conjugation assay for AC explants. *Xenopus* AC cells are pluripotent in nature and in response to specific signals, AC cells are capable of generating distinct types of tissue, making them excellent for manipulation for developmental studies (Borchers and Pieler, 2010). We designed two different sets of conjugated ACs (described in Fig. 4A). In set-1, embryos were injected with and without *foxd4l1.1(−1551)* reporter gene separately into the embryos and ACs were then dissected at stage 8. Two ACs were then conjugated as shown in Fig. 4A. In set-2, the embryos were injected with *gsc* and *foxd4l1.1(−1551)* separately and those two separately dissected ACs were then conjugated (Fig. 4A). The relative reporter activity was then measured at embryonic stage 11. The results had the reporter activity of set-2 (*foxd4l1.1(−1551)/gsc*) being significantly higher (2.5 fold) than that of set-1 (*foxd4l1.1(−1551)*/NI) (Fig. 4B, set-1 vs set-2). These results indicated that Gsc is a stimulatory molecule for neural specification to neighboring ectoderm cells, while Gsc being a direct inhibitor of neural gene expression in cell autonomous manner. We then examined whether *gsc* would indeed inhibit the endogenous mRNA transcription of neural specific markers in conjugated AC. To ascertain this inhibitory effect of Gsc, we prepared 3 different sets of conjugated ACs (*gsc/gsc*, *gsc*/NI, and NI/NI; NI was the not injected control AC) (as depicted in Fig. 4C). The expressed genes were then analyzed by RT-PCR. As expected, AC conjugates of both *gsc* injected group (*gsc/gsc*) completely abolished or strongly reduced the expression of early neural marker genes (stage 11) including *foxd4l1.1, zic3, fgf8a, chch,* and *ascl1* (Fig. 4D, lane 2 vs 3). In contrast, in AC conjugates of *gsc* injected and

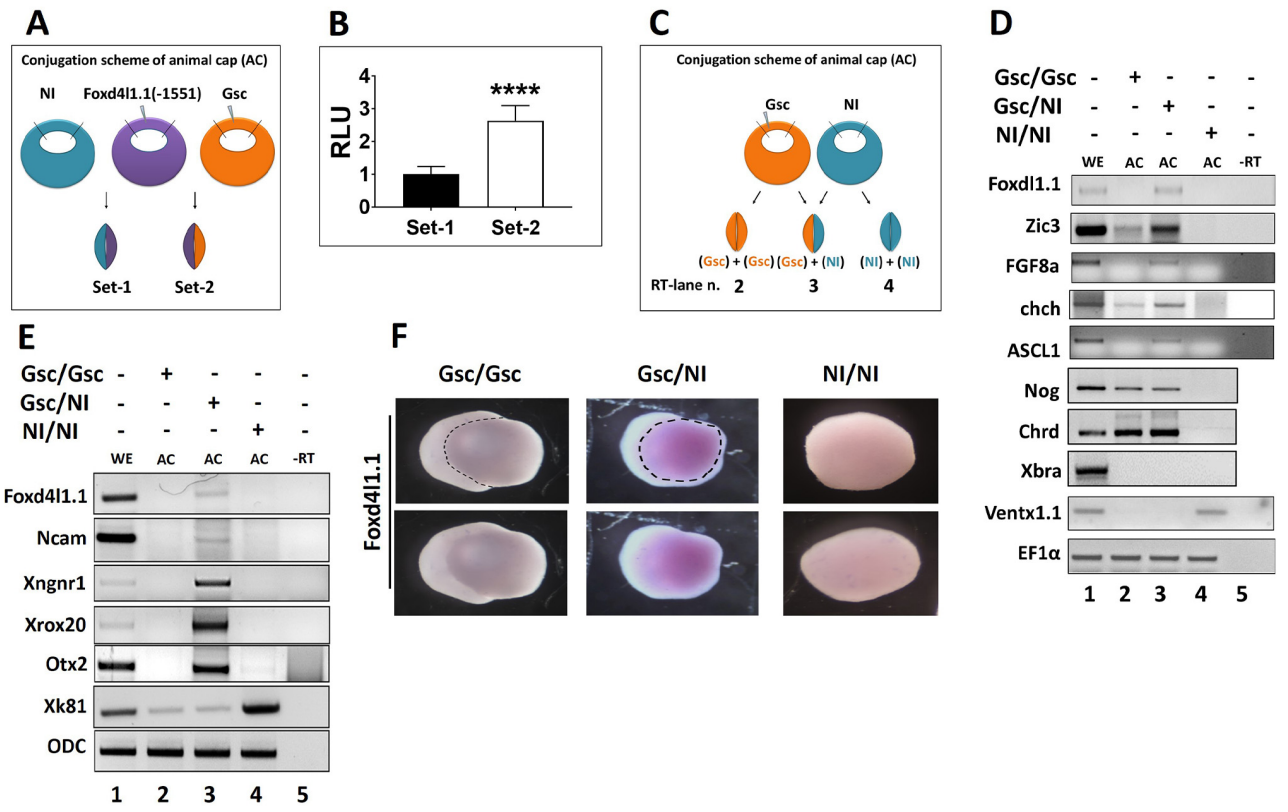

Fig. 4. A dual role of Gsc in transcriptional regulation of *foxd4l1.1*. In all experiments, *3Flag-gsc* (1 ng/embryo) and *foxd4l1.1*(*-1551*) (40 pg/embryo) were injected at one-cell stage and the ACs were dissected at stage 8 to grow until stage 11 and 24. (A) Conjugation scheme for AC explants used. (B) The relative promoter activity are measured. RLU, relative reporter activity. $****P \leq 0.0001$. (C) Schematic description of the conjugated AC explants of the embryos injected with *3Flag-gsc* and NI. The expression profiles of (D) early (stage 11) and (E) late (stage 24) neural marker genes were analyzed by RT-PCR. No RT (no reverse transcriptase added) served as a negative control while WE (whole embryos) served as positive control. (F) Whole mount *in situ* hybridization (WISH) was performed with anti-sense *foxd4l1.1* probe of at stage 11.

uninjected AC group (*gsc*/NI), the neural marker genes were markedly induced when compared to that of the uninjected control conjugates (NI/NI) (Fig. 4D, lane 3 vs 4). The results clearly show that *gsc* functions as a strong repressor of neural gene transcription in a cell-autonomous manner but a stimulator in noncell-autonomous manner. Noticeably, *chordin* and *noggin* expression were strongly increased under both conditions of *gsc/gsc* and *gsc*/NI conjugates. There were no significant differences in the expression levels of *chordin* and *noggin* in two groups of conjugates for *gsc/gsc* and *gsc*/NI (Fig. 4D, lane 2 vs 3), indicating that *chordin* and *noggin* expressions were inducible in *gsc* injected AC samples with *gsc* being a direct strong repressor of early neural target genes. Similarly, we investigated the expression pattern of late neural markers (stage 24) in the same samples. Neural markers including *ncam* and *xngnr1* (pan-neural markers), *xrox20* (mid-brain marker), and *otx2* (anterior neural marker) were significantly increased in *gsc* and not-injected conjugates (*gsc*/NI) of ACs (Fig. 4E, lane 3 vs 4) when compared to that of conjugates of all NI (NI/NI). These markers were completely abolished in conjugates of both *gsc* injected group (*gsc/gsc*) (Fig. 4E, lane 2 vs 3), indicating again that *gsc* has an inhibitory effect on neural target gene expression. All three sets of AC conjugates were further evaluated using whole-mount *in situ* hybridization (WISH) with *anti-foxd4l1.1* RNA probe. In support of RT-PCR results, the *gsc/gsc* conjugates showed no expression of *foxd4l1.1* in either portions of conjugates. On the other hand, *gsc*/NI conjugates showed that only the NI portion of conjugated ACs showed strong expression of *foxd4l1.1* (Fig. 4F). As expected, NI/NI conjugates showed no expression of *foxd4l1.1* in either portions of the conjugates (Fig. 4F). Taken together, the results confirm that Gsc has dual roles of neural inhibition in cell-autonomous and stimulation in noncell-autonomous manner, respectively.

DISCUSSION

In the present study, we sought to define the role of the homeobox repressor *gsc* in neural specification as part of its functions in the organizer. The organizer (being dorsal mesoderm) maintains its mesodermal identity while having a stimulatory effect on the naive ectoderm above its layer to become neuroectoderm during gastrula stage of embryos, and we tried to tie a role for *gsc* in the mechanism for neural gene exclusion seen in the organizer region. With the organizer as dorsal mesoderm needing a mechanism for stimulat-

ing naïve ectoderm to neuroectoderm as well as maintaining mesoderm and/or preventing it from being alternate germ layers including ectoderm and/or neuroectoderm, we adopted *gsc* as a candidate gene possibly performing such dual activity for the organizer. Gsc is a repressive TF and a known factor for enhancing *chordin* (Sasai et al., 1994), a secretory factor inhibiting BMP and leading to neighboring ectoderm cells to neuroectoderm. We found that *gsc*, as an organizer specific TF, repressed *foxd4l1.1*, a neural specific TF. We have previously found that repressive neural and non-neural TFs (*foxd4l1.1* vs *ventx1.1*) are mutually antagonistic in specifying the non-neural versus neural ectoderm activation areas of the nucleus (Kumar et al., 2020). We selected and focused on *foxd4l1.1* for the hypothesis that a TF in a distinct germ layer protects it from becoming another type of germ layer. In this view, *gsc*, a critical TF of dorsal mesoderm (organizer), protects the expression of *foxd4l1.1*, a critical TF of neuroectoderm. Thus, Gsc protects dorsal mesoderm (organizer) from becoming a neuroectoderm through *foxd4l1.1*. In addition, AC conjugation experiments demonstrated that *gsc* induction was neural stimulatory in a noncell-autonomous manner. Together, our results suggest that Gsc is an organizer specific factor performing dual roles of organizer in neural specification. Specifically, Gsc is a repressive TF to protect dorsal mesoderm from expressing neural genes as well as a stimulatory factor of neighboring neuro-ectoderm via expression of *chordin* and *noggin*. The implication and interpretation of this study is discussed below in the point of view of representative repressor TFs preserving each germ layer of dorsal and ventral mesoderm as well as dorsal (neural ectoderm) and ventral ectoderm (non-neural ectoderm).

We have previously reported on various mechanisms existing in reciprocally exclusive germ-layer specifications in early vertebrate embryogenesis (ectoderm, mesoderm and neuroectoderm) (Kumar et al., 2020). In the ventral mesoderm and ectoderm region, neural repressor *ventx1.1* is expressed and it inhibits neuroectoderm specific genes including *foxd4l1.1* and *zic3* (Umair et al., 2018). On the other hand, the neuroectoderm region requires the neuroectoderm specific repressor *foxd4l1.1* to inhibit the neural repressor *ventx1.1* expression (Kumar et al., 2020). These studies suggested reciprocal repression of *ventx1.1* and *foxd4l1.1* being at least part of specifying mechanisms for non-neural versus neural ectoderm fate specification in *Xenopus* embryos (Kumar et al., 2020; Umair et al., 2018). In the present study, we asked whether the two adjacent layers of organizer and neuro-ectoderm cells also follow such a mechanism of reciprocal repression in gastrula embryos, especially in the dorsal mesoderm territory (the Spemann organizer) (De Robertis and Kuroda, 2004; Harland, 2000; Nieuwkoop and Nigtevecht, 1954; Spemann, 1967). Here, we found that gain-of-function study of *gsc* pointed to *gsc* acting as a strong repressor in *dnbr*, *chordin*, and *noggin* mediated neuroectoderm formation in AC explants. A direct binding of Gsc in an early neural specific gene *foxd4l1.1* was also demonstrated in ChIP-seq and reporter gene analysis of gastrula embryos. Gsc-mediated repression of the neural specific repressor TF Foxd4l1.1 is important in protecting the organizer from expressing nonspecific neural genes as well as maintaining organizer as *gsc* expressing dorsal mesoderm.

From our preliminary findings, Gsc promoter also contained the direct binding response element for Foxd4l1.1 (from Chip-seq and reporter assay of Gsc promoter) (unpublished data). Whether a reciprocal repression of *gsc* and *foxd4l1.1* is the underlying mechanism for the non-neural organizer versus neural ectoderm fate specification in *Xenopus* embryos, it requires more elaboration; more immediately, any repressive functions of Foxd4l1.1 on *gsc* expression in neuro-ectoderm needs to be demonstrated. We designed and performed loss-of function studies for proof of Gsc's neural repressive role in activin treated AC system. In activin treated AC explants, one may expect increased expression for the neuroectoderm gene *foxd4l1.1*, and actually *gsc* MO injected ACs had decreased *foxd4l1.1* expression under the activin treated condition. *Gsc* MO also led to increase a neural repressor *ventx1.1* expression compared to the activin treated condition without the *Gsc* MO. The effects of *gsc* depletion on expression of *foxd4l1.1* in the organizer could have been

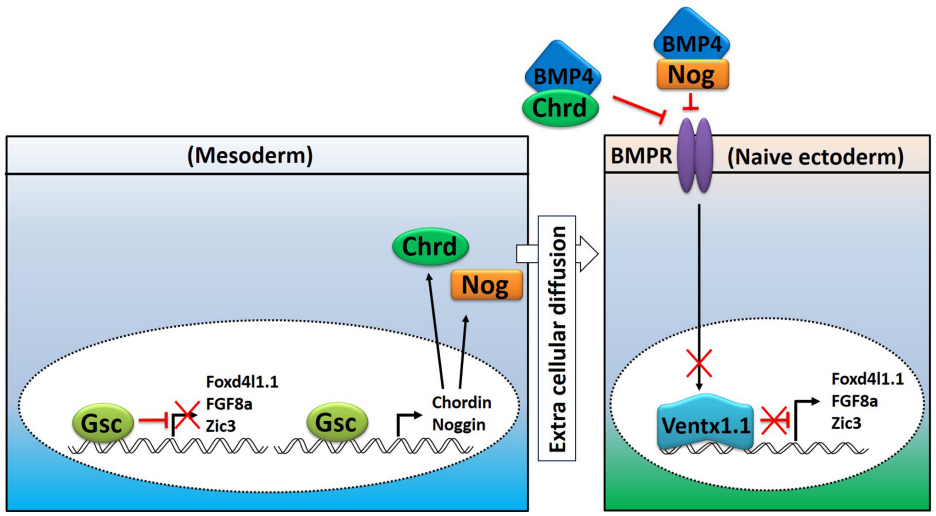

Fig. 5. Schematic diagram depicting the dual role of Gsc in early neurogenesis. In mesoderm (dorsal organizer), Gsc inhibits the neural genes while strongly induces *chordin* and *noggin* expression. Newly translated Chordin and Noggin diffuse to neighboring cells (ectoderm) where they induce neurogenesis in a BMP inhibited manner.

shown. However, previous work (Sander et al., 2007) has indicated that *gsc* depletion increases neural repressor gene *ventx1.1* and *ventx2.1* levels. A *gsc* MO experiment may lead to neural gene repression in the whole embryos. Our experiments with AC explants allowed certain conditions that were practical to be implemented (such for "activin treated" cases). The results indicated in the activin treated setup was similar to the previous experiment in whole embryos and the results using *noggin/chordin* MOs were similar to that of *Gsc* MO (Supplementary Fig. S1A, lane 1 and 2). The *Gsc* knockdown results supported *Gsc* suppressing neural genes when *Gsc* is overexpressed under *bmp* inhibition in activin treated AC explants (Supplementary Fig. S1A, lane 3 and 4). Taken together, *Gsc/noggin/chordin* depletion experiments point to a suppressive role for *goosecoid* and a stimulatory role for *noggin/chordin* in neuroectoderm formation. It is still need to investigate gsc neural repressive role along with cross-examination of other organizer TFs and signaling molecules in the developing organizer of gastrula embryos. Based on the present results, however, we propose this depicted model for Gsc inhibiting the neural TF *foxd4l1.1* in its expressed territory of the organizer, but also inducing secreted Chordin and Noggin, to induce neurogenesis in target tissues (Fig. 5).

In this study, we adopted *gsc* as an organizer specific gene TF to study the dual inhibitory and stimulatory roles of organizer in neural specification. The reasons on why we selected *gsc* are the following: First, *gsc* is the first organizer TF to be recognized as only expressed in the underlying layer of organizer but not in the above layers of the neuroectoderm in gastrula stage of embryos (Cho et al., 1991). Second, *gsc* is a repressor TF, which would protect the organizer as dorsal mesoderm from expressing neural genes including neural specific repressor TF *foxd4l1.1*. Third, *gsc* has been known to induce ectopic expression of *chordin*. Fourth, overexpression of *gsc* in ventral side of embryo leads to complete axis duplication including neural tissues of head, indicating somehow the stimulatory role of *gsc* in neural specification (Dixon Fox and Bruce, 2009; Kuroda et al., 2004; Sander et al., 2007; Seiliez et al., 2006; Yao and Kessler, 2001).

Gsc as an organizer specific repressor TF has been studied in the context of dorso-ventral specification as well as a factor in protecting the organizer by repression of signaling molecules including BMP and Wnt8 (Yao and Kessler, 2001). Gsc has been proposed as a central TF involved in mesoderm patterning (Niehrs et al.,1994). Although showing a direct reciprocal repression between dorsal and ventral specific homeobox genes *gsc* and *ventx1.1/ventx2.1* awaits more studies, involvement of the genes has been demonstrated in dorsoventral patterning of mesoderm by both gain-of function (Cho et al., 1991) and loss-of function (Steinbeisser et al., 1995) studies. Gsc has been suggested as an important TF in dorsoventral patterning, possibly through reciprocal repression of the genes to mediate a self-adjusting mechanism (Sander et al., 2007). Although the studies of *gsc* have focused on dorso-ventral specification as an organizer repressor gene as well as neuro-ectoderm specification as possibly an indirect inducer of neural stimulatory gene *chordin* (Sander et al., 2007; Sasai et al., 1994), *gsc* has not been adequately examined as a direct repressor of neural genes, and the reports have also focused on dorsoventral patterning and not much on organizer/neuroectoderm specification (Thisse et al., 1994).

Additional studies have mainly focused on neural stimulatory effect of Gsc through induction of neural stimulatory factor *chordin*, possibly through an indirect effect of *gsc* in *chordin* expression (Cho et al., 1991; De Robertis and Kuroda, 2004; Sander et al., 2007; Sasai et al., 1994). In the present work, we found that *gsc* overexpression increased ectopic expression of *chordin*, as ACs obtained from *gsc* injected embryos expressed both BMP antagonizer *chordin* and *noggin* in AC explants (Fig. 4D); this is different from the report where Gsc induced ectopic expression of *chordin* but not that of *noggin* in gsc-injected at 8 cell stage embryos (Sasai et al., 1994). At the present time, we cannot address the exact reason why *gsc* injection leads to the differences in *chordin* and *noggin* expression in AC cells and whole embryos. These may be due to differences for their mode of activation (Sasai et al., 1994). We actually elaborated the expression mode of *chordin* using the isolated 2.25 kbs in length *chordin* promoter. The isolated promoter contained a primary response elements for Smad2 and Smad3 of activin signaling, which is slightly different that from Sasai et al. (1994) where *chordin* was reported as a secondary response gene requiring de novo protein synthesis. In addition, our isolated promoter contained a strong positive cis-acting element of Gsc (GRE) adjacent to the Smad2 and Smad3 response cis-acting elements in the distal region of chordin promoter (Kumar et al., 2021). These results indicate that Gsc functions as a repressor as well as an activator in a context dependent and cell specific manner. Chordin and noggin expression needs more scrutinizing; however, ACs from gsc injected embryos expressed both chordin and noggin, supporting our hypothesis that *gsc* functions as a neural stimulatory factor via inducing *chordin* and *noggin* expression in a noncell-autonomous manner.

In the present paper, we sought to explain the dual roles of the organizer in neuroectoderm specification in the context of the functional activity of the organizer specific TF *gsc*. The present finding of cell-autonomous and noncell-autonomous regulation of organizer gene in neuroectoderm and mesoderm specification, however, needs further elaboration. The present work suggests that Gsc represses the neural specific gene *foxd4l1.1*. This, along with our preliminary findings on *foxd4l1.1* repression of Gsc promoter (from Chip-seq and reporter assays of Gsc promoter) (unpublished data), a reciprocal repression of *gsc* and *foxd4l1.1* is thought to be the underlying mechanism for the non-neural organizer versus neural ectoderm fate specification in *Xenopus* embryos. In addition, the results of Gsc repression of ventx1.1 (Fig. 1A) and our previous results of ventx1.1 repression of gsc (Hwang et al., 2003) support the premise of a reciprocal repression existing between ventx1.1 and gsc for ventral and dorsal patterning. A study on the mutual opposing roles of Gsc and Vent homeobox genes in patterning the mesoderm of *Xenopus* embryo has been reported (Sander et al., 2007). Although it requires further investigation, from our previous and current findings, we propose a reciprocal repressive mechanism for the master repressor TFs including *gsc*, *ventx1.1* and *foxd4l1.1* in protecting the organizer, the

ventral mesoderm/ectoderm and the neuroectoderm, respectively.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

Z.U. and V.K. performed the experiments and wrote the primary manuscript. J.K. and U.L. designed and supervised the study. R.S.G. and S.K. contributed to the data analysis and revision of the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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