The transcription factor NKX2-2 regulates oligodendrocyte differentiation through domain-specific interactions with transcriptional corepressors

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The homeodomain protein NK2 homeobox 2 (NKX2-2) is a transcription factor that plays a critical role in the control of cell fate specification and differentiation in many tissues. In the developing central nervous system, this developmentally important transcription factor functions as a transcriptional repressor that governs oligodendrocyte (OL) differentiation and myelin gene expression, but the roles of various NKX2-2 structural domains in this process are unclear. In this study, using in situ hybridization, immunofluorescence, and coimmunoprecipitation, we determined the structural domains that mediate the repressive functions of murine NKX2-2 and identified the transcriptional corepressors that interact with it in OL cells. Through in ovo electroporation in embryonic chicken spinal cord, we demonstrate that the N-terminal Tinman domain and C-terminal domain synergistically promote OL differentiation by recruiting distinct transcriptional corepressors, including enhancer of split Groucho 3 (GRG3), histone deacetylase 1 (HDAC1), and DNA methyltransferase 3 α (DNMT3A). We also observed that the NK2-specific structural domains suppress the function of the C-terminal domain in OL differentiation. These findings delineate the distinct NKX2-2 domains and their roles in OL differentiation and suggest that NKX2-2 regulates differentiation by repressing gene expression via multiple cofactors and molecular mechanisms.

Oligodendrocytes (OLs) are macroglial cells that form myelin sheaths around neuronal axons in the central nervous system. During early development, they originate from the ventricular neuroepithelium of neural tubes (1). In the developing spinal cord, the majority of oligodendrocytes are induced from the motor neuron progenitor domain (pMN) domain of the ventral ventricular zone (2–5), whereas a small percent of OPcs are produced in the dorsal neural progenitors independent of Sonic Hedgehog (SHH) signaling (6, 7). After OPcs are specified, they rapidly proliferate and migrate into the surrounding white matter areas, where they undergo morphological and molecular changes to become myelinating OLs. Many genes have been implicated in the regulation of OPC differentiation and maturation (8). For example, the SOX2 transcription factors SOX8 and SOX10 control terminal differentiation of oligodendrocytes (9). Myelin Regulatory Factor (MYRF), a myelin regulatory transcription factor, has been suggested to serve as a downstream rheostat for myelin growth and maintenance (10–12). The NKX2-2 homeodomain transcription factor is another key regulatory gene that initiates oligodendrocyte differentiation, as it is selectively up-regulated in OPcs during the differentiation stage (13, 14), and coexpression of Nkx2-2 and Olig2 in embryonic chick spinal cord promotes ectopic expression of myelin genes (15). Our recent studies demonstrated that forced expression of Nkx2-2 in mouse OPcs autonomously induces precocious OL differentiation by repressing transcription of downstream target genes, including Pdgfra, which promotes OPC proliferation but inhibits differentiation (16, 17).

NKX2-2 protein contains three highly conserved regions: the tinman (TN) domain, the homeodomain (HD), and the NK2-specific domain (SD) (18). Beyond the SD domain is the C-terminal region, which has been shown previously to activate transcription in luciferase reporter assays (19). The N-terminal TN domain is a 12-amino-acid sequence highly homologous to the core region of the engrailed homology 1 domain, which acts as a transcriptional repressor (20, 21). Through this domain, NKX2-2 controls spinal cord V3 interneuron development by recruiting Groucho corepressors to form a large complex (22). The TN domain also plays an important role in pancreatic β cell differentiation by recruiting different transcriptional repressors (23, 24). The most conserved HD domain carries two nuclear localization signals and functions as the DNA-binding site of full-length NKX2-2 (25, 26). The short SD domain has been suggested to mask the function of the adjacent C-terminal (CT) domain (19). Although the CT portion is highly conserved in transactivation activity, a more recent study suggested that it may possess a repressor function in inhibiting tumor growth and metastasis (27). Similarly, the C-terminal
region of the related NKX2-5 protein also has an inhibitory effect on target gene expression (28). Thus, the transcriptional nature of the CT domain is somewhat controversial and remains to be clarified.

In this study, we characterized the functions of various NKX2-2 structural domains in the oligodendrocyte differentiation process. We demonstrated that the CT domain, like the TN domain, also behaves as a transcription repressor to promote oligodendrocyte maturation and myelin gene expression. Moreover, the TN and CT regions exhibit a synergistic role in promoting OL differentiation, possibly by recruiting distinct transcriptional corepressors or epigenetic factors, including GRG3, HDAC1, and DNMT3A. The highly conserved NK2-SD domain functions to attenuate the repressive activity of the C-terminal domain. Together, our results demonstrate that the transcriptional activity of NKX2-2 protein can be modified by structural effects of specific sequences and by the transcriptional cofactors with which it interacts.

Results

The TN and CT domains are partially required for NKX2-2 induction of oligodendrocyte differentiation

NKX2-2 has been implicated as a transcriptional repressor in the control of cell differentiation and function (15, 16). Besides the DNA-binding HD domain, NKX2-2 protein also harbors two other evolutionarily conserved structural motifs (the TN and SD domains) and a less conserved CT domain. To assess their individual contribution to the regulation of OL differentiation, we first generated a series of NKX2-2 deletion mutants lacking each of the four motifs (Fig. 1A). These four derivatives were cloned into RCASBP avian retroviral vector in tandem with the mouse Olig2 gene separated by a P2A sequence to ensure their simultaneous expression in the same cells. The electroporated cells can be identified by immunostaining with the antibody, which specifically recognizes mouse but not chicken Olig2 (Fig. S1). As reported previously (15), coexpression of Olig2 and WT NKX2-2 was sufficient to induce formation of MBP and PLP mature OLs on the electroporated side (Fig. 1, B and B'). However, coexpression of NKX2-2 derivatives lacking the TN or CT domain yielded a much smaller number of mature OLs (Fig. 1, C, C', D, D', and E–G), indicating that the TN and CT domains are partially required for NKX2-2 activation of OL differentiation. In contrast, deletion of the SD domain caused stronger expression of MBP and PLP (Fig. 1, E, F, and G), suggesting that this domain normally has an inhibitory effect on NKX2-2 function. As expected, the DNA-binding HD domain was absolutely required for NKX2-2 induction of ectopic precocious OLs in electroporated tissue (Fig. 1, D, D', and G).

The TN and CT domains function synergistically to promote oligodendrocyte differentiation

As both TN and CT domains contribute to NKX2-2 function, we wondered whether each of these two structural motifs alone is sufficient for driving OL differentiation when combined with the HD domain. Although the HD domain itself was not able to activate OL differentiation (Fig. 2, B, B', and F), the chimeric
proteins consisting of HD + TN domains or HD + CT domains in the absence of other sequences were now capable of inducing the appearance of MBP + or PLP + mature OLs (Fig. 2, C–D’ and F). Noticeably, neither of these two chimeric proteins could fully recapitulate the effect of the full-length NKX2-2 protein in activating MBP/PLP expression, suggesting possible synergistic cooperation between the TN and CT domains. To test this idea, we included both domains in the expression construct, and in ovo electroporation analysis revealed that the combined TN–HD–CT construct indeed led to a higher number of mature OLs than those containing either the TN or the CT domain (Fig. 2, E–F).

The C-terminal domain acts as a transcriptional repressor for OL differentiation

It has been demonstrated previously that the CT region activated gene transcription in a cultured NIH 3T3 cell line (19). To examine whether it acts in a similar manner in activating OL differentiation, we replaced this domain with a VP16 sequence, a strong transcriptional activator, at the C-terminal position (Fig. 3A). Consistent with this notion, the NKX2-2ΔCT-EnR chimeric protein induced ectopic MBP+/PLP+ OLs, suggesting that the CT domain can be functionally substituted with a known transcriptional repressor motif (Fig. 3, E–F).

There was no evidence that showed high homology of the C-terminal sequences among various NK2 family members. However, short stretches of conserved sequences were noticed between NKX2-2 and NKX2-5, corresponding to the 252–264 position in the NKX2-2 protein (Fig. 3G). This homologous sequences exhibited a strong repressive activity in the NKX2-5 protein (28). In line with this finding, deletion of this short fragment (Tyr251-Pro264) from NKX2-5 protein dramatically decreased its ability in inducing precocious OL differentiation (Fig. 3, H–J). Taken together, these results demonstrated that the C-terminal domain acts as a transcriptional repressor in initiating OL differentiation.

The SD domain inhibits function of the TN and CT domain

The SD domain has been shown previously to mask the transcriptional activity of the C-terminal region in reporter gene assays (19), raising the possibility that the SD domain may regulate the activity of NKX2-2 during OL differentiation. To test this possibility, we inserted the SD sequence between the HD and CT domains (Fig. 4A) and then examined its effect on inducing ectopic OL formation. Compared with the HD+CT construct, the HD+S.D.+CT protein induced a significantly smaller number of MBP+/PLP+ mature OLs (Fig. 4, B–C and F), reinforcing the idea that the SD domain diminished the
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A

NKX2-2

TN

HD

SD

CT

NKX2-2ΔCT-VP16

TN

HD

SD

VP16

NKX2-2ΔCT-EnR

TN

HD

SD

EnR

B

NKX2-2(HD+CT)

HD

CT

NKX2-2(HD+CT)(Δ251-264)

HD

CT(Δ251-264)

function of the C-terminal fragment. Because the CT region acts as a transcriptional repressor, we next investigated whether the SD domain would similarly antagonize the function of EnR repressor protein and TN domain. Using the same experimental approach, we found that the HD\/H11001 SD\/H11001 EnR protein induced considerably fewer OLs than the HD\/H11001 EnR construct (Fig. 4, D–E’ and F). Similarly, insertion of the SD sequence between the TN and HD domains nearly completely abolished its ability to induce OL differentiation (Fig. 4, H–I’ and J). Interestingly, when the SD domain was placed on the other side of the HD domain, it did not compromise the induction of OL differentiation (Fig. 4, H, H’, J, F, and K), indicating its short-range inhibitory effect. Together, these results demonstrate that the SD domain negatively regulates the activity of adjacent transcriptional repressors.

The phosphorylation site regulates the function of the SD domain

A previous study revealed two important sequence motifs in the SD domain, the potential mitogen-activated protein kinase...
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(MAPK) phosphorylation site (PLPSP) and the highly hydrophobic core region (VAVPVLV) (19). We confirmed that the Ser199 site is indeed phosphorylated by mass spectrometry analysis (Fig. S2). These two motifs are highly conserved among different species (Fig. 5A), suggesting their possibly important roles in SD function. To address this possibility, we first substituted the phosphorylation residue serine with alanine (S199A) in the SD domain (Fig. 5B) and found that the S199A mutation significantly attenuated the function of the SD domain, leading to an increased number of ectopic OLs (Fig. 5C–D' and G). On the contrary, when the serine residue was replaced with aspartate (S199D), this simulated phosphorylation activated function of the SD domain (Fig. 5E, E', and G). Similarly, we replaced the hydrophobic amino acids with hydrophilic glutamates (A204E and L208E) and discovered that disruption of the hydrophobic core had little influence on the function of SD domain (Fig. 5F–G). These results indicate that the MAPK phosphorylation site Ser199, but not the hydrophobic core sequence, is critical for function of the SD domain, implying that NKX2-2 protein function may be regulated by a protein phosphorylation process.

NKX2-2 interacts with transcriptional corepressors through the TN and CT domains

A previous study demonstrated that NKX2-2 functions as a transcriptional repressor in controlling tissue development by forming a protein complex with DNMT3A, HDAC1, and GRG3 (24). Immunostaining confirmed that GRG3, HDAC1, and DNMT3A were indeed expressed in OPCs (Fig. S3). These OPC-transcribed repressor genes were then tested for their physical interaction with NKX2-2 in 293T cells by cotransfection followed by coimmunoprecipitation. In these experiments, GRG3 displayed strong binding to NKX2-2 protein, whereas HDAC1 and DNMT3A exhibited slightly weaker bindings (Fig. 6).
The portion of NKX2-2 protein that interacts with those interacting corepressors were further mapped with domain-specific deletion mutants using the coimmunoprecipitation approach. This line of study revealed that deletion of the TN domain abolished NKX2-2 binding to GRG3 but had no or little effect on its binding to DNMT3A or HDAC1 (Fig. 6, A–C). Similarly, deletion of the CT domain eliminated its binding to DNMT3A but not to GRG3 (Fig. 6B). Intriguingly, interaction between NKX2-2 and HDAC1 was significantly reduced by CT deletion but completely abolished when both the TN and CT domains were absent (Fig. 6C). Based on these findings, we postulate that NKX2-2 physically interacts with GRG3 through the TN domain, with DNMT3A through the CT domain, and with HDAC1 through both the TN and CT sequences.

Discussion

The TN and CT domains function synergistically to promote oligodendrocyte differentiation

The NKX2-2 transcription factor contains the HD domain and three other structural motifs. As the HD domain is responsible for nuclear localization and DNA-binding specificity of all homeodomain proteins, it is no surprise that deletion of this motif fails to initiate OL differentiation (Fig. 1). Previous studies demonstrated that the TN domain is necessary for development of V3 interneurons and pancreatic β cells (22, 24). This study showed that the TN domain is also partially required for NKX2-2 initiation of OL differentiation and that deletion of this structure significantly reduces NKX2-2 induction of myelin gene expression in embryonic chick spinal cords (Fig. 1, C, C’, and G). Moreover, when combined with the HD domain, the TN domain alone is sufficient for formation of MBP+ mature OLs (Fig. 2).

There is a large sequence between the TN and HD domains in the N-terminal of NKX2-2 protein. This region appears to be dispensable for NKX2-2 induction of OL differentiation. Absence of this domain from various expression constructs (e.g., TN+HD, HD+CT, and TN+HD+CT) did not abolish or even affect the NKX2-2 activation of OL differentiation (Figs. 2–4). The role of the C-terminal region in cell differentiation and tissue development has not been clearly defined. In this study, we demonstrated that NKX2-2 lacking the CT domain had a significantly weaker effect on induction of mature OLs compared with the full-length protein (Fig. 1, F and F’), indicating that the CT domain is also crucial for NKX2-2 control of OL differentiation (Fig. 2). Although the TN and CT domains take part in the OL differentiation process, neither of them could fully replace the function of intact NKX2-2 protein. However, the combined TN+CT domains exert a strong differentiation effect comparable with the full-length protein (Fig. 2), indicative of their cooperative function in initiation of OL differentiation and maturation.

The CT and TN domains function as repressor domains by recruiting corepressors

Earlier studies showed that the TN domain functions as a transcription repressor motif to control the development of V3 interneurons and pancreatic β cells (20). In contrast, the CT domain has been described previously as a strong transcriptional activator based on luciferase reporter assays (19). Unexpectedly, when this domain was replaced with VP16, which acts
as a strong transcriptional activator, the resulting fusion protein did not induce formation of mature OLs (Fig. 3, D, D', and F), implying that the CT domain does not function as a transcriptional activator in promoting OL differentiation. When the CT domain was replaced with the EnR domain, which possesses strong transcriptional repressor activity, the fusion protein mimicked the function of full-length NKX2-2 protein in stimulating OL differentiation in collaboration with OLIG2 (Fig. 3, E–F). Based on these findings, we concluded that the CT domain, like the TN domain, also acts as a transcriptional repressor in promoting OL differentiation. In keeping with this notion, the NKX2-2 and NKX2-5 proteins shared some degree of sequence homology in this region (Fig. 3G), and this homologous sequence in NKX2-5 protein possessed a strong inhibitory effect on downstream gene expression. Deletion of this homologous region in NKX2-2 greatly reduced the function of the CT domain in promoting OL differentiation (Fig. 3, H–J). In addition, it was recently reported that this C-terminal structure also functions as a repressor domain in suppressing tumor growth and metastasis (27). Together, these findings strongly suggest that the CT region is likely to function as a repressive domain in promoting OL differentiation. However, we cannot exclude the possibility that this C-terminal region could function as a transcriptional activator, depending on cell context or developmental stages.

Previous studies suggested that NKX2-2 regulates the development of other cell types by recruiting transcriptional corepressors to suppress the expression of its downstream genes (24, 29). In this study, we demonstrated that three transcriptional corepressors, GRG3, HDAC1, and DNMT3A, which are abundantly expressed in immature OPCs (Fig. S3), display physical interactions with the NKX2-2 protein. Detailed domain mapping studies revealed that NKX2-2 binds to the GRG3 repressor through the TN domain and to the DNMT3A protein through the CT domain (Fig. 6, A and B). The HDAC1 protein appears to interact with both the TN and CT domains (Fig. 6C). Based on these results, we postulate that strong repressor activity is necessary for NKX2-2 activation of the intrinsic OL differentiation program and that the TN and CT domains work synergistically to achieve robust suppression of downstream genes (e.g. Pdgfra) that normally inhibit OPC differentiation (Fig. 7).

Figure 6. Domain-specific interactions between NKX2-2 and GRG3, DNMT3A, and HDAC1. A, GRG3 binding to NKX2-2 was abolished by TN deletion. B, DNMT3A binding to NKX2-2 was abolished by CT deletion. C, HDAC1 binding was decreased by CT deletion and further reduced in the absence of the TN + CT domains. NKX2-2 proteins were tagged with FLAG in A and B but with HA in C.
It has been suggested that GRG3, HDAC1, and DNMT3A suppress gene expression through distinct mechanisms. For instance, GRGs suppress gene expression in various tissues by recruiting a variety of transcriptional corepressors (22). HDAC1 silences gene expression by histone deacetylation, with an important role in oligodendrocyte differentiation (30). DNMT3A inhibits gene expression by promoting site-specific methylation (24, 31) and collaborates with DNMT1 to regulate OL remyelination (32). These observations have raised the possibility that the NKX2-2 transcription factor promotes OL differentiation and maturation by repressing the expression of some key differentiation inhibitors (such as the Pdgfra, Hes, or ID genes) through multiple mechanisms.

**The SD domain attenuates the function of the CT repressive domain**

Although highly conserved among the members of NK-2 family, the function of the NK2-specific domain in NKX2-2 regulation of OL differentiation remains to be defined, especially under in vivo conditions. Watada et al. (19) proposed that the SD domain functionally masks the transcriptional activity of the CT domain. Consistently, we found that an NKX2-2 derivative lacking the SD domain induces a higher number of mature OLs than the full-length NKX2-2 protein (Fig. 1, E, E’, and G). Conversely, insertion of the SD domain between the HD and CT domains or between the HD and EnR domains markedly reduced the number of induced mature OLs. Interestingly, insertion of the SD domain between the TN and HD domains also significantly diminished formation of mature OLs (Fig. 4). Thus, the SD domain is capable of inhibiting the activity of various repressive domains when physically adjacent to each other. It is unclear how the short SD motif antagonizes the function of the CT domain in OL differentiation.

It has been noted previously that the SD domain contains a potential MAPK phosphorylation site and a hydrophobic core sequence (19). We found that it is the phosphorylation site, not the hydrophobic sequence, that plays a key role in the function of the SD domain (Fig. 5). This result suggests that NKX2-2 activity may be regulated by protein phosphorylation through the SD domain. It is conceivable that serine phosphorylation may cause a conformational change that destabilizes the adjacent repressive domain. It will be interesting and important to investigate how NKX2-2 function is regulated by protein phosphorylation induced by extracellular signals and signaling molecules.

**Experimental procedures**

**In ovo electroporation**

All chick embryo experiments were performed in accordance with the institutional guidelines drafted by the Laboratory Animal Facility of Hangzhou Normal University (Hangzhou, China) and were approved by the Animal Ethics Committee of this university. For expression of NKX2-2 variants in chicken spinal cords, mouse *Nkx2.2* mRNAs with deletions of various structural domains were subcloned into the chicken replication-competent retroviral vector RCASBP(B) (33). The NKX2-2 proteins were tagged with a Myc sequence at the N terminus. The procedure for in ovo electroporation has been described previously (34). Briefly, a DNA solution containing 2.5 μg/μl RCASBP(B) plasmids, 1 μg/μl pCAGGS–enhanced GFP plasmids, and 1% fast green was injected into the central lumen of embryonic chick spinal cords with a glass capillary at Hamburger and Hamilton stage HH13–HH15 (cE2). The injected embryos were then subjected to five short pulses of electrical shock (25 V, 50 ms for each pulse, 950-ms interval)
with an Electro Square Porator (BTX, ECM 830). Embryos of either sex were then allowed to develop for 5 days prior to fixation in 4% paraformaldehyde for gene expression analysis.

**In situ hybridization (ISH)**

Chicken spinal cord tissues were isolated from whole embryos and fixed in 4% paraformaldehyde overnight at 4°C in PBS. Tissues were then transferred into 30% sucrose in PBS overnight at 4°C, embedded in optimal cutting temperature compound (OCT), and sectioned on a cryostat at 16-μm thickness. Immediately adjacent sections from control and experimental tissues were used for ISH and subjected to immunohistochemistry as described previously (13). The digoxin-labeled RNA probes used in ISH corresponded to the 270–1110 nucleotides of chicken MBP mRNA (NM_205280.1) or 100–970 nucleotides of chicken PLP mRNA (NM_205277.1).

**Immunofluorescent staining**

Tissues sections were subjected to *in situ* hybridization for PLP, followed by antigen retrieval in citrate sodium buffer at 95°C for 10 min. Sections were then examined by anti-OLIG2 immunofluorescent staining. The primary anti-mouse OLIG2 antibody (Millipore, AB9610, 1:1000) only recognizes the exogenous OLIG2 protein expressed by the RCASBP constructs but not the endogenous chicken OLIG2 protein.

**Coimmunoprecipitation**

NKX2-2-FLAG-, NKX2-2ΔTN-FLAG-, NKX2-2ΔCT-FLAG-, and NKX2-2Δ(TN+CT)-FLAG- expressing vectors or control vectors were cotransfected with Grg3-HA–, Hdac1-HA–, or Dmnt3a-HA–expressing vectors into HEK293T cells by FuGENE. After 48 h, cells cultured in 10 cm dish were treated with 1 ml of lysis buffer containing 25 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, and 1 mM EDTA. 0.2 ml of cell lysate was used for input, and 40 μl of anti-FLAG magnetic beads (Sigma, catalog no. M8823) were added to the remaining samples and incubated in a vertical shaker at 4°C overnight. Beads were subsequently washed with TBS buffer (150 mM NaCl and 25 mM Tris (pH 7.4)) three times, and the FLAG-tagged proteins were eluted with 120 μl of TBS buffer containing 10 μg of 3×FLAG peptide. Both input and immunoprecipitation samples were subjected to Western blotting with anti-FLAG (Sigma, F1804, 1:10,000) or anti-HA (Abcam, ab9110, 1:10,000) antibody.

**NKX2-2 protein phosphorylation assay**

NKX2-2–FLAG protein was overexpressed in the CG4 oligodendrocyte cell line for 2 days, and then cells were lysed in lysis buffer. After centrifugation, anti-FLAG magnetic beads were added to the supernatant and incubated in a vertical shaker at 4°C overnight. Beads were subsequently washed with TBS buffer three times, and the FLAG-tagged proteins were eluted with 120 μl of TBS buffer containing 10 μg of 3×FLAG peptide. The eluent was subjected to electrophoresis and stained with Coomassie Blue. The band of 38 kDa was excised from the gel for mass spectrometry analysis (Micrometer Biotech Co., Hangzhou, China).

**Western blotting**

Equal amounts of protein were loaded on 10% BisTris/PAGE, transferred onto PVDF membranes, and blocked with 5% (w/v) milk dissolved in TBST (TBS (pH 7.4) and 0.05% Tween 20) for 1 h at room temperature. Primary antibodies were diluted in primary antibody dilution buffer (Beyotime) as follows: anti-FLAG (Sigma, F1804, 1:10,000) or anti-HA (Abcam, ab9110, 1:10,000) antibody. Secondary antibodies were from goat anti-mouse IgG (Promega, W4021) or goat anti-rabbit IgG (Promega, W4011). Blots were developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientific, TD264271D) and imaged with a medical X-ray processor (Carestream).

**Statistical analysis**

Quantitative data were presented as the mean ± S.D. and analyzed using a two-tailed paired Student’s *t* test. For each analysis, results from independent electroporated chicken tissues were treated as biological replicates (*n* ≥ 3). Statistical analyses are provided in the figure legends. Statistical significance was as follows: *p* ≤ 0.05; **p** ≤ 0.01; ***p*** ≤ 0.001; ****p*** ≤ 0.0001.

**Author contributions**—C. Z. data curation; C. Z. and M. Q. formal analysis; C. Z. investigation; C. Z. writing—original draft; H. H. and M. Q. conceptualization; Z. C. software; Z. Z. and M. Q. supervision; W. L. and M. Q. funding acquisition; M. Q. resources; M. Q. project administration; M. Q. writing—review and editing.

**References**


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