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Interaction of MTG family proteins with NEUROG2 and ASCL1 in the developing nervous system

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ABSTRACT

During neural development, members of MTG family of transcriptional repressors are induced by proneural basic helix-loop-helix (bHLH) transcription factors and in turn inhibit the activity of the bHLH proteins, forming a negative feedback loop that regulates the normal progression of neurogenesis. Three *MTG* genes, *MTG8*, *MTG16* and *MTGR1*, are expressed in distinct patterns in the developing nervous system. Various bHLH proteins are also expressed in distinct patterns. We asked whether there is a functional relationship between specific MTG and bHLH proteins in developing chick spinal cord. First, we examined if each *MTG* gene is induced by specific bHLH proteins. Although expression of *NEUROG2*, *ASCL1* and *MTG* genes overlapped, the boundaries of gene expression did not match. Ectopic expression analysis showed that *MTGR1* and *NEUROD4*, which show similar expression patterns, are regulated differently by NEUROG2 and ASCL1. Thus, our results show that expression of *MTG* genes is not regulated by a single upstream bHLH protein, but represents an integration of the activity of multiple regulators. Next, we asked if each MTG protein inhibits specific bHLH proteins. Transcription assay showed that NEUROG2 and ASCL1 are inhibited by MTGR1 and MTG16, and less efficiently by MTG8. Deletion mapping of MTGR1 showed that MTGR1 binds NEUROG2 and ASCL1 using multiple interaction surfaces, and all conserved domains are required for its repressor activity. These results support the model that MTG proteins form a higher-order repressor complex and modulate transcriptional activity of bHLH proteins during neurogenesis.

MTG8 (RUNXT1, ETO, CBFA2T1), MTG16 (CBFA2T3) and MTGR1 (CBFA2T2) are members of the MTG/ETO/CBFA2T protein family, a small group of transcriptional repressors (Fig. 1) [7,9]. They function as "protein scaffolds" that bridge various transcription factors. MTG proteins are strongly induced during neurogenesis by proneural basic helix-loop-helix (bHLH) proteins including Xngnr-1, Neurogenin2, Xash3, Xath3 (XNeuroD4), Xath5 (XAtoh7) and XNeuroD [5,8,12,13,17]. We previously showed that MTGR1 inhibits the activity of NEUROG2, and that this feedback inhibition is required for normal progression of neurogenesis [1]. Expression analysis in mice showed that the regions of *MTGR1* expression generally coincided with that of *Neruog2*, whereas expression of *MTG8* and *MTG16* was largely confined to the lineage of progenitor cells expressing *Ascl1* [2]. These differences in expression patterns suggest that (1) each bHLH protein regulates the expression of specific MTG genes, and (2) each MTG protein preferentially inhibits specific bHLH proteins [1,2,12]. Alternatively, MTG proteins may have interchangeable roles in inhibiting bHLH activity, and their different expression patterns may not be relevant in terms of generating cell-type specificity. To resolve this issue, we carried out four experiments. First, the expression patterns of genes encoding proneural bHLH proteins were compared directly to those of MTG proteins using multi-probe in situ hybridization. Next, we examined which *MTG* genes are induced or repressed by NEUROG2 and ASCL1 in a misexpression study. Third, the relative inhibitory activity of MTG proteins on NEUROG2 and ASCL1 was evaluated in a transcription assay. Finally, we identified the domains of MTGR1 that are required for the interaction with proneural bHLH proteins. Our results show that each MTG gene can be regulated by multiple bHLH proteins. We also show that some targets genes are induced by both NEUROG2 and ASCL1, but others are oppositely regulated, indicating that function of bHLH proteins is context dependent. We show that each MTG protein can inhibit the activity of multiple proneural bHLH proteins, but to varying degrees. This suggests that MTG proteins may generate local variations in the proneural bHLH protein activity during neurogenesis.

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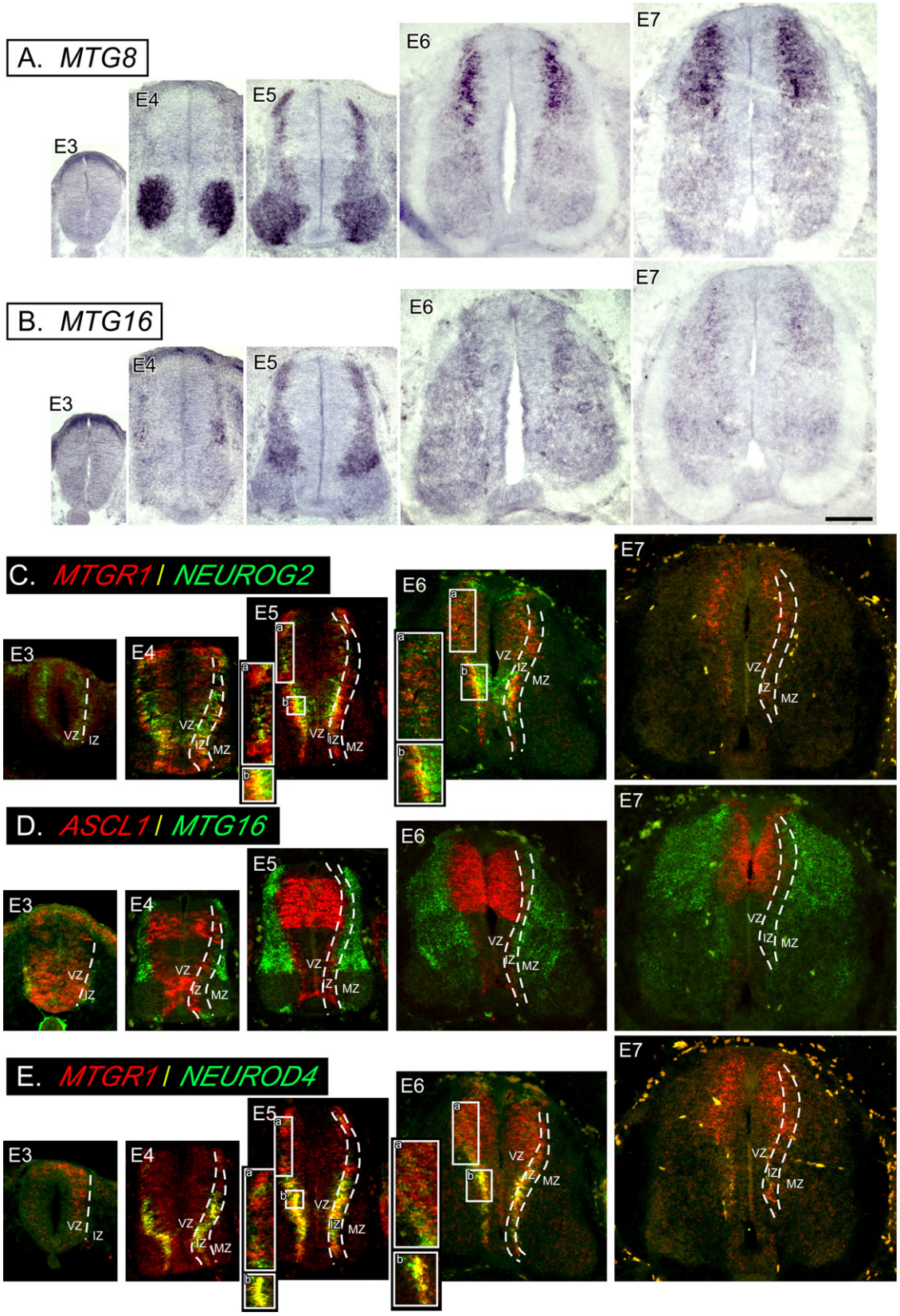

Fig. 1. Expression pattern of chicken MTGR1, MTG8 and MTG16 relative to NEUROG2 and ASCL1. Chromogenic (A and B) and fluorescent double (C–E) *in situ* hybridization. Cross sections of the spinal cord at embryonic (E) days 3–7 at the brachial level are shown. The probes used are indicated according to color in the figures. Insets are enlargements of the boxed areas in the same figure. Bar: 100 μm.

In situ hybridization, in ovo electroporation, immunohistochemistry, P19 transfection assay, Western blotting, GST pull-down assay and quantification were done as previously described [1]. Q-PCR was done according to the protocol from Applied Biosystems. Expression levels were normalized using mRNA from fetal mouse brain. Differentiation of P19 cells was induced by treatment with 1 μM all-trans retinoic acid in suspension culture [10,11]. After 4 days of induction, cells were dissociated, re-plated and allowed to differentiate.

Deletion constructs were made by PCR amplification of fragments of chicken *MTGR1* cDNA and subcloning into the *Eco*R1-*Xho*I fragments of pCS2(+) or pCS2(+)myc vectors. Chicken *ASCL1* probe and an expression plasmid are from Samuel Pfaff (Salk Institute).

We previously reported the expression patterns of *MTG* genes in developing chick and mouse nervous system [1,2,12]. Our analysis in embryonic day (E) 4 chick spinal cord suggested cell-type specific expression of different *MTG* genes, whereas our observation in mouse showed an overlapping pattern of expression of *MTG* genes, especially at later stages of development. To clarify if these different gene expression patterns reflect the difference between species or the difference in the developmental stages examined, patterns of expression of *MTG8* and *MTG16* were examined in E3 to E7 chick spinal cord by *in situ* hybridization (Fig. 1A and B). At E4, *MTG8* was expressed strongly in the differentiating motorneurons, and weakly in the position of d3 interneurons. *MTG16* was expressed in the layer of postmitotic cells excluding the motorneurons, consistent with our previous report [12]. Interestingly, both *MTG8* and *MTG16* showed a dramatic change in expression from E5 to E7. *MTG8* expression expanded dorsally to cover most postmitotic neurons at E5. After E6, however, the ventral expression was down-regulated, and by E7, expression was localized dorsally. *MTG16* was not expressed in motorneurons at all stages, but on E6 and E7, it was expressed in dorsal cell populations overlapping those expressing *MTG8*. This dynamic shift in expression domains is similar to what we observed in the mouse spinal cord [2]. Thus, our results show that, similar to the mouse spinal cord, expression domains of *MTG8* or *MTG16* in the chick spinal cord do not correlate with specific cell types throughout development, but show a dynamic ventral to dorsal shift according to progression of development.

Next, we compared expression of MTG genes with those encoding proneural bHLH proteins. Our analysis in mouse showed that, generally, *Mtgr1* was induced in areas in which *Neurog2* was expressed, while *Mtg16* expression seemed to correlate with that of *Ascl1*. We examined the spinal cord at different developmental stages by double *in situ* hybridization (Fig. 1C–E). Expression of *MTGR1* followed that of *NEUROG2* at all stages examined, particularly in the ventral spinal cord (Fig. 1C, note the yellow area indicating co-expression). In dorsal domains, however, there was less overlap of the two signals, even though *MTGR1* and *NEUROG2* were expressed generally in the same area (compare inset 'a' from the dorsal part and inset 'b' from the ventral part). This was particularly evident at E5-6. Next, we examined the extent of co-expression of *MTGR1* with *NEUROD4*, a known target of Neurog2 in mouse and frogs [8,13,17]. Interestingly, expression of *MTGR1* and *NEUROD4* heavily overlapped in ventral domains, and to a lesser degree in dorsal domains (Fig. 1E, compare insets a and b), suggesting that *MTGR1* and *NEUROD4* may be regulated by similar mechanisms in the ventral spinal cord, and by different mechanisms in the dorsal spinal cord. *MTG16* was expressed in areas with *ASCL1* expression in the ventricular zone. However, the dorsoventral boundaries of gene expression did not match, suggesting that ASCL1 is not the sole regulator of *MTG16* expression (Fig. 1D).

To determine if the proneural bHLH proteins can promote expression of specific *MTG* genes, we examined *MTG* gene expression following over-expression of NEUROG2 or ASCL1 (Fig. 2). When

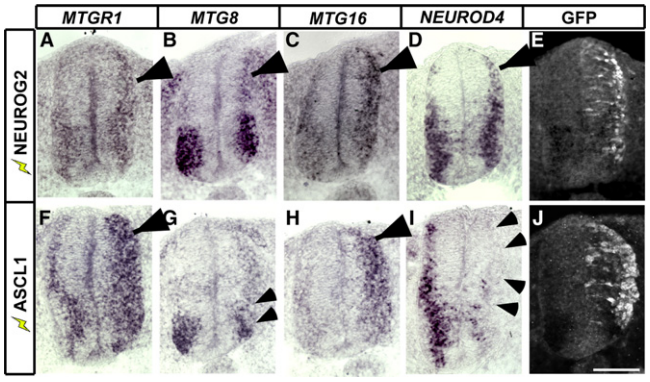

Fig. 2. Induction of MTG genes by NEUROG2 and ASCL1. E2 chick spinal cords were electroporated with expression vectors of NERUOG2 (A–E) or ASCL1 (F–J) along with the GFP expression vector and analyzed 24 h later. Adjacent sections were stained by *in situ* hybridization using *MTGR1* (A and F), *MTG8* (B and G), *MTG16* (C and H) or *NEUROD4* (D and I) probes. Electorporated areas were visualized by staining the sections with anti-GFP immunohistochemistry. (D and E) and (G and J) are from the same sections. Large arrowheads indicate ectopic expression of the markers and small arrowheads point to areas where markers are down-regulated. Unelectroporated sides (left side) serve as controls. Bar: 100 μm.

NEUROG2 was over-expressed in E2 chick spinal cord, expression of all three *MTG* genes were induced after 24 h, as was *NERUOD4* (Fig. 2A–D). In contrast, over-expression of ASCL1 led to strong induction of *MTGR1* and *MTG16* expression, but *MTG8* and *NEUROD4* were strongly repressed (Fig. 2F–I). Control experiments overexpressing the myc epitope, the GFP protein or the luciferase protein showed no change in the expression of *MTG* genes (data not shown). Therefore, *MTG* genes respond differently to ectopically expressed proneural bHLH proteins. This differential response may underlie the different patterns of *MTG* expression observed in normal development. It is interesting to note that although *MTGR1* and *NEUROD4* are expressed in highly overlapping patterns (Fig. 1E), their response to NEUROG2 and ASCL1 is different (Fig. 2A, D, F and I). This result suggests that activities of multiple bHLH factors converge on promoters of their target genes, and that genes expressed in similar patterns are not necessarily regulated by the same set of transcription factors.

We previously reported that MTGR1 can inhibit NEUROG2 activity [1]. Based on structural similarity, it is likely that other MTG proteins also can inhibit the activity of NEUROG2 and other bHLH proteins. To test if the different MTG proteins have different effects on the function of bHLH proteins, we examined the inhibitory activity of MTG proteins in a transient transfection assay in P19 cells (Fig. 3). The transcription activity of NEUROG2 and ASCL1 was assessed using a reporter construct containing E box sequences in its promoter, the known DNA binding site for proneural bHLH transcription factors. NEUROG2 alone did not activate the reporter gene, but did activate the reporter gene in combination with E47. This activity was inhibited dose-dependently by all MTG proteins. MTG16 was the most efficient, followed by MTGR1 and MTG8 (Fig. 3A). Similarly, the transcription activity of ASCL1, in combination with E47 (Fig. 3B) or ASCL1 alone (Fig. 3C), was inhibited by all MTG proteins. MTG16 was the most efficient, followed by MTGR1. Inhibitory activity of MTG8 was significantly weaker than that of MTGR1 and MTG16 in all experiments. The transcription activity of NEUROG2 and ASCL1 was inhibited only 2–3-fold at the highest dose of MTG8 tested. We did not observe further inhibition when a larger amount of MTG8 expression vector was transfected (data not shown). These results show that all MTG proteins inhibit the activity of both NEUROG2 and ASCL1, however the extent of inhibition is different.

Previously, we showed that MTGR1 physically interacts with NEUROG2, and less efficiently with E47 *in vitro* [1]. To gain insight

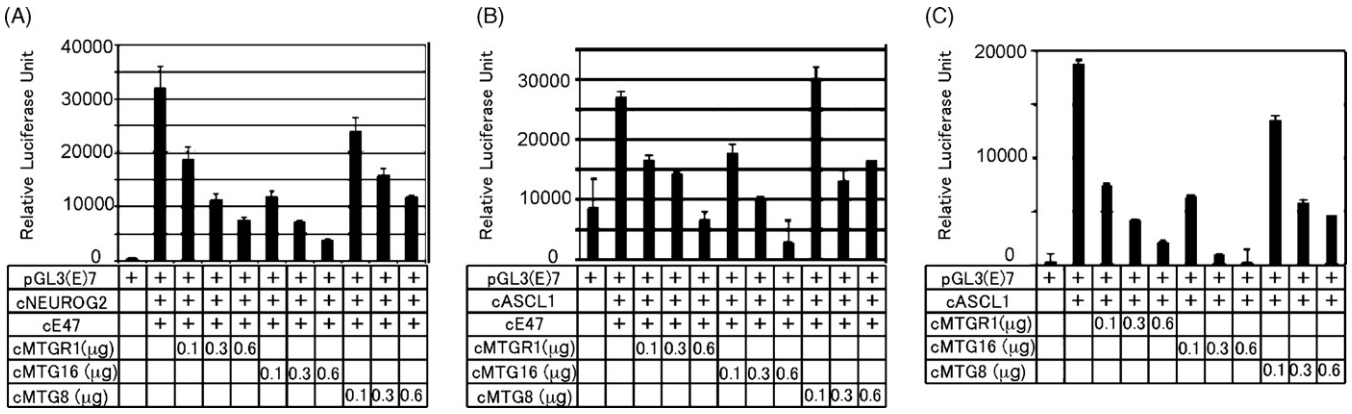

Fig. 3. Relative inhibitory activity of MTG proteins. P19 cells were transfected with the indicated vectors and analyzed 24 h later. Numbers indicate the μg amount of MTG expression vectors transfected. Data represent average of triplicate transfections and bars show standard deviation.

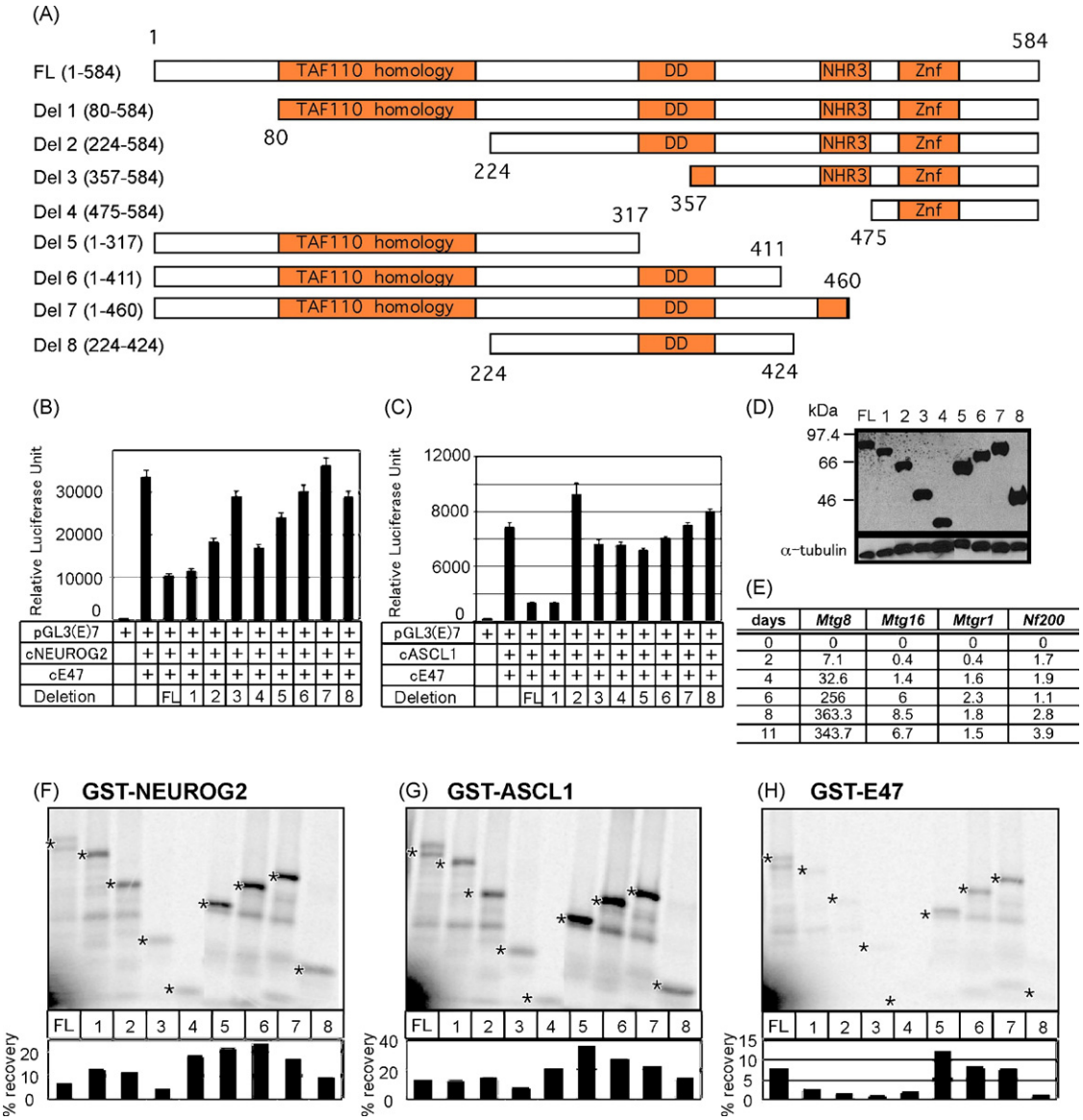

Fig. 4. Deletion assay of MTGR1. (A) Truncation mutants used in this experiment. (B and C) Reporter gene assay. Del1 showed inhibitory activity comparable to the wild-type molecule, but any other deletion constructs had weaker inhibitory activity to NEUROG2 + E47 (B) or ASCL1 + E47 (C). (D) All constructs were tagged with the myc epitope and expression levels in P19 cells were examined by Western blotting against the myc epitope tag. Western blot using anti-α-tubulin antibody is shown as a loading control. (E) Q-PCR was carried out to examine the endogenous expression level of *Mtg* genes in P19 cells. (F–H) GST pull-down assay. Deletion mutants were radiolabeled by *in vitro* translation and tested for binding to GST fusion molecules of NEUROG2 (F), ASCL1 (G) or E47 (H). Recovered radioactivity was quantified and shown as percent recovery of the input in bar graphs. Numbers in (F)–(H) correspond to the numbers of deletion constructs and asterisks indicate the positions of the bands. TAF: TATA box binding protein associated factor; DD: dimerization domain; NHR3: nervy homology region 3; Znf: zinc finger-like motif.

into the mechanism of inhibition of bHLH activity by MTGR1, we evaluated the functional domains of MTGR1 and its interaction surfaces with bHLH proteins. We made eight amino- and carboxy-terminal truncation mutants of MTGR1 and tested their abilities to inhibit NEUROG2- and ASCL1-dependent transcription (Fig. 4A–C). For both bHLH proteins, Deletion construct 1 (Del 1) showed activity equivalent to the full length MTGR1, but further truncation either from amino- or carboxy-terminal ends abolished the inhibitory activity. To confirm the expression level of truncated proteins, the myc epitope was inserted at the N-terminus and expression in P19 cells was examined by Western blot (Fig. 4D). The results showed that all constructs were expressed at equivalent levels. These constructs showed similar transcriptional activity as non-tagged proteins. To address if endogenous *Mtg* proteins affected the transcription activity, expression levels of endogenous *Mtg* genes were examined by quantitative PCR (Q-PCR; Fig. 4E). In undifferentiated P19 cells, expression levels were undetectable. In contrast, in cells induced for neuronal differentiation by aggregation and retinoic acid treatment, all *Mtg* genes were significantly induced along with a neuronal marker, *NF200*. Upregulation of *Mtg* gene expression is consistent with the endogenous expression pattern of *Mtg* genes in the spinal cord (Fig. 1) [1,2,12]. Thus, in our transient transfection experiment using undifferentiated P19 cells, it is unlikely that endogenous *Mtg* proteins affected the reporter gene activity. To address how the truncation of MTGR1 affected the interaction with NEUROG2 or ASCL1, deletion mutants were radioactively labeled by *in vitro* translation and pulled down with GST tagged NEUROG2, ASCL1 or E47 proteins (Fig. 4F–H). The results showed that all deletion constructs bound to NERUOG2 and ASCL1, suggesting that MTGR1 has multiple interaction surfaces with these bHLH proteins. In contrast, recovery of Del 1–4 was much weaker than Del 5–8 with E47, indicating that the amino-terminal half of the molecule including the TAF110 homology domain is necessary for interaction between MTGR1 and E47. Interaction between MTGR1 constructs and E47 was weaker than that between MTGR1 constructs and NEUROG2 or ASCL1 in all mutants tested. No proteins bound to GST protein alone (data not shown).

Since the first identification of *MTG* genes as targets of Xngnr-1, it has been questioned whether the difference in the expression domains is related to regionalization or cell-type specification in the nervous system [2,12]. Our analysis of expression patterns in the developing chick spinal cord showed that *MTG* genes change their expression domains as development progresses and that expression of a particular *MTG* gene does not appear to correlate with specific populations of neurons. Thus, *MTG* genes may be utilized to promote neurogenesis in multiple cell types of the developing nervous system. Alternatively, *MTG* gene expression may correspond to certain stages of neuronal maturation. Overall, *MTGR1* is expressed in cells that have just finished their final mitosis throughout the dorso-ventral axis, whereas *MTG8* and *MTG16* expression advances from ventral to dorsal areas within postmitotic neurons. Since neurogenesis takes place in a ventral to dorsal pattern, the dynamic shift in the pattern of *MTG* gene expression, especially for *MTG8*, may be related to the pattern of neurogenesis.

An interesting observation came from the epistatic analysis. *MTGR1* and *MTG16* were induced by both NEUROG2 and ASCL1, but *MTG8* and *NEUROD4* were induced by NEUROG2 and repressed by ASCL1. This differential response, particularly repression of *MTG8* expression by ASCL1 may explain the initial ventral localization of *MTG8* in the spinal cord. It is also interesting to note that although expression of *MTGR1* and *NEUROD4* show a high degree of overlap and both appear to be directly regulated by NEUROG2 [8,13,17], they are regulated differently by ASCL1. This shows that genes with similar expression patterns can be regulated differently by upstream factors. It also shows that functions of proneural bHLH factors are not interchangeable and structurally similar bHLH proteins can exert different effects on the same promoter. Similar observations have been reported in Xenopus embryos [18].

Null mice for *Mtg* genes have been reported [4,6,14]. Insertional mutation of *Mtg8* leads to deletion of midgut structure [4], whereas null mice for *Mtgr1* fail to maintain the secretory lineage in the small intestine. It was further shown that Mtgr1 associates with TCF4 and modulates the Wnt signaling pathway, thereby affecting the cell cycle of stem cells in the intestinal crypts [14]. Gene disruption of Mtg16 showed defects in proliferation and lineage allocation of hematopoietic stem cells [6]. Thus, it seems that MTG proteins are employed in various cell types to regulate stem cell proliferation and cell fate.

We observed that the ability of MTG proteins to repress NEUROG2 and ASCL1 activity, as monitored by transient transfection assays using P19 cells, was similar. However, the extent to which each MTG protein inhibits the bHLH activity was different. Whether this difference in the efficiency of inhibition is responsible for regional differences in bHLH activity in vivo requires further investigation. It is possible that by differentially modulating the activity of bHLH proteins, MTG proteins generate local differences in bHLH activity and modulate cell fate determination or cell cycle progression. Our preliminary analysis of the nervous system of the Mtgr1 null mice did not show significant alteration in cell cycle or cell fate (unpublished data). Analyzing null mice for other *Mtg* genes individually and in combination will be necessary to further identify the functional differences of individual MTG proteins.

Reported mechanisms of transcriptional inhibition by MTG proteins include recruitment of the histone deacetylase complex [16], competition for a binding partner [15,19], and inhibition of DNA binding [1], which are not mutually exclusive. Our GST pull-down assay suggested that MTGR1 interacts with NEUROG2 and ASCL1 through multiple domains, and with E47 through the TAF110 homology domain. Transcription assay, on the other hand, showed the requirement of all conserved domains of MTGR1 to repress transcriptional activity of bHLH proteins. Therefore, our data suggest that binding of MTGR1 and NEUROG2 is not sufficient for transcriptional repression, and may require interaction with additional factors such as histone deacetylases through multiple conserved domains. Given the fact that a number of proteins interact with MTG proteins, it is likely that MTGR1 forms a multi-protein complex with bHLH proteins that modulates transcription [3]. Further identification of MTG-interacting proteins will be necessary to dissect the nature of these proteins.

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