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Histone Marks and Chromatin Remodelers on the Regulation of *neurogenin1* Gene in RA Induced Neuronal Differentiation of P19 Cells

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ABSTRACT

Neurogenin1 is an important bHLH protein that plays crucial role in neurogenesis. We first show that the expression of *ngn1* increases drastically in RA induced neuronal differentiation. During which, a three successive stages of the epigenetic changes surrounding the *ngn1* gene are found correlated with a repression to activation of the gene in P19 cells. Recruiting of a repressive histone code H3K27me3 on the *ngn1* gene is the dominant change in first repression stage, which is followed by the binding of the active codes of H3K9ac, H3K14ac, and the H3K4me3 in the second and third stages of RA treatment. Additionally, BRM but not BRG1 is specifically recruited to *ngn1* gene at the third stage and is positively involved in the RA induced ngn1 expression. We propose that histone modifiers and chromatin remodelers are pivotal in the activation of the *ngn1* gene in RA induced differentiation of P19 cells. J. Cell. Biochem. 107: 264–271, 2009.

KEY WORDS: NEUROGENIN1; NEURONAL DIFFERENTIATION; HISTONE MODIFICATION; BRM; RA; P19 CELLS

Neurogenesis is a complicated multiple-step event, in which, each step-specific gene can either be induced or repressed [Diez del Corral and Storey, 2001]. Basic helix-loop-helix (bHLH) proteins exist in most stages of neural lineage, which play crucial roles in determining cell fates or differentiation. Neurogenin1 (Ngn1), an important member of the bHLH protein family, which is induced in neuronal precursor cells and is essential in neurogenesis [Sun et al., 2001]. However, the epigenetic regulation of the *ngn1* gene are still unclear.

There are two classes of enzymes that function as histone modifiers and ATP dependent chromatin remodelers in the eukaryotes. Both of them are critical in the remodeling of chromatin structure before the transcription of eukaryotic genes initiated [Chi, 2004].

Amino acid residues in the N-terminal tail as well as the globular domain of the core histones can be post-translationally modified by the histone modifiers. That the acetylation and methylation on distinct lysine residues as H3K9ac and H3K4me, and the methylation on the same H3K9 can respectively cause the same or divergent effects on the chromatin conformation. These and other specific modifications on certain unique amino acid residues in the histones are known as ''histone codes,'' which define their correlation with an active or repressive status for the transcription of genes [Jenuwein and Allis, 2001; Latham and Dent, 2007]. It has been reported that histone H3K9 and H3K14 acetylation and H3K4, K9, and K27 methylation are closely related with transcription status [Santos-Rosa et al., 2002; Barski et al., 2007; Bhaumik et al., 2007; Heintzman et al., 2007; Liu et al., 2007]. For example, H3K4me3 and H3K27me3 effectively discriminates genes that are expressed, poised for expression, or stably repressed, and therefore reflect cell state and lineage potential [Mikkelsen et al., 2007; Pan et al., 2007].

ATP-dependent chromatin remodeling complex utilizes energy from ATP hydrolyzed by its ATPase unit for the reposition of nucleosomes. Mammalian SWI/SNF chromatin remodeling complex exclusively contains either BRG1 or BRM as its ATPase subunit. BRG1 is required for neuronal differentiation that mediates the transcriptional activities of proneural bHLH proteins in *Xenopus* including *Ngn* and *NeuroD* genes [Seo et al., 2005]. BRG1 is consistently expressed throughout differentiation whereas the expression of BRM is very low in both neural precursor cells and P19 embryonal carcinoma cells that is only induced under differentiation to neurons and astrocytes [Machida et al., 2001; Itoh et al., 2008]. However, the role of BRM in neuronal differentiation is not well-understood.

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To investigate the regulation of *ngn1* gene, we used RA (*all-trans* retinoic acid)-induced P19 cells as a model for studying neuronal differentiation in this study. Here, we first report both of the activation and repression marks on core histones, and the SWI/SNF complex as the chromatin remodeler that are recruited to the promoter of *ngn1* gene. We then explore the mechanisms for these activities in the regulation of the gene at the early stage of RA induced neuronal differentiation.

MATERIALS AND METHODS

CELL CULTURE AND RA TREATMENT

P19 cells were cultured in α-minimum essential medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum. For neural differentiation, P19 cells were cultured in medium containing 0.5 μM RA (*all trans*-retinoic acid, Sigma) for 4 days, and then the aggregates were plated as a monolayer and cultured for another 3 or 4 days in the absence of RA [Jones-Villeneuve et al., 1982].

PLASMIDS

pCMV-ΔHAT$_{1,2}$-PCAF-Myc was from Dr. Qiwei Zhai as previously described [Ge et al., 2009]. pCMV-ΔHAT-p300 (HAT-defective mutant) expression plasmid was from Dr. Q. Li (National Institutes of Health, Bethesda, MD). pBJ5-BRG1K798R (an ATPase-defective K798R mutant, dominant negative) and pCG-hBRM-NTP (ATPase-defective) expression plasmid was from Dr. Anthony N. Imbalzano (University of Massachusetts Medical School). pcDNA6/BRM-NTP (ATPase-defective) was constructed from pCG-hBRM-NTP as a dominant negative expression plasmid by our laboratory.

ANTIBODIES

Monoclonal antibody against Neuronal Class III β-tubulin (TuJ1) was from Covance (MMS435P), Inc. Antibody against GAPDH was from Chemicon (MAB374); Antibody against BRM was from Abcam (ab15597). Antibodies against BRG1 (SC-10768) and PCAF (SC-13124) were from Santa Cruz Biotech; Antibodies against RNA polymerase II (05-623), acetyl-Histone H3 (Lys9, 06-942), acetyl-Histone H3 (Lys14, 06-911), dimethyl-Histone H3 (Lys9, 07-212), trimethyl-Histone H3 (Lys9, 07-442), trimethyl-Histone H3 (Lys4, 07-473), and trimethyl-Histone H3 (Lys27, 07-449) were from Upstate Biotech.

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

Quantitative Real-time RT-PCR assays were carried out as previously described [Li et al., 2007]. The relative expression of *ngn1* was normalized against *gapdh*, using the comparative CT method as the manufacturer's instructions (Rotor-Gene RG-3000A Real-time PCR System, Corbett Research, Australia). Primers used in PCR assays are as follows: *ngn1*, forward primer, 5′-CCCTGAAGACGAGGTGAAAAGT-3′, reverse primer, 5′-CTTGCCATTGTATTGTCAGCCG-3′; *gapdh*, forward primer, 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse primer, 5′-GAAGATGGTGATGGGATTT-3′. Experiments were repeated at least three times with statistical analyses for each individual experimental set. All values in the experiments were expressed as mean ± SD.

IMMUNOFLUORESCENCE

Cells were first fixed with 2% paraformaldehyde, and then permeabilized with 0.2% of Triton X-100 in PBS for 10 min, then washed and blocked in 1% goat serum. Cells were successively incubated in 1:100 mouse anti-β-tubulin (TuJ1, Covance, Inc.) antibody at 4°C overnight and the fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100) at 37°C for 30 min. Cells were then washed and incubated in DAPI (1:3,000) for 1 min followed by two PBS washings. Images were collected on a Nikon Eclipse TE2000-U microscope.

IMMUNOBLOTTING

Whole cell extracts (WCE) were prepared as described [Xiao and Lang, 2000]. Samples were separated in 8% SDS–polyacrylamide gels and analyzed by Western blotting [Xiao and Lang, 2000].

CHROMATIN IMMUNOPRECIPITATION (ChIP) AND QUANTITATIVE PCR ANALYSES

ChIP assays were carried out as previously described [Li et al., 2007]. For quantitative assays, standard curve and ChIPed DNA samples were analyzed on a Rotor-Gene RG-3000A Real-time PCR System (Corbett Research) with PCR Master Mix for SYBR Green assays (TaKaRa, Biotech). Primer pairs used for amplification of *ngn1* gene were: −2,565/−2,406, forward primer 5′-TCCGTTTCCTGCGTTTCAA-3′, reverse primer 5′-TGCTCTGGGCTGGCTGTC-3′; −2,059/−1,837, forward primer 5′-GATACTATTCCAAGGGTGCTG-3′, reverse primer 5′-TCCTGCCTTCGTGGGTC-3′; −1,616/−1,499, forward primer 5′-ACTGGTGCCGGTATTCTGGG-3′, reverse primer 5′-ATTAGGGCTTGAGGAGGTTTGT-3′; −198/+25, forward primer 5′-ATTACGGGCACGCTCCAG-3′, reverse primer 5′-CAGCTCCTGTGAGCACCAAG-3′; +955/+1,041, forward primer 5′-GACGCCCTGTTTCATCC-3′, reverse primer 5′-CAGCCAGTTCCCCATCTA-3′. Primers used for amplification of Neurod1 promoter region (−419/−64) were: forward primer 5′-TCTGCCTCTTTCACCTCTGTCC-3′, reverse primer 5′-TCACCCTCCACCCCTACCC-3′. The cycle quantity required to reach a threshold in the linear range (Qt) was determined and compared with a standard curve for the primer set generated by five 10-fold dilutions of genomic DNA samples of known concentration. The percentage of ChIPed DNA relative to input was calculated and shown as mean ± SD from three independent experiments.

LUCIFERASE REPORTER ASSAYS

The firefly luciferase reporter plasmid pGL3-ngn1-luc was constructed as follows: *ngn1* upstream regions, −2,053 to +55 relative to transcription start site, was amplified by PCR from the genome DNA of P19 cells and cloned into pGL3-Basic vector (Promega). Primers were as follows: forward, 5′-ACTATTCCAAGGGTGCTG-3′; reverse, 5′-TCTCCTGAGTGATGTCGC-3′. P19 cells plated were transfected with VigoFect Reagent (Vigorous) according to the manufacturer's instructions. To normalize firefly luciferase activity of the reporter construct (pGL3-ngn1-luc), 1/20 (mol ratio) of internal control plasmid expressing Renilla luciferase (pRL-TK vector, Promega) was co-transfected into the cells. Six hours after the transfection, the medium was replaced with fresh α-MEM (RA−) or α-MEM containing 0.5 μM RA (RA+). Promoter activity was measured 48 h later. The activities of both luciferases were

determined by means of a Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. Assays were performed 3 times each in triplicate, and all results are shown as means ± SD.

RESULTS

ngn1 WAS INDUCED IN RA-TREATED P19 CELLS

RA-induced P19 cells aggregated spontaneously on the fourth day of treatment (Fig. 1A, Aggregation), which were then plated into a fresh RA-free medium. The cells were grown in differentiation medium for another 3 or 4 days and turned into a neuron-like shape with neurite networks (Fig. 1A, Differentiation). Neuron specific class III-tubulin (TuJ1), a neuronal marker showed up in most of the cells at this stage with specific immuno-staining, (Fig. 1B). As the expression of TuJ1 dramatically increased after day 5, which indicated the cells at this stage were under neuronal differentiation (Fig. 1C).

Data from real time RT-PCR assays revealed that *ngn1* mRNA increased 162-fold after 2 days and 1,943-fold after 4 days of RA treatment as compared with control cells (Fig. 1D). The results suggested that *ngn1* gene was activated by the neuronal differentiation signals in RA treated cells.

ENHANCED HISTONE ACETYLATION ON *ngn1* GENE IN RA-TREATED P19 CELLS

To explore whether *ngn1* activation could be affected by histone acetylation, trichostatin A (TSA), an inhibitor for histone deace-

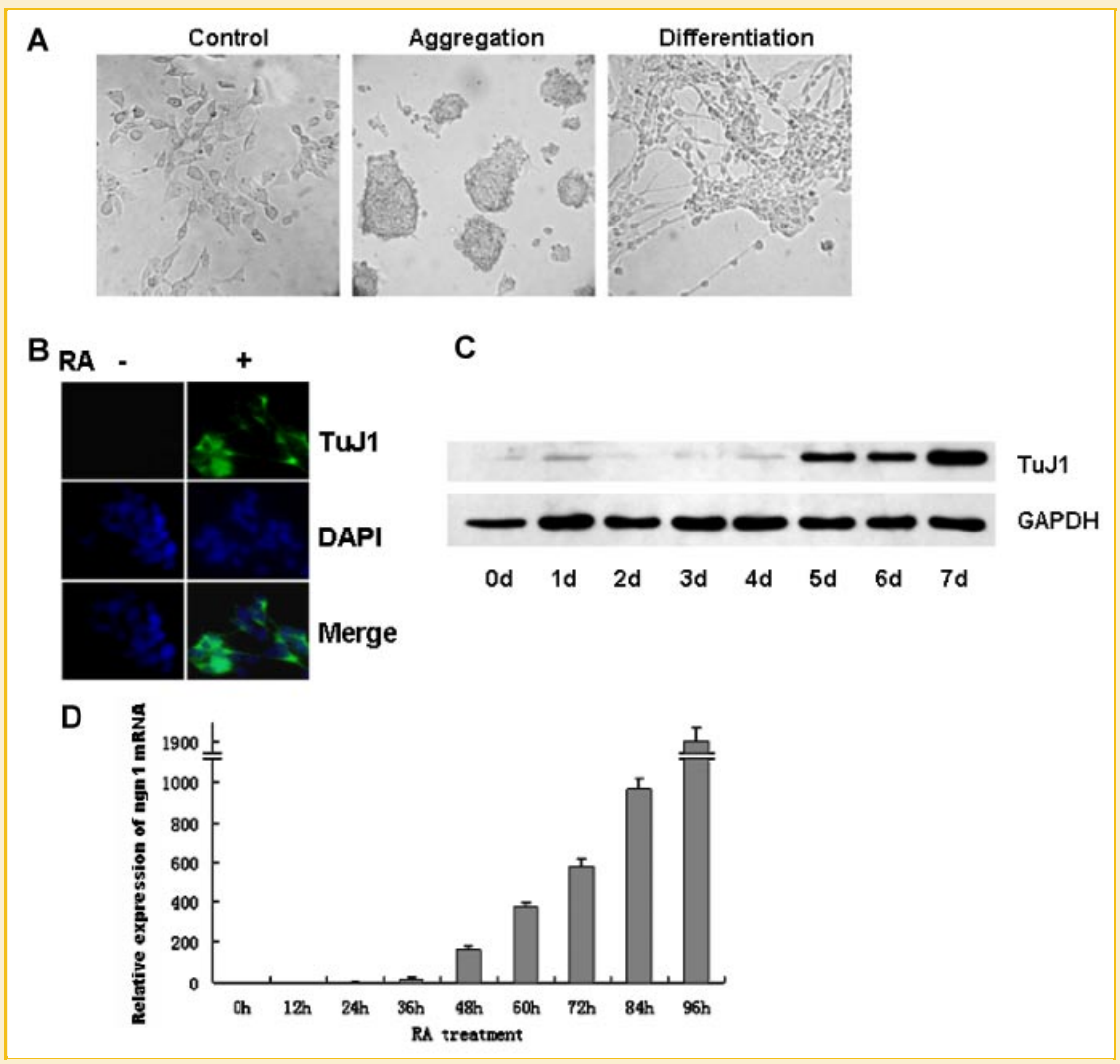

Fig. 1. RA-induced neuronal differentiation of P19 cells. A: Microscopic images (250× magnification) of P19 cells during RA-induced differentiation. P19 cells were cultured without (Control) and with RA for 4 days (Aggregation), and then cultured for another 3 days in the absence of RA (Differentiation). B: Immunostaining for neuronal β-tubulin (TuJ1, green) in P19 cells (RA−) or differentiated cells (RA+, treated with RA for 4 days, then cultured for 3 days without RA). C: Western blot assay for expression level of TuJ1. Whole cell lysates were separated on a 10% SDS–PAGE and blotted with antibodies against TuJ1 and GAPDH. D: Analysis of mRNA level of *ngn1* in RA treated P19 cells by Real-time RT-PCR. Error bar represents a normalized mean value from at least three independent experiments at each time point of RA treatment and was shown as mean ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tylase, was used to treat the cells [Liu and Xu, 2001]. We showed that the expression of *ngn1* elevated in a TSA dose dependent manner, which suggested that the increased acetylation level on histones and/or other factors facilitate the expression of *ngn1* in P19 cells (Fig. 2A).

ChIP assays were carried out with five independent primer pairs to amplify genome fragments either flanked or within the *ngn1* gene (−2,565 to +1,041) as shown in Figure 2B. It was found that while H3K9ac significantly increased with RA treatment at the proximal promoter of the *ngn1* gene, H3K14ac were not obviously changed (Fig. 2C,D). The results suggested that the efficient recruiting of H3K9ac at the promoter is prerequisite for the activation of *ngn1* gene after RA treatment.

To examine whether the two histone acetyltransferases (HATs), PCAF and p300, participated in the regulation of *ngn*1, we showed that PCAF, but not p300, was recruited to the proximal promoter of *ngn1* gene after 2 days of RA treatment (Fig. 2E,F). In the promoter activity assays, HAT domain-deleted PCAF drastically abolished the RA induction of the *ngn1* to its constitutive level of expression (Fig. 2G). Whereas no significant effect of the HAT domain-deleted

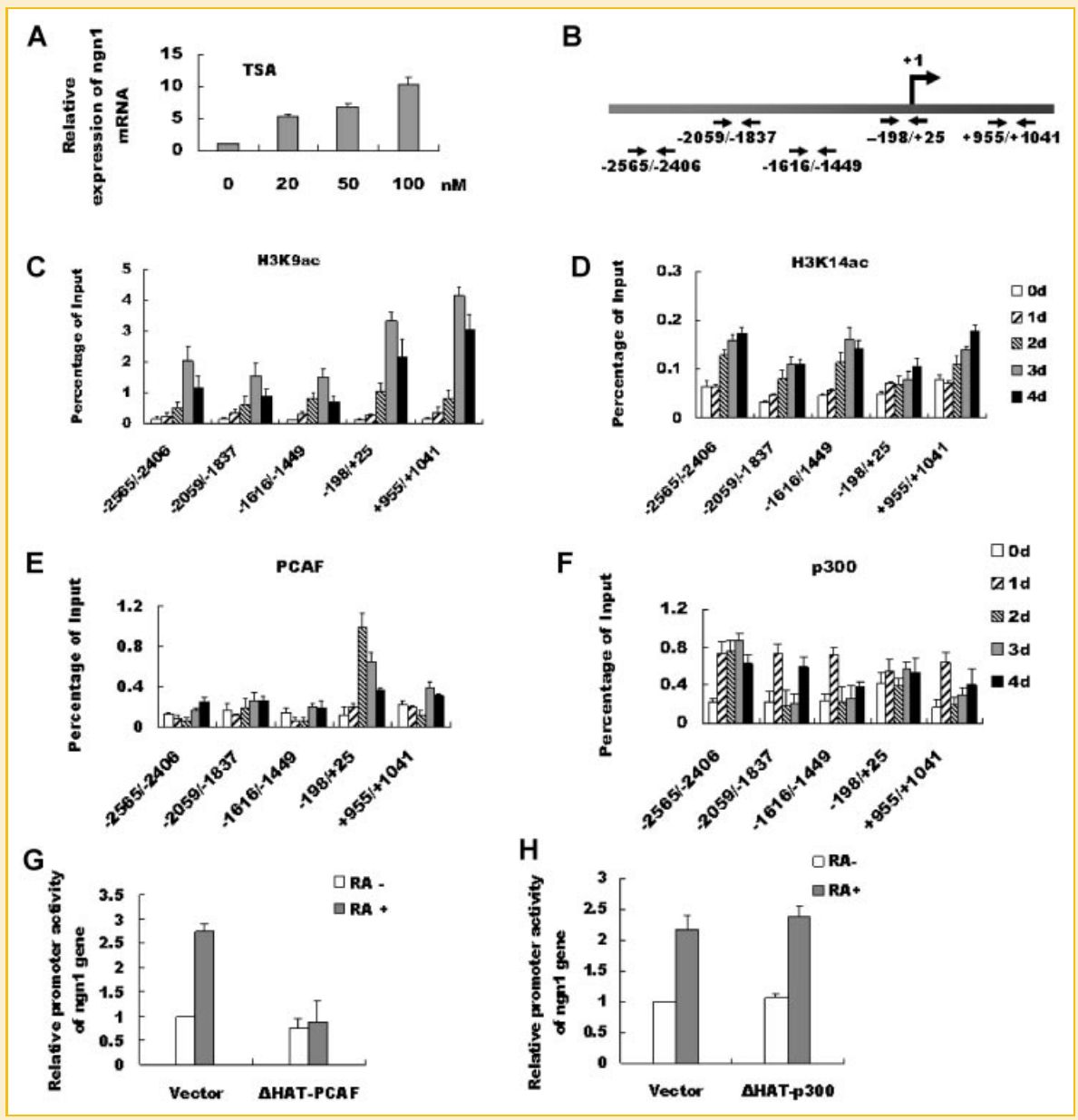

Fig. 2. Acetylation of histone H3 in *ngn1* gene region in P19 cells. A: Analysis of mRNA level of *ngn1* of P19 cells treated with different concentration of TSA (20 nM, 50 nM, 100 nM) for 2 days by Real-time RT-PCR assay. B: Schematic diagram of the positions of PCR in ChIP assays on *ngn1* gene. Five pairs of primers were used to perform real-time PCR (amplifying −2,565/−2,406, −2,059/−1,837, −1,616/−1,499, −198/+25, +955/+1,041 of *ngn1* gene). Analysis of histone acetylations and HATs in *ngn1* gene in RA treated P19 cells by ChIP assays: acetylation of histone H3K9 (C, H3K9ac), H3K14 (D, H3K14ac), PCAF (E) and p300 (F). G,H: Promoter activity assays of *ngn1* gene in P19 cells with (RA+) or without (RA−) RA treatment. Expression constructs of the dominant negative mutant pCMV-ΔHAT$_{1,2}$-PCAF-Myc (G, ΔHAT-PCAF) or pCMV-ΔHAT-p300 (H, ΔHAT-p300), and an empty vector were individually co-transfected with pGL3-ngn1-luc and pRL-TK.

p300 was observed (Fig. 2H). As the enrichment of PCAF was ahead of H3K9ac to get to its peak level, this suggested that PCAF could be involved in the acetylation of H3K9 or other remodelers at the proximal promoter of *ngn1* gene, and facilitate its expression.

EFFECTS OF RA ON THE METHYLATION OF HISTONE H3 IN *ngn1* GENE

We then analyzed the recruiting of methylated lysine in histone H3, that is, H3K9, H3K27, and H3K4 on the promoter region of *ngn1* with ChIP assay. The dimethylated H3K9 (H3K9me2) declined suddenly on the second day of RA treatment and relapsed to its previous level in the 5′ upstream regions of the gene. However, H3K9me2 accumulation further elevated on the fourth day to some twofold higher than that of the untreated control (Fig. 3A). Whereas the trimethylated H3K9 (H3K9me3) was only shown at minimal level without significant change at all sites (Fig. 3B).

By contrast, recruiting of trimethylated H3K27 (H3K27me3) enriched significantly on the first day of RA treatment with an average of some 10-fold increase in all the regions detected and then gradually decreased to the basal level of binding on the fourth day (Fig. 3C).

Trimethylated H3K4 (H3K4me3) was an activation mark [Lall, 2007], which increased steadily after the second day of RA treatment and was exclusive in the proximal promoter regions of the *ngn1* gene (Fig. 3D). The recruiting of RNA polymerase II at the proximal promoter of *ngn1* gene occurred on the first day of treatment and got to the top level at the third and fourth day (Fig. 3E). These results suggested that the H3K4me3 and RNA polymerase II recruiting and timing were comparable with the efficient expression of *ngn1* gene (Fig. 1D).

THE PARTICIPATION OF SWI/SNF CHROMATIN REMODELING COMPLEX IN THE ACTIVATION OF *ngn1* GENE

ChIP assays with antibodies against BRG1, BRM and a core subunit BAF60 of the SWI/SNF chromatin remodeling complexes were performed to analyze the recruiting profiles on the *ngn1* gene. We

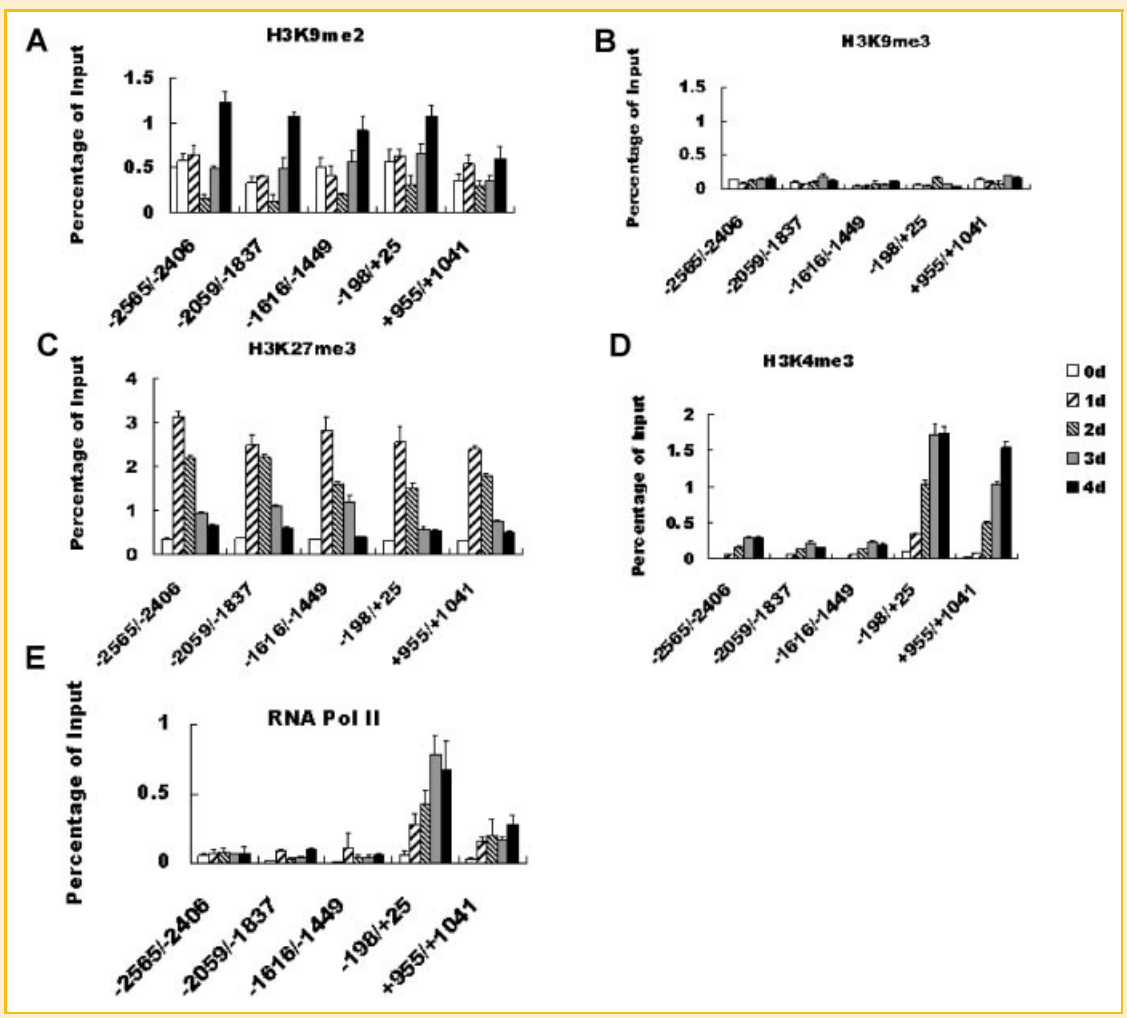

Fig. 3. Effects of RA on histone H3 methylation in *ngn1* gene region in RA treated P19 cells. Analyses of methylation of histone H3 in *ngn1* gene region in RA treated P19 cells by ChIP assays. A: dimethylation of H3K9, (B) trimethylation of H3K9, (C) trimethylation of H3K27, (D) trimethylation of H3K4. E: Analysis of the recruitment of RNA Pol II on *ngn1* gene in P19 cells during RA treatment by ChIP assay.

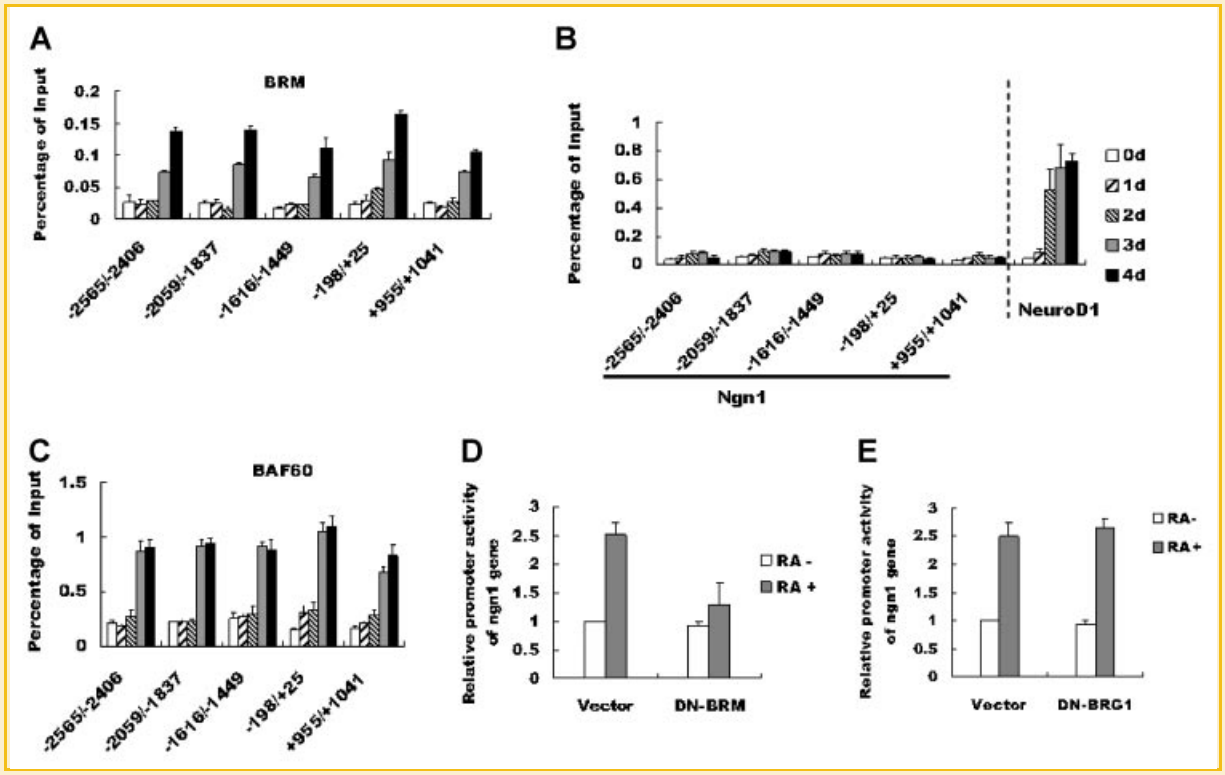

Fig. 4. Recruitment of SWI/SNF chromatin remodeling complex on *ngn1* gene in RA treated P19 cells. Analyses of the recruitment of BRM (A), BRG1 (B) and BAF60 (C) by ChIP assay. A,C: on *ngn1* gene, (B) on *ngn1* and *Neurod1* (−419/−64). D,E: Promoter activity assays of *ngn1* gene in P19 cells with (RA+) or without (RA−) RA treatment. Expression constructs of the dominant negative mutant BRM (pcDNA6/BRM-R) or BRG1 (pBJ5-BRG1K798R), and an empty vector were individually co-transfected with pGL3-ngn1-luc and pRL-TK.

showed that BRM was efficiently recruited to the regulatory region of *ngn1* after 3 days of RA treatment, in particular, on the fourth day (Fig. 4A). However BAF60 was recruited to *ngn1* gene more efficiently on the third day in all the regions of the gene detected (Fig. 4C). By contrast, the binding of BRG1 on *ngn1* gene was not induced by RA treatment at all comparing with another neural specific gene *Neurod1*, as a positive control here (Fig. 4B left vs. right panels). It was further shown that the dominant-negative BRM drastically abolished the RA induction of the promoter activity of *ngn1* (Fig. 4D), but not that of the BRG1 (Fig. 4E). These data suggested the BRM-SWI/SNF chromatin remodeling complex was functional in RA induced activation of *ngn1*.

DISCUSSION

We have shown that *ngn1* is activated in RA induced neuronal differentiation of P19 cells, which is in accordance with the modifications on histone 3 and the recruitment of SWI/SNF chromatin remodeling complex on *ngn1* gene. The results indicate that chromatin remodeling is essential in the RA induced expression of *ngn1* gene in P19 cells.

According to the timing of recruiting histone marks or chromatin remodelers to *ngn1* gene in the cells, three stages are roughly defined (Fig. 5). The first stage covers the changes from untreated cells to the first day after RA treatment, which is characterized by a significant increase of H3K27me3 surrounded the promoter of *ngn1* gene. The second stage is specified by a crossing between the increasing of activation mark H3K4me3 and the declining of the repression H3K9me2 from the first to the second day of treatment. In the third stage of days 3 and 4, H3K4me3 and H3K9ac eventually get to their peak levels that is accompanying by the efficient recruiting of RNA Pol II at the proximal promoter region of the gene. Meanwhile, as the BRM-chromatin remodeling complex is recruited to all the detected regions of *ngn1* gene, we suggests that it play a role to ensure the histone modifications required on the activation of the gene. Summarizing the above, each of the epigenetic changes happens on the *ngn1* gene is either temporal or spatial specific that sums up to make the multi-stage regulation of the *ngn1* gene in RA induced differentiating P19 cells as shown in the scratched model of Figure 5.

To our surprise, in the first stage of chromatin remodeling (the first day of RA treatment), the recruiting of H3K27me3, a repressive histone mark is significantly induced on the *ngn1* gene (Fig. 3C). Although it is discrepant to the general idea of gene activation, we suggest that the methylation of H3K27 could only be an early responding event to RA in the P19 cells when *ngn1* gene is not functional and need to be kept in a silence. Therefore, the recruiting of the repression mark H3K27me3 in the case of *ngn1* gene is appropriate before the cells are committed to differentiation. In other words, the presence of H3K27me3 may indicate the selection of genes that needs to be repressed immediately after RA treatment to make sure that the appropriate timing of differentiation occurs in

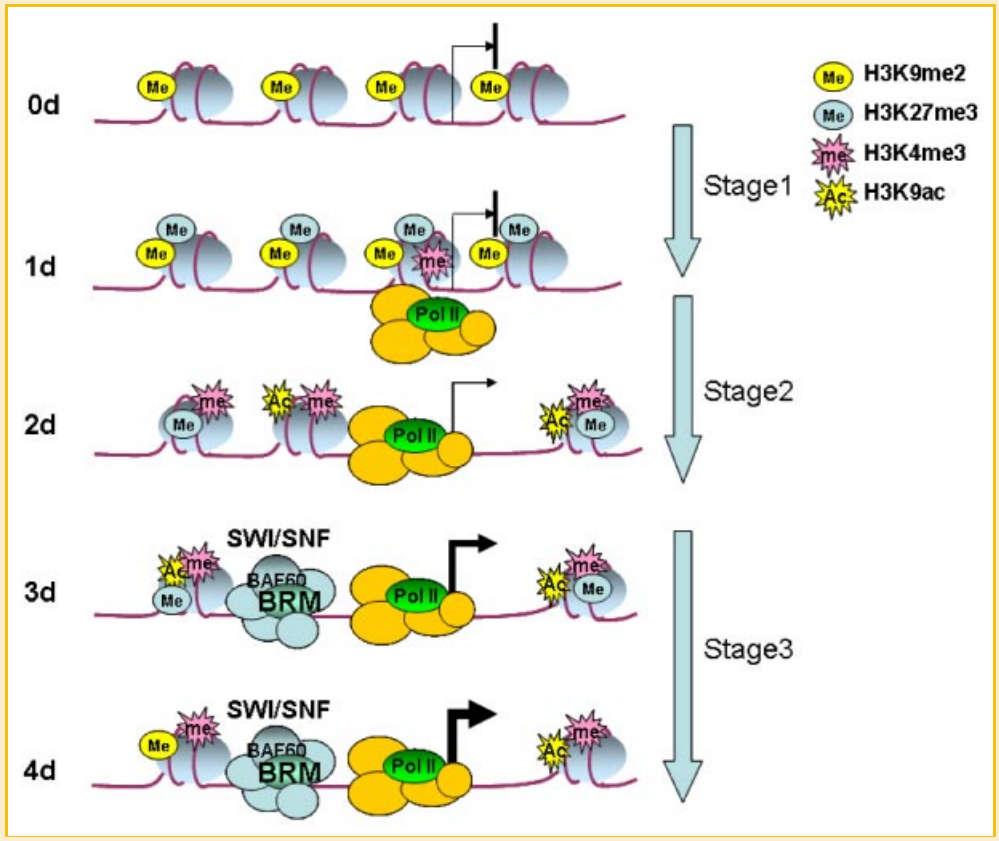

Fig. 5. A schematic model for stages of epigenetic regulation on ngn1 in RA treated P19 cells. ✸ or ⬭ represent an activation or repression histone mark, respectively. Arrows represent the transcription efficiency of the gene. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the cells. A recent opinion shows that a repressive and an activation mark can be co-exist to determine the expression state of the gene [Barski et al., 2007], which explains the coexistence of H3K27me3 and H3K4me3, and that of the H3K9ac and H3K9me2 even on the same lysine site at the *ngn1* gene.

In this study, the recruiting of BRM on *ngn1* provides novel evidence to the function of BRM in neuronal differentiation. As the dominant negative BRM reduces the promoter activity of *ngn1* in RA-induced cells, which indicates BRM takes important parts in activating the *ngn1* gene. However, unlike the case of other neuronal marker gene *NeuroD* in RA induced cells (Fig. 4B, right panel) [Seo et al., 2005], BRG1 is neither recruited to *ngn1* gene, nor affected the promoter activity of *ngn1* (Fig. 4D, left panel). This result shows that BRG1 and BRM, although highly homologous in protein structure, are case by case specific in selecting target genes in neuronal differentiation. Furthermore, we suggest that BAF60 may take parts in mediating BRM to the *ngn1* gene as it does in recruiting the SWI/SNF complex via MyoD to the specific promoter during myogenic differentiation [Li et al., 2007]. As BRM is involved in both differentiation and cell cycle exit [Das et al., 2007], the recruitment of BRM in the third stage of RA treatment may indicate that the cells are committed to differentiation.

In summary, we have shown for the first time on the sequential recruiting of specific histone marks to the promoter region surrounding the *ngn1* gene in the early stage of RA induced differentiation of P19 cells. Our results suggest that these epigenetic changes could be summarized in three successive stages: (1) elevation of H3K27me3 as a repression mark on *ngn1* gene to control the proper timing for the initiation of RA induced differentiation. (2) The crossing between the increasing activating marks and the declining of the repressive ones that facilitates the RNA Pol II recruiting on the promoter of *ngn1* gene. (3) The recruiting of late responding activation marks H3K4me3 and H3K9ac accompanying by the BRM-SWI/SNF chromatin remodeling complex on the *ngn1* gene is coincident with the activation of *ngn1* gene. These results show novel links on the comprehensive modifications of core histone with the activation of neuronal specific gene and the commitment of the P19 cells to RA induced differentiation.

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