Synergy between the RE-1 Silencer of Transcription and NFκB in the Repression of the Neurotransmitter Gene TAC1 in Human Mesenchymal Stem Cells*

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The RE-1 silencer of transcription (REST) is a transcriptional regulator that represses neuron-specific genes in non-neuronal tissues by remodeling chromatin structure. We have utilized human mesenchymal stem cells (MSCs) as a research tool to understand the molecular mechanisms that regulate a neurogenic program of differentiation in non-neuronal tissue. MSCs are mesoderm-derived cells that generate specialized cells such as stroma, fat, bone, and cartilage. We have reported previously the transdifferentiation of MSCs into functional neuronal cells (Cho, K. J., Trzaska, K. A., Greco, S. J., McDade, J., Wang, F. S., Ye, J.-H., and Rameshwar, P. (2005) Stem Cells 23, 383–391). Expression of the neurotransmitter gene TAC1 was detected only in neuronal cells and thus served as a model to study transcriptional regulation of neuron-specific genes in undifferentiated MSCs. Bone marrow stromal cells are known to transiently express TAC1 following stimulation with the microenviron-mental factor interleukin-1α. We thus compared the effects of interleukin-1α stimulation and neuronal induction of MSCs on TAC1 regulation. Transcription factor mapping of the 5’-flanking region of the TAC1 promoter predicted two REST-binding sites adjacent to one NFκB site within exon 1. Chromatin immuno-nprecipitation, mutagenesis, and loss-of-function studies showed that both transcription factors synergistically mediated repression of TAC1 in the neurogenic and microenvironmental models. Together, the results support the novel finding of synergism between REST and NFκB in the suppression of TAC1 in non-neuronal cells.

Adult and embryonic stem cells both show therapeutic potential for many diseases (1–7). In addition, stem cells provide invaluable experimental resources to study developmental processes, including embryogenesis, cancer development, and aging (8–10). These in vivo processes are recapitulated in vitro in investigational models. Mesenchymal stem cells (MSCs) are found in fetal tissues and in adults (11). Bone marrow (BM) is the primary residence of MSCs in adults (11). MSCs can trans-differentiate into several specialized cells, including functional neuronal cells, in vitro methods (12).

Transdifferentiation is the process whereby a cell of one defined germ layer, such as mesoderm, differentiates into a cell of a different germ layer, such as ectoderm. MSCs differentiate along multilineages to generate cells of osteogenic, chondrogenic, and adipogenic types (11). MSCs have been shown to exhibit plasticity by generating specialized cells of all germ layers. However, as stem cells, it is unclear how MSCs can prevent premature expression of tissue-specific genes. The RE-1 silencer of transcription (REST), also known as neural restrict-ing silencing factor, is a transcription factor that represses target gene transcription by binding regulatory elements containing a consensus 21-bp RE-1 sequence (13). Repression occurs through assembly of a repressor complex at the N and C termini of the transcription factor (14). The N terminus recruits Sin3A/histone deacetylase (HDAC), and the C terminus recruits the corepressor Co-REST to mediate chromatin remodeling and repression of gene expression (14). REST expression has been demonstrated in non-neuronal, neural stem, and neural progenitor cells, where its function has been ascribed to repressing the expression of pan-neuronal genes in non-neuronal or immature neuronal tissues (15). Recently, REST has been shown to exhibit both tumor suppressor and oncogenic activities, thus implicating its role in developmental processes (16, 17). In non-neuronal, neural stem, and neural progenitor cells, REST suppresses genes exclusive to mature neurons (18). More specifically, REST silences target genes involved in synaptogen-esis, progression of the cell cycle, neurotransmitter biosynthe-sis, and electrophysiological function (18). Down-regulation of REST is consistent with neuronal maturation and increased expression of neuron-specific genes.

REST has been shown to modulate the expression of the neuro-transmitter gene TAC1 (also known as preprotachykinin A) in a rodent hippocampal seizure model (19). One of the major encoded peptides of TAC1 is the neurotransmitter substance P, which belongs to the tachykinin family of neuropeptides (20). Repression of TAC1 has also been reported to occur through an NFκB-dependent mechanism in non-neuronal BM stromal cells (21).

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3 The abbreviations used are: MSCs, mesenchymal stem cells; BM, bone mar-row; REST, RE-1 silencer of transcription; HDAC, histone deacetylase; IL-1α, interleukin-1α; FCS, fetal calf serum; RT, reverse transcription; ChIP, chro-matin immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA.
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We have shown previously the suppression of TAC1 in human MSCs, but up-regulation following transdifferentiation to neuronal cells (12). This observation is significant in light of MSCs showing potential for application in neuronal tissue injuries (22). MSCs have also been proposed as stem cells that can be used in gene delivery not only for tissue repair, but also to sites of tumor metastasis (23). This led us to ask whether microenvironmental factors might affect neuron-specific gene expression in stem cells. This question is significant because expressed genes could potentially affect the surrounding tissues, thereby leading to microenvironment-stem cell crosstalk. This type of communication could be harmful and/or beneficial. Regardless, an understanding of the mechanism by which suppressed genes are affected by microenvironmental factors will lead to insights on communication between stem cells and their surrounding microenvironment. We addressed this general question with a focus on TAC1 expression in undifferentiated MSCs, their transdifferentiated neuronal progeny, and BM stromal cells because the latter are known to transiently express TAC1 following stimulation with the microenvironmental factor interleukin-1α (IL-1α). In the study, we focused on the roles of the transcription factors REST and NFkB. The latter was selected because of a functional binding site close to the binding region for REST within exon 1 of TAC1. Here we report on the synergistic roles for REST and NFkB in the repression of TAC1 transcription in undifferentiated MSCs and their suppression following neuronal induction or microenvironmental influence.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Dulbecco’s modified Eagle’s medium with high glucose, Dulbecco’s modified Eagle’s medium/nutrient mixture F-12, α-minimum essential medium, 1-glutamine, B-27 supplement, and hygromycin B were purchased from Invitrogen. Fetal calf serum (FCS), all-trans-retinoic acid, and Ficoll-Hypaque were obtained from Sigma, and defined FCS was from Atlanta Biologicals (Lawrenceville, GA).

Recombinant human IL-1α was obtained from Hoffmann-La Roche, and recombinant human basic fibroblast growth factor was purchased from R&D Systems (Minneapolis, MN). Protease inhibitor mixture and human brain nuclear extract were from Active Motif (Carlsbad, CA), and HeLa cell nuclear extract was obtained from Upstate (Charlottesville, VA). Rabbit anti-REST antibody was purchased from Upstate; rabbit anti-p65 and goat anti-riboosomal protein L28 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-RNA polymerase II monoclonal antibody from Active Motif; and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody from Sigma.

Vectors—pGL3-Basic with the 5′-flanking region of TAC1 (pGL3-TAC1–1.2, 1.2 kb) was described previously and includes intron 1, exon 1, and upstream sequences (24). pCMV-IxBa-wt and pCMV-IxB-mut (where wt and mut are wild-type and mutant, respectively) are part of the Mercury IxB dominant-negative vector set from Clontech. The mutant sequesters NFkB in the cytosol by preventing its phosphorylation. pβ-gal and pTK-Hyg were purchased from Clontech.

Culture of Human MSCs—Human MSCs were cultured from BM aspirates as described (25). Unfractionated BM aspirates (2 ml) were diluted in 12 ml of Dulbecco’s modified Eagle’s medium containing 10% FCS (D10 medium) and then transferred to vacuum-gas plasma-treated tissue culture Falcon 3003 Petri dishes (BD Biosciences). The plates were incubated, and at day 3, mononuclear cells were isolated on a Ficoll-Hypaque density gradient and replaced in the culture plates. 50% of the medium was replaced with fresh D10 medium at weekly intervals until the adherent cells were ~80% confluent. After four cell passages, the adherent cells were symmetric: CD14−, CD29+, CD44+, CD34−, CD45−, SH2+, and prolyl 4-hydroxylase− (25). All experiments utilizing MSCs were repeated with different BM donors.

Culture of BM Stromal Cells—BM aspirates were obtained from healthy donors between 18 and 25 years old. Nucleated cells (107) were added to 25-cm2 tissue culture flasks (Falcon 3109) in stromal culture medium (α-minimum essential medium with 20% FCS). Flasks were incubated at 37 °C. At day 3, the red blood cells and neutrophils were removed on a Ficoll-Hypaque density gradient, and the mononuclear cells were resuspended in fresh stromal medium. The flasks were incubated until the cells attained maximal confluence, with weekly replacement with 50% fresh stromal medium. At confluence, the trypsin-sensitive adherent cells were passaged. Cells were passaged at least five times before being used in experiments. Flow cytometry studies indicated that >99% of the cells were negative for CD14 and positive for prolyl 4-hydroxylase (26). All experiments utilizing BM stromal cells were repeated with different BM donors.

Neuronal Induction of MSCs—MSCs at 70–80% confluence were trypsinized and then subcultured in 60-mm Falcon 3002 Petri dishes or in Falcon 353046 6-well plates (BD Biosciences). For Western analyses, reverse transcription (RT)-PCR, and chromatin immunoprecipitation (ChIP), 105 MSCs were seeded in 60-mm tissue culture dishes. For transfection studies, 103 MSCs were seeded in 6-well tissue culture plates. All cells were allowed to adhere to the culture surface overnight in D10 medium. At 20% confluence, D10 medium was replaced with neuronal induction medium containing Ham’s Dulbecco’s modified Eagle’s medium/nutrient mixture F-12, 2% FCS, B-27 supplement, 20 μM all-trans-retinoic acid, and 12.5 ng/ml basic fibroblast growth factor. A stock solution of all-trans-retinoic acid was diluted in dimethyl sulfoxide to 20 mM. The induction by undefined FCS is permissive to commitment to other cells as opposed to the retention of pluripotency. In parallel studies, MSCs were cultured in medium with vehicle used for reconstitution of all-trans-retinoic acid and growth factors, hereafter referred to as uninduced MSCs (called D0 cells). The data in all experiments were similar for D0 MSCs and those cultured in expansion medium (D10 medium), with the latter referred to as undifferentiated MSCs. During induction, the medium was unchanged for a maximum of 12 days. Concurrent experiments replacing 50% of the neuronal induction medium after 4 days of induction yielded similar results. All experimental endpoints were performed with a maximal confluence of 70% to control for contact inhibition. Experimental endpoints for induced MSCs were 6 and 12 days of induction (called D6 and D12 cells). Previous studies have shown that these endpoints correspond...
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Semiquantitative and Real-time RT-PCR—Total RNA (2 μg) was reverse-transcribed, and 200 ng of cDNA was used to amplify TAC1, REST, or p65. For real-time RT-PCR, REST was amplified with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The primers for REST and TAC1 span +2777 to +2985 (GenBank™ accession number NM_005612) and +60 to +328 (accession number NM_003182), respectively, with the following sequences: REST, 5′-ttc caa aaa gcc ttc tct-3′ (forward) and 5′-ggt ctc ctg atg ctc ct-3′ (reverse); and TAC1, 5′-act gtc gct ggc aaa atc-3′ (forward) and 5′-ggg cca ctt gtt ttt caa-3′ (reverse). PCRs were normalized by amplifying the same sample of cDNA with primers specific for β-actin. The primers for β-actin span +842 to +1037 (GenBank™ accession number NM_001101), with the following sequences: 5′-tgc cct gag gca ctc ttc-3′ (forward) and 5′-ggt cca ggc gga tga tct-3′ (reverse). Real-time PCRs were performed with an Applied Biosystems 7500 real-time PCR system. The cycling profile for real-time PCR for REST (40 cycles) and β-actin (40 cycles) was 94 °C for 15 s and 60 °C for 45 s. Gene expression analysis was performed using the Applied Biosystems 7500 system SDS software. Normalizations were performed with β-actin, and values were arbitrarily assigned a value of 1. For semiquantitative RT-PCR, REST, TAC1, and p65 were amplified. The primers for REST and TAC1 were also used for real-time quantitative RT-PCR, REST, TAC1, and p65 were amplified. Values were normalized to a housekeeping gene (GAPDH) and values were arbitrarily assigned a value of 1. For semiquantitative PCR, GAPDH amplification was used for all other binding reactions (13). Electrophoresis of reaction mixtures was carried out at 400 V for 3–4 h at 4 °C on a 6% polyacrylamide gel. In the supershift assays, nuclear extracts were incubated with 2 μg of anti-REST or anti-p65 antibody prior to incubation with labeled probes. 25 μg of HeLa cell nuclear extract was found to be optimal for the wild-type REST1 and RE-1-binding element (positive control probe) oligonucleotides, whereas the maximal concentration of extract was used for all other binding reactions (13). Electrophoresis of reaction mixtures was carried out at 400 V for 3–4 h at 4 °C on nondenaturing 5% polyacrylamide gels in 0.25× Tris borate/EDTA. Gels were dried and visualized by autoradiography or exposed to Kodak XAR-5 film with an intensifying screen for 12 h at −80 °C.

Rest1 and NFκB Mutation—Mutations of the REST1 and NFκB sites were performed with a site-directed mutagenesis kit from Stratagene (La Jolla, CA) following the manufacturer’s instructions. Briefly, forward and reverse primers for the desired mutant sequence (see Table 1) were synthesized and used in PCR to amplify pGL3-TAC1–1.2. The resultant mutant REST1 and NFκB vectors were given the designations pGL3-TAC1–1.2/REST1-mut and pGL3-TAC1–1.2/NFκB-mut, respectively. Mutant DNA sequencing was performed at the Molecular Core Facility of the New Jersey Medical School.

ChIP—ChIP was performed using the ChIP-IT enzymatic kit (Active Motif) according to the manufacturer’s specific guidelines. Briefly, uninduced (D0) and induced (D12) MSCs (stimulated for 16 h with 10 ng/ml IL-1α or left unstimulated) were fixed with 3.7% formaldehyde. Next, chromatin was extracted and enzymatically sheared into 500–1000-bp fragments. Sheared chromatin was separated by electrophoresis on 1.5% agarose containing ethidium bromide to verify fragment size. Chromatin was incubated overnight with 2 μg of anti-REST, anti-p65, anti-IgG (negative control), or anti-RNA polymerase II antibody and then precipitated with protein G-agarose. Genomic DNA was then eluted with buffers provided with the kit. Enriched chromatin was analyzed by PCR using primers flanking the REST1- and NFκB-binding sites within exon 1/TAC1. The primers span +689 to +801 (GenBank™ accession number AF252261), with the following sequences: 5′-act gag cag gcc aaa g-3′ (forward) and 5′-agt ccc gcc ggg tgt gat-3′ (reverse). Control chromatin enriched with anti-RNA polymerase II antibody for the GAPDH gene was analyzed by PCR using primers included with the kit. Total chromatin served as the input loading control, and water served as the non-template negative control for PCR. All PCRs (10 μl) were analyzed by electrophoresis as described above.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA for REST and NFκB binding was performed as described (27). Briefly, REST- and NFκB-binding sites within the 5′-flanking region of the TAC1 promoter (−722 to +498; GenBank™ accession number AF252261) were predicted using the MatInspector transcription factor analysis tool from Genomatix. Double-stranded oligonucleotides containing the predicted binding sites (REST1, REST2, and NFκB) were synthesized at the Molecular Core Facility of the New Jersey Medical School (see Table 1). Both sense and antisense sequences have TG overhangs that served as end fillings with reverse transcriptase (SuperScript, Invitrogen) and [32P]dCTP and dATP (50 μCi of 3000 Ci/mM; PerkinElmer Life Sciences). REST and NFκB consensus sequences are shown in boldface. Base pair changes within the mutant sequences are underlined and italicized. Extracts from HeLa cells and human brain nuclei served as positive and negative controls, respectively, for REST protein, and extracts from the breast cancer cell line MDA-MB-231 served as a positive control for NFκB protein. For binding reactions, 5, 10, 25, or 50 μg of nuclear extract was incubated with 1 ng of double-stranded 32P-labeled oligonucleotide for 25 min at room temperature in 12.5 μl of binding reaction containing 10 mM Tris HCl (pH 8.0), 150 mM KCl, 0.1% Triton X-100, 12.5% (v/v) glycerol, 0.2 mM dithiothreitol, and 13 ng/ml poly(dI·dC) (Sigma). In the supershift assays, nuclear extracts were incubated with 2 μg of anti-REST or anti-p65 antibody prior to incubation with labeled probes. 25 μg of HeLa cell nuclear extract was found to be optimal for the wild-type REST1 and RE-1-binding element (positive control probe) oligonucleotides, whereas the maximal concentration of extract was used for all other binding reactions (13). Electrophoresis of reaction mixtures was carried out at 400 V for 3–4 h at 4 °C on nondenaturing 5% polyacrylamide gels in 0.25× Tris borate/EDTA. Gels were dried and visualized by autoradiography or exposed to Kodak XAR-5 film with an intensifying screen for 12 h at −80 °C.

Stable Suppression of REST in MSCs—Construction of the small interfering RNA (siRNA) vector pMPSKH1 was described previously (28). The wild-type REST siRNA sequence (GenBank™ accession number NM_005612) spans +1550 to +1568 (5′-ggt taa tct aca gta tca c-3′; pMPSKH1-REST-wt). The negative control siRNA sequence contains three nucleo-
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A. Normalized Band Density

B. S' -722 Tac1 5' Exon 1 Intrum 1 3' TAC1-1.2

C. REST GAPDH

D. Fold Change REST mRNA

E. REST β-Actin

F. ChIP

G. REST1' REST2' REST1' REST2' REST1' REST2'

Probe wt mut wt mut wt mut wt mut wt mut wt mut
Extract + + + + - - - - + + + +
Antibody - - - - + + + + - - - -

Complex Free Probe

Antibody Complex

Complex

Free Probe
tide mutations within the gene-specific insert (underlined and italic): 5′-gtg tta tat aa gtt ctc c-3′ (pPMSKH1-REST-mut). At 50% confluence, undifferentiated MSCs orstromal cells were cotransfected with pTK-Hyg andpPMSKH1-REST-wt or pPMSKH1-REST-mut. Stable transfectants were selected with 25 μg/ml hygromycin. Stable knockdown of REST was evaluated by RT-PCR with REST-specific primers previously utilized for PCR. All knockdown cultures were maintained in medium containing hygromycin.

**Transient Transfection and Reporter Gene Assay**—At 50% confluence, MSCs (uninduced and induced) and/or BM stromal cells were cotransfected with pβ-gal and pG3-TAC1–1.2 (wild-type or mutant REST1; wild-type or mutant NFκB) using Effectene transfection reagent (Qiagen Inc., Valencia, CA). In additional studies, undifferentiated MSCs stimulated for 16 h with 10 ng/ml IL-1α or left unstimulated were cotransfected with pβ-gal, pG3-L-TAC1–1.2, and IκB (wild-type or mutant). After 48 h, cell-free lysates were prepared as described previously (24). The lysates were quantified for luciferase and β-galactosidase activities using the Dual-Luciferase assay system (Promega Corp.) and the β-galactosidase detection kit II (Clontech), respectively. Normalizations were performed using the luciferase/β-galactosidase activity ratio in cells transfected with the pG3 vector alone, arbitrarily assigning a value of 1. Transfections were repeated if β-galactosidase activities were <100 relative luciferase units. Total protein was determined with the Bio-Rad DC protein assay kit.

**Western Analysis**—Nuclear and cytoplasmic proteins were extracted with the NXTACT kit (Sigma) according to the manufacturer’s specified guidelines. Total protein was determined with the Bio-Rad DC protein assay kit. Experiments (15 μg) were treated with 1 μl of protease inhibitor mixture and analyzed by Western blotting on 4–20% SDS-polyacrylamide precast gels (Bio-Rad). Proteins were next transferred onto polyvinylidene difluoride membranes (PerkinElmer Life Sciences) and incubated overnight with primary antibodies. Detection was performed the following day by a 2-h incubation with horseradish peroxidase-conjugated IgG. All primary and secondary antibodies were used at final dilutions of 1:1000 and 1:2000, respectively. Horseradish peroxidase was developed with chemiluminescence detection reagent (PerkinElmer Life Sciences). The membranes were stripped with Restore stripping buffer (Pierce) for reprobing with other antibodies. Cytoplasmic contamination of nuclear extracts was determined by reprobing the membranes with anti-ribosomal protein L28 antibody. HeLa cell nuclear extract served as a positive control for REST detection.

**Data Analyses**—Statistical analyses were performed by analysis of variance and the Tukey-Kramer multiple comparison test. p < 0.05 was considered significant.

## RESULTS

**TAC1 Expression in Uninduced and Induced MSCs**—Human MSC-derived neuronal cells express the neurotransmitter gene TAC1 (12). This study therefore focused on TAC1 as a model to understand suppression of neuron-specific genes in MSCs. The first set of experiments determined timeline expression of TAC1 in MSCs induced to generate neurons. The results show a proportional correlation between TAC1 mRNA expression and days of induction as determined by semiquantitative RT-PCR (Fig. 1A, inset). No band was detected in uninduced MSCs (D0 cells). The results (presented as normalized densities with GAPDH mRNA) show significant (p < 0.05) increases in band density at 6 and 12 days of induction (D6 and D12 cells) (Fig. 1A).

**Interaction of REST with TAC1 in Uninduced and Induced MSCs**—Exon 1 of TAC1 is a noncoding region with regulatory elements (24). In these experiments, we assessed the mechanism by which TAC1 transcription is suppressed in undifferentiated MSCs (12). We focused on the consensus sequences for

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**TABLE 1**

| Sequence (5′→3′) | TAC1 region | Sequence description |
|-----------------|-------------|---------------------|
| tga acc aga gaa ctc agc acc ccg cgg gac tg | +54 to +82 | Wild-type REST1 |
| tga acc aga gaa ccg agc cgc ctg cgg gac tg | +54 to +82 | Mutant REST1 |
| tgg gga ctg tcc gtc gca gta act ggc cgc tg | +78 to +105 | Wild-type NFκB |
| tgg gga ctg tcc gtc gaa gga cgg gcc cgc tg | +78 to +105 | Mutant NFκB |
| tgc tca gaa ggg tgg aca ggc ccc g | NA | RE-1-binding element |
| tgc tca gca ccc cgc ggg act ctc gtc ctg cgc agt aat g | +64 to +97 | Wild-type NFκB |
| tgc tca gca ccc cgc ggg act ctc ggc cgc agt aat g | +64 to +97 | Mutant NFκB |

**FIGURE 1. REST interaction and function in MSCs.** A, total RNA from uninduced (D0) and induced (D6 and D12) MSCs was studied for TAC1 mRNA by RT-PCR (inset). Band densities were normalized with GAPDH, and uninduced values were arbitrarily assigned a value of 1. The results are presented as the mean ± S.D. (n = 5). B, shown is a schematic diagram of the 5′-flanking region of the TAC1 promoter, hereafter referred to as TAC1–1.2. TAC1–1.2 contains the noncoding exon 1, intron 1, and upstream sequences. Exon 1 contains two predicted binding sites for REST (REST1 and REST2) and one site for NFκB (κB–1). C, total RNA from D0, D6, and D12 cells was studied for REST mRNA by RT-PCR. Normalizations were performed with oligonucleotide specific for GAPDH (n = 5). D, REST mRNA levels were determined by real-time RT-PCR. Normalizations were performed with β-actin, and D12 values were arbitrarily assigned a value of 1. Results are presented as the mean ± S.D. of the fold change (n = 5). E, nuclear extracts from D0, D6, and D12 cells were prepared and analyzed by Western blotting with anti-REST antibody. Normalizations were performed with anti-β-actin antibody (n = 3). HeLa cell nuclear extract served as a positive control. F, D0 and D12 cells were fixed with 3.7% formaldehyde, and chromatin was extracted and sheared into 500–1000-bp fragments. ChiP assay was performed with anti-REST antibody and oligonucleotides flanking the exon 1/TAC1 REST sites. Normalizations were performed with chromatin enriched with anti-RNA polymerase II antibody (for GAPDH) or total chromatin (Input) (n = 3). Chromatin enriched with anti-IgG antibody or water served as a negative control for the PCs. G, shown are representative blots of EMSA performed with REST-positive extract (HeLa), anti-REST antibody, and wild-type (wt) and mutant (mut) REST1 and REST2 probes. Parallel control studies were performed with REST-negative extract (brain) and probe containing the known REST-binding element (RE-1) (n = 3). **, p < 0.05 versus uninduced (D0) cells; ***, p < 0.05 versus induced (D12) cells.
two REST sites identified in exon 1 and a flanking functional NFκB site (Fig. 1B) (21). These two sites are relevant to this study based on their links to TAC1 repression in non-neuronal and neuronal cell types, respectively (19, 21).

Semiquantitative RT-PCR for REST mRNA using total RNA from uninduced (D0) and induced (D6 and D12) MSCs showed strong band intensities in D0 cells. Following induction, band intensities were decreased in D6 cells and undetectable in D12 cells (Fig. 1C). Using a quantitative approach by real-time RT-PCR, we observed significantly (p < 0.05) elevated levels of REST mRNA in D0 cells compared with D6 and D12 cells (Fig. 1D).

The changes in REST mRNA were next studied at the protein level. Western blotting was performed with nuclear extracts from uninduced (D0) and induced (D6 and D12) MSCs. Dense bands were observed in D0 cells, reduced bands in D6 cells, and undetectable bands in D12 cells (Fig. 1E).

The expression of REST in uninduced MSCs does not determine the status of REST interaction with the TAC1 gene. To address this question, we performed ChIP assay with immunoprecipitated chromatin from uninduced (D0) and induced (D12) MSCs. The precipitated genomic DNA was subjected to RT-PCR with primers flanking the REST-binding regions within TAC1. Intense bands were observed for D0 cells (Fig. 1F, upper panel, REST1/Tac1 lane), but were undetectable in D12 cells (lower panel, REST1/Tac1 lane). In summary, uninduced MSCs show elevated expression of REST, which binds TAC1. Neuronal induction of MSCs leads to REST downregulation and its release from TAC1.

REST Binding to Two Consensus Regions in Exon 1/TAC1—The experiments discussed above indicated binding of REST to TAC1. However, the data did not determine whether REST binds to one or both predicted consensus sequences (Fig. 1B). Here we performed EMSA with wild-type and mutant oligonu-
cleotides spanning both REST regions (Table 1). EMSA was performed with HeLa cell nuclear extract as a source of REST protein at the optimal concentration of 25 μg of protein. We observed a band for the wild-type REST1 probe (Fig. 1G, lane 1), whereas no band was observed for the mutant REST1 and wild-type and mutant REST2 probes (lanes 2–4). The bands were specific because probe alone showed no band (Fig. 1G, lanes 5–8), and incubation with anti-REST antibody produced a supershift for the wild-type REST1 probe (Fig. 1G, lane 9).

Because REST expression is decreased in brain tissues, we used extracts from human brain nuclei as negative controls in EMSA. The experiment was performed with the probe that showed a band with HeLa cell extracts (Fig. 1G, lane 13). No band was detectable with brain nuclear extract (Fig. 1G, lane 14). To reaffirm the experimental band, we performed additional experiments. We performed EMSA with HeLa cell extract and known probes that bind REST, referred to as the RE-1-binding element (Table 1), as a positive control. We observed a strong band for the positive control, whereas no band was observed when the probe was incubated with human brain nuclear extract (Fig. 1G, lanes 15 and 16). In summary, the results authenticate REST1 as a REST-binding site, but not REST2.

Role of REST in TAC1 Activation—Because the mutant REST1 oligonucleotide was unable to bind REST protein (Fig. 1G), we next applied a similar mutation to study the role of REST1 in TAC1 expression. This question was addressed with a reporter gene assay using pGL3-TAC1–1.2 (Fig. 1B) with mutant or wild-type REST1. Uninduced (D0) and induced (D6 and D12) MSCs were transfected with the aforementioned vectors, and after 48 h, cell extracts were quantitated for luciferase activities. The data show a significant (p < 0.05) increase in activities in D0 and D6 cells for the mutant compared with the wild type (Fig. 2A). There was no significant (p > 0.05) difference between wild-type and mutant REST1 in D12 transfec-tants (Fig. 2A). These results are consistent with the Western blots for REST protein (Fig. 1E). In summary, the results demonstrate a functional REST1 site within exon 1 of TAC1.

TAC1 Reporter Gene Activity in REST Knockdown MSCs—In these experiments, we explored the cause-effect relationship between endogenous REST and TAC1 activity by reporter gene assay. We stably knocked down REST in undifferentiated MSCs with REST siRNA. Controls were transfected with mutant REST siRNA. REST knockdown was validated at the mRNA and protein levels, as shown in Fig. 2B (B and C, respectively). RT-PCR was normalized with GAPDH-specific primers, and Western blotting was normalized with anti-β-actin antibody.

The next set of experiments was based on the premise that REST represses TAC1 expression. Thus, if REST is knocked down, then TAC1 reporter gene activity is expected to be increased in undifferentiated MSCs. We therefore transiently transfected REST knockdown MSCs (uninduced (D0) or induced (D6 and D12)) with pGL3-TAC1–1.2 (wild type). Parallel experiments were performed with mutant REST siRNAs. After 48 h, REST knockdown cells showed significantly (p < 0.05) greater activities in uninduced and D6 cells compared with similar experimental points with mutant siRNA or cells not stably transfected with siRNA (Fig. 2D). REST knockdown MSCs induced for 12 days showed no significant (p > 0.05) difference in luciferase activities compared with mutant siRNAs or cells not stably transfected with siRNA (Fig. 2D). In summary, siRNA knockdown leads to increased luciferase activity at time points (D0 and D6) at which pGL3-TAC1–1.2 activities are expected to be at the base line or relatively low.

Cause-Effect Relationship between Endogenous REST and TAC1 in MSCs—In these experiments, we aimed to verify that the reporter gene activities (Fig. 2D) were consistent with endogenous association between REST and TAC1. We studied TAC1 mRNA in REST knockdown MSCs that were uninduced (D0) or induced (D6 and D12). Control analyses were performed with cells stably transfected with mutant REST siRNA or with untransfected cells. Real-time PCR with TAC1-specific primers showed a timeline increase (p < 0.05) in TAC1 mRNA at 0 and 6 days in REST knockdown cells compared with mutants and untransfected MSCs (Fig. 2E). In the case of D12 cells, the mRNA levels were similar for untransfected cells and cells transfected with mutant and wild-type siRNAs (Fig. 2E, D12 bars). In summary, REST knockdown correlates with an increase in TAC1 mRNA in uninduced (D0) and induced (D6) MSCs.

Mutant Promoter Activity in REST Knockdown MSCs—We next compared mutant REST1 promoter activity in REST knockdown MSCs versus untransfected MSCs (Fig. 2F). No significant (p > 0.05) difference in TAC1 activity was observed between siRNA knockdown MSCs, mutant siRNAs, and untransfected cells.

Nuclear Localization of NFκB in MSCs—NFκB has been shown to repress TAC1 transcription in BM stromal cells (21). Because an NFκB-binding site is located adjacent to REST1 (Fig. 1B), we investigated whether the activated form of this transcription factor might be down-regulated in induced MSCs, when TAC1 mRNA levels are increased. To this end, we studied cytoplasmic and nuclear extracts of the p65 subunit of NFκB in uninduced (D0) and induced (D6 and D12) MSCs.

FIGURE 2. REST repression of TAC1 in MSCs. A, pGL3-TAC1–1.2/REST1-wt was modified to include the REST1 mutation. Uninduced (D0) and induced (D6 and D12) MSCs were transfected with wild-type (wt) or mutant (mut) REST1, and after 48 h, luciferase and β-galactosidase activities were measured. Results are presented as the mean ± S.D. of normalized luciferase (n = 5). Normalizations were performed with the luciferase/β-galactosidase activity ratio in uninduced cells transfected with the pGL3 vector alone, arbitrarily assigning a value of 1.8. MSCs were stably transfected with siRNA vectors containing either wild-type or mutant REST siRNA inserts. Total RNA from transfected stromalants and MSCs not transfected with siRNA was studied for REST mRNA by RT-PCR. Analyses were performed as described in the legend of Fig. 1C. C, nuclear extracts from stable transfec-tants and MSCs not transfected with siRNA were prepared and analyzed by Western blotting with anti-REST antibody. Analyses were performed as described in the legend of Fig. 1E. D, stable transfec-tants and MSCs not transfected with siRNA were induced (D6 and D12) or left uninduced (D0) and then transiently transfected with pGL3–TAC1–1.2. Analyses were performed as described for A. E, stable transfec-tants and MSCs not transfected with siRNA were induced (D6 and D12) or left uninduced (D0), and TAC1 mRNA was quantified by real-time RT-PCR. Results are presented as the mean ± S.D. of the fold change (n = 5). Normalizations were performed with β-actin, and untransfected values were arbitrarily assigned a value of 1. F, stable transfec-tants and MSCs not transfected with siRNA were transiently transfected with mutant REST1. Analyses were performed as described for A, *p < 0.05 versus pGL3–TAC1–1.2/REST1-wt alone; **, p < 0.05 versus untransfected cells.
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observed a bright band in the D0 cell nuclear extract and a relatively lighter band at 6 days of induction (Fig. 3A, middle panel). No band was detectable at 12 days of induction. Bands of light-to-moderate density were detected in cytoplasmic extracts during the entire period of induction (Fig. 3A, upper panel). Loading proteins were normalized with anti-β-actin antibody. The blots were studied for cytoplasmic contaminants by stripping and reprobing with anti-ribosomal protein L28 antibody, which showed no band, indicating no major contaminants (data not shown). The data indicate an inverse correlation between activated NFκB and TAC1 expression.

Interaction of REST and NFκB with TAC1 in Unstimulated and IL-1α-stimulated MSCs—We next determined whether NFκB binds TAC1 in uninduced (D0) and induced (D12) MSCs. Chromatin was extracted from both cell types and analyzed by ChIP assay with anti-p65 antibody. PCR for the TAC1 region flanking the NFκB-binding site revealed intense bands in the uninduced (but not induced) cells (Fig. 3B, upper two panels, p65/Tac1 lanes).

IL-1α has been reported to induce TAC1 in BM stromal cells (24). Because IL-1α is a pro-inflammatory cytokine and would be expected to be present within the microenvironment of a tissue injury, we investigated whether IL-1α blunts the interaction of REST and NFκB with TAC1 in undifferentiated MSCs. Chromatin was extracted from uninduced (D0) and induced (D12) MSCs stimulated for 16 h with 10 ng/ml IL-1α and analyzed by ChIP assay with anti-REST and anti-p65 antibodies. PCR for the TAC1 region flanking the REST- and NFκB-binding sites revealed undetectable bands in the stimulated cells (Fig. 3B, lower two panels, REST/Tac1 and p65/Tac1 lanes). In summary, IL-1α suppresses the interaction of REST and NFκB with TAC1.

Effects of NFκB on TAC1 Expression in MSCs—We next assessed the effect of activated NFκB on TAC1 expression. Because NFκB was not activated in induced MSCs (Fig. 3A), we addressed this question in undifferentiated MSCs. To this end, cells were transfected with pGL3-TAC1–1.2 and wild-type IκBa or mutant IκB. After 48 h, luciferase activities were determined and showed significant (p < 0.05) increases in mutant IκB transfectants compared with wild-type IκBa (Fig. 3C, left bars).

Because IL-1α has been shown to induce TAC1 in the differentiated progeny of MSCs, viz. stromal cells (24), we next determined whether IL-1α (10 ng/ml, 16-h stimulation) also induces TAC1 expression in MSCs. We observed significant (p < 0.05) increases in luciferase activity following IL-1α stimulation compared with unstimulated MSCs (Fig. 3C, open bars) by reporter gene assay with pGL3-TAC1–1.2. To determine whether NFκB can reverse the inducing effects of IL-1α, we repeated the reporter gene assay with cells cotransfected with wild-type and mutant IκB. The results showed a significant (p < 0.05) decrease with wild-type IκBa compared with transfectants with pGL3-TAC1–1.2 alone (Fig. 3C, right bars). It is interesting that mutant IκB showed significant (p < 0.05) increases in luciferase activities compared with transfectants with pGL3-TAC1–1.2 alone (Fig. 3C, right hatched bar versus open bar). Detection of NFκB in the nuclear and cytoplasmic fractions was determined in all transfected cells to correlate with reporter activity (Fig. 3C, upper and lower panels below the graph). In summary, IL-1α alleviates NFκB-mediated repression of TAC1 in undifferentiated MSCs.

Effects of NFκB on TAC1 Mutant (REST1) Promoter Activity in MSCs—We next assessed whether overexpression of NFκB or its sequesteration within the cytoplasm could alter TAC1 mutant promoter activity in MSCs (Fig. 3D). No significant difference (p > 0.05) in TAC1 activity was observed between wild-type and mutant IκB or untransfected cells.

Mutation of the NFκB-binding Region and Effect on TAC1 Activation—In these experiments, we investigated the synergistic relationship between NFκB and REST in TAC1 regulation. We mutated the NFκB-binding site within the TAC1 promoter (Table 1) to inhibit NFκB binding without affecting the binding affinity for REST (Fig. 3E). We performed EMSA with wild-type and mutant oligonucleotides spanning the NFκB-binding region, HeLa cell nuclear extract as a source of REST protein, and MDA-MB-231 nuclear extract as a source of NFκB protein. We observed a band for NFκB extract incubated with the wild-type probe (Fig. 3E, lane 3), but not the mutant probe (lane 4). Bands were also observed for REST extract incubated with both wild-type and mutant probes (Fig. 3E, lanes 5 and 6). The bands were specific because probe alone showed no band (Fig. 3E, lanes 1 and 2), and incubation with anti-REST and anti-p65 antibodies produced a supershift (lanes 8, 10, and 11).

Because the mutant NFκB oligonucleotide was able to bind REST, but not NFκB protein (Fig. 3E), we applied a similar mutation to study the role of NFκB in TAC1 expression. We addressed this question using pGL3-TAC1–1.2 with mutant or wild-type NFκB. Undifferentiated MSCs were transfected with mutant and wild-type NFκB reporter vectors, and after 48 h, cell extracts were quantitated for luciferase activities. The data show a significant (p < 0.05) increase in activity for mutant NFκB compared with wild-type NFκB (Fig. 3F). In summary, the results demonstrate that abrogation of NFκB binding is sufficient to increase TAC1 activation even with bound REST.

Effects of REST on the Repression of TAC1 in Differentiated MSCs (Stroma)—In the last set of experiments, we explored whether TAC1 is similarly regulated in BM stromal cells, which are the normal lineage-differentiated progeny derived from MSCs (11). RT-PCR and Western blotting were performed in the stroma for REST and p65 mRNAs and proteins, respectively (Fig. 4, A and B). Parallel studies were performed with undifferentiated MSCs as a positive control. Dense bands were observed in the stroma for REST and p65 mRNAs (Fig. 4A, left) and proteins (Fig. 4B, left).

Because NFκB has been reported to repress TAC1 in BM stromal cells, we investigated whether REST is also involved in TAC1 repression (21). We stably transfected the stroma with wild-type or mutant REST siRNA. These transfecants were transiently transfected with pGL3-TAC1–1.2. After 48 h, luciferase activities in REST knockdown stroma showed significantly (p < 0.05) greater activities compared with cells transfected with mutant REST siRNA or cells not transfected with siRNA (Fig. 4C). The stroma transiently transfected with pGL3-TAC1–1.2 alone and then stimulated for 16 h with IL-1α served as a positive control for TAC1 activity (Fig. 4C). In summary,
FIGURE 3. NFκB repression of TAC1 in MSCs. A, nuclear (nuc) and cytoplasmic (cyto) extracts from uninduced (D0) and induced (D6 and D12) MSCs were prepared and analyzed by Western blotting with anti-p65 antibody. Normalizations were performed with anti-β-actin antibody (n = 3). B, uninduced (D0) and induced (D12) MSCs were stimulated for 16 h with 10 ng/ml IL-1α or left unstimulated, and chromatin was extracted and analyzed by ChIP assay. Chromatin enriched with anti-REST and anti-p65 antibodies was analyzed as described in the legend of Fig. 1F. C, undifferentiated MSCs were transiently cotransfected with pGL3-TAC1–1.2 and wild-type IκBα or mutant IκBα. In parallel studies, MSCs were transfected with pGL3-TAC1–1.2 alone. After 24 h, transfectants were stimulated for 16 h with IL-1α or left unstimulated, and luciferase and β-galactosidase activities were measured. All analyses were performed as described in the legend to Fig. 2A. E, shown are representative blots of EMSA performed with REST (R)-positive extract (HeLa), NFκB (N)-positive extract (MDA-MB-231), anti-REST and anti-p65 antibodies, and wild-type (wt) and mutant (mut) NFκB probes (n = 3). F, pGL3-TAC1–1.2-NFκB-wt was modified to include the NFκB mutation. Undifferentiated MSCs were transfected with the wild-type or mutant NFκB construct, and analyses were performed as described for Fig. 2A. **, p < 0.05 versus unstimulated cells transfected with pGL3-TAC1–1.2-NFκB-wt; ***, p < 0.05 versus stimulated cells transfected with pGL3-TAC1–1.2.
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A.

B.

C.

FIGURE 4. REST repression of TAC1 in BM stromal cells. A, total RNA from undifferentiated MSCs and the stroma was studied for REST and p65 mRNAs by RT-PCR. Normalizations were performed with oligonucleotides specific for GAPDH (n = 5). B, nuclear extracts from undifferentiated MSCs and the stroma were prepared and analyzed by Western blotting with anti-REST and anti-p65 antibodies. Normalizations were performed with anti-β-actin antibody (n = 3). C, stromal cells were stably transfected with siRNA vectors containing either wild-type (wt) or mutant (mut) REST siRNA inserts. Stable transfectants and the stroma not transfected with siRNA were transiently transfected with pGL3-TAC1–1.2, and after 48 h, luciferase and β-galactosidase activities were measured. In parallel experiments, the stroma not transfected with siRNA was transiently transfected with pGL3-TAC1–1.2 and then stimulated for 16 h with IL-1α. All analyses were performed as described in the legend of Fig. 2A. The potential for REST and NFκB to bind exon 1/TAC1 is listed for each variable. *, p < 0.05 versus unstimulated cells transfected with pGL3-TAC1–1.2 alone.

the results demonstrate that TAC1 is regulated by REST and NFκB in BM stromal cells.

DISCUSSION

Non-neuronal cells exhibit defined as well as undefined mechanisms to prevent constitutive pan-neuronal gene expression (15). However, non-neuronal cells have been reported to express inducible neuronal genes, albeit transiently, in several cases (24). MSCs represent the non-neuronal type. These cells have shown promise in neural repair and involve a developmental transition of MSCs from the non-neuronal to neuronal type (12, 22). As such, the ability of MSCs to transdifferentiate into functional neuronal cells expressing TAC1 at the transcriptional (but not translational) level is transient through cytokine stimulation (24).

We focused on REST-binding sites because this transcription factor is linked to silencing of neuronal genes in non-neuronal tissues (13). Although computational analyses identified consensus sequences for two REST-binding sites within exon 1/TAC1, we have shown only the REST1 site to be functional (Fig. 1). However, we did not prove that REST2 cannot bind to REST with low affinity. These are ongoing studies, including the implication for low binding affinity of REST for the second site. This also does not imply that the REST2 site is not involved in the regulation of TAC1. Once we determined REST1 to be a site with efficient functions in TAC1 regulation and given its proximity to the NFκB locus, we continued to focus on this site. Mutation of REST1 has been demonstrated to inhibit binding of NFκB as well as REST (21). This observation seems logical given the proximity of the REST1 and NFκB sites in exon 1 (Fig. 1B). Reporter gene activity in the TAC1 5′-flanking region was low in MSCs, but high following the mutation of REST1 in both uninduced and induced (D6) MSCs (Fig. 2A). Mutation of REST1 was irrelevant at 12 days of induction because, at this time, REST was undetectable (Figs. 1E and 2A). This is consistent with the fact that D12 cells are ectodermal with a pan-neuronal phenotype and would require constitutive expression of TAC1. The inability of REST and NFκB to bind the mutant REST1 site was confirmed because overexpression of IκB (Fig. 3D) did not suppress and REST siRNA knockdown (Fig. 2F) did not enhance mutant REST1 activity.
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A. Undifferentiated MSCs; Stroma

B. Undifferentiated MSCs + IL-1α; Induced MSCs (D6)

C. Stroma + IL-1α; Induced MSCs (D12)

Because REST1 mutation also blocks binding of NFκB, it is expected that blunting of NFκB would cause an increase in TAC1 reporter gene activities in undifferentiated and induced (D6)MSCs (21). Indeed, after reducing the efficiency of NFκB binding with mutant IκB, we observed increased activity in undifferentiated MSCs (Fig. 3C). The gels in Fig. 3C demonstrate that the ability of REST or NFκB to bind TAC1 correlated with its activity.

Our studies expanded on the above results to verify that there is synergy between REST and NFκB in TAC1 regulation as determined by loss-of-function studies. Consistent with another report on the repressive effects of REST in non-neuronal cells, immature neurons, and some post-mitotic neurons (19), our studies also showed changes in REST expression as the cells matured (Figs. 1 and 2). Before concluding synergistic functions between REST and NFκB on TAC1, we determined whether these two transcription factors can bind simultaneously to exon 1/TAC1. Indeed, ChIP assays showed binding to endogenous TAC1 using primers spanning the REST1/NFκB sites (Figs. 1B and 3B). Synergism was determined in REST knockdown MSCs, in which NFκB can still bind to exon 1/TAC1, and then studied in parallel experiments in which wild-type MSCs were transfected with mutant IκB or with a mutant NFκB reporter (Figs. 2D and 3, C and F). The data show synergistic effects of REST and NFκB on TAC1 regulation. This premise was based upon comparative studies in which maximal TAC1 activity was observed upon mutation of the REST1 and NFκB sites (Fig. 2A) compared with studies for either transcription factor alone (Figs. 2D and 3, C and F). Although NFκB typically has a positive rather than a suppressive effect on gene expression, a recent report has ascribed a repressive role for the transcription factor under specific circumstances (29).

The developmental processes of this study were expanded to incorporate how microenvironmental factors can affect MSCs as differentiated or undifferentiated cells. This question was important because stem cells are planned for tissue regeneration. We performed studies with the pro-inflammatory cytokine IL-1α because it is expected to be present in injured tissues. We observed suppression of REST expression in undifferentiated MSCs following IL-1α stimulation, which concomitantly led to the expression of TAC1. This result has clinical significance because, as stem cells such as MSCs are applied to patients, premature expression of neuron-specific genes could be harmful or beneficial to the insult.

A question addressed in this study is whether there are parallel mechanisms in BM stromal cells, in which TAC1 expression is transient, compared with neuronal cells, in which the gene is constitutively expressed at the level of transcription. NFκB mediates suppression of TAC1 in the stroma (21). In this study, we observed similar suppression by REST (Fig. 4C).

IL-1α activates NFκB in immune cells, yet we observed enhanced TAC1 activity in stromal cells (Fig. 4C). In MSCs, IL-1α stimulation repressed activation of NFκB through sequestration of the transcription factor in the cytoplasm (Fig. 3C). Overexpression of IκB in IL-1α-stimulated MSCs resulted in activated NFκB in the nuclear fraction. These results explain why TAC1 activity was suppressed in these cells because nuclear NFκB acts to repress TAC1 at the REST1 site (21). In-depth studies are required to determine the intracellular mechanisms involved in IL-1α-mediated activation of TAC1 in the stroma because it is unclear whether NFκB is operative in stromal cells, as indicated by the question marks below Fig. 4C. Perhaps IL-1α could induce TAC1 reporter gene activity via two cAMP-responsive element sites, as reported previously (24). Transient repression of TAC1 by REST and NFκB has physiological significance because excess production of substance P by BM stromal cells could disrupt BM homeostasis by prematurely causing immune cell proliferation, including MSCs, which have been linked to clinical disorders in BM (20).

Fig. 5 depicts the observed mechanism of TAC1 regulation in undifferentiated MSCs, the stroma, and differentiating neuronal cells. In undifferentiated MSCs and the stroma, REST and NFκB act synergistically to mediate repression of TAC1 transcription (Fig. 5A). IL-1α stimulation or 6-day neuronal induction of MSCs suppresses REST and NFκB expression, thereby partially alleviating repression of TAC1 (Fig. 5B). IL-1α stimulation of the stroma or 12-day neuronal induction of MSCs results in undetectable expression of REST and NFκB, thereby eliminating repression of TAC1 (Fig. 5C).

The regulatory actions of REST include both stable gene silencing and transient transcriptional repression (15). The findings of this study suggest the latter with respect to TAC1 regulation in undifferentiated MSCs and BM stromal cells. The
N terminus of REST recruits Sin3A/HDAC2 to mediate epigenetic silencing and stable suppression of REST target genes (30, 31). This mechanism modifies chromatin structure to tightly regulate target gene transcription (32). The C terminus of REST assembles a repressor complex with Co-REST to mediate HDAC-dependent or -independent repression (33). The HDAC-dependent mechanism would favor stricter gene regulation, as would be predicted in non-neuronal cells that never express neuronal genes. The HDAC-independent mechanism would predict a more transient level of regulation because no epigenetic changes are made to remodel chromatin structure. This latter mechanism would fit stem cells with neurogenic potential and non-neuronal cells that transiently express neuronal genes. It would be interesting to determine whether an HDAC inhibitor such as trichostatin A could increase TAC1 transcription in undifferentiated MSCs or the stroma. Future studies will utilize an anti-REST antibody known to detect REST4.

Neuronal differentiation of MSCs was not observed in REST knockdown MSCs without neuronal induction. REST knockdown MSCs exhibited morphology similar to that of wild-type cells during consecutive passages and did not demonstrate observable differences during neuronal induction. These findings seem logical because a neurogenic program of differentiation is extremely complex and may not be dictated by a single developmental switch. It is interesting that REST knockdown in mammary epithelial cells is sufficient to induce transcription (17). Future studies will address this issue by examining REST knockdown MSCs for a transformed phenotype.

In summary, this work has demonstrated that REST and NFκB act synergistically to mediate repression of TAC1 in MSCs and the stroma. These findings are novel and implicate similar developmental mechanisms regulating transient and constitutive neuronal gene expression in non-neuronal cells.

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