Chromosome 21-derived MicroRNAs Provide an Etiological Basis for Aberrant Protein Expression in Human Down Syndrome Brains*

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Down syndrome (DS), or Trisomy 21, is the most common genetic cause of cognitive impairment and congenital heart defects in the human population. Bioinformatic annotation has established that human chromosome 21 (Hsa21) harbors five microRNA (miRNAs) genes: miR-99a, let-7c, miR-125b-2, miR-155, and miR-802. Our laboratory recently demonstrated that Hsa21-derived miRNAs are overexpressed in DS brain and heart specimens. The aim of this study was to identify important Hsa21-derived miRNA/mRNA target pairs that may play a role, in part, in mediating the DS phenotype. We demonstrate by luciferase reporter, mRNA 3′-untranslated region reporter assays, and gain- and loss-of-function experiments that miR-155 and -802 mediate down-regulation of MeCP2, a methyl-CpG-binding protein that participates in the regulation of gene expression. Importantly, silencing of endogenous miR-155 or -802, by antisense single-stranded interfering RNA (ASO) delivered intravenicularly, resulted in the normalization of MeCP2 and MeCP2 target gene expression. Taken together, these results suggest that selective inactivation of Hsa21-derived miRNAs may provide a novel therapeutic tool in the treatment of DS.

The presence of three copies of all, or part, of human chromosome 21 (Hsa21) results in the constellation of physiologic traits known as Down syndrome (DS) or Trisomy 21 (1). With an incidence of approximately one in 750 live births, DS is the most frequently survivable congenital chromosomal abnormality (2, 3). The phenotypes of DS are complex and variable; they include craniofacial anomalies, congenital heart defects, craniofacial malformations, gastrointestinal anomalies, leukemial-lynphoid pathologies (4–14). The phenotypes of DS are complex and variable; they include craniofacial anomalies, congenital heart defects, craniofacial malformations, gastrointestinal anomalies, leukemial-lynphoid pathologies (4–14). The phenotypes of DS are complex and variable; they include craniofacial anomalies, congenital heart defects, craniofacial malformations, gastrointestinal anomalies, leukemia, and Alzheimer disease (1–3). Experimental studies utilizing animal models with DS have confirmed that the DS phenotype is increased by ~50% (i.e. >50% of the wild type, or ∼50% of the wild type, or ∼50% of the wild type). Recent bioinformatic analysis revealed that humans more than 500 miRNAs (miRNA, microRNA; UTR, untranslated region; RT, reverse transcription) are predicted to control the activity of all protein-coding genes, and functional studies indicate that microRNAs are processed from precursor molecules (pri-miRNAs), which are further processed to mature miRNAs by basepairing interactions between nucleotides 2 and 8 of the 3′-UTR (3′-UTR) of mRNAs. miRISCs sub-sequently inhibit gene expression by targeting mRNAs for silencing complex to specifically recognize and regulate particular mRNAs. Mature miRNAs recognize their target mRNAs by basepairing interactions between nucleotides 2 and 8 of the miRNA (the seed region) and complementary nucleotides in mRNA 3′-untranslated region (3′-UTR) of mRNAs. miRISCs subsequently inhibit gene expression by targeting mRNAs for translational repression or destabilization (12–14). In mammals, miRNAs are predicted to control the activity of ~30% of all protein-coding genes, and functional studies indicate that miRNAs participate in the regulation of almost every cellular process investigated. Importantly, alterations in miRNA expression have also been observed in a number of human pathologies (9–14).

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§ The abbreviations used are: Hsa21, human chromosome 21; DS, Down syndrome; miRNA, microRNA; UTR, untranslated region; RT, reverse transcription; MeCP2, methyl-CpG-binding protein; ASO, antisense single-stranded chemically enhanced oligonucleotides; ChIP, chromatin immunoprecipitation; IV, intracerebroventricular; CREB, CAMP response element-bind-ing protein; MEF2C, myocyte enhancer factor 2C; CHO, Chinese hamster ovary; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA.
Bioinformatic annotation has established that Hsa21 harbors five miRNA genes (miR-99a, let-7c, miR-125b-2, miR-155, and miR-802). We have previously demonstrated, by miRNA expression profiling experiments, that of the 424 human mature miRNAs investigated, only 10 miRNAs were overexpressed in human brain DS specimens when compared with age- and sex-matched controls (15). Importantly, RT-PCR, and miRNA in situ hybridization experiments validated that all five Hsa21-derived miRNAs were overexpressed in these brain specimens (15). In this study, we test the hypothesis that Trisomy 21 gene dosage overexpression of Hsa21-derived miRNAs result in the decreased expression of specific target proteins in both individuals with DS, and in a mouse model of DS. We demonstrate that the Hsa21-derived miRNA predicted the mRNA target, the transcription factor methyl-CpG-binding protein 2 (MeCP2) (16, 17), is underexpressed in DS brain specimens. Furthermore, we demonstrate that two MeCP2 target genes that play key roles in neuronal plasticity and development (18–21), CREB1/Creb1 and MEF2C/Me2fc, are also aberrantly regulated in these same samples. We conclude from these data that the improper attenuation of MeCP2 expression ultimately results in the dysregulation of important “regulatory circuits” that contribute, in part, to the cognitive defects that occur in DS individuals.

EXPERIMENTAL PROCEDURES

Human Brain Specimens—Human brain cerebellum, hippocampus (HIPP), and pre-frontal cortex (Pre-FCTX) samples, age- and sex-matched, were obtained from the Tissue Bank for Developmental Disabilities Research of the University of Maryland at Baltimore, in contract with the National Institutes of Health, NICHDD. Additional brain samples for this project were provided by the Institute for Brain Aging and Dementia and the University of California Alzheimer Disease Research Center (UCI-ADRC). The fetal samples ranged from 18 to 22 weeks of gestation. Fetal, children, adolescent, and adult brain samples were obtained from the Brain and Dementia and the University of California Alzheimer Disease Research Center (UCI-ADRC). The fetal samples ranged from 18 to 22 weeks of gestation. Children, adolescent, and adult brain samples used in this project were provided by the Institute for Brain Aging and Dementia and the University of California Alzheimer Disease Research Center (UCI-ADRC). Additional human brain samples used in this project were provided by the Institute for Brain Aging and Dementia and the University of California Alzheimer Disease Research Center (UCI-ADRC). The fetal samples ranged from 18 to 22 weeks of gestation. Children, adolescent, and adult brain samples used in this project were provided by the Institute for Brain Aging and Dementia and the University of California Alzheimer Disease Research Center (UCI-ADRC).

Cell Culture—The human neuroblastoma cell line, SK-N-BE(H2), was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 80 units/ml of penicillin, and 80 μg/ml of streptomycin. All cultured cells were maintained in a humidified atmosphere of 5% CO₂.

Hsa21-derived miRNA Bioinformatic Analyses—To predict putative Hsa21-derived miRNA target mRNAs, multiple computational algorithms were utilized at the default settings (miRBase (22, 23), TargetScan (24–26), PicTar (27, 28), and PITA (29)). These computational analyses demonstrated that Hsa21-derived miRNAs could theoretically interact with thousands of distinct mRNA targets and, unfortunately, many of the identified targets did not overlap between analyses. Given that the combinations of computational analyses perform worse than the prediction of a single algorithm (58), we chose to focus on TargetScan-predicted miRNA targets because this algorithm has a precision rate of around 50% with a sensitivity of ~12% (58). To reduce the number of TargetScan putative targets, the list of candidate mRNAs was subsequently prioritized with respect to their potential clinical relevance to DS and the number of multiple Hsa21-derived miRNA recognition sites harbored in possible mRNA targets (supplemental Tables S2 and S3).

Real Time PCR—Total RNA was isolated from frozen human control and DS brain, or transfected cell, samples using TRIzol (Invitrogen). The RNA was subsequently treated with RNase-free DNase I, and mature human let-7c, miR-99a, miR-125b, miR-155, and miR-802 were quantified utilizing TaqMan® microRNA assay kits specific for each Hsa21-derived miRNA (Applied Biosystems, Foster City, CA) as previously described (15, 30–33). Briefly, 100 ng of total RNA was heated for 5 min at 80 °C with 2.5 μM 18S rRNA antisense primer followed by 5 min at 60 °C then cooling to room temperature. The resulting solution was then added to a reverse transcriptase mixture and transcription was performed in 20 μl according to the manufacturer’s recommendations. Subsequent real time PCR (20 μl total reaction) was performed by adding 1:50 dilution of cDNA. Gene expression was calculated relative to 18S rRNA and Ct values were normalized to control and DS brain, or transfected cell, samples using TriZOL® reagent (Invitrogen) as template DNA was used as template. The Ct values were subsequently isolated from recombinant colonies and sequenced to ensure authenticity. The MeCP2 3’-UTR inserts were removed from the pCR 2.1 plasmid by EcoRI digestion. The fragments were subsequently gel purified, filled in, and blunt-end ligated into a filled-in XhoI site that is located downstream of the Renilla luciferase (r-luc) reporter gene (psCHECK-2™, Promega). The authenticity and orientation of the inserts relative to the Renilla luciferase gene were confirmed by dideoxy sequencing. The resulting recombinant plasmid was designated psCHECK/MeCP2. The mutant reporter constructs, psCHECK/155mut1 and psCHECK/155mut2, and psCHECK/155mut1&2 were generated by utilizing the psCHECK/MeCP2 plasmid as template and mutating the first (located at 4693–4699 bp) and/or second (located at 6321–6327 bp) miR-155 recognition site (Fig. 1C) harbored in the MeCP2 3’-UTR using the QuikChange site-directed mutagenesis kit (Stratagene). Briefly, a forward miR-155 number 1 mutagenic primer (5’-GGCCTGAGATG-CCTGATATAAAAACAGGCAAGGGAATCTG-3’) and a complementary reverse miR-155 number 1 mutagenic...
added protease and phosphatase inhibitors. Equal quantities of specimens were solubilized with RIPA buffer using freshly made protease inhibitors. Expression levels of Hsa21-derived miRNAs and MeCP2, CREB1, and MEF2C mRNA levels were quantitated by RT-PCR as described above. Protein lysates were also isolated for Western blot experiments. Western Blot Analyses—Frozen human control and DS brain specimens were solubilized with RIPA buffer using freshly added protease and phosphatase inhibitors. Equal quantities (10 μg/well) of cell lysate were separated by 10% SDS-PAGE. Following transfer to nitrocellulose membrane and blocking, the blot was incubated with an anti-MeCP2 antibody (Upstate Biotechnology, number 07-013), anti-CREB1 antibody (Upstate Biotechnology, number 06-863), anti-MEF2C antibody (Santa Cruz, number SC-132660), or anti-GAPDH antibody (Santa Cruz, number SC-20357). The immunoblots were incubated with a secondary antibody conjugated with horseradish peroxidase, visualized with enhanced chemiluminescence (ECL), and the autoradiographs were quantitated by densitometric analysis. The blots were subsequently stripped and re-probed with a GAPDH-specific antibody (Cell Signaling) to normalize the level of MeCP2, CREB1, or MEF2C proteins to total protein. Western blots probing MeCP2 levels in fetal brain samples were routinely exposed to film an additional 5–10 min so that the MeCP2 bands could be more easily detected and quantitated. All of the antibodies utilized detected the appropriate protein based on molecular mass of the protein visualized (e.g. CREB1, 43 kDa; GAPDH, 37 kDa; MeCP2, 75 kDa; MEF2C, 44 kDa).

Immunohistochemistry—Immunohistochemical testing was performed using the Ventana Benchmark System (Ventana Medical Systems, Tucson, AZ). All human control and DS brain sections were either stained histologically (with hematoxylin and eosin) or a forward miR-155 mutagenic 3′-UTR using the QuikChange site-directed mutagenesis kit (Stratagene). Transfection and Luciferase Assay—Hsa21-derived miRNA mimics (partially double-stranded RNAs that mimic the Dicer cleavage product and are subsequently processed to their respective mature miRNAs), scrambled non-targeting control mimics, Hsa21-derived miRNA (antisense single-stranded chemically modified oligonucleotides, ASO), and negative control miRNAs, all obtained from Dharmacon (La Jolla, CA), were utilized for transfection. CHO and SK-N-SH cells were transiently transfected with the Lipofectamine 2000 reagent (Invitrogen). Once control (e.g. SK-N-SH) or transfected CHO cells were washed and lysed with Passive Lysis Buffer (Promega), and the luciferase signal is normalized to the firefly Renilla luciferase signal. Alternatively, SK-N-SH cells were transiently transfected utilizing Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Twenty-four hours after transfection total RNA was isolated and Hsa21-derived miRNAs and MeCP2, CREB1, and MEF2C mRNA levels were quantitated by RT-PCR as described above. Protein lysates were also isolated for Western blot experiments.

Chromatin Immunoprecipitation (ChIP)—Control or DS brain tissue (75–125 mg) was minced into very fine pieces on a glass plate using a sterile razor blade. The pieces were scraped into a 1.8-ml microcentrifuge tube containing 1.2 ml of 1% formaldehyde in phosphate-buffered saline, and rocked for 10 min at room temperature. The tissue was pelleted by pulse centrifugation and re-suspended in 1 ml of phosphate-buffered saline containing 0.125 M glycine and protease inhibitors (all remaining solutions contained protease inhibitors). Samples were placed on ice for several minutes before being either stained histologically (with hematoxylin and eosin) or a forward miR-155 mutagenic 3′-UTR using the QuikChange site-directed mutagenesis kit (Stratagene).
were incubated at room temperature for 5 min, pelleted by pulse centrifugation, and washed twice with 1 ml of phosphatebuffered saline containing protease inhibitors. Following the final wash the samples were re-suspended in 500 μl of homogenization buffer containing 10 mM Tris-HCl (pH 7.5), 4 mM MgCl2, and 20 μM P-40. The samples were homogenized using a glass-ground hand-held homogenizer (Wheaton Industries, Millville, NJ). Fifty microliters of the resulting solution was removed and used in real time PCR for input quantity normalization. Samples were subsequently incubated on ice for 10 min and pelleted by centrifugation at 3000 × g for 5 min. The pellets were washed twice with ice-cold homogenization buffer, and re-suspended in 500 μl of micrococcal nuclease buffer containing 10 mM Tris-HCl (pH 7.5), 4 mM MgCl2, and 1 mM CaCl2. The samples were then sonicated in a Branson Sonifier 450 at a power setting of 6 at 50% duty cycle for 6 s. Micrococcal nuclease (Sigma) was added to a concentration of 5 units/ml, and the samples incubated at 37 °C for 7 min. Fifty microliters was removed and used to check for nuclease digestion efficiency on a 3% agarose gel after DNA extraction. The nuclease reaction was terminated by the addition of 1 μl of 1 mM EDTA, 45 μl of 10% SDS, and 45 μl of 100 mM NaCl. Samples were again sonicated as described above. The resulting solution was centrifuged at 13,000 × g for 10 min at 4 °C and the supernatant collected. The supernatant was separated into two aliquots of 200 μl each and used as input for a ChIP Assay Kit from Upstate Cell Signal (Wheaton, NJ). Briefly, each 200-μl aliquot was incubated on a rotating platform overnight for 15 min on a rotating platform for 1 h. DNA was recovered from this solution using a ChIP DNA Clean & Concentrator kit from Zymol Resarch (Orange, CA). The resulting DNA pellets were re-suspended in nuclease-free distilled water and used in real time PCR to quantify captured CREB1 and MEF2C promoter products.

Antagomirs—Based on the optimization strategies of Krutzfeldt et al. (34, 35), chemically modified (all ribonucleotide basepairs were 2′-O-methyl modified, six phosphorothioate backbone modifications were also included with two phosphorothioates located at the 5′-end and four at the 3′-end and a cholesterol moiety at the 3′-end) single-stranded RNA analogs complementary to mouse miR-155 (5′-CCCUACUACAAUUGAGCUUAAGCUUAAC-3′, designated antagomir-155), and miR-802 (5′-AAGGAUGAAUCUUUGUACUGA-3′, designated antagomir-802), were synthesized and reverse phase-high pressure liquid chromatography purified for in vivo use (Dharmacco, Lafayette, CO). A scrambled control antagomir (5′-GACUACCUCUAGCUUAAACGAUAUACGA-3′) was also synthesized with the same modifications as described above, however, a biotin moiety was included at the 5′-end of the oligonucleotide so that proper location after in vivo injection and analysis could be performed. Biotin-rich oligonucleotide sequences were obtained from The Jackson Laboratory (Bar Harbor, ME), housed in pairs in a temperature-controlled room (25 °C), and maintained on a 12-h light/dark cycle with free access to food (Teklad Rodent Diet 8604 pellets, Harlan, Madison, WI) and water. All procedures were approved by the National Animal Care and Use Committee and are consistent with AAALAC guidelines and the Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23, revised 1996). Significant efforts were also made to minimize pain or discomfort in the experimental animals and number of animals used while maintaining statistically valid group numbers.

Stereotaxic Intracerebroventricular (ICV) Injection—The method of Vanderwolf (36) was used as a technical guide for animal care and stereotaxic surgery. An intraperitoneal injection mixture (1 ml/kg) of ketamine (100 μg/ml), xylazine (10 mg/ml) was used to deeply anesthetize each mouse before shaving its head and positioning it in a dual Ultra Precise Small Animal Stereotactic Instrument (model 962, David Kopf Instruments, Tujunga, CA). On the dorsal surface of the head, a midline incision was made to separate the subcutaneous fascia to expose the skull and visualize bregma and lambda. Stereotaxic coordinates (37) for injection into the lateral ventricles were: 1.0-mm lateral from bregma (x plane), −0.34-mm rostral from bregma (y plane), and −2.5-mm ventral from bregma (z plane). After dialing in the coordinates, an 18-gauge needle (catalog number 305196, BD Biosciences) was used to make a bur hole for the syringe needle to pass through the skull. A gas-tight 10-μl Hamilton syringe was used with a microsyringe holder (model 1772-F1, David Kopf Instruments, Tujunga, CA) to inject 10 μl of miR-155 antagomir at a rate of 5 μl/min. The injection needle was left in place for 5 min after the injection,
then withdrawn. The bur hole was covered by bone wax and the scalp sutured. Following injections, subjects were returned to their home cages to recover and allowed food and water ad libitum. The mice were sacrificed on day 7 after antagonim injection by isofurane inhalation and decapitation. The brains were removed from the skull and placed in a mouse brain blocker and sectioned in 1.0-mm blocks. From the subsequent blocks, the prefrontal cortex, striatum, hippocampus, and cortex (remaining regions combined) were dissected out. The brain regions were placed in individual 0.5-ml flat top microcentrifuge tubes, flash frozen in liquid nitrogen, and stored at −80 °C until further use.

Statistical Analysis—All data are reported as mean ± S.E. When comparisons were made between two different groups, statistical significance was determined using Student’s t test. When multiple comparisons were made, statistical significance was determined using two-way analysis of variance. All statistical analysis was performed using the software package Prism 4.0b (GraphPad Software, San Diego, CA).

RESULTS

Hsa21-derived miRNAs Are Overexpressed in Human DS Brain Specimens—To extend our previous observations that demonstrated that Hsa21-derived miRNAs were overexpressed in human DS fetal heart and hippocampus specimens, mature RT-PCR assays specific for these miRNAs were performed utilizing total RNA isolated from human DS fetal heart and hippocampus specimens from brains of fetuses, infants, and adults with DS (supplemental Table S1, Postmortem Specimen Information). These experiments revealed that all five Hsa21-derived miRNAs were overexpressed at least 50% in prefrontal cortex specimens from brains of fetuses, infants, and adults with DS compared with age- and sex-matched control brains (supplemental Fig. S1, A–E).

MeCP2 Is a Target of Hsa21-derived MiRNAs—The overexpression of MeCP2 was observed in DS brains utilizing immunohistochemistry and Western analysis (supplemental Fig. S1, A–E) or miR-802 (Fig. 1A) or miR-155 (Fig. 1B) expression. To determine whether or not miR-155 and/or -802 could regulate the expression of MeCP2, we chose to utilize a luciferase reporter assay. The rationale for utilizing this assay is that the binding of a given miRNA to its target mRNA results in repression thereby reducing activity and expression of the target mRNA (10). Therefore, the functional importance of these sites in repressing MeCP2 expression is questionable. To begin to determine whether or not miR-155 and/or -802 could regulate the expression of MeCP2, we chose to utilize a luciferase reporter assay. The rationale for utilizing this assay is that the binding of a given miRNA to its target mRNA results in repression thereby reducing activity and expression of the target mRNA (10).

To validate that miR-155 and/or -802 interacted with specific target sequences localized within the MeCP2 3′-UTR, additional luciferase reporter constructs were generated in which the 7-bp “seed” sequences, which are complementary to the 5′-end of miR-155 (Fig. 1C) or miR-802 (Fig. 1E), were mutated. The resulting constructs were subsequently co-transfected with miR-155 or -802 into CHO cells and luciferase activity was measured. Importantly, miR-155 could no longer decrease the luciferase activity of only the psiCHECK/155mut1,2-transfected cells (Fig. 1D), suggesting that miR-155 can interfere with luciferase expression via direct interaction with both miR-155 recognition sites (located at positions 4679–4700 and 6305–6328 bp) harbored within the MeCP2 3′-UTR. Additionally, the data suggest that the effect of multiple miR-155 recognition sites is additive because luciferase activity is lowest when both miR-155 sites are present. In contrast, miR-802 and the mutant construct co-transfection experiments demonstrated that
miR-802 can interfere with luciferase expression via direct interaction with only the second miR-802 site (i.e. 6875–6896 bp) in this in vitro surrogate assay (Fig. 1F).

To further demonstrate that MeCP2 mRNA is a true target of miR-155 and -802, the endogenous expression levels of these miRNAs in a human neuronal cell line (SK-N-SH) were
individually manipulated by transfection of miRNA mimics (gain-of-function experiment) or miRNA inhibitors (ASO, loss-of-function experiment) and changes in mature miR-155, -802, MeCP2 mRNA, and protein levels were determined. With transfection of either the miR-155 or -802 mimic, endogenous miR-155 and -802 levels increased by 200–310% (Fig. 1G) and MeCP2 mRNA (Fig. 1G) and MeCP2 protein (Fig. 1H) levels were significantly decreased (30–50% at the mRNA level and 40–50% at the protein level) when compared with non-transfected or scrambled miRNA-transfected cells. In contrast, subsequent to transfection with either miR-155 or -802 ASO inhibitors, endogenous miR-155 and -802 levels decreased by 60–70% at the protein level. Collectively, these results strongly support the hypothesis that MeCP2 mRNA is a target of both miR-155 and -802 and suggest that these miRNAs markedly decrease MeCP2 expression by targeting MeCP2 mRNAs for degradation.

**MeCP2 Is Underexpressed in Human DS Brain Specimens**—To demonstrate the potential significance of MeCP2 as a target of Hsa21-derived miRNAs *in vivo*, we investigated whether or not MeCP2 was underexpressed in brain samples isolated from individuals with DS. RT-PCR experiments demonstrated that, independent of the age or brain region investigated, MeCP2 mRNA levels were decreased by 60–70% in brain specimens from DS individuals relative to age- and sex-matched controls (Fig. 2, A and B). Consistent with these results, Western blot analyses of the same human brain specimens showed that...
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MeCP2 protein levels were also attenuated 50–70% in the DS samples relative to controls (Fig. 2, C and D).

To further validate our Western blot results, and determine which cell type(s) expressed MeCP2 protein, immunohistochemistry experiments were performed utilizing a MeCP2-specific antibody and formalin-fixed age- and sex-matched control and DS samples generated from the same brain specimens used in the RT-PCR and Western studies. Representative photomicrographs of the human control brain samples (Fig. 2, E and G) demonstrated many positive MeCP2-stained neurons (fetal, 9–12%; adult, 11–20%). The positive signal was evident in both the cytoplasm (large arrow) as well as in the nucleus of neurons (small arrow). In contrast, there were far less positive MeCP2 staining cells (fetal, <1%; adult, 1–5%) in brain specimens from individuals with DS (Fig. 2, F and H). Quantitative analysis of age- and sex-matched DS and control brain specimens demonstrated that MeCP2 expression was decreased at least 4-fold in DS samples.

_Mature miR-155 and -802 Manipulation or MeCP2 siRNA Knockdown Modulate MeCP2 Target Gene Expression—_Initially identified on the basis of this ability of the protein to bind methylated DNA, MeCP2 was thought to only transcriptionally repress target genes (16, 17). Recently, however, Chahrour et al. (39) demonstrated that MeCP2 can activate and repress the transcription of a large number of genes that play a role in neurobiology. Based on these observations, we now hypothesize that as a consequence of Trisomy 21-mediated downregulation of MeCP2 protein expression, genes that are transcriptionally repressed by MeCP2 would be underexpressed in DS relative to controls. To begin to test this hypothesis we have chosen to focus on one transcriptionally activated (cAMP response element-binding protein; CREB1) and one transcriptionally silenced (myocyte enhancer factor 2C; MEF2C) target gene that are identified as Mecp2 target genes in vivo. To further substantiate the hypothesis that CREB1 and MEF2C are transcriptional targets of MeCP2, the endogenous levels of MeCP2 were manipulated by reducing or increasing MeCP2 protein levels, MeCP2 target gene expression is modulated.

**CREB1 Target Genes Are Aberrantly Expressed in Human DS Brain Specimens—**Because MeCP2 was underexpressed in human brain samples (Fig. 2, A–H), we investigated whether or not a dysregulation of CREB1 and MEF2C also occurred in brain specimens isolated from individuals with DS. RT-PCR assays demonstrated that, independent of age or brain region investigated, CREB1 mRNA was underexpressed (Fig. 4, A and B, 45–60%) and MEF2C mRNA was overexpressed (Fig. 5, A and B, 160–210%) in DS samples when compared with controls. In addition, Western blot analyses demonstrated that CREB1 protein levels were also decreased 40–60% in DS fetal hippocampus and adult prefrontal cortex samples (Fig. 4C). In contrast, MEF2C protein levels were increased 60–70% in identical DS brain samples (Fig. 5C). To further substantiate the Western blot results, and determine which cell type(s) expressed CREB1 and MEF2C, and also immunohistochemistry experiments were performed. Representative photomicrographs of the human brain specimens demonstrated positive staining for CREB1 (Fig. 4D; fetal, 25–33%; adult, 45–60%) and MEF2C (Fig. 5D; fetal, 160–210%; adult, 160–210%). The positive signal was evident in both the cytoplasm (large arrow) and the nucleus of neurons (fetal, 1%; adult, 11–20%). The positive signal was evident in both the cytoplasm (large arrow) as well as in the nucleus of neurons (small arrow).

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**Mature miR-155 and -802 Manipulation or MeCP2 siRNA Knockdown Modulate MeCP2 Target Gene Expression—**Initially identified on the basis of this ability of the protein to bind methylated DNA, MeCP2 was thought to only transcriptionally repress target genes (16, 17). Recently, however, Chahrour et al. (39) demonstrated that MeCP2 can activate and repress the transcription of a large number of genes that play a role in neurobiology. Based on these observations, we now hypothesize that as a consequence of Trisomy 21-mediated downregulation of MeCP2 protein expression, genes that are transcriptionally repressed by MeCP2 would be underexpressed in DS relative to controls. To begin to test this hypothesis we have chosen to focus on one transcriptionally activated (cAMP response element-binding protein; CREB1) and one transcriptionally silenced (myocyte enhancer factor 2C; MEF2C) target gene that are identified as Mecp2 target genes in vivo. To further substantiate the hypothesis that CREB1 and MEF2C are transcriptional targets of MeCP2, the endogenous levels of MeCP2 were manipulated by reducing or increasing MeCP2 protein levels, MeCP2 target gene expression is modulated.

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demonstrated that of the five Hsa21-derived miRNAs only the mouse orthologs of miR-155 and -802 were overexpressed by 40–60% in the Ts65Dn mice thereby validating computational data (Fig. 6A). As miR-155 and -802 were overexpressed in the Ts65Dn and human DS brain specimens, we hypothesized that MeCP2 and CREB1 would be underexpressed and MEF2C would be overexpressed in these mice, similar to what was observed in human brain samples. To test this hypothesis RT-PCR and Western blot experiments were performed on hippocampus samples isolated from Ts65Dn and euploid control mice. Importantly, these experiments demonstrated that MeCP2 and CREB1 protein (Fig. 6, E and F) levels were underepressed in the Ts65Dn samples compared with the euploid controls (30–40% at the mRNA level and 40–90% at the protein level). In contrast, in these same samples, MEF2C mRNA (Fig. 6B) and protein
levels were overexpressed (220–235% at the mRNA level and 200–300% at the protein level). Taken together, these results further support the premise that MeCP2 mRNA is a target for miR-155 and -802 and underexpression of MeCP2 may be involved, in part, in mediating DS.

Silencing of miR-155 or -802 in Vivo with Antagomirs Normalizes miR-155, miR-802, MeCP2, and MeCP2 Target Gene Expression—Previous studies demonstrated that chemically modified, cholesterol-conjugated, single-stranded RNA analogs complementary to miRNAs, designated “antagomirs,” can silence endogenous miRNAs in vivo (34, 35, 45). To determine whether or not the silencing of miR-155 or -802 expression in the brains of Ts65Dn mice resulted in augmented MeCP2 expression levels, Ts65Dn and euploid control littermates were ICV injected with antagomir-155 or miR-802. Due to the biological stability of antagomirs (34, 35), Ts65Dn brains were harvested 7 days after injection to maximize their effect. ICV injection of antagomir-155 resulted in the attenuation (30–40%) of endogenous expression of mature miR-155 in the hippocampus of the Ts65Dn animals and no changes were observed in miR-802 expression levels (Fig. 6C). Importantly, the decrease in miR-155 expression resulted in augmented MeCP2 (65–170%)}
and CREB1 (150–200%) mRNA (Fig. 6D) and MeCP2 (70–90%) and CREB1 (40–70%) protein levels (Fig. 6E), and attenuated MEF2C mRNA (150–165%) and protein levels (180–250%) (Fig. 6, D and E) in the hippocampus of the Ts65Dn animals. Similar results were obtained in Ts65Dn animals ICV injected with antagomir-802 (Fig. 6C–F). Importantly, no changes were observed in the mir-155, mir-802, MeCP2, CREB1, or MEF2C expression levels in Ts65Dn animals ICV injected with a control scrambled antagomir (Fig. 6, C–E). In summary, these cumulative results clearly suggest that MeCP2 mRNA is a direct target of miR-155 and -802 in vivo, and that silencing of endogenous miRNAs may have therapeutic value.

DISCUSSION

The major findings in the present study are that Hsa21-derived miRNAs (miR-155, and -802), and proteins MeCP2, CREB1, and MEF2C are all aberrantly expressed in a cascade-dependent manner in brain specimens isolated from DS individuals. Similar results were obtained utilizing Ts65Dn mouse brain samples that were trisomic for only miR-155 and -802.
These findings occur regardless of the age or brain region investigated when compared with age- and sex-matched controls in our human and mouse studies. Importantly, precision in vivo silencing of miR-155 or -802 with antagonirs resulted in the normalization of the appropriate miRNA, MeCP2, CREB1, and MEF2C expression in Ts65Dn mice. These results suggest that...
Trisomy 21-induced, Hsa21-derived miR-155 and -802 overexpression directly inhibits MeCP2 expression which, in turn, leads to the aberrant expression of MeCP2-activated and -silenced target genes (e.g. Creb1 and Mef2c) in vivo.

Although bioinformatic analyses demonstrated that Hsa21-derived miRNAs could theoretically interact with thousands of distinct mRNA targets, we chose to initially focus on MeCP2 as a potentially important DS target mRNA because mutations in this gene have already been shown to cause the postnatal neurodevelopmental disorder Rett syndrome (16, 17). Specifically, MeCP2 is a transcription factor that binds to methylated CpG dinucleotides and induces the recruitment of protein complexes that are involved in histone modifications and chromatin remodeling (16, 17). Therefore, MeCP2 was thought to play an important role in the transcriptional silencing of specific target genes. However, Chahrour et al. (39) recently demonstrated that MeCP2 could activate and repress the transcription of a large number of genes. MeCP2 is expressed in most tissues and cell types with the highest expression levels detected in the brain, where it is present primarily in neurons (46, 47). MeCP2 is spatially and developmentally regulated, and is characterized by heterogeneous expression in subpopulations of neurons in the brain. The timing of MeCP2 expression correlates with the maturation of the central nervous system (47, 48), and recent reports suggest that MeCP2 may be involved in synaptic plasticity (49). Finally, transgenic mouse models have demonstrated that either underexpression or overexpression of MeCP2 are detrimental to cognitive development indicating that levels of MeCP2 in the central nervous system are regulated and crucial for neuronal function.

Consistent with our observation that MeCP2 is underexpressed in DS brain specimens, several investigations have demonstrated that, although a large number of Hsa21-derived mRNAs are overexpressed, many non-Hsa21 genes are also down-regulated (50, 51). Furthermore, Haghighat et al. (50) demonstrated that the MeCP2 protein was underexpressed in DS samples, but also showed that MeCP2 expression was reduced in brain specimens isolated from DS fetuses, individuals with Rett syndrome, Angelman syndrome, Cri-du-chat syndrome, autism, and attention deficit hyperactivity disorder (52). Finally, Samaco et al. (53) demonstrated that precise control of MeCP2 is critical for normal behavior and they predicted that human neurodevelopmental disorders would result from a subtle reduction in MeCP2 expression. Therefore, in the DS setting, the documented underexpression of MeCP2 may play a major role in mediating the observed neurodevelopmental disorders. Additionally, decreased MeCP2 expression may represent a common thread in a number of neurodevelopmental disorders.

Traditionally, MeCP2 was thought to play an important role in transcriptional silencing of specific target genes (16, 17). Recently, however, MeCP2 was shown to activate and repress the transcription of a large number of genes (39). Although MeCP2 is known to regulate many downstream target genes (39), we have chosen to focus on CREB1 and MEF2C, as they have been shown to play a critical role in various aspects of neural development (18–21). We believe that these key proteins are aberrantly expressed in DS brains as a result of the dysregulation of MeCP2 expression mediated by Hsa21-derived miRNAs. This may represent, in part, the next crucial sequence of steps in this neural gene network. Specifically, CREB factors are critical to a variety of functions in the nervous system (including functions that are especially relevant to conditions such as DS), including neurogenesis and neuronal survival, development, and differentiation, axonal outgrowth, synaptic plasticity, and memory formation (18, 19). Our CREB expression and ChIP experimental data supports the study published by Chahrour et al. (39) where they also demonstrated that MeCP2 regulated CREB1 gene expression. Interestingly, these investigators also established that MeCP2 and CREB1 were direct binding partners and act synergistically on the promoter region of the somatostatin gene (i.e. a MeCP2-activated target gene) (39). Finally, Chen et al. (54) demonstrated that MeCP2 expression was induced by a CREB-induced miR-132-mediated mechanism. Whether these observations support the notion of a critical relationship between MeCP2 and CREB is an open question despite the fact that these investigators demonstrated that MEF2C plays a role in transcriptional enhancement and proper distribution of expression in the layers of the neocortex (21) and facilitates MEF2C-mediated striatal remodeling (16, 17). In contrast to these findings, the appropriate CREB family member factor signals may be crucial for normal regulation in a disease state plays a role in establishment of disease, specific reduction of the miRNA in question would be therapeutically desirable. Inhibition of miRNA activity can be achieved through the use of chemically modified, strand-reverse complement oligonucleotides or ASOs. In general, an effective ASO is resistant to nonspecific cellular nucleases, resistant to miRNA-directed cleavage by RISC, and binds miRNAs in RISCs with high affinity, effectively out-competing binding to target mRNAs (55). ASO inhibitors containing exclusively 2′-O-methyl (2′-O-Me) ribose sugars are resistant to cleavage by both RISC and other cellular ribonucleases and 2′-O-methyl-modified RNA-RNA hybrids are more thermodynamically stable than either RNA-RNA or DNA-RNA duplexes (56). Nuclease-resistant phosphorothioate backbone linkages, in combination with 2′-O-Me ribose modifications, have also been employed in ASOs (34, 35). Finally, a 3′-terminal cholesterol group conjugation appears to aid delivery of ASOs into cells; however, it may have properties that further enhance ASO activity, such as improved intracellular escape from liposomes, relocalization of the targeted miRNAs, or enhancement of ASO stability (55). Utilizing these ASO strategies, previous studies have demonstrated that chemically modified, cholesterol-conjugated, single-stranded RNA analogs complementary to miRNAs, designated antagonirs, can silence endogenous miRNAs in vivo (34, 35, 45). The mech-
anism(s) by which ASOs affect miRNA expression can theoretically occur at multiple levels (57): 1) by binding to the mature miRNA within the RISC and acting as a competitive inhibitor; 2) by binding to the pre-miRNA and preventing its processing or entry into the RISC; 3) by interfering with the processing or export of the pre- or pri-miRNA from the nucleus. Regardless of the mechanism, the net result is a reduction in the concentration of a specific miRNA-programmed RISC.

In conclusion, our data show that the Hsa21-derived miRNAs are overexpressed due to Trisomy 21, and results in the underexpression of the target protein, MeCP2. The attenuation of this protein, and subsequent aberrant expression of the CREB1 and MEF2C transcription factors, may lead to abnormal brain development through anomalous neuronal gene expression during the critical period of synaptic maturation (i.e. alterations in neurogenesis, neuronal differentiation, myelination, and synaptogenesis), which are thought to result in the cognitive impairment of DS patients (2, 3). Although we have demonstrated that miR-155 and -802 can directly regulate the expression of MeCP2, Hsa21-derived miRNAs may regulate thousands of mRNA targets. However, therapeutically this is not a disadvantage because inhibition or knock-down of these overexpressed miRNAs should normalize the expression levels of all miRNA/mRNA targets back to non-trisomic 21 levels.

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