Journal of Cellular Biochemistry 103:1877–1885 (2008)

A Case Report: Bone Marrow Mesenchymal Stem Cells From a Rett Syndrome Patient Are Prone to Senescence and Show a Lower Degree of Apoptosis

Tiziana Squillaro,[1,2] Giuseppe Hayek,[3] Ernesto Farina,[2] Marilena Cipollaro,[2] Alessandra Renieri,[1] and Umberto Galderisi[2,4]*

[1]Medical Genetics, University of Siena, Siena, Italy
[2]Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Excellence Research Center for Cardiovascular Diseases, Second University of Naples, Naples, Italy
[3]Childhood Neuropsychiatry, Azienda Ospedaliera Senese, Siena, Italy
[4]Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Temple University, Philadelphia, Pennsylvania

Abstract Rett syndrome (RTT) is one of the most common genetic diseases responsible for a progressive disabling neurodevelopmental disorder. Mutations in the MeCP2 gene were identified in the great majority of RTT patients. MeCP2 protein binds to methylated DNA and produces changes in chromatin structure. This is a key event in regulation of gene expression. It has been suggested that MeCP2 might be important for neuronal development. Moreover, the frequent occurrence of osteoporosis and scoliosis in RTT patients suggests impaired bone formation and/or remodeling. Mesenchymal stem cells (MSCs) can differentiate as mesodermal cells such as bone, cartilage cells, and adipocytes. MSCs have been shown to possess great somatic plasticity; in fact, they can differentiate as neurons and astrocytes. We studied RTT patients' MSCs because they are progenitors of osteocytes, and it has been suggested that RTT patients' osteogenesis could be impaired. Moreover, MSCs might represent a useful model for the study of neurogenesis. MSCs from RTT patient showed precocious signs of senescence in a comparison with the MSCs of healthy-patient control groups. This was in agreement with the reduced gene-expression in the control of stem cell self-renewal and upregulation of lineage specific genes, such as those involved in osteogenesis and neural development. Control groups enabled us to observe a lower degree of apoptosis in RTT patient cells. This means that aberrant stem/progenitor cells, instead of being eliminated, can survive and become senescent. Our research provides a new insight into RTT syndrome. Senescence phenomena could be involved in triggering RTT syndrome-associated diseases. J. Cell. Biochem. 103: 1877–1885, 2008.

Key words: MECP2 gene; marrow stromal stem cells; differentiation; apoptosis; senescence; cell cycle

Rett syndrome (RTT) is one of the most common genetic causes of mental retardation in girls, with an estimated prevalence of 1:10,000–1:15,000 [Weaving et al., 2005]. It is a progressive disabling neurodevelopmental disorder, which presents with regression, loss of speech, purposeful hand movements, acquired microcephaly, ataxia, stereotypic hand-washing activities, and growth retardation [Weaving et al., 2005]. In addition to classic RTT, a number of variants have been described [Weaving et al., 2005]. In 1999, mutations in the *MECP2* gene were identified in RTT patients; mutations are found in up to 90% of classic RTT cases, in a lower percentage of RTT variants but also in males with both syndromic and non-syndromic X-linked mental retardation [Weaving et al., 2005]. Since pathogenic mutations had been identified in such a large percentage of patients, RTT was considered a monogenic disorder.

Transcriptional silencing is associated with methylation of CpG DNA islands and with the action of proteins that bind methylated DNA. The MeCP2 protein binds to methylated DNA and mediates gene silencing by causing changes

Grant sponsor: Sbarro Institute.

*Correspondence to: Umberto Galderisi, Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Second University of Naples, Via Costantinopoli 16, 80138 Napoli, Italy.
E-mail: umberto.galderisi@unina2.it

Received 22 June 2007; Accepted 17 August 2007
DOI 10.1002/jcb.21582

in chromatin structure through the interaction with co-repressors such as Sin3A and the histone deacetylase complex [Jones et al., 1998]. Moreover, MeCP2 interacts directly with DNA methyltransferase DNMT1 and can contribute to maintenance of DNA methylation [Kimura and Shiota, 2003]. The transcriptional repression process, involving the methylation of lysine 9 of histone H3, has been linked to DNA methylation. In detail, MeCP2 was shown to promote methylation of lysine 9 of H3 [Kimura and Shiota, 2003]. Overall, MeCP2 could play a key role in regulation of gene expression.

The MeCP2 protein is found in most tissues and cell types, but its highest expression is detected in the brain [Tudor et al., 2002; Luikenhuis et al., 2004]. It is present in different regions of developing rat brains, starting from late embryonic stages. Protein levels correlate with neural maturation and synapse formation, since MeCP2 expression increases as cells acquire a mature neural phenotype [Jung et al., 2003; Mullaney et al., 2004]. Several knock-out models for MeCP2 have been generated [Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002]. One of these models has shown that gene deletion in post-mitotic neurons also gives RTT-like phenotype, suggesting that MeCP2 might be important for neuronal late differentiation rather than development [Chen et al., 2001; Tudor et al., 2002; Luikenhuis et al., 2004].

The frequent occurrence of osteoporosis and scoliosis in RTT patients suggests that bone formation and/or remodeling were impaired in these patients. Several authors have suggested that MeCP2 mutations in RTT not only affect brain development but could also affect osteogenesis [Haas et al., 1997; Budden and Gunness, 2001, 2003; Ager et al., 2006; Zysman et al., 2006].

Stem cells within normal tissues are defined by common characteristics: self-renewal to maintain the stem cell pool over time; regulation of stem cell numbers through a strict balance between cell proliferation, cell differentiation, and cell death; and the ability to give rise to a broad range of differentiated cells [Morrison et al., 1997; Gage, 2000; Temple, 2001].

Chromatin state is fundamental for gene expression. In fact, euchromatin is permissive for transcription, while eterochromatin is repressive. Epigenetic mechanisms, including DNA methylation and histone modifications, can affect the balance between eu- and eterochromatin acting as main regulators of gene expression. Self-renewal, proliferation and differentiation properties of stem cells are controlled by key transcription factors. However, their activity is modulated by epigenetic mechanisms that operate at highest hierarchical level. Studies on these factors can be especially important to dissect molecular pathways governing the biology of stem cells [Edlund and Jessell, 1999; Rasmussen, 2003; Buszczak and Spradling, 2006].

In RTT patients MeCP2 inactivation can impair epigenetic mechanisms regulating stem cell biology this in turn could alter the physiological development of tissues and organs.

Based on these premises, we decided to study the biology of mesenchymal stem cells (MSCs) obtained from bone marrow of a RTT patient.

In addition to hematopoietic stem cells, bone marrow contains cells called marrow stromal stem cells that meet criteria for stem cells of non-hematopoietic tissues. These stem cells are currently referred to as either MSCs, because of their ability to differentiate into mesenchymal cells (such as bone and cartilage cells, adipocytes), or marrow stromal cells, because they appear to stem from the complex array of supporting structures found in marrow [Prockop, 1997; Bianco and Gehron Robey, 2000; Colter et al., 2000]. MSCs have shown to possess great somatic plasticity since they can differentiate into non-mesenchymal lineages. In fact, it has been demonstrated that MSCs are capable of differentiating into neurons and astrocytes in vitro and in vivo [Jori et al., 2005; Krabbe et al., 2005; Wislet-Gendebien et al., 2005].

We hypothesized that studying MSCs in RTT patients could be of interest for several reasons: (i) MSCs are the progenitors of osteocytes and it has been suggested that osteogenesis could be impaired in RTT patients. (ii) In neurodevelopmental disorders, such as RTT, neural stem cells should be analyzed to detect possible alterations in neuronal/glial commitment and differentiation. Obviously, neural stem cells cannot be obtained from RTT patients. MSCs could represent a valid alternative to study neurogenesis since they can differentiate in neurons and glia. (iii) MSCs are easily isolated from bone marrow cells with a procedure that causes limited uncomfortable pain to patients.

EXPERIMENTAL PROCEDURES

Molecular Analysis of the Identified Patient

Blood samples were obtained after informed consent. DNA was extracted from peripheral blood using a QIAamp DNA Blood Kit (Qiagen, Italy). DNA samples were screened for mutations in the four exons coding for *MECP2* using Transgenomic WAVE denaturing high performance liquid chromatography (DHPLC). The analysis of the *MECP2* gene for deletions/duplications was performed as previously described [Ariani et al., 2004]. PCR products resulting in abnormal DHPLC profiles were sequenced on both strands using PCR primers with fluorescent dye terminators on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, CA).

MSC Cultures

Bone Marrows were obtained from a female child with RTT syndrome and two healthy female children after informed consent. All children (patient and controls) showed no statistically significant differences in the body mass index and had the same age range (4–6 years old). Bone marrow aspirates were collected from iliac crests, separated on Ficoll density gradient (GE Healthcare, Italy), and the mononuclear cell fraction was harvested and washed in phosphate buffer saline (PBS). We seeded $1–2.5 \times 10^5$ cells per cm^2 in 100 mm dishes with α-modified Eagle's medium (αMEM) containing 10% fetal bovine serum (FBS), 2 ng/ml basic FGF, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (proliferating medium). After 24–48 h, non-adherent cells were discarded, and adherent cells representing MSCs along with committed progenitors were washed twice with PBS. Cells were then incubated for 7–10 days in proliferating medium to reach confluence and extensively propagated for further experiments. We used cells till the 4th passage, each time we plated 2×10^3 cells per cm^2. All cell culture reagents were obtained from Euroclone Life Sciences (Italy) and Hyclone (UT) unless otherwise stated.

Cell Cycle Analysis

For each assay 3×10^5 cells were collected and resuspended in 500 μl of a hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate and 50 μg/ml propidium iodide, RNAse A). Cells were incubated in the dark for 30 min and then analyzed. Samples were acquired on a FACS-Calibur flow cytometer using the Cell Quest software (Becton Dickinson, NJ) and analyzed with standard procedure using the Cell Quest software and the ModFitLT software version 3 (Becton Dickinson).

Detection of Apoptotic Cells

Apoptotic cells were detected taking advantage of Alexa 568-conjugated Annexin V (Roche, Italy), which binds to phosphatidylserine translocated from the inner part of the plasma membrane to the outer layer of the cell during the early stages of apoptosis. To stain apoptotic cells, the culture medium was discarded and cells were incubated with Alexa 568-conjugated Annexin V, diluted 1: 50 in a solution containing 10 mM HEPES, pH 7.4 (Sigma-Aldrich, Italy), 140 mM NaCl (Sigma-Aldrich, Italia), 5 mM $CaCl_2$ (Sigma-Aldrich, Italia), for 15 min at room temperature. Then, cells were washed with PBS, and were counterstained with Hoechst 33342 (Sigma-Aldrich, Italy) 100 μg/ml for 5 min, washed with water, mounted on glass slides with Mowiol (Calbiochem, San Diego, CA) containing 2.5% DABCO (Sigma-Aldrich, Italia) and observed at a fluorescence microsope (Leica Mycrosystems Italia, Milano, Italy). In every experiment, at least 1,000 cells were counted in different fields to calculate the percentage of dead in culture.

Senescence-Associated β-Galactosidase Assay

Senescence-associated β-galactosidase is useful marker to detect replicative senescence, since this enzyme, active at pH 6, is differentially expressed in senescent cells compared to younger ones [Kurz et al., 2000].

Cells were fixed for 10 min with a solution of 2% (v/v) formaldehyde and 0.2% (w/v) glutaraldehyde. Cells were washed with PBS and then incubated at 37°C for at least 2 h with a staining solution (30 mM citric acid/phosphate buffer (pH 6), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 150 mM NaCl, 2 mM $MgCl_2$, 1 mg/ml X-Gal solution). The percentage of senescent cells was calculated by the number of blue cells (β-galactosidase positive cells) out of at least 500 cells in different microscope fields.

TRAP Assay

TRAP assay was carried out according Kim et al. [1994] and Kim and Wu [1997]. Briefly, 5×10^5 cells were lysed in 10 mM Tris–HCl (pH 7.5),

2.5 mM $MgCl_2$, 1 mM EGTA (ethylene glycol bis (2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid), 0,5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonatehydrate) 10% glycerol, 5 mM β-mercaptoethanol, 1 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) for 30 min at 4°C. The lysates were then centrifuged for 10 min at 10,000*g* at 4°C. After centrifugation, protein concentration was determined by Bradford Assay. Reaction mixture for TRAP assay was done as it follows: 1X Taq DNA polymerase buffer (Promega, Italy), 1.5 mM $MgCl_2$ 50 μM dNTPs, primer M2(TS) (5′-AATCCGTCGAGCAGAGTT-3′) TRAP internal control (5′-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3′). This mixture was incubated for 30 min at 25°C, then we added primer ACX primer (5′-GCGCGG[CTTACC]$_3$CTAACC-3′), primer NT (5′-ATCGCTTCTCGGCCTTTT-3′), 2.5 U Taq DNA polymerase (Promega). The reaction was denatured for 3 min at 94°C, then amplified for 30–35 cycles (94°C for 15 s, 60°C for 15 s, 72°C for 15 s). Primers ACX and M2(TS) will amplify the telomerase products, while primers M2(TS) and NT will amplify the TRAP internal control. PCR products were resolved on 20% polyacrylamide gels stained with Gelstar (Cambrex Bioscience, Denmark).

Micrococcal Nuclease Assay

Cells were permeabilized with 0.01% L-α-lysophosphatidylcholine (Sigma-Aldrich, Italy), in 150 mM sucrose, 80 mM KCl, 35 mM HEPES pH 7.4, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ for 90 s, followed by digestion with 2 U/ml micrococcal nuclease (Sigma-Aldrich, Italy) in 20 mM sucrose, 50 mM Tris–HCl pH 7.5, 50 mM NaCl and 2 mM $CaCl_2$ at room temperature for various times. Digested DNA was resolved on 1% agarose gel electrophoresis.

RNA Extraction and RT-PCR and Real Time PCR

Total RNA was extracted from cell cultures using TRI REAGENT (Molecular Research Center, Inc., OH) according to the manufacturer's protocol. The mRNA levels of the genes analyzed were measured by RT-PCR amplification, as previously reported [Galderisi et al., 1999].

Sequences for mRNAs from the nucleotide data bank (National Center for Biotechnology Information) were used to design primer pairs for RT-PCR reactions (Primer Express, Applied Biosystems, CA). Primer sequences are available on request. Appropriate regions of HPRT (hypoxanthine-guanine phosphoribosyltransferase) cDNA were used as controls. PCR cycles were adjusted to have linear amplification for all the targets. Each RT-PCR reaction was repeated at least three times. A semiquantitative analysis of mRNA levels was carried out by the "GEL DOC UV SYSTEM" (Biorad Company, CA).

When minimal differences in gene expression were detected, experiments were repeated using Real Time PCR approach. The Real Time PCR assays were run on Opticon 4 machine (MJ Research, Waltham, MT). Reactions were performed according to the manufacturer's instructions by using SYBR green PCR Master mix. Primer sequences were designed with Primer express software.

Statistical Analysis

Statistical significance was evaluated using ANOVA analysis followed by Student's *t*-test and Bonferroni's test. Data were expressed as average value ± standard deviation. Generally speaking, comparison between patient and each of the two controls were in good agreement, when significant differences were observed we indicated them.

RESULTS

An enrolled RTT patient (6-years-old) had the clinical manifestations of fruste Rett Syndrome. She carried a de novo mutation (c.1164-1207del) in the MeCP2 gene.

MSCs From RTT Patient Show a Lower Degree of Apoptosis

MSCs from RTT patient and controls were analyzed by FACS analysis. In several experiments, we observed a significant reduction of S-phase cells, along with an increase of G_2/M cells in RTT samples compared with controls. Table I shows a representative experiment.

FACS analyses allowed us to also detect apoptic cells having a subdiploid DNA content

TABLE I. A Representative FACS Analysis of MSCs From RTT Patient and Control

	G_1 phase (%)	S-phase (%)	G_2/M phase (%)
RTT patient	49.80	8.98	41.14
Control	44.34	33.64	21.70

Analyses were carried out on actively proliferating cells.

(pre-G_1 phase). In RTT cells, we observed a decrease of apoptic cells compared with the two controls (−24% and −80%, respectively).

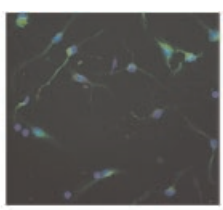
These data were confirmed by annexin assay that evidenced a reduced percentage of apoptotic cells in cultures from RTT samples compared with controls (Fig. 1).

MSCs From RTT Patient Are Prone to Senescence

We observed signs of senescence in cells from RTT patient, as detected by in situ acid-beta-galactosidase compared with cells from healthy donors (Fig. 2). To extend this finding, we measured telomerase activity by a primer extension assay in which telomerase reverse transcriptase (TERT) synthesizes telomeric repeats onto oligonucleotide primers (TRAP assay) [Kim et al., 1994; Kim and Wu, 1997].

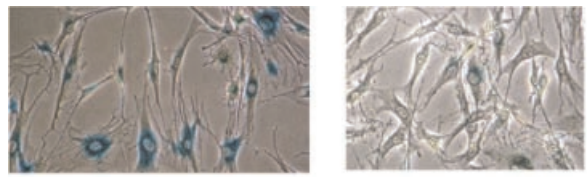
Telomerase-positive extracts were obtained from MSCs of healthy children, as expected for stem cell samples. However, the telomerase activity was reduced in RTT patient (Fig. 3).

It has been demonstrated that distinct heterochromatin structures accumulate during senescence and could represent a hallmark of this process. In particular, Narita et al. [2003] evidenced heterochromatin formation during cellular senescence and demonstrated that DNA from senescent cells was more resistant to limited micrococcal nuclease digestion compared to normal cells. Cells from RTT patient showed an increased resistance to nuclease digestion compared with cells from controls (Fig. 4).



	APOPTIC CELLS (%)
RTT PATIENT	3,10 ± 0,45
CONTROL	14,7 ± 2,02

Fig. 1. Detection of apoptic cells by annexin assay. Fluorescence photomicrograph represents cells stained with annexin V (green), which binds to phosphatidylserine residues exposed on the outer layer of the cell membrane during the early stages of apoptosis. Nuclei were counterstained with Hoechst 33342 (blue). The table shows, for RTT patient and control, the percentage of apoptotic cells in cultures. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



	SENESCENT CELLS (%)
RTT PATIENT	18,1 ± 2,15
CONTROL	10,7 ± 1,08

Fig. 2. Senescence-associated β-galactosidase assay performed on MSCs obtained from RTT patient and control as indicated in the table. The picture shows several senescent cells as detected with β-galactosidase assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Molecular Pathways Involved in Cell Cycle Regulation, Apoptosis, and Senescence

We analyzed the expression of key genes involved in cell cycle regulation, apoptosis, and senescence. In MSCs from RTT patient, the reduction of cells in S-phase is in agreement with the decrease of cyclin E mRNA (−74% ± 9%) (Fig. 5A). Expression of Rb (−47% ± 7%), Rb2/p130 (−43% ± 6%), p107 (−43% ± 8%), p27 (−54% ± 8%), and p53 (−55% ± 8%) mRNA was downregulated in RTT patient compared to controls (Fig. 5A). This is an unexpected result since these genes are related to cell cycle arrest

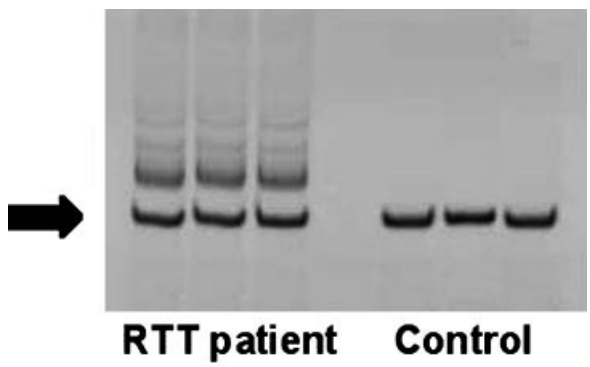


Fig. 3. Polyacrylamide gel electrophoresis of TRAP assay products obtained from RTT and control cells. The picture shows the PCR amplified products of telomerase activity. Three different reactions for RTT patient and control were shown. Black arrow: TRAP internal control. The assay measures enzymatic activity of telomerase. In the first step of the reaction, active telomerase in cell extracts adds a varied number of telomeric repeats (TTAGGG) onto the 3′ end of the substrate oligonucleotide M2(TS). After which PCR is used to amplify the extended products. The M2 primer serves as forward primer while ACX is the reverse primer for PCR. The products are then electrophoresed on a polyacrylamide gel. The internal control of reaction is to demonstrate that PCR reactions were properly carried out.

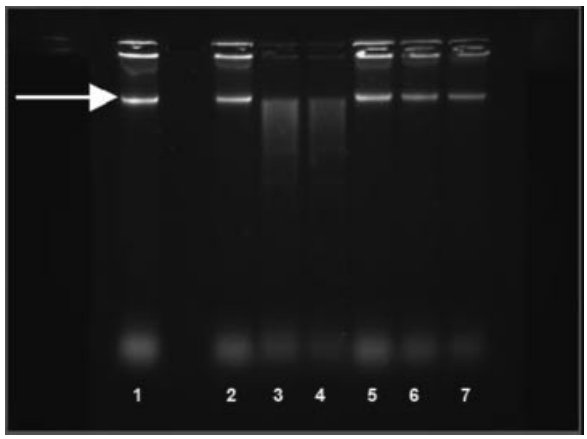


Fig. 4. Micrococcal nuclease assay. Agarose gel electrophoresis of genomic DNA from RTT and control sample. DNA was digested with micrococcal nuclease for various times, as indicated below. It is evident in the picture that a high molecular weight DNA band is more resistant to nuclease digestion in MSCs from RTT patient compared with the control. **Lane 1**: undigested control DNA; **lanes 2–4** control DNA that was digested for 1′, 3′, and 6′, respectively; **lanes 5–7** DNA from RTT sample that was digested for 1′, 3′, and 6′, respectively. White arrow: high molecular weight DNA.

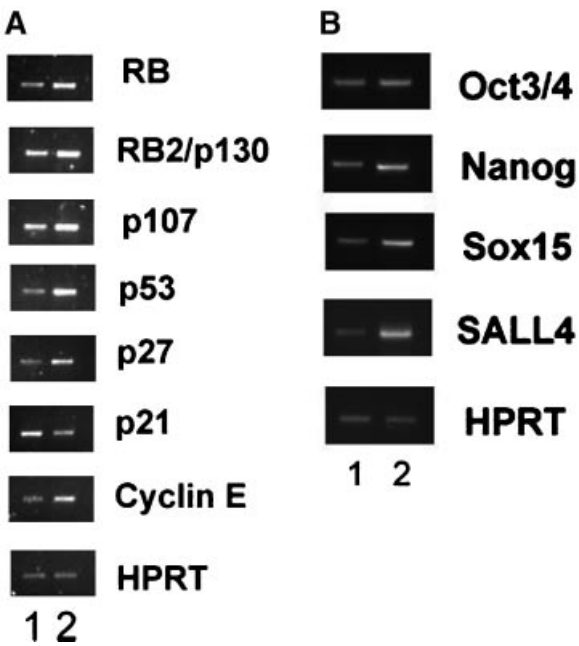


Fig. 5. Semiquantitative RT-PCR analysis of mRNA expression of the indicated genes from RTT patient (column 1) and healthy control (column 2). mRNA levels were normalized with respect to HPRT, chosen as an internal control. Each experiment was repeated at least three times. **A**: cell cycle related genes; (**B**) genes involved in stem cell self-renewal and uncommitted state.

and senescence. It is possible that other genes could promote these processes in RTT cells. On the other hand, we detected an upregulation of the cyclin kinase inhibitors p21 ($+78\% \pm 6\%$) and p16 ($+230\% \pm 20\%$). This last gene plays a key role in blocking cell cycle progression and promoting senescence phenomena. Interestingly, we did not observe TERT expression in RTT cells while its expression was easily detected in control MSCs (data not shown). Absence of TERT expression further confirms that RTT cells were prone to senescence.

In MSCs from RTT patient, we detected a significant decrease of Bax/Bcl-2 ratio ($-61\% \pm 8\%$) compared to controls. This is in agreement with the reduction of apoptic cells as detected with annexin assay (Fig. 1).

Genes Involved in Stem Cell Self-Renewal

Studies on transcriptional profiling of stem cells allowed a preliminary identification of "stemness" genes participating in control of stem cell properties, such as self-renewal ability and retention of an uncommitted state [Cai et al., 2004; Mikkers and Frisen, 2005; Takahashi and Yamanaka, 2006]. We compared the expression of some "stemness" genes in MSCs from RTT patients and healthy individuals. In controls and RTT samples, we did not detect the expression of Sox2, GDF3, UTF1, TCL1, and Zfp42. In MSCs from RTT patient, we observed a significant reduction of Oct 3/4 ($-30\% \pm 5\%$), Sox15 ($-37\% \pm 7\%$), SALL4 ($-89\% \pm 8\%$), and Nanog ($-50\% \pm 6\%$) (Fig. 5B).

Expression of Lineage Specific Gene

In MSCs, low-level transcritption of lineage-specific genes has been detected along with highly expressed housekeeping and stemness genes. During differentiation, silencing of chromatin domains and the formation of heterochromatin prevents this promiscuous transcription and the activity becomes limited to lineage-specific genes. According to this transcription model, it can be hypothesized that MSC differentiation is biased to those lineages whose key genes are already expressed in uncommitted cells.

We compared classical mesodermal lineage specific markers in MSCs from RTT patient and in those from healthy donors. In MSCs from the RTT child, we detected an increase of osteopontin ($+76 \pm 8\%$) that is an osteogenic marker, while we did not detect modification of

PPAR-γ, an adipocyte differentiation marker. The somatic plasticity of MSCs allows their differentiation also in ectodermal derivatives, such as neurons. For this reason we also analyzed the expression of neural specific markers (Table II). Overall, these markers were upregulated in RTT samples compared with MSCs from healthy children.

DISCUSSION

RTT is a genetic disorder affecting mainly neural development. Several reports also showed that osteogenesis process could be impaired in RTT patients [Haas et al., 1997; Budden and Gunness, 2001, 2003; Ager et al., 2006; Zysman et al., 2006]. Improper tissue development and maturation may arise from alterations in stem cell reservoirs [Ohlstein et al., 2004; Scadden, 2006]. For these reasons, we decided to investigate the biology of bone marrow MSCs. These cells are of great interest for studies on RTT syndrome since they represent the physiological pool for generation of osteocytes. Moreover, they can represent a useful model to study neural development.

MeCP2 Inactivation Could Alter the Biology of MSCs

MSCs from RTT patient showed precocious signs of senescence compared with healthy controls. This was in agreement with a reduced expression of genes involved in control of stem cell self-renewal and upregulation of lineage specific genes, such as those involved in osteogenesis and neural development. These data are in agreement with research showing that genomic DNA methylation decreases gradually during cell culture progression and, in general, in aging of organisms [Nilsson et al., 2005; Zheng et al., 2006]. In fact, MeCP2 inactivation could contribute to decrease in the levels of DNA methylation [Fuks, 2005].

We observed a lower degree of apoptosis in RTT patient cells compared with controls. This means that aberrant stem/progenitor cells, instead of being eliminated, could survive and become senescent.

Overall, these data suggest that in RTT patient, the bone marrow reservoir of MSCs could be composed of stem cells with impaired self-renewal ability and modifications of uncommitted state. Alternatively, in the reservoir, a change in stem cells/progenitors ratio could occur. These aberrations could be involved in development of bone pathologies observed in some RTT patients.

On the basis of our research data, we could hypothesize that MeCP2 inactivation may be responsible for alterations in other stem cell reservoirs, such as the neural stem cell pool. These abnormalities could generate symptoms and diseases that are associated with RTT syndrome.

TABLE II. Semiquantitative RT-PCR Analysis of mRNA Expression of the Indicated Genes From RTT Patient and Healthy Control

Gene	RTT patient	Control
GFAP	Not detected	1
MAP2	10.0 ± 1.50	1
Glutamine synthetase	1.48 ± 0.14	1
NAIP	3.30 ± 0.30	1
CNPase	6.20 ± 1.22	1
Vimentin	0.30 ± 0.08	1
Neuron specific enolase	1.64 ± 0.29	1

mRNA levels were normalized with respect to HPRT, chosen as an internal control. Each experiment was repeated at least three times. Arbitrary control value is 1.

MeCP2 and Gene Expression

Most of genes we analyzed can be silenced through methylation of their own promoters. At first glance, MeCP2 mutation should disrupt silencing of these genes and lead to their expression. Nevertheless, several genes showed a decreased or unchanged expression in MSCs from RTT patient compared with controls. These data are in agreement with studies showing that MeCP2 inactivation can induce both gene upregulation and silencing [Colantuoni et al., 2001; Nuber et al., 2005; Delgado et al., 2006]. This may be explained considering that (i) methylated DNA is a docking site for several proteins besides MeCP2 and (ii) MeCP2 inactivation may induce the expression of (co)-repressors acting on methylated promoters to trigger gene downregulation through epigenetic mutations that do not require the presence of MeCP2 on methylated CpG islands.

CONCLUSION

Our research provides a new insight on RTT syndrome. Senescence phenomena could be involved in triggering RTT syndrome-associated diseases.

ACKNOWLEDGMENTS

This study was partially supported by a Sbarro Institute grant to U.G. We thank Dr. Monica Bocchia for bone marrow aspirates.

REFERENCES

Ager S, Fyfe S, Christodoulou J, Jacoby P, Schmitt L, Leonard H. 2006. Predictors of scoliosis in Rett syndrome. J Child Neurol 21:809–813.

Ariani F, Mari F, Pescucci C, Longo I, Bruttini M, Meloni I, Hayek G, Rocchi R, Zappella M, Renieri A. 2004. Real-time quantitative PCR as a routine method for screening large rearrangements in Rett syndrome: Report of one case of MECP2 deletion and one case of MECP2 duplication. Hum Mutat 24:172–177.

Bianco P, Gehron Robey P. 2000. Marrow stromal stem cells. J Clin Invest 105:1663–1668.

Budden SS, Gunness ME. 2001. Bone histomorphometry in three females with Rett syndrome. Brain Dev 23 (Suppl 1):S133–S137.

Budden SS, Gunness ME. 2003. Possible mechanisms of osteopenia in Rett syndrome: Bone histomorphometric studies. J Child Neurol 18:698–702.

Buszczak M, Spradling AC. 2006. Searching chromatin for stem cell identity. Cell 125:233–236.

Cai J, Weiss ML, Rao MS. 2004. In search of "stemness". Exp Hematol 32:585–598.

Chen RZ, Akbarian S, Tudor M, Jaenisch R. 2001. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nat Genet 27:327–331.

Colantuoni C, Jeon OH, Hyder K, Chenchik A, Khimani AH, Narayanan V, Hoffman EP, Kaufmann WE, Naidu S, Pevsner J. 2001. Gene expression profiling in postmortem Rett Syndrome brain: Differential gene expression and patient classification. Neurobiol Dis 8:847–865.

Colter DC, Class R, DiGirolamo CM, Prockop DJ. 2000. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci USA 97:3213–3218.

Delgado IJ, Kim DS, Thatcher KN, LaSalle JM, Van den Veyver IB. 2006. Expression profiling of clonal lymphocyte cell cultures from Rett syndrome patients. BMC Med Genet 7:61.

Edlund T, Jessell TM. 1999. Progression from extrinsic to intrinsic signaling in cell fate specification: A view from the nervous system. Cell 96:211–224.

Fuks F. 2005. DNA methylation and histone modifications: Teaming up to silence genes. Curr Opin Genet Dev 15:490–495.

Gage FH. 2000. Mammalian neural stem cells. Science 287: 1433–1438.

Galderisi U, Di Bernardo G, Cipollaro M, Peluso G, Cascino A, Cotrufo R, Melone MA. 1999. Differentiation and apoptosis of neuroblastoma cells: Role of N-myc gene product. J Cell Biochem 73:97–105.

Guy J, Hendrich B, Holmes M, Martin JE, Bird A. 2001. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet 27:322–326.

Haas RH, Dixon SD, Sartoris DJ, Hennessy MJ. 1997. Osteopenia in Rett syndrome. J Pediatr 131:771–774.

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP. 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19:187–191.

Jori FP, Napolitano MA, Melone MA, Cipollaro M, Cascino A, Altucci L, Peluso G, Giordano A, Galderisi U. 2005. Molecular pathways involved in neural in vitro differentiation of marrow stromal stem cells. J Cell Biochem 94:645–655.

Jung BP, Jugloff DG, Zhang G, Logan R, Brown S, Eubanks JH. 2003. The expression of methyl CpG binding factor MeCP2 correlates with cellular differentiation in the developing rat brain and in cultured cells. J Neurobiol 55: 86–96.

Kim NW, Wu F. 1997. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res 25:2595–2597.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. 1994. Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015.

Kimura H, Shiota K. 2003. Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase Dnmt1. J Biol Chem 278:4806–4812.

Krabbe C, Zimmer J, Meyer M. 2005. Neural transdifferentiation of mesenchymal stem cells—A critical review. APMIS 113:831–844.

Kurz DJ, Decary S, Hong Y, Erusalimsky JD. 2000. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. J Cell Sci 113(Pt 20):3613–3622.

Luikenhuis S, Giacometti E, Beard CF, Jaenisch R. 2004. Expression of MeCP2 in postmitotic neurons rescues Rett syndrome in mice. Proc Natl Acad Sci USA 101:6033–6038.

Mikkers H, Frisen J. 2005. Deconstructing stemness. EMBO J 24:2715–2719.

Morrison SJ, Shah NM, Anderson DJ. 1997. Regulatory mechanisms in stem cell biology. Cell 88:287–298.

Mullaney BC, Johnston MV, Blue ME. 2004. Developmental expression of methyl-CpG binding protein 2 is dynamically regulated in the rodent brain. Neuroscience 123:939–949.

Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113:703–716.

Nilsson O, Mitchum RD, Jr., Schrier L, Ferns SP, Barnes KM, Troendle JF, Baron J. 2005. Growth plate senescence is associated with loss of DNA methylation. J Endocrinol 186:241–249.

Nuber UA, Kriaucionis S, Roloff TC, Guy J, Selfridge J, Steinhoff C, Schulz R, Lipkowitz B, Ropers HH, Holmes MC, Bird A. 2005. Up-regulation of glucocorticoid-regulated genes in a mouse model of Rett syndrome. Hum Mol Genet 14:2247–2256.

Ohlstein B, Kai T, Decotto E, Spradling A. 2004. The stem cell niche: Theme and variations. Curr Opin Cell Biol 16: 693–699.

Prockop DJ. 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74.

Rasmussen TP. 2003. Embryonic stem cell differentiation: A chromatin perspective. Reprod Biol Endocrinol 1:100.

Scadden DT. 2006. The stem-cell niche as an entity of action. Nature 441:1075–1109.

Shahbazian M, Young J, Yuva-Paylor L, Spencer C, Antalffy B, Noebels J, Armstrong D, Paylor R, Zoghbi H. 2002. Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. Neuron 35:243–254.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676.

Temple S. 2001. The development of neural stem cells. Nature 414:112–117.

Tudor M, Akbarian S, Chen RZ, Jaenisch R. 2002. Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain. Proc Natl Acad Sci USA 99:15536–15541.

Weaving LS, Ellaway CJ, Gecz J, Christodoulou J. 2005. Rett syndrome: Clinical review and genetic update. J Med Genet 42:1–7.

Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. 2005. Plasticity of cultured mesenchymal stem cells: Switch from nestin-positive to excitable neuron-like phenotype. Stem Cells 23:392–402.

Zheng QH, Ma LW, Zhu WG, Zhang ZY, Tong TJ. 2006. p21Waf1/Cip1 plays a critical role in modulating senescence through changes of DNA methylation. J Cell Biochem 98:1230–1248.

Zysman L, Lotan M, Ben-Zeev B. 2006. Osteoporosis in Rett syndrome: A study on normal values. Sci World J 6: 1619–1630.