University of Siena

Ph.D. in Medical Genetics

New perspectives on Rett syndrome: the role of MECP2 gene in cellular senescence and neural differentiation

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Index

Index

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1. Introduction

1.1 Rett Syndrome

Rett syndrome (RTT, OMIM#312750) is a progressive neurodevelopmental disorder and one of the most common causes of mental retardation in girls, with an extimated prevalence of approximately 1 in 10,000-15,000 females. Its name comes from Andreas Rett who first described it in 1966 (94) but the syndrome was internationally recognized after Hagberg et al. (41) described 35 cases in 1983.

RTT is an X-linked dominant disorder caused by mutation in methyl-CpG-binding protein 2 (MECP2).

This syndrome is characterized by apparently normal development for the first 6–18 months of life, followed by a period of regression in language and motor skills. The patients lose purposeful hand use and replace them with repetitive stereotyped hand movements. They usually have normal head circumference at birth followed by postnatal deceleration of head growth. Social withdrawal, communication dysfunction, loss of acquired speech and cognitive impairment are also characteristic of RTT patients. The impairment of locomotion is also very common. Additional characteristics include autistic features, panic-like attacks, respiratory dysfunctions (episodic apnea and/or hyperpnea), bruxism, impairment of sleeping patterns, progressive kyphosis or scoliosis, decreased somatic growth and hypotrophic small and cold feet and/or hands (113) (116). After initial regression, the condition stabilizes. Patients may recover some skills and usually survive into adulthood, but the incidence of sudden, unexplained death is significantly higher than in controls of a similar age (75).

In addition to classic RTT, a number of variants have been described. In 1999, mutations in the MECP2 gene were identified in RTT patients; mutations are found in up to 70% of classic RTT cases, in a lower percentage of RTT variants but also in males with both syndromic and nonsyndromic X-linked mental retardation (113). Since pathogenic mutations had been identified in such a large percentage of patients, RTT was considered a monogenic disorder.

1.1.2 Clinical Features

The established diagnostic criteria for RTT have been recently revised in order to clarify previous ambiguities in interpretation of clinical features (42). In its classical form it causes regression of speech and purposeful hand movements after a few months of relatively normal development. Postnatal microcephaly, hand dyspraxia, stereotypic “hand-washing” activities, ataxia, abnormal breathing, and growth retardation are among its characteristic features. The disease progresses according to a predictable course that comprises four stages (108).

During stage I (6-18 months) patients cease to acquire new skills; they show decelerating head growth and autistic features. In stage II (1-4 years), RTT girls lose the ability to speak and the purposeful use of the hands. During this stage, patients show the classic “hand-washing” stereotypic activity, irregular breathing patterns, truncal and gait ataxia/apraxia. In stage III (4-7 years), girls become more alert and interested both in people and their surroundings, however inability to speak, hand apraxia and stereotypic hand activities persist. Other somatic and neurologic handicaps, such as severe scoliosis, reduced somatic growth and epilepsy, become evident. During stage IV (5-15 years and older), seizures become less frequent, but somatic and neurologic deterioration continues, resulting in spastic quadriparesis.

In addition to the classic form, five distinct categories of atypical RTT have been delineated on the bases of clinical criteria (41). These variants have some, but not all diagnostic features of RTT and can be milder or more severe. They include:

I) the infantile seizure onset variant, with seizure onset before regression;

II) “forme fruste” with a miled and incomplete clinical course;

III) congenital variant, lacking the normal perinatal period;

IV) the late regression variant, witch is rare and still controversial;

V) the preserved speach variants (PSV), in which girls recover the ability to speak in single words or third person phrases and display an improvement of purposeful hand movements at stage III of disease progression (122).

1.1.3 Structure and function of the MECP2 gene and protein

MECP2 gene was mapped to the X chromosome, in the Xq28 region (22) and consists of 4 exons. It encodes two separate isoforms depending on the use of alternative splice variants: a protein of 486 amino acids (MeCP2_E2; encompassing part of exon 2 but not exon 1) and the recently identified protein of 498 amino acids (MeCP2_E1; encompassing exon 1 but not exon 2) (67) (79) (Fig. 1A-B).

The MeCP2_E1 isoform is more abundant in brain, while the MeCP2_E2 isoform predominates in other tissues.

MeCP2 protein contains four functional domains: (1) a methyl-CpG-binding domain (MBD, 85 amino acids) which binds to 5-methyl cytosine with a high affinity; (2) a transcriptional repression domain (TRD, 104 amino acids) which interacts with histone deacetylase and transcriptional corepressor SIN 3A; (3) the nuclear localization signal which may be responsible for the transport of MeCP2 into the nucleus, and (4) the C-terminal segment which facilitates its binding to the nucleosome core (81) (56) (62) (Fig. 1B).

Interactions between the transcription repressor complex and chromatin-bound MeCP2 lead to deacetylation of core histones H3 and H4 by histone deacetylases resulting in compaction of the chromatin, making it inaccessible to components of the transcriptional machinery. In addition, MeCP2 can perform histone deacetylase-independent transcriptional repression (120) (Fig. 2a-b). DNA-methylation-dependent repression is important for *X-chromosome inactivation* (XCI) and genomic imprinting.

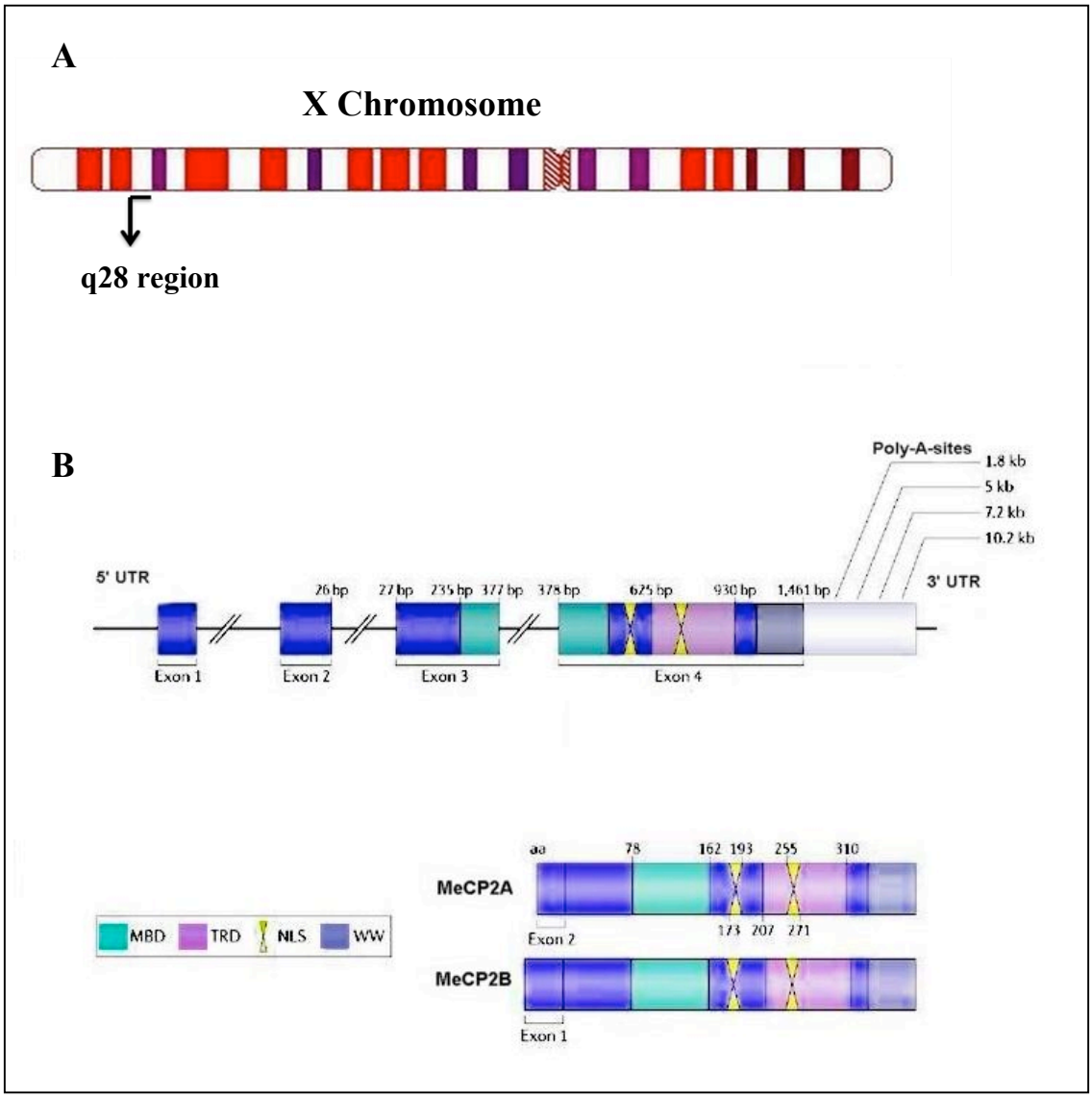

Fig.1 : **A)** Representative scheme of X chromosome q28 region in which MECP2 gene is located.

B) MECP2 (methyl-CpG-binding protein 2) gene structure (on the top). The two main protein isoforms, MeCP2A (486 amino acids) and MeCP2B (498 amino acids), are produced by alternative splicing of the MECP2 transcript and differ in their N-terminal regions, which are encoded by exon 2 of the gene in the case of MeCP2A and exon 1 in MeCP2B (in bottom on the right). MBD, methyl-CpG binding domain; NLS, nuclear localization signal; poly(A), polyadenylation; TRD, transcriptional repression domain; X, stop codon (in bottom on the left) (Figure adapted from 109) .

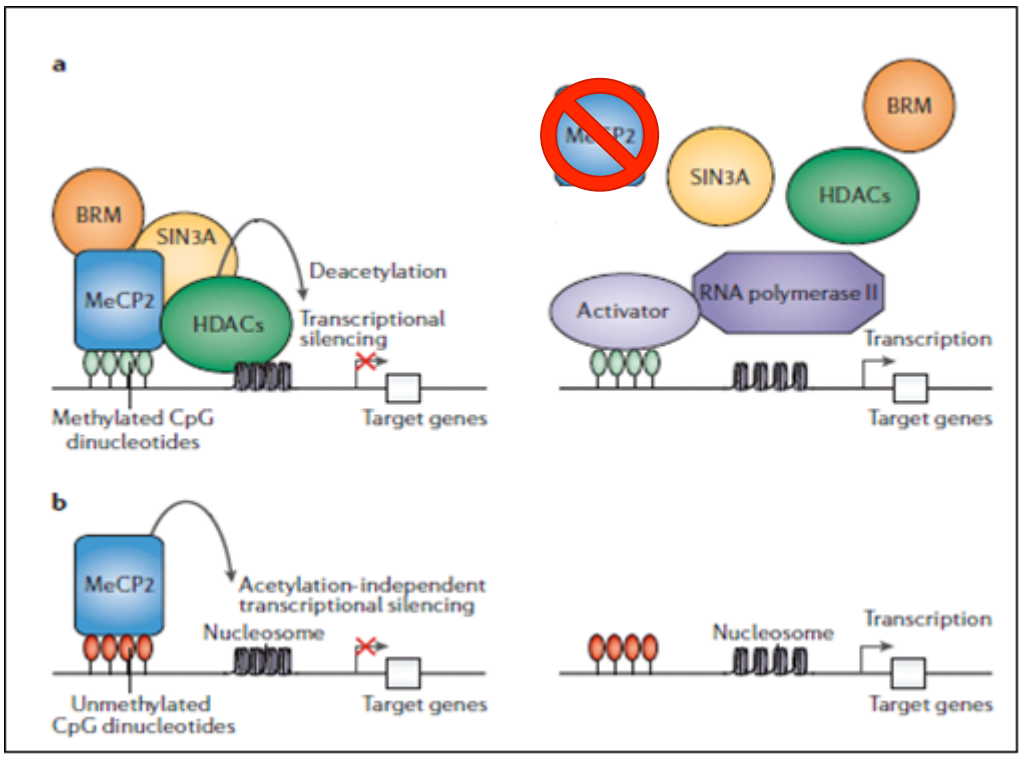

Fig. 2: a) MeCP2 binds methylated DNA and recruits chromatin-remodelling complexes that contain SIN3A (a transcriptional co-repressor), BRM (a SWI/SNF-related chromatin remodelling protein) and histone deacetylases (HDACs) (left panel). This leads to chromatin condensation owing to histone deacetylation, which results in a limited accessibility of the transcriptional machinery to promoter regions. When MeCP2 is not bound to methylated DNA (right panel), the complex that usually contains MeCP2, BRM, SIN3A and HDACs is not recruited. This lack of MeCP2 binding to DNA could be due to missense mutation in the methyl-CpG-binding domain or loss of expression of MeCP2. In this case, histones remain acetylated and the DNA at the promoter remains in an open conformation, allowing transcription factors to bind DNA and initiate transcription.

b) MeCP2 is also a potent chromatin-condensing protein and can repress gene expression independently of DNA methylation, at least in vitro (left panel). At promoters where this DNA-methylation-independent function of MeCP2 is involved in regulating expression, a deficiency or absence of MeCP2 leads to a disorganization of chromatin structure (indicated here by increased spacing between nucleosomes), making transcription more likely to occur (right panel) (Figure adapted from 109) .

MECP2 belongs to a family of methyl-CpG-binding proteins consisting of MBD1, MBD2, MBD3, and MBD4, all of which contain the conserved methyl-CpG-binding domain (MBD) (9) (109) (Fig. 3).

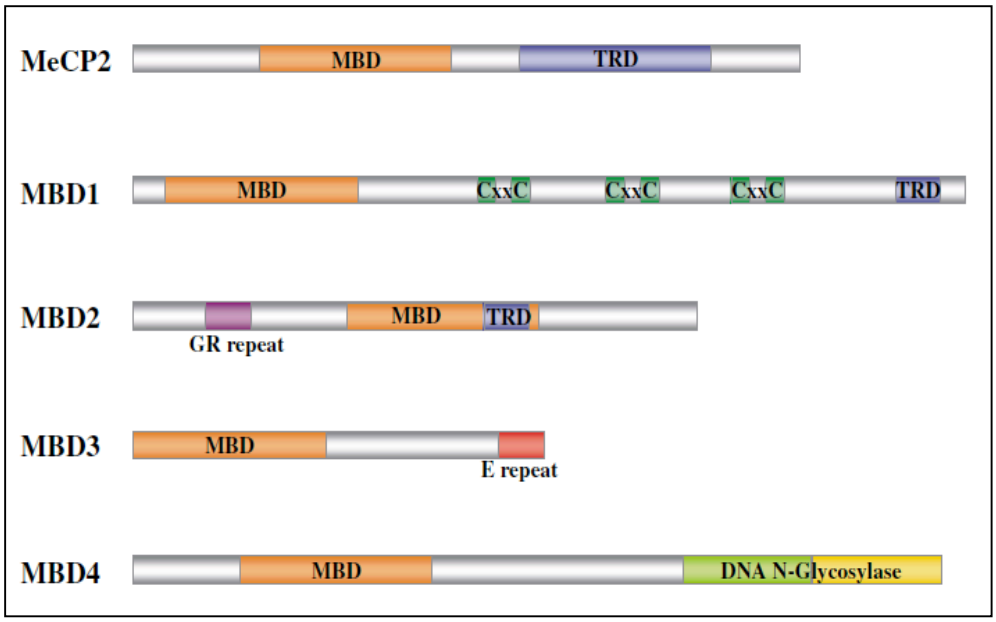

Fig.3: Characteristic domains of the methyl CpG binding (MBD) protein family. The predominant isoform of MeCP2 expressed in human brain has 498 residues. MBD1 has multiple isoforms, ranging in size from 50 to 70 kDa (605 residues, 586 residues, 556 residues, 549 residues and 503 residues). MBD2 contains 414 residues (44 kDa); MBD3 has two splice variants: 285 residues and 253 residues (27 and 32 kDa, respectively). MBD4 contains 554 residues (63 kDa). The MBD sequence motif is depicted as an orange box in each protein. Other defined sequence motifs in the individual MBD family members are also depicted (Figure adapted from 109).

MeCP2, the most extensively studied member of MBD family, is a nuclear protein dynamically expressed during postnatal mammalian brain development and is a marker for neuronal maturity. Elevated MeCP2 expression is

hypothesized to be required for neuronal differentiation by the regulation of multiple target genes (89).

The dominant model of MeCP2 function hypothesizes that MeCP2 binds to CpG methylated promoters and recruits histone deacetylase along with co-repressor activities, thereby silencing gene transcription (Fig. 4A). Yasui et al. in 2007 demonstrated that CpG islands and transcriptionally silent promoters constitute only a minority of MECP2 binding sites. MECP2 binds a large number of intergenic sites, suggesting a key role in long-range chromatin modifications. This research group showed a correlation between MECP2 and RNA polymerase II binding sites, which suggests that MECP2 can bind transcriptionally active promoters in order to modulate gene expression. For these reasons, MECP2 may be considered a transcriptional modulator rather than a transcriptional repressor (118).

Moreover, Yasui and colleagues, demonstrated that dense promoter methylation does not correlate with MeCP2 binding. Recent findings indicate that the promoters with the highest density of potential CpG methylation sites are mostly unmethylated even when inactive (115). Therefore these promoters are probably not represented in the methylation data. These results may also be explained by previous findings demonstrating the importance of A/T sequences adjacent to methylated CpGs in determining binding of MeCP2 (61) and the methylation- independent binding and chromatin compaction by MeCP2 *in vitro.*

In addition, Young and colleagues (119) demonstrated that MeCP2 acts as a splicing regulator.

It is tempting to propose that the two activities are economically coordinated, such that when a gene becomes reactivated by releasing MeCP2 from its promoter (through posttranslational modifications, for example), splicing of the nascent transcript is modulated by MeCP2. This proposal is consistent with the recent finding that MeCP2 binds RNA as avidly as it binds methylated DNA and that these two activities are mutually exclusive (53). Moreover, the identification of aberrantly spliced p16INK4a transcripts due to promoter methylation supports a link between DNA methylation and splicing. Interestingly, a recently identified transcriptional target of MeCP2, *Dlx5*, was identified by microarray experiments as an *in vivo* target of MeCP2-dependent splicing regulation (119) (Fig. 4B). Because MeCP2 binds preferentially methylated CpGs, this hypothesis adds an

extra level of complexity, posttranscriptional splicing modulation, for epigenetic control of gene expression.

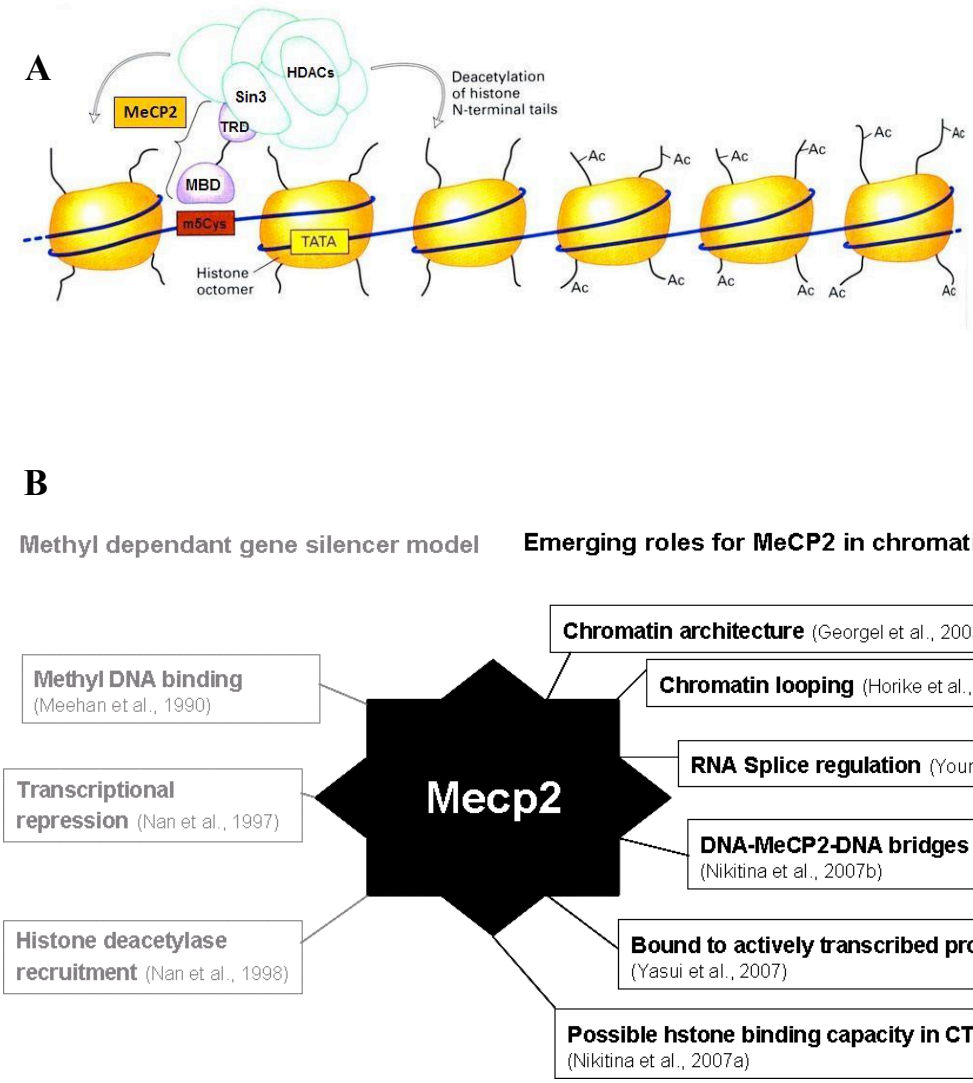

Fig. 4: **A)** A representative scheme of the dominant model of MeCP2 function. **B)** Chronological representation of known MeCP2 functions. The progression of traditional understanding of MeCP2 as a methyl-dependent proximal gene silencer is shown in grey on the left, while studies implicating MeCP2 as having additional functions are listed on the right. CTD, carboxyl terminal domain (Figure adapted from 119) .

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1.1.4 MECP2 mutation in RTT phenotypes

Mutations in MECP2 account for approximately 70% of females with classic RTT, 50% of PSV cases and a lower percentage of other variants (23) (93) (123).

Since is been demonstrated MECP2 involvement in RTT, several mutation data bases have been developed (http://homepages ed.ac.uk/ skirmis/; http://mecp2.chw.edu.au/ ; http://www.biobank.unisi.it).

Studies aimed at establishing a genotype/phenotype correlation have yielded conflicting results (123). This discrepancy may result from differences in study design such as assessment scale of phenotype severity and classification of MECP2 mutations. In addition, biological factors such as the pattern of X inactivation and the effects of genetic background and modifier genes may account for the discrepancy (93).

MECP2 mutations have been classified according to two systems. Some studies group mutations in truncating and missense (48) (3) while others divide mutations more accurately into early-truncating, late-truncating, and missense (47). Early truncating mutations occur in the MBD and TRD domains and lead to complete loss of MeCP2 function. Late-truncating mutations occur in the C-terminal domain, and therefore the MBD and TRD domains are intact. To date missense mutations have been described exclusively in the MBD and the TRD domains (Fig. 5).

There is some agreement that missense mutations have a milder effect than nonsense mutations and that early truncating mutations (located in the MBD or TRD), by causing complete loss of MeCP2 function, are preferentially associated with a more severe phenotype than late truncating mutations (located in the C-terminal domain) (93). Furthermore, the common Arg270X mutation seems to be associated with increased mortality (54).

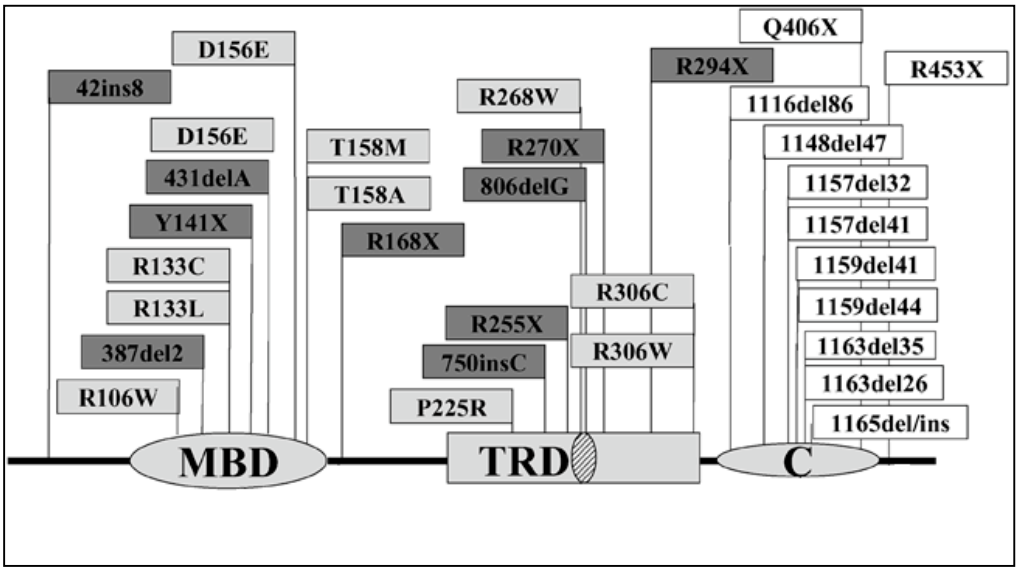


Fig. 5: MeCP2 and location of mutations causing Rett syndrome. *Striped circle* Nuclear localization signal. Early truncating (*gray flags*), late-truncating (*white flags*), and missense mutations (*light gray flags*) are reported. *MBD* Methyl binding domain; *TRD* transcription repression domain; *C* C-terminal domain (figure adapted from 93)

1.1.5 Effect of X-chromosome inactivation

The inactivation of one of the X chromosomes occurs randomly in differentiating embryonal cells in females, resulting in cells that are mosaic with respect to which chromosome is active. The purpose is to equalize X-linked gene products between XX females and XY males.

The *MECP2* gene is located at q28 on the X chromosome and it has been demonstrated to be subjected to X-inactivation in mice and humans (2) (22).

Females with the MECP2 mutations exhibit a broad spectrum of clinical presentations ranging from classical Rett syndrome to asymptomatic carriers, which can be explained by differences in X chromosome inactivation. In support of this hypothesis, skewed XCI (presumably favouring inactivation of the mutant allele) has been observed in healthy carrier mothers of RTT patients and partially skewed XCI in less severe RTT phenotypes (7) (50).

However, several studies showed that carrier mothers were asymptomatic and presented non-random XCI in the peripheral blood cells, which resulted in the X chromosome harboring the mutant allele that was predominantly active. Thus, the presence of non-random XCI in the peripheral blood cells did not provide an explanation for the normal phenotype of the carrier mothers (66)(33).

Furthermore, it has been demonstrated that XCI may vary remarkably between tissues (101). Thus, the extrapolations of results based on sampling peripheral tissues, such as lymphocytes, to other tissues, such as brain, may be misleading. Studies performed on RTT brain tissues suggest that balanced XCI patterns are prevalent (69) (100).

This results suggests that mechanisms other than XCI may contribute to the phenotypic heterogeneity associated with MECP2 mutations (69).

1.2. DNA methylation

Among the epigenetic mechanisms that are involved in regulation of mammalian gene expression, DNA methylation is the most widely studied (6).

This process is involved in X chromosome inactivation in females and DNA imprinting, events which result in monoallelic gene expression. It also contributes to genome stability by preventing translocations of repetitive and transposable sequences, and likely plays a dynamic role in development (77) (49).

DNA methylation is catalysed by DNA cytosine-5-metyltransferase (DNMTs) and occurs at the 5' position of cytosine within CpG islands (GC-rich regions) that are present at the 5' ends of about 40% of mammalian genes (9).

These regions are generally unmethylated, but the methylation of specific sites in CpG islands is used as a regulatory mechanism for some genes, where it is usually associated with transcriptional down-regulation (6).

DNA methylation may impact the transcription of genes in two ways:

I) the methylation of DNA may itself physically impede the binding of transcriptional proteins to the gene;

II) methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodelling proteins that can modify histones, thereby forming compact, inactive chromatin termed silent chromatin.

This link between DNA methylation and chromatin structure is very important. In particular, loss of methyl-CpG-binding protein 2 (MeCP2) has been implicated in Rett Syndrome and methyl-CpG binding domain protein 2 (MBD2) mediates the transcriptional silencing of hypermethylated genes in cancer (97).

However, additional mechanisms by which CpG methylation represses gene transcription have been proposed. These include the inhibition of RNA-polymerase elongation, impairment of RNA-polymerase loading, interfering with RNA polymerase initiation that involves localized histone deacetylation. However, several studies provided evidence that DNA methylation may be secondary events in transcriptional silencing and that DNA methyltransferases

may take cues from histone modifications, which may serve as the primary epigenetic mark (109).

In mammalian cells, DNA methylation is carried out by two general classes of enzymatic activities: maintenance methylation and *de novo* methylation (Fig. 6).

Maintenance methylation activity is necessary to preserve DNA methylation after every cellular DNA replication cycle. Without the DNA methyltransferase (DNMT), the replication machinery itself would produce daughter strands that are unmethylated and over time would lead to passive demethylation. DNMT1 is responsible for copying DNA methylation patterns to the daughter strands during DNA replication.

DNMT3a and DNMT3b are the *de novo* methyltransferases that set up DNA methylation patterns early in development.

Cytosine methylation is an essential mechanism for the normal development of different organisms including mammals and plants. For example, disruption of any of the three major DNMTs in mouse (*Dnmt1, Dnmt3a and Dnmt3b*) results in lethality, either during or just after embryonic development, and is associated with global demethylation (70) (52). Disruption of DNMT1 in human cancer cells results in abnormal mitotic progression and cell death (36). Moreover, DNMT3A and 3B seem to be required for neoplastic transformation and tumour progression (96).

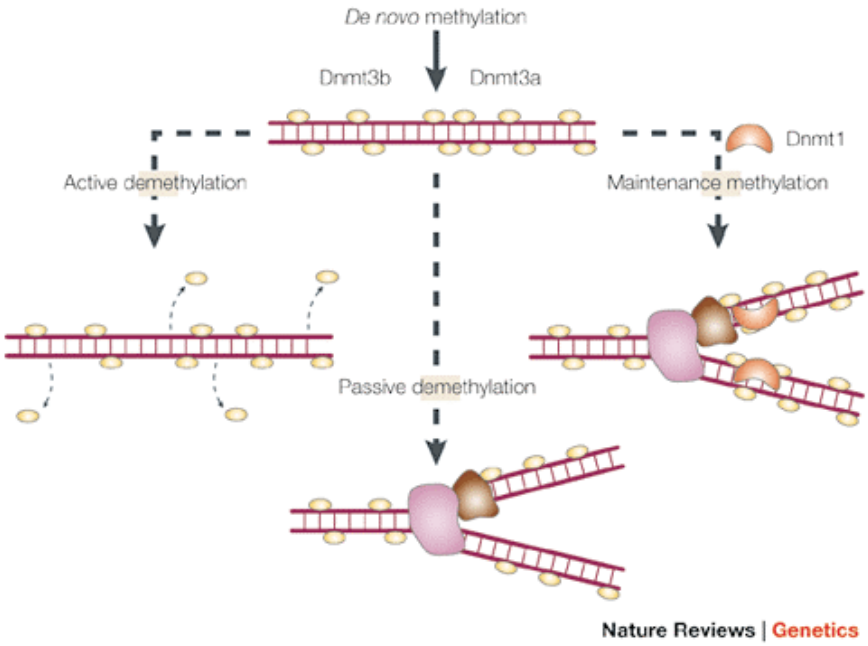


Fig. 6: A representative scheme of maintenance methylation and *de novo* methylation. (figure adapted from Nature Reviews, 2005).

1.3 Stem cells

Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce mature progeny cells, including cells both nonrenewing progenitors and terminally differentiated effector cells.

Like the differentiated state, the stem cell state requires active maintenance and is dependent on the composition of proteins and the balance of those proteins present in that cell at any given time. So, the stem cell state is dynamic since it can respond to a variety of signals that dictate distinct differentiated paths (10).

Stem cells can be isolated both from embryo and adult mammalian tissues and organs, and have been classified by their developmental potential as totipotent (able to give rise to all embryonic and extra-embryonic cell types), pluripotent (able to give rise to all cell types of the embryo proper), multipotent (able to give rise to a subset of cell lineages), oligopotent (able to give rise to a more restricted subset of cell lineages than multipotent stem cells), and unipotent (able to contribute only one mature cell type) (113).

More in depth, embrionic stem cells (ES) are pluripotent cells derived from the inner cell mass of blastocysts (27). Nevertheless, their use give rise to ethical questions, which caused an opened international debate focused on the need to control their utilization by established laws.

Adult stem cells, instead, are able to give rise only to progeny cells corresponding to their tissue of origin. Such tissue-specific stem cells have been described to varying degrees for the adult intestine, skin, muscle, blood and nervous system (113).

While some tissues, such us the blood, skin, gut, testis, must perpetually renew, the majority of cells and tissues in adult mammals exhibit very low turnover under normal circumstances; some of these respond poorly to regenerative pressure (e.g., heart), while other respond quite well (e.g., liver). These observation have been interpreted as indicating the existence and function of stem cells within some highly regenerative tissues and a lack of stem cells functions in other one (73).

Nonetheless, multiple organs, including the brain and the heart, once thought of as nonrenewing, postmitotic tissues, actually have been shown to exhibit previously unappreciated cell turnover.

These observation have opened the door for studies aimed at identifying additional tissue-resident adult stem cell populations and evaluating their regenerative potential (113) .

The main cell lineages, generating from some of the most important adult stem cell sources, are:

Hematopoietic stem cells (HSC): provide a continuous source of progenitors for red cells, platelets, monocytes, granulocytes, and lymphocytes.

Mesenchymal stem cells (MSC): give rise to multiple mesenchymal lineage, such as osteocytes, condrocytes and adipocytes.

Neural stem cells (NSC): give rise to neurons, astrocytes, and oligodendrocytes.

Epidermal stem cells : located predominantly in or near the bulge region of the hair follicle, give rise to keratinocytes.

Muscle satellite cells: reside beneath the basal lamina of mature muscle fibers.

Intestinal stem cells: are believed to reside near the bottom of the intestinal crypts.

For other tissues, including the liver and pancreas, the identity of resident stem cell populations, and even whether regeneration of these tissues in adults is stem cell mediated, is still debated.

Preston and colleagues in 2003 (90) demonstrated that adult stem cells live the native tissue to maintain the homeostasis of diseased or injured tissues revealing an high grade of somatic plasticity. Moreover several studies indicated that stem cells derived from one organ could be induced to differentiate into cells of another both *in vitro* and *in vivo*. This process has been referred to by some as *transdifferentiation* even if scientific community prefer the term *somatic plasticity* to distinguish it from the process whereby one differentiated cells can be induced to assume characteristics of another (38).

These findings aroused expectations for therapeutical application of stem cells derived from adult tissues; they could be harvested from an accessible district (such as bone marrow) and then induced to differentiate in cell types of other organs and tissues (such as neurons). To this rationale somatic plasticity represents an important feature to identify a characteristic stem cell population useful for therapeutic applications.

1.3.1 Mesenchymal stem cells (MSCs)

Because circulating blood cells survive for only a few days or months, hematopoietic stem cells (HSCs) in bone marrow must provide a continuous source of progenitors for red cells, platelets, monocytes, granulocytes, and lymphocytes (43). However, bone marrow also contains cells that meet the criteria for stem cells of nonhematopoietic tissues. The stem-like cells for nonhematopoietic tissues are currently referred to either as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as marrow stromal cells (MSCs), because they appear to arise from the complex array of supporting structures found in marrow (91).

Friedenstein and colleagues, in 1976, were able to isolate for the first time MSC cell population because of their capacity to grow as an adherent layer in colture .

Other reports (45) (19) demonstrated that single-cell-derived colonies of human MSCs are heterogeneous in that they contain at least two morphologically distinct kinds of cells: spindle-shaped cells and large cuboidal or flattened cells. Colter and colleagues in 2001 (20) extended these previous observations to demonstrate that MSCs colonies also contain extremely small cells that are rapidly self-renewing (RS cells).

The RS cells appear to be the earliest progenitors in the cultures and have the greatest potential for multilineage differentiation. They differ from more mature cells in the same cultures by a series of surface epitopes and expressed proteins.

MSCs are relatively easy to isolate from the small aspirates of bone marrow that can be obtained under local anesthesia; they are also relatively easy to expand in culture and can differentiate into mesenchymal lineage such as osteocytes, chondrocytes, adipocytes and myocytes when exposed to the appropriate stimuli *in vitro* (Fig.7).

Moreover the discovery that stem cells in adults can first reside in one tissue and then contribute to another suggests a previously unrecognized degree of plasticity in stem cell function. Indeed, it now appears that cell fate changes are a natural property of stem cells and may be involved in ongoing physiological repair of tissue damage throughout life.

Several study demonstrated that after systemic infusion of MSCs, progeny of the cells appeared in a variety of tissues, including bone, cartilage, lung, spleen, and thymus (91) (83). Ferrari and colleagues in 1998 (131) for first showed that engraftment of donor MSCs into repairing muscle was observed after either local injection or systemic injection. Engraftment into muscle of a dystrophin-deficient mouse was seen after systemic infusion of rare marrow cells defined as a ‘‘side population’’ (SP) or SP cells that may be precursors of MSCs.

Moreover is been demostred that MSCs can also differentiate into non-mesenchymal lineage such as astrocytes, oligodendocytes and neurons both *in vitro* with appropriate stimuli (65) (117) (55) and *in vivo* following transplantation (85) (25) (16) (Fig. 7).

MSCs high somatic plasticity is of interest as concern the possibility of generating differentiated cells having different embryological origin.

For these reasons, these cells are currently being tested for their potential use in cell and gene therapy for a number of human disease. In fact, a lot of studies had been conduce to evaluate the MSC engraftment effects on promoting neuronal regeneration in neurodegenerative diseases like Huntington and Parkinson’s diseases (46) (16) (39).

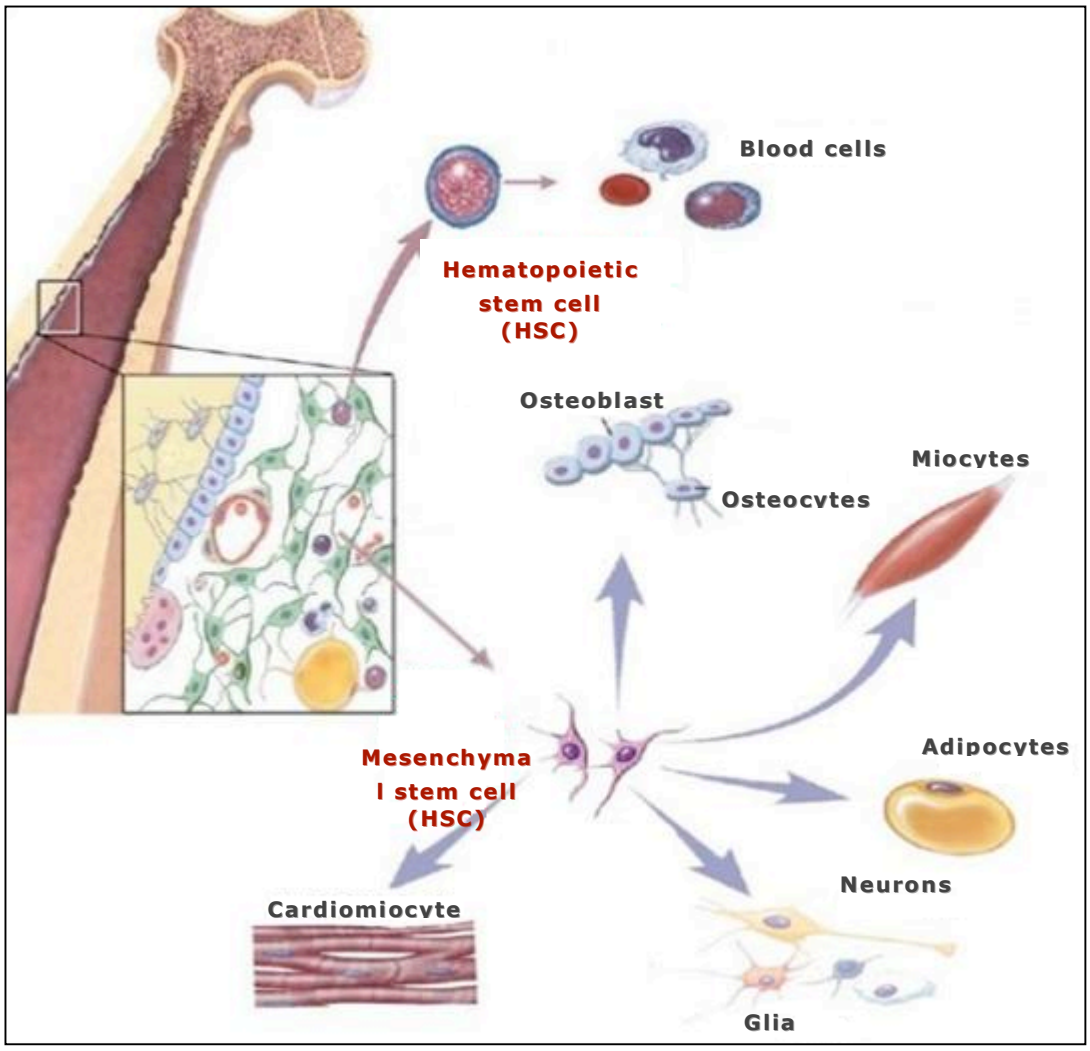


Fig. 7: Bone marrow stem cell populations and derivated cell lineages. (figure adapted from Winslow et al., 2001).

In addition, MSCs or related cells were also shown to engraft into the central nervous system. The presence of donor-derived astrocytes was observed after systemic infusion of whole marrow into immunodeficient mice. After direct infusion into basal ganglia of rats, either rat or human MSCs integrated and migrated in a manner similar to paraventricular astrocytes that have many of the properties of neural stem cells (19)

In conclusion, the numerous recent findings suggest that the concept of stem cells is in a state of flux and that the commonly held view of a tissue-specific adult stem cell may need to be expanded.

2. Rationale, aim and outline of the study

2. Rationale, aim and outline of the study

Rett syndrome (RTT) is a neurodevelopmental disorder and one of the most common genetic causes of mental retardation in girls. Mutation in MECP2 gene are found in up to 70% of classic RTT cases and in a lower percentage of RTT variants.

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

The frequent occurrence of osteoporosis and scoliosis in RTT patients suggests that bone formation and/or remodelling is impaired in these patients. Several authors have suggested that MeCP2 mutations in RTT not only affect brain development but could also affect osteogenesis (40) (11-12) (1) (125).

Based on these premises, I decided to study the biology of mesenchymal stem cells (MSCs) obtained from bone marrow of two RTT patients and from healthy controls.

I hypothesized that studying MSCs in RTT patients could be of interest for several reasons:

- 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.
- MSCs, among all the stem cell reservoirs in the body, play a key role in maintaining body's homeostasis in several tissues and organs.
- MSCs are the progenitors of osteocytes and it has been suggested that osteogenesis could be impaired in RTT patients.
- MSCs are easily isolated from bone marrow cells with a procedure that causes limited uncomfortable pain to patients.

I have investigated more in depth the effects of MeCP2 inactivation on the physiology of RTT patient MSCs, with a specific analysis of cell cycle progression, apoptosis, senescence and differentiation.

Moreover as a key characteristic of stem cells is self-renewal capacity to maintain the stem cell pool over the time, they must have an uncommitted state and avoid the specification of any differentiation process. For this reason my research was also devoted to analyze the expression of genes involved in control of stem cell self-renewal and of lineage specific genes, such as those involved in osteogenesis and neural development.

Another topic of my study consisted to investigate the role played by MeCP2 in neuronal development. The lack of functional MECP2 gene impairs physiologic differentation of neural cells altering the normal regulation of genes involved in neuronal development.

An impaired differentiative process may derive by stem cells/progenitors altered proliferative behaviors, by an unregulated apoptosis or by acquisition of an aberrant phenotype.

To this issue I induced MSCs obtained from RTT patient and controls to differentiate in neurons *in vitro* to evaluate which of above mentionated process would be altered.

Finally to have a general picture on the role of MECP2 in stem cell biology, I decided to silence MECP2 gene expression in human cultures of MSCs by siRNA technologies.

In RTT patients the great majority of MeCP2 mutations determines impairment of MeCP2 binding to methylated DNA. The mutated protein are generally inactive and do not acquire extra functions, such as dominant negative activity. For this reason, impairment in cell physiology following MeCP2 mutations is associated with a reduction in the functional MeCP2 protein level that is lower compared with that of healthy people. Nevertheless, some MeCP2 mutations found in RTT patients could be associated with a specific patient presentation (phenotype). Silencing of MeCP2 with siRNA should demonstrate that alterations in stem cells biology observed in MSC from RTT patients analyzed (carrying on different mutations in MECP2 gene) were related to MeCP2 protein downregulation and not to specific patient phenotypes.

My study could represent a good topic for RTT research: a better understanding of the consequences of MeCP2 inactivation and the effects on the biology of stem cells will be essential to shed light on RTT pathogenic mechanisms.

3. Materials and Methods

3.1 Molecular analysis of the identified patients.

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 (5). 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).

3.2 MSC cultures

In a first set of experiments, bone marrows were obtained from two female children with RTT syndrome (RTT-1; RTT-2) and two healthy female children after informed consent. All children (patients and controls) showed no statistically significant differences in the body mass index and had the same age range (6-8 years old). For other experiments (RNA interference) bone marrows were obtained from healthy female donors.

Bone marrow aspirates were collected from iliac crests, separated on Ficoll density gradient (GEHealthcare, Italy), and the mononuclear cell fraction was harvested and washed in phosphate buffer saline (PBS). I seeded 1–2.5 x 10^5 cells per cm^2 in 100 mm dishes with a-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 mg/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. All cell reagents were obtained from Euroclone Life Sciences (Italy) and Hyclone (UT, USA) unless otherwise stated.

3.2.1 Gene silencing

Twenty-one nucleotide long siRNAs targeted to MeCP2 mRNA were designed with 3' overhanging uridine dimers, following the procedure described by Reynolds (95). Target sequence were aligned to the human gene database in a BLAST search to eliminate those with significant homology to other genes. Four target sequences for each gene corresponding to sequences located in the 5', 3' or medial regions of each transcript were chosen to asses which region is more susceptible to siRNA-induced target mRNA degradation. Twenty-one nucleotide-long siRNAs with a random sequence having no known targets in the human genome were chosen as a control for specificity of gene silencing experiments.

I followed manufacturer's protocol (pSilencer adeno 1.0-CMV from Ambion, TX, USA) to produce adeno-siRNAs. To obtain enough viruses for cell transduction the recombinant adenoviruses were expanded by infection of 293 cells and then purified (Fig. 8).

Once obtained adeno-siRNAs targeting MeCP2, MSC cell cultures were transduced at different multiplicity of infection (MOI) to obtain a good silencing effect. Forty-eight to seventy-two hours post-transduction, the gene downregulation were evaluated by western blots and RT-PCR.

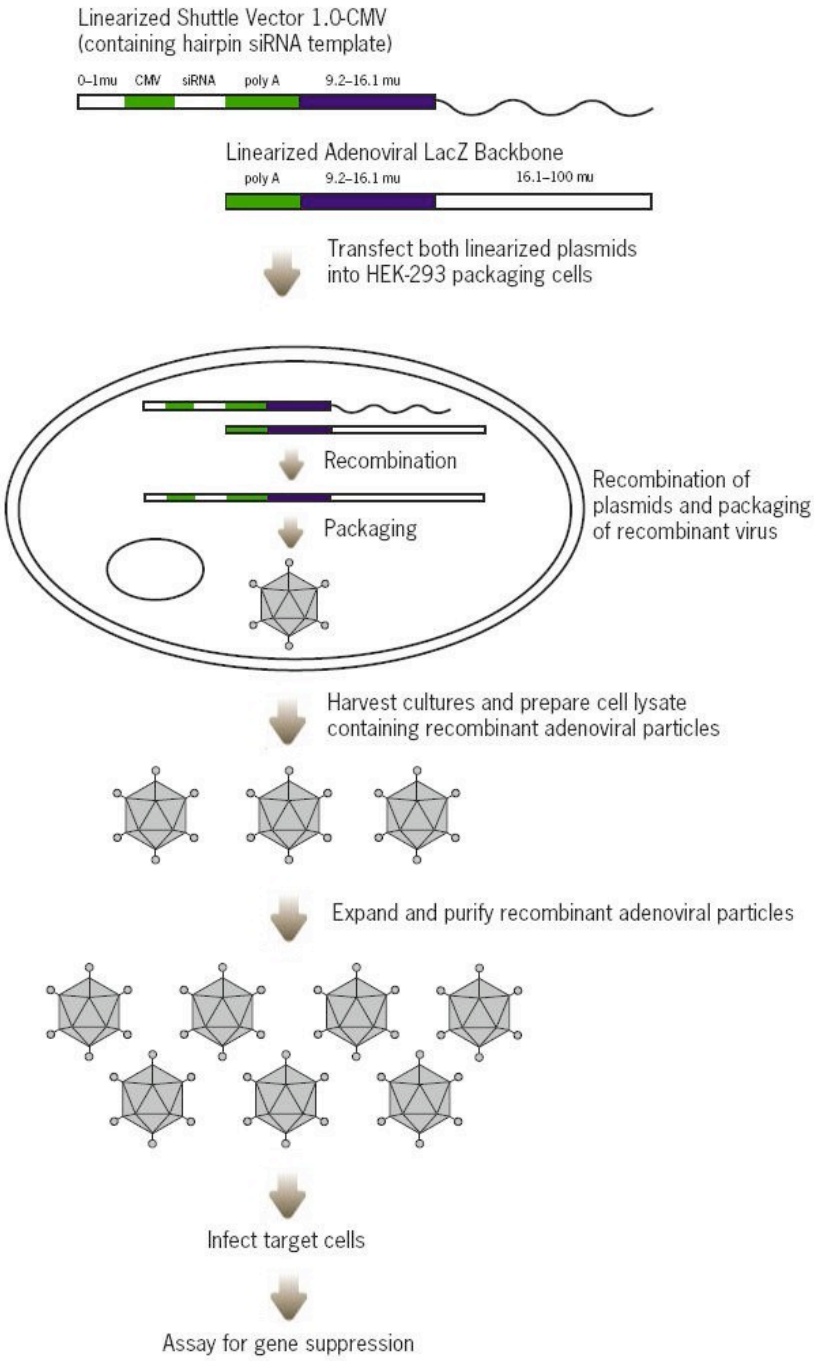


Fig. 8: Representative scheme of recombinant adenovirus production.

3.2.2 MSC neuronal differentiation

2.5 x 10^5 cells, obtained from RTT-2 patient and an healthy control, were seeded in 100 mm dishes with a-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 mg/ml streptomycin (proliferating medium).

After 24 h proliferating medium was discarded, cells were washed twice with PBS and neural differentiation medium (Hyclone, Thermo-Scientific, Waltham, MA) was added following manufacturer's protocol.

The cultures were incubated at 37°C in 5% CO_2 atmosphere and medium chanced every 3 days. The cultured was maintained for 7 days.

3.2.3 Ne-N immunocytochemistry detection

7 x 10^3 cells, obtained from RTT-2 patient and an healthy control were grown on slide chambers in neuronal differentiation medium. After 5 days medium was discard and cells were fixed in 4% phormaldeyde for 10 min. Following three washes in PBS, cells were incubated with primary antibody anti-Neu N (mouse anti-NeuN 1:1000, Chemicon International, Temecula, CA) for 4 h. After three washes in PBS, cells were incubated with secondary antibody (donkey anti-mouse IgG, alexa fluor 568) for 1h at room temperature. The cells were counterstained with HOECHST 33342 and observed at fluorescent microscope (Leica Mycrosystems Italia, Italy).

The percentage of Neu-N positive cells was calculated by counting at least 500 cells in different microscope fields.

3.2.4 Cell cycle analysis

For each assay 3 x 10^5 cells were collected and resuspended in 500 ml of a hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate and 50 mg/ml propidium iodide, RNAse A). Cells were incubated in the dark for 30 min and then analyzed. Samples were acquired on a FACSCalibur flow cytometer using

the Cell Quest software (Becton Dickinson, NJ) and analyzed with standard procedure using the Cell Quest software.

3.2.5 BrdU proliferation assay

- BrdU administration

7 x 10^3 cells, obtained from RTT-2 patient and an healthy control were grown on slide chambers in proliferating medium. After 24h 10 μM bromodeoxyuridine (BrdU) (Sigma –Aldrich, Italy) was added to the culture medium over nigth.

- BrdU incorporation detection

Cells on slide were fix in absolute methanol for 10 minutes at 4 °C and then rehydrated in PBS for 3 minutes.

The slides were incubated in 2N HCl for 1h at 37°C to denature DNA and then immersed in 0,1 M borate buffer (pH 8,5), changing the buffer twice over 10 minutes, to neutralize the acid treatment.

Then the slides were places in a humidified chamber and incubated with primary antibody anti-BrdU (Roche-Italy, monoclonal anti-mouse, 6 μg/ml) for 1 h at room temperature.

After three washes in PBS, slides were incubated with IgG-FITC conjugated secondary antibody (Jackson ImmunoResearch, anti-mouse, West Grove, PA, USA, dil. 1:100) for 1h in dark at room temperature. The cells were counterstained with HOECHST 33342 and observed at fluorescent microscope (Leica Mycrosystems Italia, Italy).

The percentage of BrdU positive cells was calculated by counting at least 500 cells in different microscope fields.

3.2.6 Apoptosis detection

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 mg/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 microscope (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.

3.2.7 Evaluation of caspase 9 activity

I evaluated caspase 9 activity following manufacturer's instructions (B-Bridge international, CA, USA). Briefly, after 1 hour of incubation of $2x10^5$ cells with the caspase 9 substrate FAM-LEHD-FMK and two washes in PBS, all samples were analyzed with FACS using a FACScalibur (Becton Dickinson, NJ, USA) and the Cell Quest Technology (Becton Dickinson, NJ, USA).

3.2.8 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 (68). 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 K4Fe(CN)6, 5 mM

K3 Fe(CN)6, 150 mM NaCl, 2 mM MgCl2, 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.

3.2.9 TRAP (Telomeric Repeat Amplification Protocol) assay

TRAP assay was carried out according Kim et al. (1994) (58) and Kim and Wu (1997) (126). Briefly, 5 x 10^5 cells were lysed in 10 mM Tris–HCl (pH 7.5), 2.5mM MgCl2, 1mM EGTA, 0,5% CHAPS, 10% glycerol, 5mM b-mercaptoethanol, 1mM AEBSF [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride] for 30 min at 4 °C. The lysates were then centrifuged for 10 min at 10,000g at 4°C. After centrifugation, protein concentration was determined by Bradford Assay (Biorad). Reaction mixture for TRAP assay was done as it follows: 1X Taq DNA polymerase buffer (Promega, Italy), 1.5mM MgCl2, 50 mM dNTPs, primer M2(TS) (5'-AATCCGTCGAGCAGAGTT-3') TRAP internal control (5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3').
This mixture was incubated for 30 min at 25°C, then I added primer ACX (5'-GCGCGG[CTTACC]$_3$CTAACC-3'), primer NT (5'-ATCGCTTCTCGGCCT TTT-3'), 2.5 U Taq DNA polymerase (Promega, Italy). 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).

3.2.10 Micrococcal nuclease assay

Cells were permeabilized with 0.01% L-alysophosphatidylcholine (Sigma-Aldrich, Italy), in 150mM sucrose, 80mM KCl, 35mM 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 20mM sucrose, 50mM Tris–HCl pH7.5, 50mM NaCl and 2 mM $CaCl_2$ at room temperature for various times. Digested DNA was resolved on 1% agarose gel electrophoresis.

3.2.11 8-oxo-dG detection

8-oxo-dG (7,8-dihydro-8-oxo-2'-deoxyguanosine) within DNA was detected by immunocytochemistry with the anti-8oxodG (clone 2E2) primary antibody (Trevigen, MD, USA) according the manufacturer's protocol. A Hoechst 33342 staining was performed and then cells were observed at a fluorescence microscope (Leica Mycrosystems Italia, Italy). The percentage of 8-oxo-dG positive cells was calculated by counting at least 500 cells in different microscope fields.

3.2.12 Detection of Reactive Oxygen Species (ROS)

The generation of ROS in MSC was monitored by conversion of hydroethidine (HEt) to ethidium (Et) by anion superoxyde as described previously (129). In brief, 24 and 48 hours after adenosiRNA transductions, MSC cultures were incubated for 1 hour at 37°C with 1 μg/ml HEt. Cells were then trypsinized and resuspended in PBS. Flow cytometry analysis was performed using a FACScan flow cytometer (Becton Dickinson, NJ, USA) interfaced with a Hewlett Packard computer (mod.310) for data analysis.

3.2.13 Plasmid-based assay for in vitro DNA repair activity

The assay was carried out according Diggle et al. with modifications (130). In brief, the pcDNA3 plasmid (Promega Italia, Italy) was digested with ECoRI enzyme (Invitrogen, Italy), then purified with phenol and ethanol precipitated. MSCs transduced either with Ad-siMeCP2 or Ad-siCTRL were lysed in 50 mM Tris-HCl pH 7,5, 1M KCl, 2 mM EDTA, 1 mM DTT. 5 μg of protein lysates were added to 200 ng of digested and purified pcDNA3 in a buffer containing 200 mM HEPES pH 7.5, 800 mM KCl, 100 mM $MgCl_2$. This reaction allowed in vitro DNA end-joining of digested plasmid and was carried for 2 hours at 37 °C. End-joined plasmids were treated with Proteinase K and SDS. The reactions were phenol purified and then electrophoresed on 1% agarose gel.

3.2.14 RNA Extraction and RT-PCR and Real Time PCR

Total RNA was extracted from cell cultures using OMNIZOL (EuroClone, Italy) according to the manufacturer's protocol. The mRNA levels of the genes analyzed were measured by RT-PCR amplification, as previously reported (30). 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), (Tab. 1-4). Appropriate regions of HPRT (hypoxanthine-guanine phosphoribosyltransferase) and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) 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 (Applied Biosystems). Primer sequences were designed with Primer express software.

GENE	*POSITION*	*SEQUENCE*
RB	2443 2549	5'- TTGCAGTATGCTTCCACCAGG-3' 5'-ATGTTCCCTCCAGGAATCCGT-3'
RB2/p130	3958 4091	5' TGACAAAGGAGAGCACAGCCA-3' 5'-TGCACACCCGAACAATCAAG-3'
P107	2949 3073	5'-TGATGGATGCTCCACCACTCT-3' 5'-TGGTGTAAGGCCTGACCCATT-3'
P16	118 275	5'-TTCCTGGACAGCGTCGTGGT-3' 5'-TGGTTACTGCCTCTGGTCCC-3'
ARF	415 492	5'-GCCCTCGTGCTGATGCTAC-3' 5'-CCCATCATCATGACCTGGTCT-3'
MDM2	286 396	5'-GGAGCAGGCAAATGTGCAA-3' 5'-GCAATGGCTTTGGTCTAACCA-3'
P53	1892 1992	5'-TGGCCTTGAAACCACCTTTT-3' 5'-AACTACCAACCCACCAGCCAA-3'
P21	632 802	5'-CAAAGGCCCGCTCTACATCTT-3' 5'-AGGAACCTCTCATTCAACCGC-3'
P27	913 1019	5'-GGAATAAGGAAGGCACCTGCA-3' 5'-TCCACAGAACCGGCATTTG-3'
CycE	268 381	5'-AGGAAGAGGAAGGCAAACGTG-3' 5'-TGCATTATTGTCCCAAGGCTG-3'
MECP2	6308 6408	5'-TGAGATGCCTGGTGAGCATTACAG-3' 5'-TCCACCTTCCATACCACTCCCA-3'
MECP2 (II-iso)	51 376	5'-GAGGCGAGGAGGAGAGACT-3' 5'-GAGCCTGACCCTTCTGATGT-3'
HPRT	223 323	5'-TGAACGTCTTGCTCGAGATGTG-3' 5'-CCAGCAGGTCAGCAAAGAATTT-3'
GAPDH	121 281	5'-GGAGTCAACGGATTTGGTCGT-3' 5'-ACGGTGCCATGGAATTTGC-3'
TERT	1682 1813	5'-AAGTTCCTGCACTGGCTGATG-3' 5'-GCTTTGCAACTTGCTCCAGAC-3'
BAX	227 357	5'-ATGGTGCCACCAAGAAGCTGA-3' 5'-TGCCACTCGGAAAAAGACCTC-3'
BCL2	4414 4595	5'-GCTGCACAAATACTCCGCAAG-3' 5'-TGTGGAGAGAATGTTGGCGTC-3'
PPARγ	844 945	5'-TCAACCAGCTCAATCCAGAGT-3' 5'-TCGCCTTTGCTTTGGTCAG-3'
Osteopontin	463 606	5'-GGTCACTGATTTTCCCACGGA-3' 5'-TGGATGTCAGGTCTGCGAAAC-3'
Aggrecan	6427 6528	5'-TCCTCAAGCCTCCTGTACTCAG-3' 5'-TTCACGTTTCCATTCCGGA-3'

Table 1: Primer sequences of cell cycle, apoptosis and mesenchymal differentiation related genes.

GENE	*POSITION*	*SEQUENCE*
SOX2	1563 1701	5’-CCATCCACACTCACGCAAAA-3’ 5’-TATACAAGGTCCATTCCCCCG-3’
NANOG	1169 1310	5’-TGGACACTGGCTGAATCCTTC-3’ 5’-CGCTGATTAGGCTCCAACCAT-3’
OCT 3/4	1121 1223	5’-TCCCATGCATCCAAAGTCAGG-3’ 5’-CCAAAAACCCTGGCACAAACT-3’
KLF4	1508 1689	5'-CTGCGGCAAAACCTACACAA -3' 5'-GGTCGCATTTTTGGCACTG -3'
SOX15	315 441	5’-GAACAGGTTGGAAGCAAAGGC-3’ 5’-GGCTCGATCCTGAAAATGGA-3’
SALL4	2394 2508	5’-GCCCAGATATCCTGGAAACCA-3’ 5’-TTCTGCGAGCTCTCTGCTTTG-3’
BMI1	437 575	5'-AATGTCTTTTCCGCCCGCT -3' 5'-ACCCTCCACAAAGCACACACAT -3'
DPPA2	798 905	5'-AGCCATGTTGGCATCATGG -3' 5'-GAGGCTTGCAGCAAAAAGGC -3'
ERAS	969 1103	5'-AATGTAGACCTTTCCCCAGGC -3' 5'-AAAGCCCCTCACCAAGTGAA -3'
GDF3	778 887	5’-AAAAGGAGAGCAGCCATCCCT-3’ 5’-GCAATGATCCACTTGTGCCAA-3’
TCL1	667 793	5’-CTCGGCTTTTCTCAGCTGGAT-3’ 5’-GGTGAATGCGCGTGTTCTCA-3’
UTF1	876 992	5’-CGACATCGCGAACATCCTG-3’ 5’-AGAATAAGCCCACGGCCA-3’
ZFP42	953 1085	5’-ATGACATGCTGAGCGCAATCG-3’ 5’-AACGCTTTCCCACATTCCG-3’

Table 2: Primer sequences of “stemness” related genes.

GENE	*POSITION*	*SEQUENCE*
MAP2	565 675	5’TGCACACTCCACATCCACCTGA3’ 5’TCTCCAAAGGCACCCTCTTCA3’
ENO2	1633 1741	5’GCCCAGAACTTCCCTGATTGA3’ 5’AAGTGCGGAACCCCAATGA3’
CNPase	3151 3346	5’ATTCCACCATGGCTAAGGCAC3’ 5’TGTCCTGTCAAGCGTGGTGTT3’
TH	767 960	5’CTGATTGCTGAGATCGCCTTC3’ 5’ATATTGTCTTCCCGGTAGCCG3’
RET	1261 1371	5’TCTCAACCGGAACCTCTCCAT3’ 5’TTGAAGTGGAGCAAGAGGACG3’
GLUT	775 942	5’GGGTGGCCCGTTTCATCTTGC3’ 5’GTGCCGCTTGCTTAGTTTCTC3’
VIM	1164 1264	5’TGGAAGAGAACTTTGCCGTTG3’ 5’AAGGTGACGAGCCATTTCCTC3’

Table 3: Primer sequences of neuronal differentiation related genes.

DNA REPAIR PATHWAY	*GENE*	*POSITION*	*SEQUENCE*
DBS (double strand break)	**BRCA2**	3732 3884	5’-AATGCCCCATCGATTGGTC-3’ 5’-AAAGCCCCTAAACCCCACTTC-3’
	MRE11A	675 775	5’GGCAATCATGACGATCCCA-3’ 5’-TCCACAGACATTGAACGTCCA-3’
	XRCC4	781 881	5’-AAA ACG AGC ACC TGC AGA AGG-3’ 5’-GCC TCC AAG GCT TCT TTG TCA-3’
MMR (mismatch repair)	**MLH1**	1517 1634	5’-ATC AGT CCC CAG AAC GTG GAT-3’ 5’-GCC CAA TAG CTT GCT CTC GAT-3’
	TREX1	1195 1305	5’-ACACAATGGTGACCGCTACGA-3’ 5’-TTCAGCGCAGTGATGCTATCC-3’
	MSH2	2058 2160	5’-TTCCACATCATTACTGGCCCC-3’ 5’-CACATGGCACAAAACACCCA-3’
	MSH5	2353 2454	5’-GCCACCAACTTTCTGAGCCTT-3’ 5’-GACAAGATCGTTGCCATCCTC-3’
	PRKDC	3141 3242	5’-TTA CCA CAA CCC TGC TCG TCA-3’ 5’-ACC TGG ACG TCG CCA ATA TTG-3’
	POLD3	178 305	5’-ACC TCC TGA TCC TGT GCC AAA-3’ 5’-ACC ATG CAG CCT TCT TCA TCC-3’
BER (base excision repair)	**MUTYH**	1601 1701	5’-TTG GAC AGG AGA GCC TAT GCA-3’ 5’-TTG TAG CGT TGG CCA CTT CTG-3’
	MPG	341 479	5’-CAGCATCTATTTCTCAAGCCCA-3’ 5’-TCGGAGTTCTGTGCCATTAGG-3’
	NTHL1	331 443	5’-CAAAAAGGATGCACCTGTGGA-3’ 5’-TTTGGCTGGAGAGCATCAGTG-3’
	FEN1	464 590	5’-TGGCCATTGATGCCTCTATGA-3’ 5’-CGAATGGTGCGGTAGAACATG-3’
	OGG1	2074 2180	5’-CATATGTGGCACATTGCCCA-3’ 5’-CTCCGGAAAAAGTTTCCCAGTT-3’
	MBD4	384 487	5'-GCTTCTGCTCAGTTTGGTGCT-3’ 5’-CCTGCTGTCTTCCCAAATAACC-3’
NER (nucleotide excision repair)	**XPA**	738 873	5’-GAATTGCGGCGAGCAGTAA-3’ 5’-ATGTCAGTTCATGGCCACACA-3’
	RAD23A	146 255	5’-CAGACCTTCAAGATCCGCATG-3’ 5’-ATGAGTTTCTGTCCAGCCACG-3’
	ERCC3	1222 1324	5’-CCCAGTTCAAGATGTGGTCCA-3’ 5’-TAGGTGCTAATGGCAACGGAG-3’
	SLK	2607 2713	5’-AAATTTTCCGGCGCTTTGAG-3’ 5’-TTCCAGGCGTTCGATAGTCTG-3’

Table 4: Primer sequences of “DNA repair” related genes.

3.2.15 Western blotting

Cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.1% Triton X-100, a protease inhibitor cocktail (Roche, Italy) for 30 min at 4°C. The lysates were then centrifuged for 10 minutes at 10,000 g at 4°C. Protein concentration in the supernatants was assessed using the Bradford assay (Biorad).

10-40 μg of each sample were loaded, electrophoresed in polyacrylamide gel and electroblotted onto a nitrocellulose membrane. All the primary antibodies were used according to the manufacturers' instructions.

Immunoreactive signals were detected with a horseradish peroxidase–conjugated secondary antibody (Santa Cruz, CA, USA) and reacted with ECL plus reagent (GE Healthcare, Italy).

3.2.16 DNA Extraction

Total DNA was extracted by MSC cultures according to classical phenol DNA extraction protocol (127).

3.2.17 Cytosine extension assay to detect sequence specific changes in DNA Methylation

2 μg of genomic DNA was digested with 10-fold excess of HpaII, MspI, BsHII and NotI endonucleases according to manufacturer's protocol (New England Biololabs, MA, USA). The single nucleotide extension reaction was performed in a 25 μl reaction containing 0.25 μg of DNA, 1X PCR Buffer, 1 mM $MgCl_2$, 0,25 Units Taq Polymerase (Promega Italia, Italy, USA) [^{32}P]dCTP (10 mCi/ml) (Perkin Elmer Italia, Italy) and incubated at 56°C for 1 hour. To separate labeled DNA from unincorporated dCTP, duplicate 10 μl aliquots from each reaction were purified on Nucleospin extract 2 kit (Machery-Nagel, Germany) and then processed for scintillation counting.

3.2.18 HS-PCR

I examined the DNA methylation status of several gene promoters by HpaII restriction sensitive PCR (HS-PCR). Sequences of promoter regions, obtained from the Cold Spring Harbor promoter database (Cold Spring Harbor, NY, USA), were used to design primer pairs for HS-PCR reactions (Primer Express, Applied Biosystems, CA, USA).

I used Methprimer software to classify promoters, in CpG poor promoters, having a CpG frequency that is the same of genome average, and in CpG islands-promoters with high frequency of CpG dinucleotides.

DNA for HS-PCR was extracted from MSCs treated either with Ad-siRNA-MECP2 or Ad-siRNA-CTRL. In brief, cells were lysed in 0.5% SDS, 0,1M EDTA, 10mM Tris-HCl pH 8.0, 10 μg/ml RNAse and incubated at 37°C for 1 hour. Lysates were then treated with 100 ng/ml proteinase K at 55°C for 3 hours. For HS-PCR 2 μg of genomic DNA was digested with MspI or HpaII restriction enzymes (New England Biolabs, MA, USA) for 18 hours at 37°C, then phenol/chloroform purified. 100 ng of digested DNA was PCR amplified.

3.3 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.

4.Results

4. Results

I report the experimental results of my study in three sections.

The first part is focused on the study of MSCs biology from a RTT patient compared to an healthy control; in particular I analyzed the main cellular processes (e.g. cell proliferation, apoptosis, senescence, cell commitment and self-renewal properties) to understand more in depth the effects of MeCP2 inactivation on the physiology of RTT patient MSCs.

Because RTT is a disorder impairing the physiological neuronal development, in the second part of my study I set up a neuronal differentiation protocol to analyze the differentiative ability of RTT patient MSCs.

In the last part, I silenced MECP2 gene expression in human cultures of healthy donor MSCs by siRNA technology to have a general picture on the role of MECP2 in stem cell biology independent of the result of a specific patient MECP2 mutation.

4.1 Study of RTT-1 patient MSC biology

MSCs were obtained from RTT-1 patient (6 years old) and from an healthy control of the same age.

The enrolled RTT patient had the clinical manifestations of "forme fruste" of Rett Syndrome. She carried a *de novo* mutation (c.1164-1207del) in the MECP2 gene.

RTT-1 patient MSCs and control one were isolated from whole bone marrow aspirate, expanded *in vitro* and utilized to analyze in depth cell proliferation, apoptosis, senescence, cell commitment and self-renewal properties.

4.1.1 MSCs from RTT-1 patient show a lower degree of apoptosis

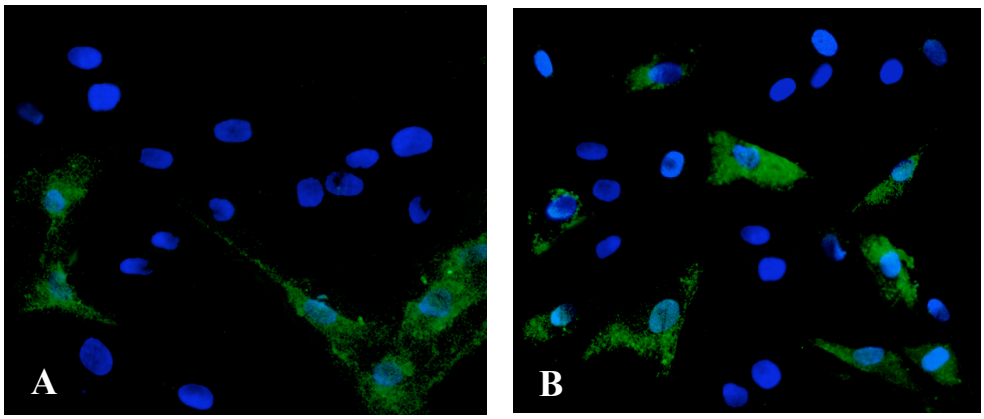
MSCs from RTT-1 patient and control were analyzed by FACS analysis. In several experiments, I observed a significant reduction of S-phase cells, along with an increase of G2/M cells in RTT-1 samples compared with control. Table 5 shows a representative experiment.

	G_1-Phase (%)	S-Phase (%)	G_2/M-Phase (%)
RTT-1 Patient	49.80	8.98	41.14
Control	44.34	33.64	21.70

Table 5 : A representative FACS analysis of MSCs from RTT-1 patient and control.

FACS analysis allowed me to also detect apoptotic cells having a subdiploid DNA content (pre-G1 phase). In RTT-1 cells, I observed a decrease of apoptotic cells compared with the control (- 48% ± 2.1%).

These data were confirmed by annexin assay that evidenced a reduced percentage of apoptotic cells in cultures from RTT-1 samples compared with controls (Fig. 9).



	Apoptic cells (%)
RTT-1 Patient	3.10±0.45
Control	14.7±2.02

Fig. 9: Detection of apoptotic cells by annexin assay. Fluorescence photomicrographs 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).

A: RTT-1 patient cells; **B:** control cells.

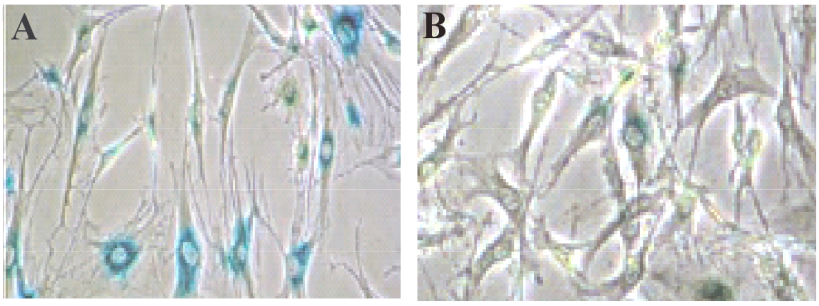
The table shows the percentage of apoptotic cells in cultures (±SD; n=3).

4.1.2 MSCs from RTT-1 patient are prone to senescence

Senescence-associated acid beta-galactosidase is widely used as a biomarker of replicative senescence (68) (51).

I observed signs of senescence in cells from RTT patient, as detected by in situ acid beta-galactosidase compared with cells from healthy donor (Fig. 10). To extend this finding, I measured telomerase activity by a primer extension assay in which telomerase reverse transcriptase (TERT) synthesizes telomeric repeats onto oligonucleotide primers (TRAP assay) (58) (126). Telomerase-positive extracts were obtained from MSCs of healthy child, as expected for stem cell samples. However, the telomerase activity was reduced in RTT-1 patient (Fig. 11).

It has been demonstrated that distinct heterochromatin structures accumulate during senescence and could represent a hallmark of this process. In particular, Narita et al.in 2003 (82) 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-1 patient showed an increased resistance to nuclease digestion compared with cells from controls (Fig. 12).



	Senescent cells (%)
RTT-1 Patient	***18.1 ± 2.15***
Control	***10.7 ± 1.08***

Fig. 10: Senescence-associated β-galactosidase assay performed on MSCs obtained from RTT- patient (A) and control (B).
The table shows the percentage of senescent cells in cultures (±SD; n=3).

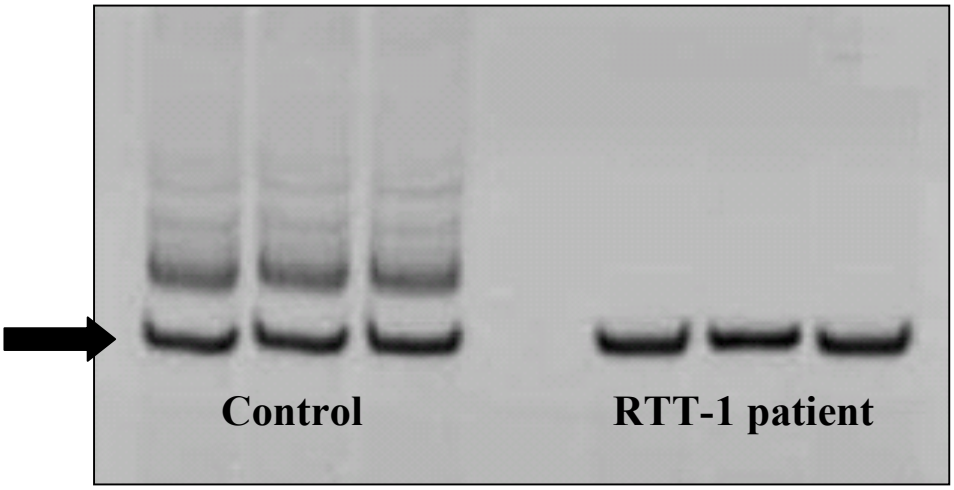


Fig. 11. Polyacrylamide gel electrophoresis of TRAP assay products obtained from RTT-1 and control cells. The picture shows the PCR amplified products of telomerase activity. Three different reactions for RTT patient and control were shown. Black arrow: TRAP internal control. The assay measures enzymatic 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.

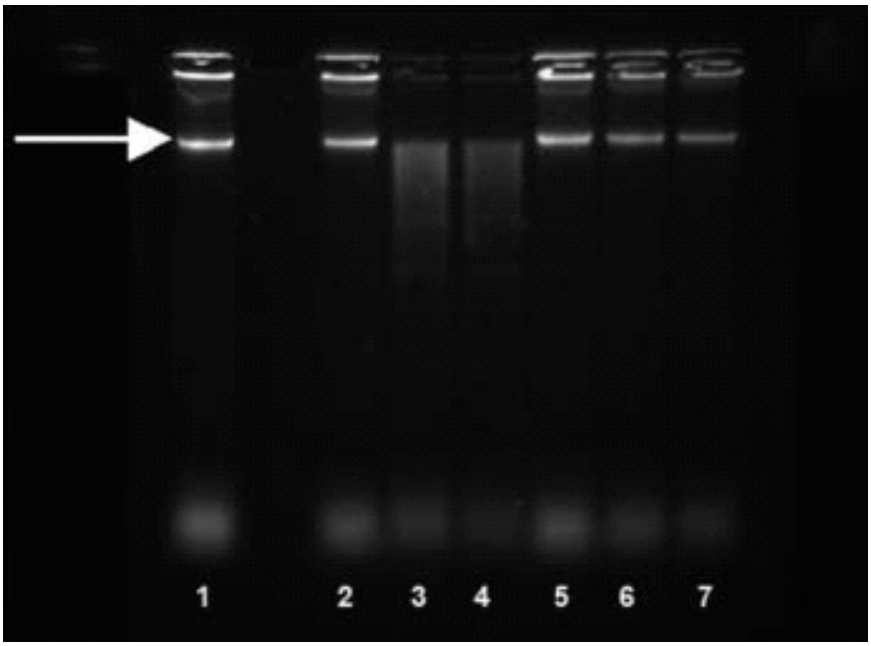


Fig. 12: Micrococcal nuclease assay. Agarose gel electrophoresis of genomic DNA from RTT and control sample. DNA was digested with micrococcal nuclease for various times, as indicated below. It is evident in the picture that a high molecular weight DNA band is more resistant to nuclease digestion in MSCs from RTT-1 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-1 sample that was digested for 1', 3', and 6', respectively. White arrow: high molecular weight DNA.

4.1.3 Molecular pathways involved in cell cycle regulation, apoptosis, and senescence

I analyzed the expression of key genes involved in cell cycle regulation, apoptosis, and senescence. In MSCs from RTT-1 patient, the reduction of cells in S-phase is in agreement with the decrease of cyclin E mRNA (-74% ± 9%) (Fig. 13A). Expression of RB (- 47% ±7%), RB2/P130 (- 43% ± 6%), P107 (- 43% ± 8%), $P27^{KIP1}$ (- 54% ± 8%), and P53 (- 55% ± 8%) mRNAs were downregulated in RTT-1 patient compared to control (Fig. 13A-13B). This is an unexpected result since these genes are related to cell cycle arrest and senescence. It is possible that other genes could promote these processes in RTT-1 cells. On the other hand, I detected an upregulation of the cyclin kinase inhibitors $P21^{CIP1}$ (+ 78% ± 6%) and $P16^{INK4A}$ (+230% ± 20%) (Fig.13B). This last gene plays a key role in blocking cell cycle progression and promoting senescence phenomena. Interestingly, I did not observe TERT expression in RTT-1 cells while its expression was easily detected in control MSCs. TERT gene encodes for the proteic component with reverse transcriptase activity of telomerase enzyme.

Absence of TERT expression further confirms that RTT-1 cells were prone to senescence. In MSCs from RTT-1 patient, I detected a significant decrease of Bax/Bcl-2 ratio (- 61% ± 8%) compared to control (Fig. 14). This is in agreement with the reduction of apoptotic cells as detected with annexin assay (Fig. 9).

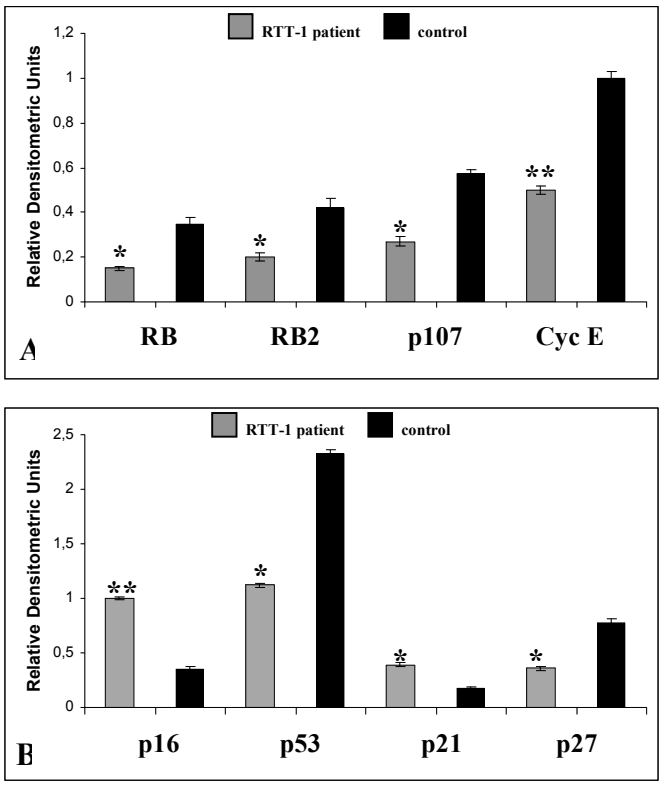


Fig 13: A-B: Semiquantitative RT-PCR analysis of mRNA expression of cell cycle and senescence related genes from RTT-1 patient and control. mRNA levels were normalized with respect to HPRT chosen as an internal control (±SD; n=3) (*p<0.05; **p<0.01).

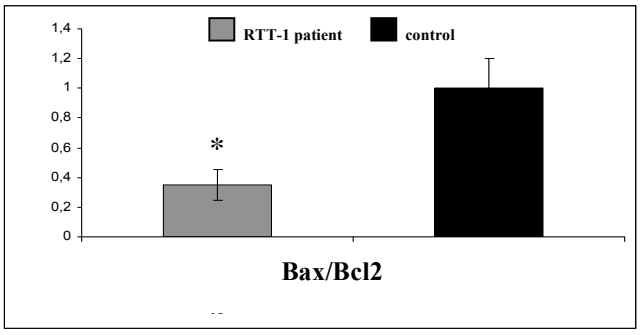


Fig 14:: Bax and Bcl2 ratio calculate by semiquantitative RT-PCR analysis of mRNA expression of Bax and Bcl2 genes from RTT-1 patient and control. mRNA levels were normalized with respect to HPRT chosen as an internal control (±SD; n=3) (*p<0.05).

4.1.4 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 (13) (76) (105). In adult stem cells, some “stemness genes” are not expressed. I analyzed a panel of embryonic stemness genes to evaluate which of them were active in MSCs and the effects of MECP2 mutation on their expression.

I compared the expression of some ‘‘stemness’’ genes in MSCs from RTT-1 patient and healthy individual. In control and RTT-1 samples, I did not detect the expression of SOX2, GDF3, UTF1, TCL1, ZFP42 and ERAS genes. In MSCs from RTT-1 patient, I observed a significant reduction of OCT 3/4 (-30% ±5%), SOX 15 (-37% ±7%), SALL4 (-89% ±8%), and NANOG (-50% ±6%) (Fig. 15). Moreover I did not detected any significative changes in the expression levels of DPPA2, KLF4 and BMI 1.

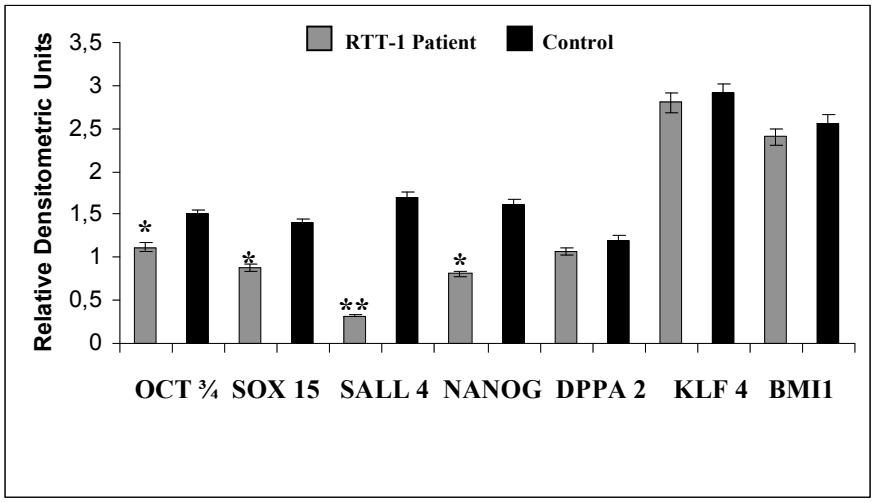


Fig. 15: Semiquantitative RT-PCR analysis of mRNA expression “stemness” related genes from RTT-1 patient and control. mRNA levels were normalized with respect to HPRT chosen as an internal control (±SD; n=3) (*$p<0.05$; **$p<0.01$).

4.1.5 Expression of lineage specific genes

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 MSCs differentiation is biased to those lineages whose key genes are already expressed in uncommitted cells.

I compared classical mesodermal lineage specific markers in MSCs from RTT-1 patient and in those from healthy donor. In MSCs from the RTT-1 child, I detected an increase of osteopontin (+76 ± 8%) that is an osteogenic marker, while I did not detect modification of PPAR-γ and Aggrecan, an adipocyte and a condrocyte differentiation marker, respectively (Fig. 16)

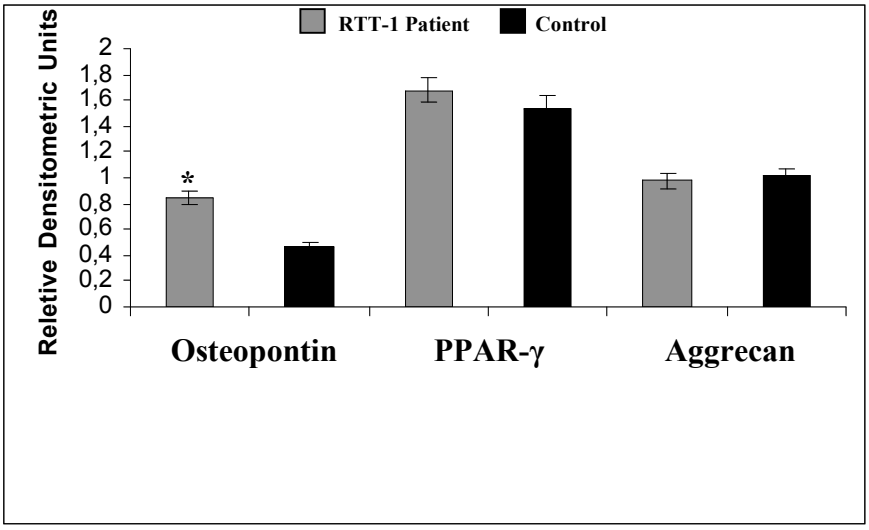


Fig. 16: Semiquantitative RT-PCR analysis of mRNA expression mesenchymal lineage specific related genes from RTT-1 patient and control. mRNA levels were normalized with respect to HPRT chosen as an internal control. (±SD; n=3) (*$p<0.05$).

4.2 Study of RTT-2 patient MSC biology and neuronal differentiation

MSCs were obtained from RTT-2 patient (8 years old) and from an healthy control of the same age.

RTT-2 patient carried a *de novo* mutation (R270X) in the MeCP2 gene.

RTT-2 patient MSCs and control one were isolated from whole bone marrow aspirate and expanded *in vitro.*

Because the amount of MSCs obtained from a single bone marrow aspirate is too exiguous to perform experiments both for neuronal differentiation and MSCs biology study, I decided to use MSCs obtained from RTT-2 patients to evaluate MECP2 gene effects on neuronal differentiation, since it represents an important target of my research.

The analysis of RTT-2 patient MSCs biology was evaluated by appropriate assays in order to confirm previous data obtained from RTT-1 patient.

4.2.1 MSCs from RTT-2 patient are prone to senescence and show a lower degree of apoptosis

MSCs from RTT-2 patient and control were analyzed by FACS analysis. In several experiments, we observed a significant reduction of S-phase cells, along with an increase of G2/M cells in RTT-2 samples compared with control. Table 6 shows a representative experiment. In order to confirm these data I performed BrdU proliferation assay.

BrdU is commonly used in the detection of proliferating cells; it can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication.

MSCs from RTT-2 patient show a lower percentage of BrdU positive cells respect to control (fig 17).

Moreover, FACS analyses allowed me to also detect apoptotic cells having a subdiploid DNA content (pre-G1 phase).

In RTT-2 cells, I observed a decrease of apoptotic cells compared with the control (- 45% ± 1.9%).

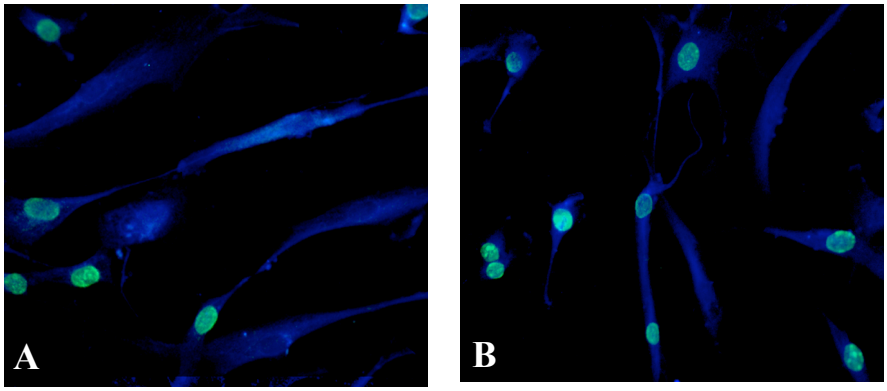
These data were confirmed by annexin assay that evidenced a reduced percentage of apoptotic cells in cultures from RTT-2 samples compared with controls (Fig. 18).

Finally I evaluated the percentage of senescent cells by acid β-galactosidase assay. Table 7 shows an higher number of β-galactosidase positive cells in MSCs RTT-2 samples then control.

These results are in agreement with the previous ones obtained from RTT-1 patient.

	G_1Phase (%)	*S Phase(%)*	*G_2/M Phase(%)*
RTT-2 Patient	*44,02*	*35,34*	*23,05*
Control	*27,75*	*55,16*	*16,01*

Table 6 : A representative FACS analysis of MSCs from RTT-2 patient and control.

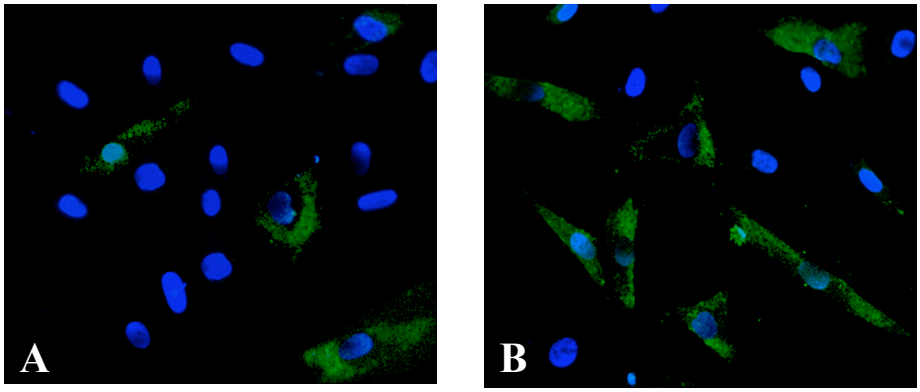


	BrdU positive cells (%)
RTT-2 Patient	*48,20±3,70*
Control	*73,90±5,80*

Fig. 17: Detection of proliferating cells by BrdU incorporation assay. Fluorescence photomicrographs represents BrdU positive cells (green); BrdU can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle). Nuclei were counterstained with Hoechst 33342 (blue).

A: RTT-2 patient cells; **B:** control cells.

The table shows the percentage of BrdU positive cells in cultures (±SD; n=3).



	Annexine positive cells (%)
RTT-2 Patient	*2,70±0,73*
Control	*10,70±1,10*

Fig. 18: Detection of apoptotic cells by annexin assay. Fluorescence photomicrographs 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).

A: RTT-2 patient cells; **B:** control cells. The table shows, the percentage of apoptotic cells in cultures (±SD; n=3).

	Senescent cells (%)
RTT-2 Patient	*19,8±2,3*
Control	*11,1±1,2*

Table 7: Senescence-associated β-galactosidase assay results performed on MSCs obtained from RTT- patient and control. The table shows the percentage of senescent cells in cultures (±SD; n=3).

4.2.2 RTT-2 MSCs neuronal differentiation

MSCs can differentiate into mesenchymal lineage such as osteocytes, chondrocytes, adipocytes; moreover is been demostred that these stem cells can also differentiate into non-mesenchymal lineage such as neurons, astrocytes and oligodendrocytes (65) both *in vitro,* by appropriate differentiative protocols (106) (85), and after transplantation (55) (25).

To induce neuronal differentiation in RTT-2 patient and control MSCs, I followed Hyclone (Thermo Scientific) manufacturer's protocol.

Following differentiation induction, a block in cell cycle progression is usually observed. FACS analysis led on MSCs from RTT-2 patient and control, 5 days after neuronal induction, revealed an increase of cells of both samples in G_0 phase followed by a concomitant reduction in S phase (Tab. 8).

These findings were extended by immunocytochemistry analysis of Neu-N protein (Neuronal Nuclei), 5 days after induction.

Neu-N is a neuron-specific nuclear protein; its expression is observed in most neuronal cell types (80) and it is commonly used as neuronal specific marker.

Table 9 shows percentage of Neu-N positive cells both for RTT-2 patients and control.

The neuronal induction protocol allows MSCs to acquire a neuronal-like phenotype 4-5 days following induction, as shown by cellular body thinning and production of branch extensions.

RTT-2 patient MSCs exhibit a limited production of branch extensions compared to control ones. (Fig 19).

Observations at light microscope revealed the death of most neuronal-like phenotype cells in both RTT-2 and control, 8 days after the induction. This data was confirmed by Annexin V assay (Tab. 10) .

	G_1Phase (%)	S Phase(%)	G_2/M Phase(%)
RTT-2 Patient neur.	93,07	1,37	4,66
Control neur.	93,77	1,91	2,83

TABLE 8 : A representative FACS analysis of MSCs from RTT-2 patient and control.

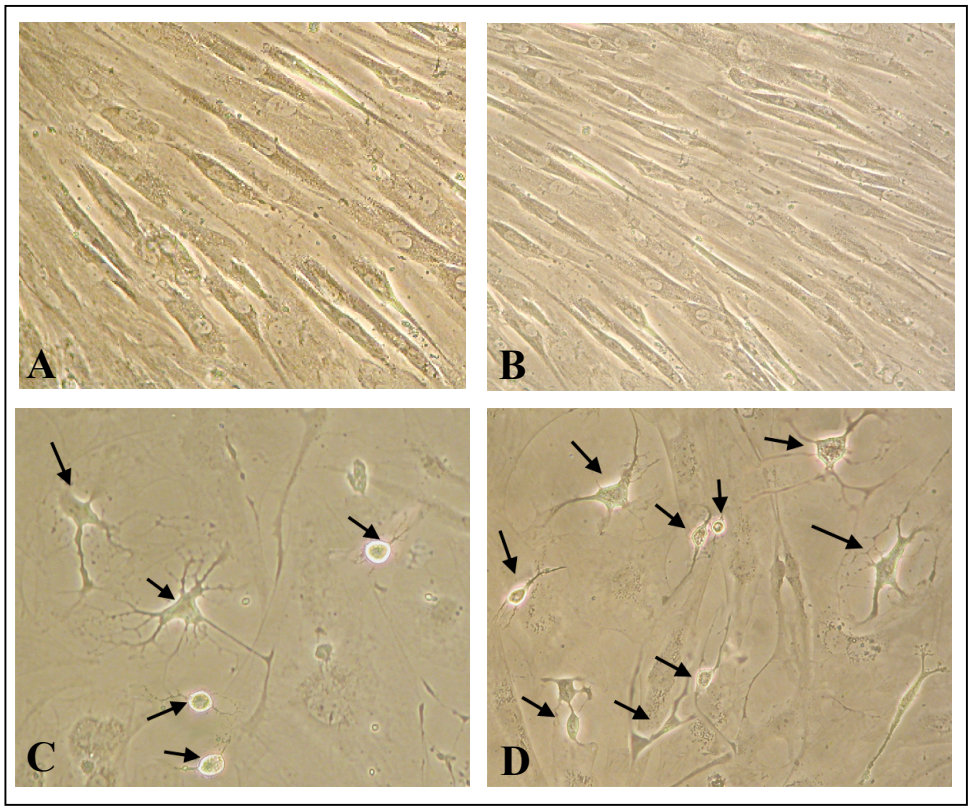


Fig. 19: Light microscope pictures of: undifferentiated MSCs from RTT-2 patient (A) and control (B); MSCs 5 days after neuronal differentiation from RTT-2 patient (C) and control (D), black arrows indicate neuronal-like cells.

	Neu-N positive cells(%)
RTT-2 patient neur.	*37±1,9*
Control neur.	*41±2,7*

Table 9: The table shows, the percentage of Neu-N positive cells in cultures of RTT-2 patient (top lane) and control (bottom lane) following neuronal differentiation.

	Annexine positive cells (%)
RTT-2 Patient neur.	*5,1±0,43*
Control neur.	*7,9±0,70*

Table 10: The table shows, the percentage of apoptotic cells in cultures of RTT-2 patient (top lane) and control (bottom lane) following neuronal differentiation.

4.2.3 Study of genes involved in neuronal differentiation

To confirm the morphological differences observed in RTT-2 patient and control I did molecular analysis of genes involved in neuronal differentiation.

These experiment were performed by semiquantitative RT-PCR on RNA from RTT-2 and control MSCs 7 days following neuronal differentiation (RTT-2 neur. and CTRL neur., respectively) and from undifferentiated controls (RTT-2 and CTRL).

I analyzed the expression of several neuronal differentiation markers: Microtubule-associated protein 2 (MAP2); Enolase 2 (ENO2); 2',3'-Cyclic nucleotide 3' phosphodiesterase (CNPase); Thyrosine hydroxylase (TH); ret proto-oncogene (RET) (Tab. 11). I did not detected the expression of TH and RET both in differentiated (RTT-2 neur. and CTRL neur.) and undifferentiated (RTT-2 and CTRL) MSCs.

For the other markers I detected an increase of mRNA expression levels in MSCs induced to neuronal differentiaton respect to undifferentiated controls (Tab. 11).

On the other hand I observed a decrease of MAP2 (-63% ± 2%), ENO2 (-95%± 5,7%) and CNPase (-52% ± 3%) mRNA expression levels in RTT-2 neur. respect to CTRL neur.

Moreover the analysis of glial specific marker genes: Glutamine synthetase (GLUT) and Vimentin (VIM), did not revealed statistically significative changes in their expression levels in both differentiated and undifferentiated samples (Tab. 11).

GENE	RTT-2	CTRL	RTT-2 neur.	CTRL neur.	FUNCTION
MAP2	0,22±0,04	0,37±0,06	0,85±0,05(*)	1,39±0,07	Microtubule assembly in neurogenesis
ENO2	0,40±0,04	0,53±0,03	0,98±0,04(**)	1,92±0,08	Neural development
CNPase	1,10±0,09	1,28±0,08	1,95±0,07(*)	2,97±0,1	Microtubule assembly
TH	nd	nd	nd	nd	Dopamine metabolism
RET	nd	nd	nd	nd	Neural crest development
GLUT	0,69±0,06	0,81±0,08	0,71±0,08	0,79±0,05	Glial glutamate metabolism
VIM	1,30±0,08	1,26±0,05	1,21±0,07	1,25±0,09	Glial cytoskeleton stabilization

Table 11: Semiquantitative RT-PCR analysis of mRNA expression of neuronal differentiation related genes from undifferentiated samples (RTT-2 and CTRL) and neuronal differentiated samples (RTT-2 neur. and CTRL neur.). mRNA levels were normalized with respect to HPRT chosen as an internal controls. (±SD; n=3) (*$p<0,05$) (**$p<0,01$).

4.3 Silencing of MECP2 gene in hMSCs

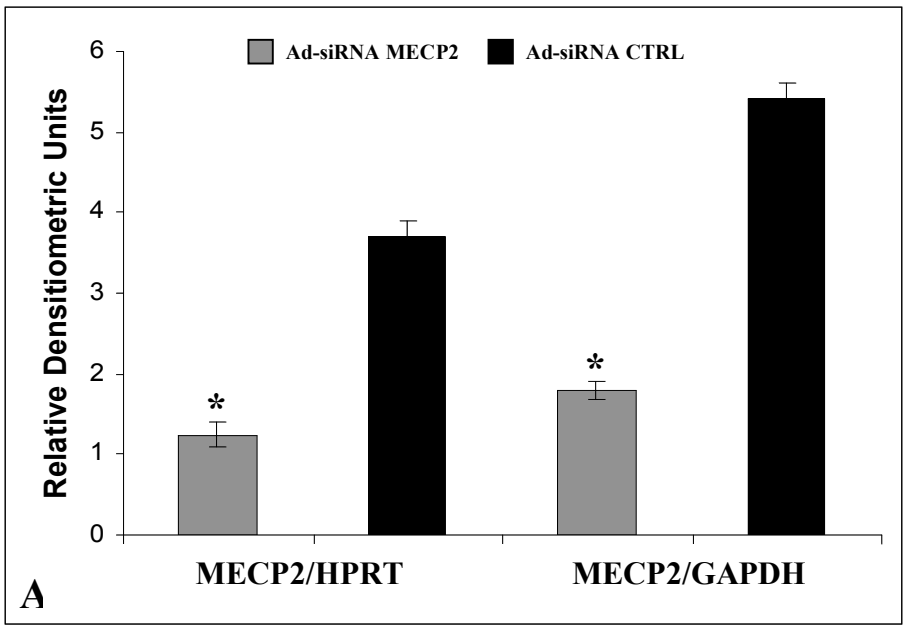
MECP2 gene is composed of four exons, and two alternative spliced transcripts have been characterized: MECP2A (also called MECP2_E2) and MECP2B (or MECP2_E1). The E1 isoform is composed by exons 1, 3 and 4, whereas the E2 isoform by exons 1, 2, 3 and 4.

In many studies only global expression of MECP2 proteins has been described, since western blot analysis do not allow a easy resolution of the two MECP2 protein isoforms; in fact, the E1 isoform is 498 aminoacids in length, whereas the E2 isoform is 486 aminoacids long. Moreover, it is not known, if protein isoforms are truly redundant or whether each of them may have some unique function. For this reason, I analyzed overall MECP2 protein expression.

I obtained a transduction rate of 80-90% with 50-100 MOIs of Ad-siRNAs. MSCs were tested for MECP2 knockdown 48 hours after Ad-siRNA transductions. Among the different Ad-siRNAs I analyzed that I labeled as Ad-siRNA-MECP2 was effective in silencing.

I obtained a maximal 70% decrease of MECP2 mRNA detected by RT-PCR using the primer pair to detect both isoforms (fig. 20A). The normalization of MECP2 mRNA levels with respect to GAPDH or HPRT resulted in similar values. Silencing experiment induced downregulation of both E1 and E2 isoforms.

The effect of silencing was further verified also by analyzing the protein levels of MECP2 through Western Blotting. I obtained a 50% decrease of MECP2 protein in MSCs transduced with Ad-siRNA-MECP2 in comparison controls (Fig. 20B). This partial silencing induced striking biological effects suggesting that maintaining MECP2 levels in a narrow range is essential for normal functions of cells.



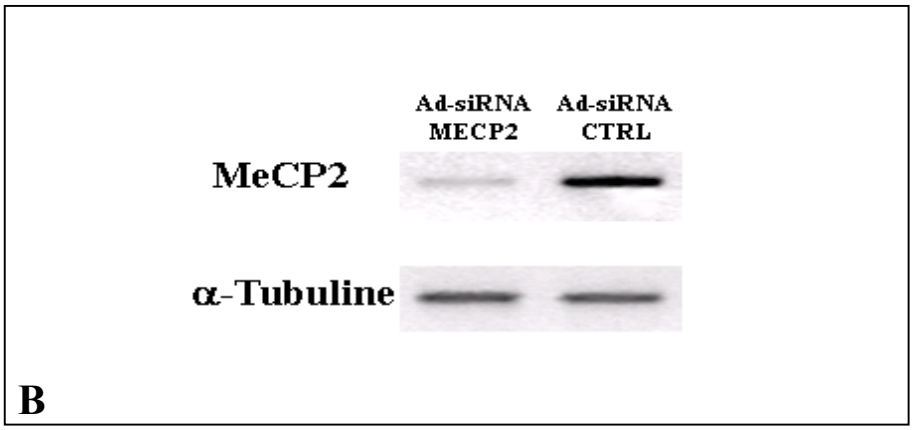


Fig. 20. **A**: Semiquantitative RT-PCR analysis of mRNA expression of MECP2 gene from hMSC Ad-siRNA MECP2 and Ad-siRNA CTRL mRNA levels were normalized with respect to HPRT (grey histogram), and to GAPDH (black histogram) chosen as internal controls (±SD; n=3) (*p<0,05).

B: Western blot analysis of MeCP2 protein expression from hMSC Ad-siRNA MECP2 and Ad-siRNA CTRL (top lane). Protein levels were normalized with respect to α–Tubuline (bottom lane) chosen as an internal control.

4.3.1 Downregulation of MECP2 induced a decrease of cell proliferation and apoptosis

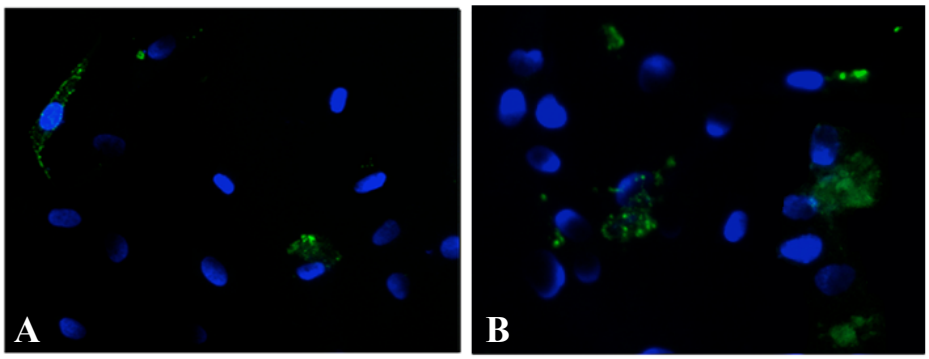
MSCs with silenced MECP2 gene and controls were analyzed by flow cytometry. In several experiments, I observed a significant reduction of S-phase cells, along with an increase of G_1 cells in samples with silenced MECP2 compared with controls. Table 12 shows a representative experiment.

Annexin assay evidenced a reduced percentage of apoptotic cells in cultures from MECP2 silenced samples compared with controls (Fig. 21). This is in line with a reduction of caspase 9 activity in cells treated with Ad-siRNA-MECP2 compared with controls (Tab. 13).

Results are in agreement with expression analysis of apoptosis-related genes. In MSCs with reduced level of MECP2, I detected a decrease of Bax/Bcl-2 ratio compared to controls. Overall, these data are consistent with my studies on MSCs from RTT-1 and RTT-2 patients.

	G_1 Phase (%)	**S Phase (%)**	**G_2/M Phase (%)**
Ad-siRNA-MECP2	***70,3***	***29,2***	***0,5***
Ad-siRNA-CTRL	***51,6***	***48,2***	***0,2***

Table 12: A representative FACS analysis of MSCs from Ad-siRNA MECP2 and Ad-siRNA CTRL.



	Annexin V positive cell (%)
Ad-siRNA-MECP2	***13,0 ± 0,9***
Ad-siRNA-CTRL	***19,5 ± 1,5***

Fig. 21: Detection of apoptic cells by annexin assay. Fluorescence photomicrographs represents cells stained with annexin V (green), which binds to phosphatidylserine residues exposed on the out erlayer of the cell membrane during the early stages of apoptosis. Nuclei were counterstained with Hoechst 33342 (blue).

A: hMSC Ad-siRNA MECP2; **B:** hMSC Ad-siRNA CTRL.

The table shows the percentage of apoptotic cells in cultures (±SD; n=3).

	Caspase 9 activity (%)
Ad-siRNA-MECP2	***4,86 ± 0,7***
Ad-siRNA-CTRL	***12,63 ± 1,2***

Table 13*:* The table shows, the percentage of caspase 9 activity in Ad-siRNA MECP2 and Ad-siRNA CTRL cells.

4.3.2 MSCs with reduced expression of MECP2 are prone to senescence

I observed signs of senescence in cells treated with Ad-siRNA-MECP2, as detected by *in situ* acid-beta-galactosidase compared with cells transduced with Ad-siRNA-CTRL (Tab. 14). To extend this finding, I measured telomerase activity by a primer extension assay in which telomerase reverse transcriptase (TERT) synthesizes telomeric repeats onto oligonucleotide primers (TRAP assay).

Extracts obtained from MSCs transduced with Ad-siRNA-CTRL were telomerase-positive, as expected in stem cell samples. However, the telomerase activity was strongly reduced in MSC extracts from cultures treated with Ad-siRNA-MECP2 (Fig. 22). These data were in agreement with the level of TERT expression. In fact, in cells with silenced *MECP2*, the TERT mRNA level was decreased compared with controls (-80% ± 15%) (Fig. 23).

I analyzed a panel of embryonic stemness genes to evaluate which of them were active in MSCs and the effects of *MECP2* silencing on their expression.

I evaluated changes in the expression of these genes, (Tab. 15) during MSC *in vitro* cultivation. I did not detect any expression of *SOX2, GDF3* and *ZFP42* in MSCs under the different experimental conditions I used. All the other genes but *BMI1* showed a decline of expression in MSCs treated with Ad-siRNA-MECP2 (Fig. 24). These data strongly suggest that MSCs lose their “stemness” while becoming senescent.

	Senescent cells (%)
Ad-siRNA-MECP2	***76 ± 3,15***
Ad-siRNA-CTRL	***35 ± 2,90***

Table 14: Senescence-associated b-galactosidase assay performed on hMSC Ad-siRNA MECP2 (A) and Ad-siRNA CTRL (B). The table shows, the percentage of senescent cells in cultures

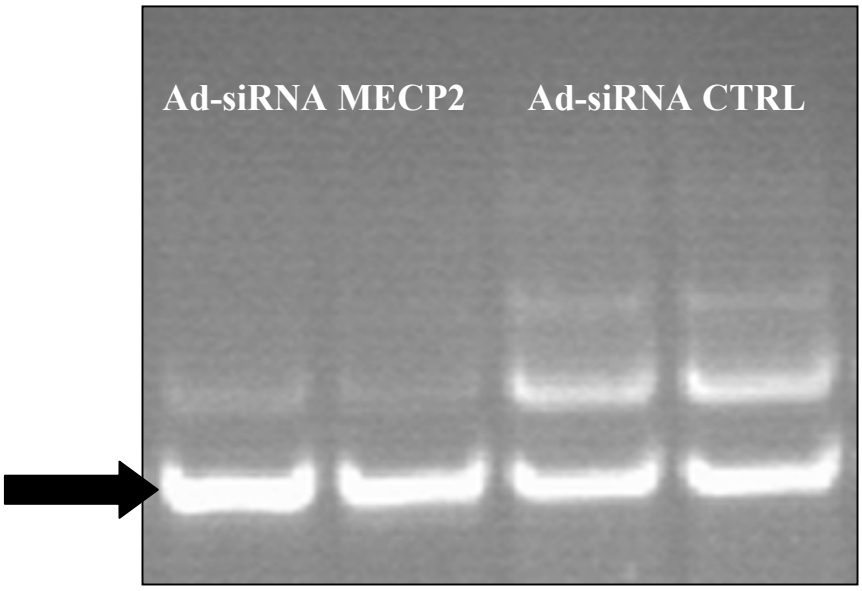


Fig. 22 : Polyacrylamide gel electrophoresis of TRAP assay products obtained from RTT and control cells. The picture shows the PCR amplified products of telomerase activity. Three different reactions for RTT patient and control were shown. Black arrow: TRAP internal control. The assay measures enzymatic 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.

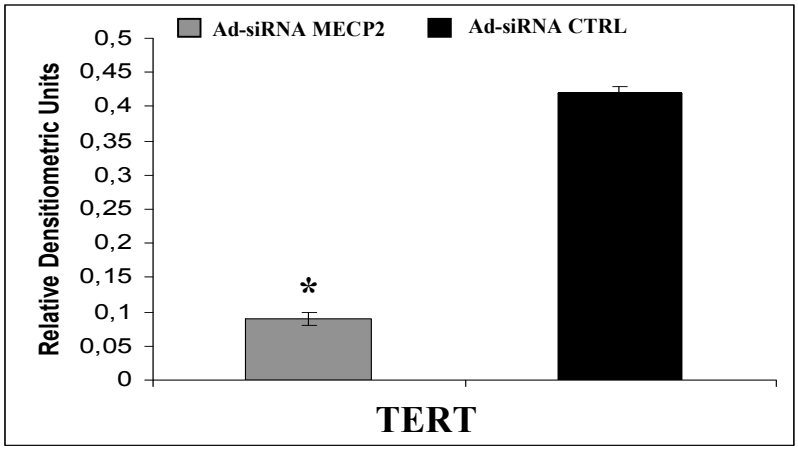


Fig 23: Semiquantitative RT-PCR analysis of mRNA expression of TERT gene from hMSC Ad-siRNA MECP2 and Ad-siRNA CTRL mRNA levels were normalized with respect to HPRT chosen as an internal control (±SD; n=3) (*p<0,05).

GENE	*GENE FUNCTION*
SOX2	*Embryonic transcription factor*
NANOG	*Embryonic transcription factor*
OCT 3/4	*Embryonic transcription factor*
KLF4	*Transcription factor*
SOX15	*Embryonic transcription factor*
SALL4	*Transcription factor*
BMI1	*Chromatin remodeling*
DPPA2	*Embryonic transcription factor*
ERAS	*GTPase*
GDF3	*Transcription factor*
TCL1	*Oncogene*
UTF1	*Embryonic coactivator*
ZFP42	*Transcription factor*

Table 15: "Stemness" related genes function

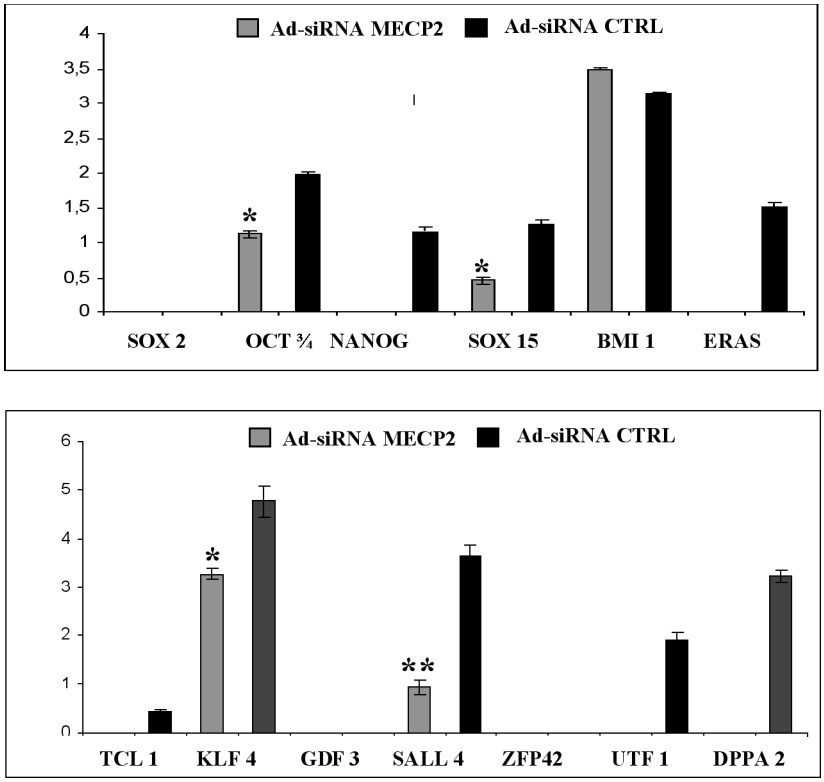


Fig 24: Semiquantitative RT-PCR analysis of mRNA expression of "stemness" gene from hMSC Ad-siRNA MECP2 (grey histograms) and Ad-siRNA CTRL(black histograms). mRNA levels were normalized with respect to HPRT chosen as an internal control (±SD; n=3) (*p<0,05; **p<0,01).

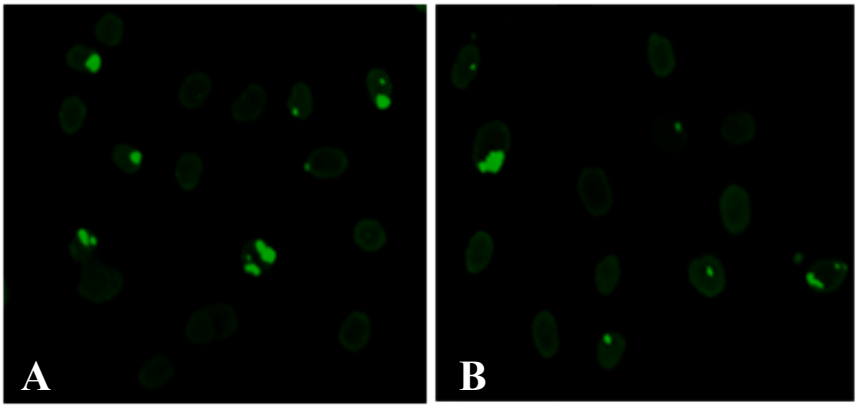
4.3.3 Senescence induced by reduced level of MECP2 is associated with damaged DNA

Cellular senescence involves damage to various cellular constituents, such as proteins, lipids and DNA. The imperfect maintenance of DNA represents the critical contributor to aging (112) (74). There are several sources of DNA damage, such as extrinsic factors (ionizing radiations, pollution, genotoxic drugs, etc) and cell-intrinsic sources, such as replication errors, spontaneous chemical changes and reactive oxygen species (ROS). As normal byproducts of metabolism, ROS are a chronic persistent damaging agent that greatly contributes to aging. A major product of oxidative damage to DNA is the 8-oxo-2'-deoxyguanosine (oxo8dG) that increases during cellular senescence.

I analyzed the effects of Ad-siRNA-MECP2 on the level oxidative damage to DNA. Immunocytochemistry experiments showed that the percentage of oxo8dG

positive MSCs was significantly higher ($p < 0.05$) in samples having reduced expression of *MECP2* compared with controls (Fig. 25).

The increase of oxo8dG DNA damage was not associated with a general increment of intracellular ROS in MSCs treated with Ad-siRNA-MECP2 in comparison with controls, as detected by flow cytometry analysis of hydroethidine (HEt) conversion to ethidium (Et) (Fig. 26). On the other hand, MECP2 downregulation did not affect the expression of manganese-dependent superoxide dismutase (SOD2) and catalase, which are two fundamental ROS reducing agents into cells (35) (Fig. 27).



	8-oxo-dG positive cells (%)
RTT-2 Patient neur.	*6,0±0,3*
Control neur.	*4,0±0,2*

Fig 25: Fluorescence photomicrographs represents cells positive for anti-8-oxo-dG primary antibody (green); A) Ad-siRNA MECP2, B) Ad-siRNA CTRL. The table shows percentage of 8-oxo-dG positive cells (±SD; n=3).

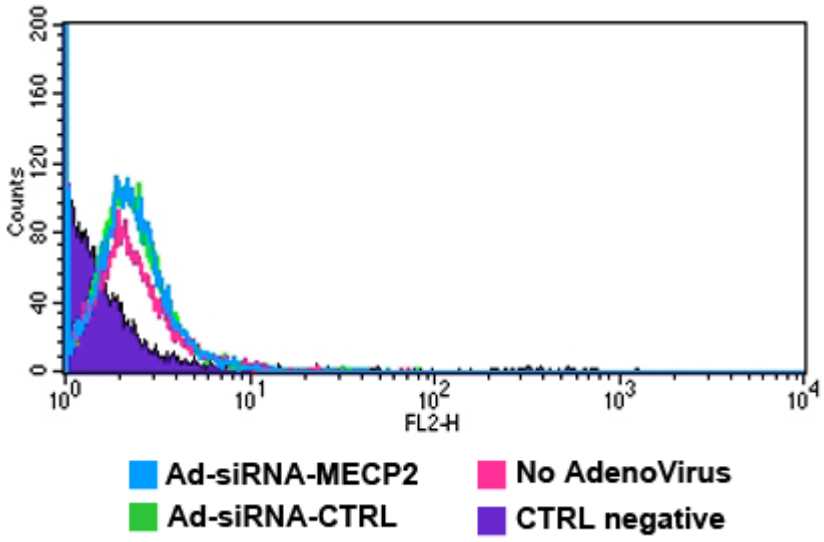


Fig 26: Flow cytometry analysis of hydroethidine (HEt) conversion to ethidium (Et) 24h after Ad-siRNA transductions.

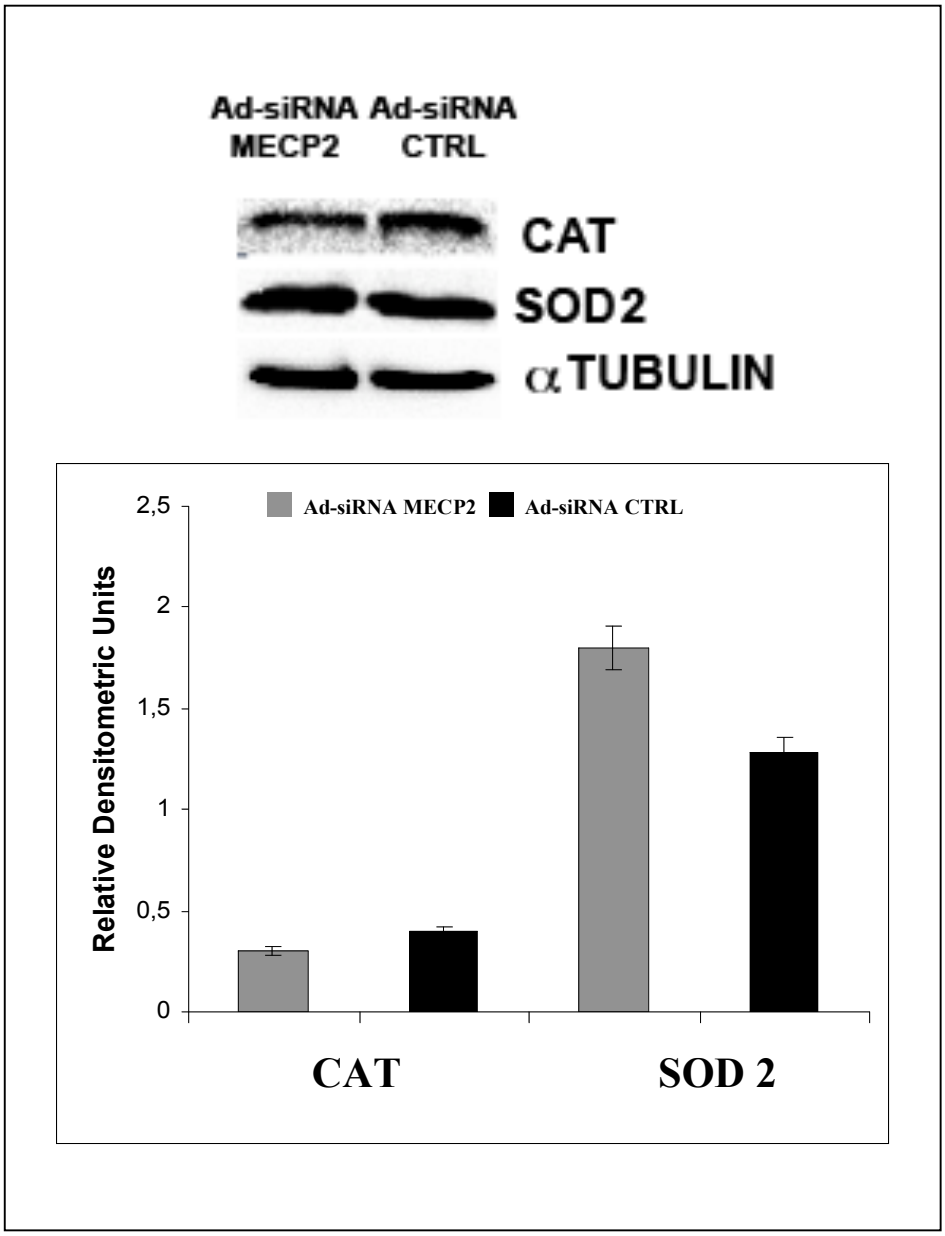


Fig. 27: Western blot analysis of SOD2 and Catalase levels from hMSC Ad-siRNA MECP2 and Ad-siRNA CTRL. Protein levels were normalized with respect to α–Tubuline chosen as an internal control (±SD; n=3).

4.3.4 Genes involved in DNA repair

ROS and other DNA damaging agents can cause cells to enter into senescence, impairing their physiological role. The DNA repair system is one of the major mechanisms that cells use to minimize DNA damage (44) (60).

On these premises, I decided to verify if senescence of MSCs was accompanied with changes in the expression of genes involved in different types of DNA repair. I selected a panel of genes involved in the regulation of base and nucleotide excision repair (BER and NER, respectively), mismatch repair (MER), and double strand break repair (DSBR) (98) (Tab. 16). Of interest, all the analyzed genes but *RAD23A* displayed a significant downregulation following transduction of MSCs with Ad-siRNA-MECP2 (Tab. 17). In particular, I observed a strong decrease of POLD3 that belongs to MER pathway, NTHL1 for NER pathway, BRCA2 and MRE11A for DSB (Tab. 17).

I decided also to analyze the ability of MSCs to repair double strand breaks that are the most dangerous form of DNA damage. Plasmid-based assay for in vitro DNA repair activity showed an impairment of this activity in MSCs with reduced expression of *MECP2* (Fig. 28).

Altogether, these data suggest that increase of oxo8dG damage may be attribute to a failure of maintenance of DNA rather than to an increase in ROS production.

DNA REPAIR PATHWAY	*GENE*	*GENE FUNCTION*
DBS (double strand break)	**BRCA2**	DNA and protein binding
	MRE11A	Endonuclease, exonuclease, hydrolase activity
	XRCC4	DNA and protein binding
MMR (mismatch repair)	**MLH1**	ATP and protein binding
	TREX1	Exonuclease
	MSH2	Mismatch binding
	MSH5	Mismatch binding
	PRKDC	DNA-dependent protein kinase and protein binding
	POLD3	Protein binding
BER (base excision repair)	**MUTYH**	DNA glycosylase
	MPG	DNA glycosylase
	NTHL1	AP endonuclease activity and DNA glycosylase
	FEN1	Endonuclease activity
	OGG1	DNA glycosylase
	MBD4	DNA glycosylase
NER (nucleotide excision repair)	**XPA**	A zinc finger protein involved in DNA excision repair
	RAD23A	DNA and protein binding
	ERCC3	ATP-dependent DNA helicase
	SLK	Nuclease activity

Table 16: DNA repair related genes function.

	GENE	Ad-siRNA *MECP2*	Ad-siRNA *CTRL*
MER	*MSH2*	*1.20±0.13 (*)*	*1.92±0.28*
	POLD3	*0.03±0.01 (**)*	*0.33±0.06*
	PRKDC	*0.53±0.07 (**)*	*1.58±0.08*
	TREX1	*1.85±0.10 (*)*	*3.57±0.49*
	MLH1	*1.29±0.17*	*1.60±0.24*
	MSH5	*n.d.*	*n.d*
BER	*MBD4*	*1.23±0.18*	*1.94±0.31*
	MUTYH	*n.d.*	*n.d.*
	OGG1	*0.62±0.08 (**)*	*3.50±0.59*
	FEN1	*1.16±0.15 (**)*	*3.76±0.64*
	MPG	*1.71±0.20 (**)*	*3.66±0.51*
	NTLH1	*n.d. (**)*	*0.31±0.05*
NER	*XPA*	*1.41±0.14*	*1.71±0.27*
	ERCC3	*0.83±0.16 (**)*	*3.00±0.36*
	SLK	*0.31±0.06 (*)*	*1.45±0.16*
	RAD23A	*1.56±0.26*	*1.57±0.29*
DSB	*XRCC4*	*n.d.*	*n.d.*
	BRCA3	*0.04±0.01 (**)*	*0.71±0.08*
	MRE11A	*0.08±0.01 (**)*	*0.78±0.09*

Table 17: Semiquantitative RT-PCR results of mRNA expression of DNA repair genes from hMSC Ad-siRNA MECP2. mRNA levels were normalized with respect to HPRT chosen as an internal control (±SD; n=3) (*p<0,05; **p<0,01).

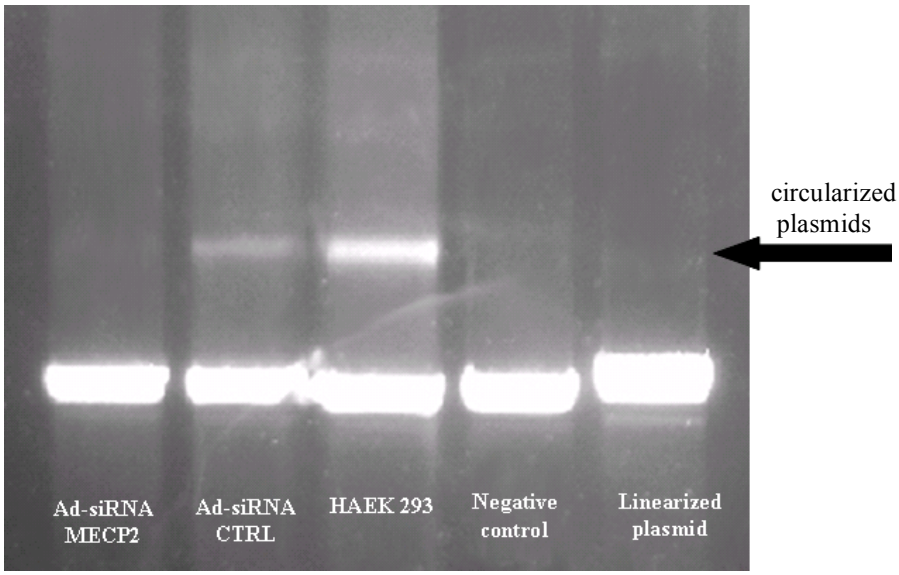


Fig. 28: *In vitro* DNA repair assay. The picture shows agarose gel electrophoresis of pcDNA3 plasmids that were digested with ECoRI enzyme and then treated with protein lysates to allow *in vitro* DNA end-joining. Protein lysates were obtained from MSCs treated with Ad-siRNA-MECP2 and Ad-siRNA-CTRL, respectively. Human Embrionic Kidney 293 cells (HEK) were chosen as the positive control for the DNA end-joining activity. Repair of double strand breaks produces circularized plasmids that migrate in agarose distinctly from linear DNAs of tha same mass.

4.3.5 Molecular pathways involved in cell cycle regulation, apoptosis, and senescence: RB and P53 crosstalk

Once I analyzed the biological phenomena affected by *MECP2* silencing, I tried to dissect its relationship to molecular pathways involved in several processes. In particular, I analyzed the expression of Retinoblastoma gene family members (*RB* and *RB2/P130*), which control cell cycle arrest, differentiation, apoptosis, and/or senescence (31) (14) (87).

I observed an increase of *RB* gene expression at both mRNA and protein levels in cells treated with Ad-siRNA-MECP2 (Fig. 29-30). *RB2/P130* was upregulated at mRNA level while I did not observe change in protein expression, although I noticed a decrease of hyperphosphorylated inactive form in Ad-siRNA-MECP2 transduced cells (Fig. 30).

Physiology of MSCs could also be affected by another gene that plays a key function in cell homeostasis: *P53*. The tumor suppressor *P53* gene is involved in cell cycle regulation, apoptosis, senescence, and differentiation in several biological systems (34). Over *in vitro* MECP2 downregulation, I obtained evidence for significant changes in *P53* mRNA while no modification of protein level occurred (Fig. 30).

Some cyclin kinase inhibitors (CKIs), such as $P21^{CIP1}$, $P27^{KIP1}$, and $P16^{INK4A}$, have overlapping pathways with the RB family and P53. In particular, $P21^{CIP1}$ and $P16^{INK4A}$ are often expressed in senescent cells. It has been suggested that $P21^{CIP1}$ is mainly associated with cell cycle arrest and inhibition of apoptosis, whereas $P16^{INK4A}$ plays a major role in senescence (14). After *in vitro* silencing of *MECP2*, I observed a significant increase in $P21^{CIP1}$ expression while $P27^{KIP1}$ evidenced a significant decline of mRNA, whereas no change was detected at protein level (Fig. 29-30). Of interest, $P16^{INK4A}$ showed an upregulation of mRNA and protein expression (Fig. 29-30).

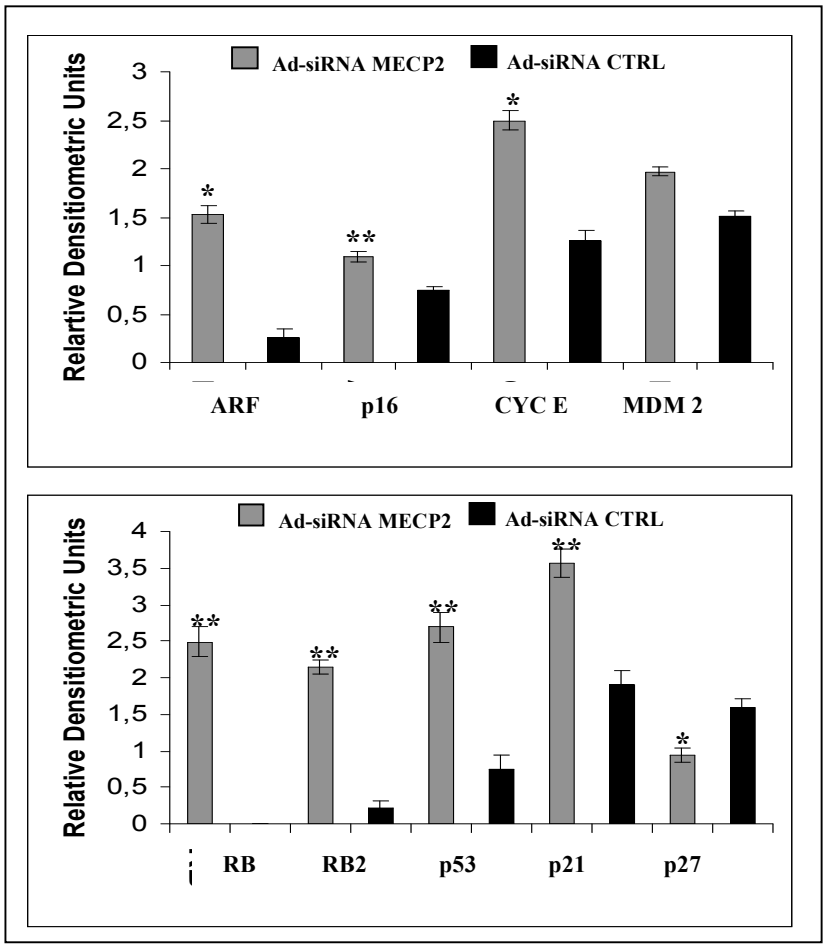
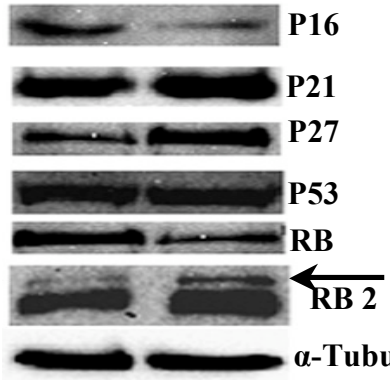


Fig. 29: Semiquantitative RT-PCR analysis of mRNA expression of cell cycle gene from hMSC Ad-siRNA MECP2 (grey histograms) and Ad-siRNA CTRL(black histograms). mRNA levels were normalized with respect to HPRT chosen as an internal control (±SD; n=3) (*p<0,05; **p<0,01).



	AdsiRNA MECP2	*AdsiRNA CTRL*
P16	*0,24±0,03(∗∗)*	*0,07±0,01*
P21	*0,66±0,08*	*0,63±0,09*
P27	*0,06±0,01(∗)*	*0,12±0,02*
P53	*0,08±0,01*	*0,09±0,02*
RB	*0,22±0,04(∗)*	*0,11±0,01*
RB2	*0,21±0,04*	*0,28±0,05*

Fig. 30: Western blot analysis of cell cycle protein levels from hMSC Ad-siRNA MECP2 and Ad-siRNA CTRL (left panel). In table (right panel) are reported protein levels normalized with respect to α–Tubuline chosen as an internal control (±SD; n=3) (*p<0,05; **p<0,01).

4.3.6 MECP2 reduction affects the level of cytosine methylation

Effects of MECP2 silencing on the in vitro behavior of MSCs could be related to modification of DNA methylation status. I evaluated the global level of cytosine methylation by a rapid cytosine extension assay. This method is based on the selective use of methylation sensitive restriction enzymes that leave a 5'-guanine overhang after DNA cleavage followed by single nucleotide primer extension with [^{32}P]dCTP. DNA obtained from MSCs treated with Ad-siRNA-MECP2 showed a reduced incorporation of cytosine following digestion with *HpaII* and *BshII* enzymes compared with controls (Fig. 31). This result is in agreement with agarose gel electrophoresis of genomic DNA digested with methylation sensitive restriction enzymes.

In a second step, I decided to analyze the methylation status of promoters of genes whose expression was modified by *MECP2* silencing. Promoter regions were classified according to their C+G content and frequency of CpG dinucleotides. Promoters of stemness-related genes under investigation were classified as CpG poor promoters (having a CpG frequence that is the same of genome average) with Methprimer software. On the contrary, genes belonging to *RB* and *P53* pathways were classified as CpG islands-promoters, since they have high frequence of CpG dinucleotides. I carried out HS-PCRs to detect methylation of promoters under analysis. The method uses methylation-sensitive HPAII enzyme, to cleave DNA at specific methylated-cytosine residues which have lost their methyl group, that are followed by a guanine, CpG, followed by amplification of the resultant products. The amplification products are only detected when digestion of the products are inhibited by methylation being present. Results of HS-PCRs evidenced that MECP2 silencing did not change the methylation status of both classes of promoters. In fact, no significant differences in PCR amplifications were observed in samples from MSCs treated with Ad-siRNA-MECP2 compared to control (Fig. 32).

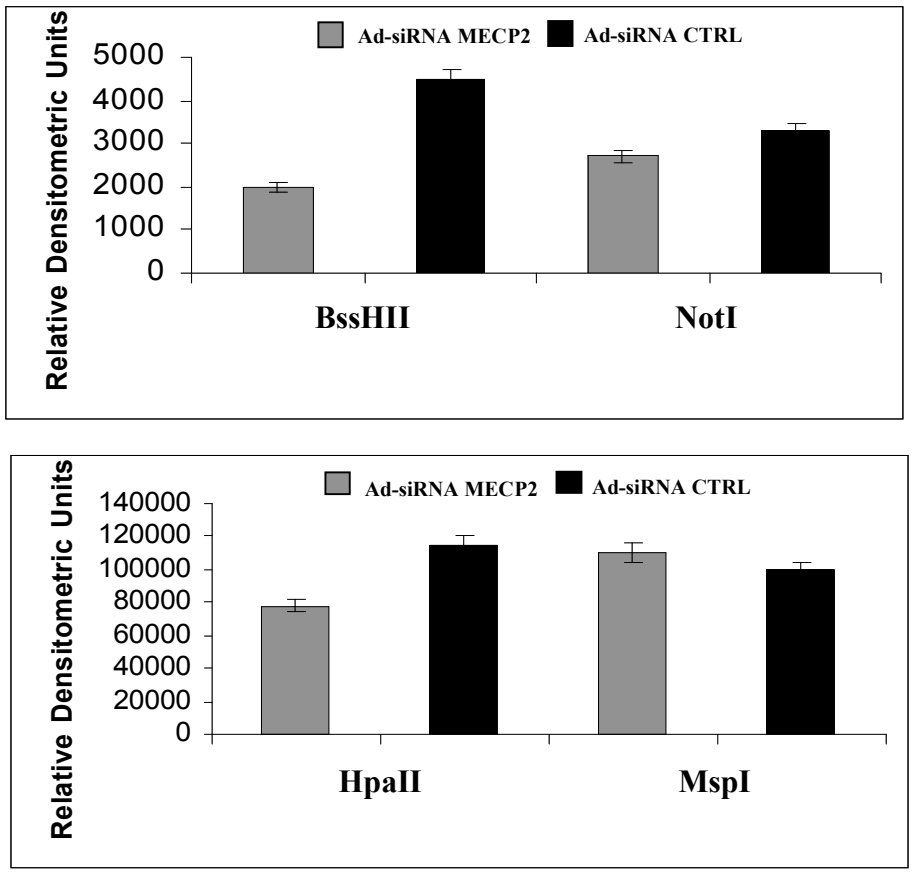


Fig. 31: Cytosine extension assay. The method is based on the selective use of methylation-sensitive restriction enzymes that leave a 5' guanine overhang after DNA cleavage, followed by single nucleotide extension with radiolabelled dCTP. Histograms show the dCTP incorporation following digestion with HpaII and BssHII methylation sensitive enzymes alongside their isoschizomers MspI and NotI, respectively; which cut the DNA irrespective of methylation status. Following HpaII and BssHII digestion, the decrease in dCTP incorporation in Ad-siRMA-MECP2 treated cells indicate that the percentage of methylated cytosines in higher than the control group.

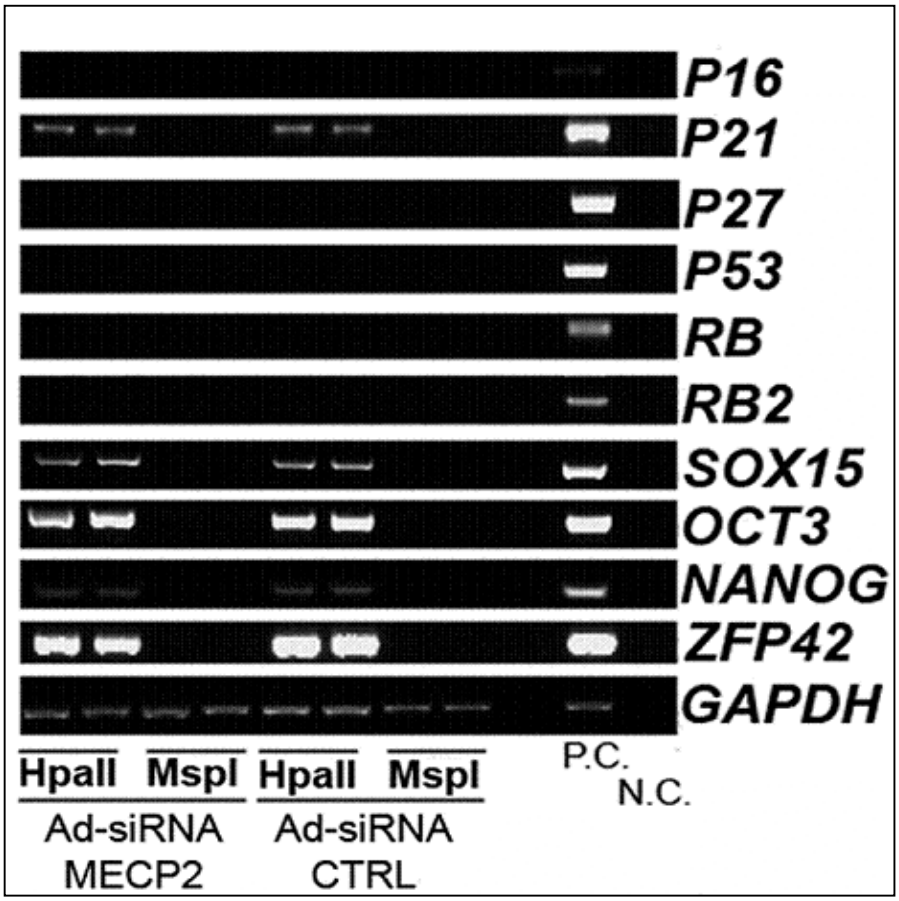


Fig. 32: Analysis of the methylation status of several gene promoters by HpaII restriction sensitive PCR (HS-PCR). Genomic DNA was initially digested to completion with either HpaII or MspI and subjected toPCR to amplify promoter regions that are indicated in the picture. The amplification of HpaII digested genomic DNA provide evidence of cytosine methylation at the recognition site CCGG. In control cells (Ad-siRNA-CTRL) the promoters with CpG islands, which are essentially unmethylated, were not amplified following HpaII digestion (see P16, P21, P27, P53, RB and RB2); whereas CpG poor promoters, having a variable degree of methylation, were successfully amplified (see SOX 15, OCT 3, NANOG, ZFP42). In both cases downregulation of MeCP2 did not modify the amplification pattern. No amplifications are expected for MspI-digested genomic DNA. A promoter region of GAPDH gene that does not contain HpaaII/MspI recognition sites was amplified as the reaction control.
Positive control (P.C.) is amplifivation with genomic DNA not digested with restriction enzyme. Negative control (N.C.) is PCR amplification without DNA.

5. Discussion

RTT is a genetic disorder affecting mainly neural development. Several reports also showed that osteogenesis process could be impaired in RTT patients (40) (11-12) (1) (125). Improper tissue development and maturation may arise from alterations in stem cell reservoirs (88) (99). For these reasons, I decided to investigate the biology of bone marrow MSCs from two RTT patients. These cells are of great interest for studies on RTT syndrome since they represent the physiological pool for generation of osteocytes. Moreover, they represent a useful model to study neural development since they can also differentiate into non-mesenchymal lineage such as neurons, astrocytes and oligodendrocytes (65) both *in vitro* (106) (85) and after transplantation (71) (25). Nevertheless to have a general picture on the role of MECP2 in stem cell biology, I silenced MECP2 gene expression in human cultures of MSCs.

5.1.1 MeCP2 Inactivation Could Alter the Biology of MSCs

MSCs from RTT-1 patient showed precocious signs of senescence compared with a healthy control. This was in agreement with a reduced expression of genes involved in control of stem cell self-renewal and upregulation of a lineage specific gene (Osteopontin) involved in osteogenesis (Fig. 15-16).

I observed a lower degree of apoptosis in RTT-1 patient cells compared with control. This means that aberrant stem/progenitor cells, instead of being eliminated, could survive and become senescent.

Overall, these data suggest that in RTT-1 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 these research data, I 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.

5.1.2 MeCP2 and Gene Expression

Most of genes I 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-1 patient compared with control. These data are in agreement with studies showing that MeCP2 inactivation can induce both gene upregulation and silencing (21) (86) (24).

This may be explained considering that:

I. methylated DNA is a docking site for several proteins besides MeCP2

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.

These findings suggest that MeCP2 inactivation is not always accompanied by target gene activation; in order to clarify this observation should be useful a depth analysis of MeCP2 binding promoters.

5.2 MeCP2 inactivation and neuronal differentiation

Results obtained from the study of RTT-2 patient MSCs biology are in agreement with RTT-1 patient one; in depth I observed a lower degree of apoptosis and an increase of senescence in RTT-2 patient with respect to healthy control. These data as result of two specific patients MECP2 mutations, allow me to hypothesize that the inactivation of MECP2 could affect the "functioning" of MSCs with triggering of senescence phenomena.

To induce neuronal differentiation in RTT-2 patient and control MSCs, I followed Hyclone (Thermo Scientific) manufacturer's protocol.

FACS analysis led on MSCs from RTT-2 neur. and CTRL neur., 5 days after neuronal induction, revealed an increase of cells of both samples in G_1 phase followed by a concomitant reduction in S phase (Tab. 8). These data confirmed that cells of both samples leave the undifferentiated state to a committed one. This finding was confirmed by morphological analysis that revealed a transition of

RTT-2 neur. and CTRL neur. samples to cells with asymmetrical morphology compared with the symmetrical morphology of untreated cells (RTT-2 and CTRL). This change progressed until they acquired a neuronal-like phenotype 6 days following differentiative induction as shown by cellular body thinning and production of branch extensions.

I also observed the expression of a neuron-specific protein (NeuN) in RTT-2 neur. and CTRL neur. MSCs (Tab. 9), testing the neuronal differentiation.

Interestingly, depth analysis showed differences between RTT-2 neur. and CTRL neur. as far as morphological and molecular characteristics.

Indeed, the morphological observations, which showed a limited production of branch extensions in RTT-2 neur. compared to CTRL neur., were confirmed by molecular analysis of neuronal differentiation marker genes (Tab. 11).

I observerd a significative increase of MAP2, ENO2 and CNPase mRNA expression levels in CTRL. neur. with respect to RTT-2 neur. (Tab 11).

Morphological and molecular data are in agreement with previous findings indicating a critical cell biological function for MeCP2 in mediating the final stages of neuronal development (128).

This idea can be further supported also by the not significative difference in mRNA expression levels of the above mentioned genes in undifferentiated controls (RTT-2 and CTRL) (Tab 11).

So the lack of functioning MeCP2 probably affect neuronal phenotype maintenance in MSCs following differentiative induction.

Moreover the absence of changes in expression levels of glial-specific marker genes (GLUT and VIM) between differentiated and undifferentiated samples, showed that my differentiative protocol give rise only to neurons.

5.3 MECP2 gene in the regulation of MSC physiology

Epigenetic modifications to genome consist in DNA methylation that occurs almost exclusively in the context of CpG dinucleotides (CpGs). MeCP2 is a member of a family of proteins that preferentially bind to methylated CpGs. Mutations in MECP2 gene underlie RETT syndrome; for this reason, several studies have been focused on the ability of MECP2 to regulate gene expression in

several systems (102) (6). Nevertheless, the specific contribution of MECP2 in stem cell physiology is less well understood. This last issue is of great interest since several reports evidenced that chromatin modifiers have a key part in the control of stem cell functions by affecting self-renewal, pluripotency, commitment and senescence (111) (78). In this context, I sought to investigate the role of MECP2 in the regulation of MSC physiology also to better understand the complexity of RETT syndrome. In order to confirm and extend above-mentioned results I took advantage of adeno-siRNA technique to silence MECP2 in MSCs from healthy donors.

5.3.1 Partial silencing of MECP2 gene.

Treatment of MSCs with adenosiRNAs directed against MECP2 mRNA induced a 70% decrease of target molecule detected by RT-PCR using the primer pair to detect both mRNA isoforms (Fig. 20A). This in turn, induced a 50% decrease of MeCP2 protein in MSCs transduced with Ad-siRNA-MECP2 in comparison to MSCs transduced with control Ad-siRNA-CTRL (Fig. 20B). This partial silencing induced striking biological effects suggesting that maintaining MECP2 levels in a narrow range is essential for normal functions of cells. The importance of a fine tuning of MECP2 expression is in line with the researches of Collins et al. (18). They demonstrated that mild overexpression of MECP2 cause a progressive neurological disorder in mice. Generally speaking, there are several genes having a tightly regulated expression, and even subtle alterations may disrupt the normal function of cells (64) (120). One explanation for why certain genes may require precise control is if they regulate or are involved in balancing disparate downstream pathways possessing mutually opposing activities (32). This may be the case of MECP2, which can modulate gene expression in either positive or negative manner as evidenced by Yasui et collaborators (118).

5.3.2 Downregulation of MECP2 induced a decrease of cell proliferation and apoptosis along with trigger of senescence

Partial silencing of MECP2 in MSCs induced a significant reduction of S-phase cells, along with an increase of G_1 cells. This was accompanied by a reduction of apoptotic cells (Tab. 12).

Noteworthy, I evidenced signs of senescence in cells treated with Ad-siRNA-MECP2, as detected by *in situ* acid-beta-galactosidase compared with cells transduced with Ad-siRNA-CTRL (Tab. 14). This was associated with the reduced expression and activity of telomerase reverse transcriptase (TERT) (Fig. 22). These data are consistent with the studies on MSCs from RTT patients suggesting that MECP2 plays a role in the regulation of these key events for cell's life.

Analysis of stem cell transcriptional profiles allowed the identification of "stemness" genes participating in the control of stem cell properties, such as self-renewal ability and retention of an uncommitted state (92) (76) (104). Studies on stem cells obtained from RTT-1 patient with mutated MECP2 demonstrated that during *in vitro* replicative senescence, MSCs lose their "stemness" while becoming senescent. This phenomenon is accompanied by a downregulation of several stemness genes (32). This result is in line with data obtained in MSCs treated with Ad-siRNA-MECP2. In fact, the majority of analyzed stemness-related genes showed a decline of expression in MSCs with silenced MECP2 (Fig. 24).

5.3.3 Senescence induced by reduced level of MECP2 is associated with damaged DNA

Accumulation of DNA damage has long been suggested as one of the major form of contribution to the ageing of cells and organisms (100) (74). Reactive oxygen species (ROS), which are normal byproducts of cell's metabolism, are a chronic persistent damaging agent that greatly contributes to aging. 8-oxo-2'-deoxyguanosine (oxo8dG) that increases during cellular senescence is the major product of ROS action (100) (74).

Partial silencing of MECP2 augmented significantly the percentage of oxo8dG positive MSCs (Fig. 25). This phenomenon was associated neither with a general increment of intracellular ROS (Fig. 26) nor with changes in the expression of manganese-dependent superoxide dismutase (SOD2) and catalase, which are two fundamental ROS reducing agents into cells (Fig. 27) (35). Senescence induced by partial silencing of MECP2 appears to rely upon impairment of DNA damage repair mechanisms. In fact, following transduction of MSCs with Ad-siRNA-MECP2, I observed a downregulation in the expression of several genes belonging to base and nucleotide excision repair, mismatch repair, and double strand break repair (Tab. 17). In agreement with this result, I observed a reduced ability of MSCs transduced with Ad-siRNA-MECP2 to repair double strand breaks (Fig.28).

Altogether, these findings indicate that increase of oxo8dG damage may be attribute to a failure of maintenance of DNA rather than to an increase in ROS production.

5.3.4 Cell cycle arrest and senescence induced by MECP2 silencing appear to be governed by activation of RB pathway

Usually P53 and retinoblastoma-associated pathways are engaged in regulation of multiple aspects of cell's life, such as cell cycle progression, apoptosis, senescence, and differentiation (34) (31) (87) (14). I decided to address the role of these pathways in biological processes induced by MECP2 silencing.

Following transduction of MSCs with Ad-siRNA-MECP2, I detected an upregulation of RB gene expression along with a decrease of hyperphosphorylated inactive form of RB2/p130 (Fig. 29-30). The activation of RB-pathways seems to be confirmed by the observation that the cyclin kinase inhibitor $P16^{INK4A}$, which prevents RB phosphorylation and inactivation, showed an upregulation of mRNA and protein expression (Fig. 29-30).

Effect of *in vitro* MECP2 downregulation on P53 expression was more complex: I observed a significant changes in P53 mRNA while no modification of protein level occurred (Fig. 29-30). Notwithstanding, I may not exclude that P53 was activated by post-translational modifications. On the other hand, after *in vitro*

silencing of MECP2, I detected a significant increase in the expression of cyclin kinase inhibitor $P21^{CIP1}$, a direct target of P53 (Fig. 29).

Taken together, these data suggest that cell cycle arrest and senescence process induced by MECP2 downregulation could be sustained by activation of RB and P53 pathways.

5.3.5 MECP2 reduction affects the level of cytosine methylation

MECP2 binds methylated CpG dinucleotides and can regulate gene expression by recruiting several complexes, such as histone deacetylases and DNA methyltransferases (72) (6). I analyzed the global level of cytosine methylation with a rapid assay to detect alteration in methylation of DNA. Results indicate that silencing of MECP2 induces an increase of methylated cytosines in the genome (Fig. 31). To have a closer look on the methylation status of DNA, I carried out HS-PCRs to amplify promoter regions of several genes whose expression was affected by MECP2 downregulation. Results of HS-PCRs evidenced that MECP2 silencing did not change the methylation status of analyzed promoters (Fig. 32).

I cannot exclude that subtle changes in cytosine methylation were overlooked by experimental assays. In depth analysis such as DNA methylation arrays may give comprehensive information on the effects of MECP2 downregulation in these cells. Notwithstanding, my data suggest that a decrease in MECP2 level may specifically affect methylation of intergenic cytosines. This hypothesis is in agreement with the first large scale mapping of MECP2 binding sites. This study showed that MECP2 binds preferentially intergenic or intronic regions (118). A striking observation was the increase of methylated cytosines following silencing of MECP2. It should be considered that MECP2 interacts with maintenance DNA Methyltransferase (DNMT1) and could direct methylation activity on MECP2-responsive genes (59). I may hypothesize that absence and/or reduced expression of MECP2 could allow DNMT1 to methylate much more targets other than MECP2-binding sites. In fact, other Methyl-CpG binding proteins may direct DNA methyltransferases on their own sites. Interestingly, Tatematsu et al. (107)

demonstrated that MBD2-MBD3 heterodimer complexes seem to bind hemimethylated DNA and recruit DNMT1. Moreover, the lack of MECP2 may render DNA methylases available to interact with other factors, which in turn can promote methylation activity, such as Ets transcription factor PU.1 (103).

6. Conclusions and Perspectives

6.1 Conclusions

MeCP2 protein binds to methylated DNA and produces changes in chromatin structure. This is a key event in regulation of gene expression. My results suggest that in RTT patients MeCP2 inactivation could impair epigenetic mechanisms regulating stem cell biology altering the physiological development of tissues and organs.

MECP2 can be identified as a chromatin modifier whose expression must be tightly regulated to avoid alteration in cell's homeostasis.

More in depth, the experimental data of my research show that MECP2 downregulation induces senescence phenomena. Senescence of MSCs following MECP2 silencing is associated with failure of maintenance of DNA.

Furthemore, senescence seems to occur through canonical RB- and P53-related pathways.

My research could provide also a new insight on RTT syndrome. Senescence phenomena could be involved in triggering RTT syndrome-associated diseases.

6.2 Perspectives

My future research will aim to clarify the relationship between MeCP2 inactivation and the methylation state of its target genes promoters. It will be investigated by chip on chip analysis.

This assay will allow to determine the locations of MeCP2 binding sites on DNA and their methylation status.

Furthermore to better understand the role played by MeCP2 in the on-going stages of neuronal development, I will set up a protocol of inducible MECP2 gene silencing.

In this way it will be possible to obtain a MECP2 downregulation in the early and tardive stages of MSC neuronal differentiaton process, by activation of silencing mechanism at appropriate time courses.

7. References

1. Ager S, Fyfe S, Christodoulou J, Jacoby P, Schmitt L, Leonard H (2006) Predictors of scoliosis in Rett syndrome. J Child Neurol. 2006 Sep;21(9):809-13.
2. Alder, J.T., Chessell, I.P., Bowen, D.M. (1995) A neurochemical approach for studying response to acetylcholine in Alzheimer's disease. Neurochem Res 20, 769-771
3. Amir, R., Dahle, E.J., Toriolo, D., Zoghbi, H.Y. (2000) Candidate gene analysis in Rett syndrome and the identification of 21 SNPs in Xq. Am J Med Genet 90, 69-71
4. Anvret, M., Zhang, Z.P., Hagberg, B. (1994) Rett syndrome: the bcl-2 gene--a mediator of neurotrophic mechanisms? Neuropediatrics 25, 323-324.
5. 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. 200424(2):172-7.
6. Bienvenu, T., Chelly, J. (2006) Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. Nat Rev Genet 7, 415-426.
7. Bienvenu, T., des Portes, V., McDonell, N., Carrie, A., Zemni, R., Couvert, P., Ropers, H.H., Moraine, C., van Bokhoven, H., Fryns, J.P., Allen, K., Walsh, C.A., Boue, J., Kahn, A., Chelly, J., Beldjord, C. (2000) Missense mutation in PAK3, R67C, causes X-linked nonspecific mental retardation. Am J Med Genet 93, 294-298.
8. Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH. (2003) Visualizing superoxide production in normal and diabetic rat islets of Langerhans. J Biol Chem. 278(11):9796-801.
9. Bird, A.P., Wolffe, A.P. (1999) Methylation-induced repression--belts, braces, and chromatin. Cell 99, 451-454
10. Blau, H.M., Brazelton, T.R., Weimann, J.M. (2001) The evolving concept of a stem cell: entity or function? Cell 105, 829-841.
11. Budden SS, Gunness ME. (2001) Bone histomorphometry in three females with Rett syndrome. Brain Dev. Suppl 1:S133-7.
12. Budden SS, Gunness ME. (2003) Possible mechanisms of osteopenia in Rett syndrome: bone histomorphometric studies. J Child Neurol. 18(10):698-702.
13. Cai J, Weiss ML, Rao MS. (2004) In search of "stemness". Exp Hematol. ;32(7):585-98.
14. Campisi J, d'Adda di Fagagna F. (2007) Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 8(9):729-40.
15. Chen Y, Teng FY, Tang BL. (2006) Coaxing bone marrow stromal mesenchymal stem cells towards neuronal differentiation: progress and uncertainties. Cell Mol Life Sci. 63(14):1649-57.
16. Choi CB, Cho YK, Prakash KV, Jee BK, Han CW, Paik YK, Kim HY, Lee KH, Chung N, Rha HK. (2006) Analysis of neuron-like differentiation of human bone marrow mesenchymal stem cells. Biochem Biophys Res Commun. Nov 10;350(1):138-46.
17. Christodoulou, J., Weaving, L.S. (2003) MECP2 and beyond: phenotype-genotype correlations in Rett syndrome. J Child Neurol 18, 669-674.

18. Collins AL, Levenson JM, Vilaythong AP, Richman R, Armstrong DL, Noebels JL, David Sweatt J, Zoghbi HY. (2004) Mild overexpression of MeCP2 causes a progressive neurological disorder in mice. Hum Mol Genet. 13(21):2679-89.
19. 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 U S A. Mar 28;97(7):3213-8.
20. Colter DC, Sekiya I, Prockop DJ. (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci U S A. 98(14):7841-5.
21. 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(5):847-65.
22. D'Esposito, M., Ciccodicola, A., Gianfrancesco, F., Esposito, T., Flagiello, L., Mazzarella, R., Schlessinger, D., D'Urso, M. (1996) A synaptobrevin-like gene in the Xq28 pseudoautosomal region undergoes X inactivation. Nat Genet 13, 227-229
23. De Bona, C., Zappella, M., Hayek, G., Meloni, I., Vitelli, F., Bruttini, M., Cusano, R., Loffredo, P., Longo, I., Renieri, A. (2000) Preserved speech variant is allelic of classic Rett syndrome. Eur J Hum Genet 8, 325-330
24. 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.
25. Dezawa M, Kanno H, Hoshino M, Cho H, Matsumoto N, Itokazu Y, Tajima N, Yamada H, Sawada H, Ishikawa H, Mimura T, Kitada M, Suzuki Y, Ide C. (2004) Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. J Clin Invest. 113(12):1701-10.
26. Diggle CP, Bentley J, Kiltie AE. (2003) Development of a rapid, small-scale DNA repair assay for use on clinical samples. Nucleic Acids Res. 31(15):e83
27. Evans, M.J., Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154-156
28. Fuks, F., Hurd, P.J., Wolf, D., Nan, X., Bird, A.P., Kouzarides, T. (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. J Biol Chem 278, 4035-4040.
29. Fuks F. (2005) DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev. 15(5):490-5.
30. 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(1):97-105.
31. Galderisi U, Cipollaro M, Giordano A. (2006) The retinoblastoma gene is involved in multiple aspects of stem cell biology. Oncogene. 25(38):5250-6.
32. Galderisi U, Helmbold H, Squillaro T, Alessio N, Komm N, Khadang B, Cipollaro M, Bohn W, Giordano A. (2008) In Vitro Senescence of Rat Mesenchymal Stem Cells is Accompanied by Downregulation of Stemness-Related and DNA Damage Repair Genes. Stem Cells Dev. [Epub ahead of print].

33. Gibson, J.H., Williamson, S.L., Arbuckle, S., Christodoulou, J. (2005) X chromosome inactivation patterns in brain in Rett syndrome: implications for the disease phenotype. Brain Dev 27, 266-270.
34. Giaccia AJ, Kastan MB. (1998) The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev. 1998 Oct 1;12(19):2973-83.
35. Giorgio M, Trinei M, Migliaccio E, Pelicci PG. (2007) Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? Nat Rev Mol Cell Biol. 8(9):722-8.
36. Gonzalo, S., Jaco, I., Fraga, M.F., Chen, T., Li, E., Esteller, M., Blasco, M.A. (2006) DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 8, 416-424.
37. Gordon D, Scolding NJ. (2009) Human mesenchymal stem cell culture for neural transplantation. Methods Mol Biol. 549:103-18.
38. Greco B, Recht L., (2003) Somatic plasticity of neural stem cells: fact or fancy? J Cell Biochem. Jan 1;88(1):51-6.
39. Greene P. (2009) Cell-based therapies in Parkinson's disease. Curr Neurol Neurosci Rep. 9(4):292-7.
40. Haas RH, Dixon SD, Sartoris DJ, Hennessy MJ. (1997) Osteopenia in Rett syndrome. J Pediatr. 131(5):771-4.
41. Hagberg, B., Aicardi, J., Dias, K., Ramos, O. (1983) A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. Ann Neurol 14, 471-479
42. Hagberg, B., Romell, M. (2002) Rett females: patterns of characteristic side-asymmetric neuroimpairments at long-term follow-up. Neuropediatrics 33, 324-326.
43. Hay, E. (1966) Regeneration. Holt, Rinehart and Winston, New York).
44. Hoeijmakers JH. (2001) Genome maintenance mechanisms for preventing cancer. Nature. 411(6835):366-74
45. Horwitz AR, Parsons JT., (1999) Cell migration--movin' on. Science. Nov 5;286(5442):1102-3.
46. Hou L, Hong T. (2008) Stem cells and neurodegenerative diseases. Sci China C Life Sci. 51(4):287-94.
47. Huppke, P., Held, M., Laccone, F., Hanefeld, F. (2003) The spectrum of phenotypes in females with Rett Syndrome. Brain Dev 25, 346-351
48. Huppke, P., Laccone, F., Kramer, N., Engel, W., Hanefeld, F. (2000) Rett syndrome: analysis of MECP2 and clinical characterization of 31 patients. Hum Mol Genet 9, 1369-1375
49. Illingworth, R., Kerr, A., Desousa, D., Jorgensen, H., Ellis, P., Stalker, J., Jackson, D., Clee, C., Plumb, R., Rogers, J., Humphray, S., Cox, T., Langford, C., Bird, A. (2008) A novel CpG island set identifies tissue-specific methylation at developmental gene loci. PLoS Biol 6, e22
50. Ishii, T., Makita, Y., Ogawa, A., Amamiya, S., Yamamoto, M., Miyamoto, A., Oki, J. (2001) The role of different X-inactivation pattern on the variable clinical phenotype with Rett syndrome. Brain Dev 23 Suppl 1, S161-164
51. Itahana K, Campisi J, Dimri GP. (2007) Methods to detect biomarkers of cellular

senescence: the senescence-associated beta-galactosidase assay. Methods Mol Biol. 371:21-31. Review.
52. Jaenisch, R., Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33 Suppl, 245-254
53. Jeffery, L., Nakielny, S. (2004) Components of the DNA methylation system of chromatin control are RNA-binding proteins. J Biol Chem 279, 49479-49487
54. Jian, L., Archer, H.L., Ravine, D., Kerr, A., de Klerk, N., Christodoulou, J., Bailey, M.E., Laurvick, C., Leonard, H. (2005) p.R270X MECP2 mutation and mortality in Rett syndrome. Eur J Hum Genet 13, 1235-1238.
55. Levy YS, Bahat-Stroomza M, Barzilay R, Burshtein A, Bulvik S, Barhum Y, Panet H, Melamed E, Offen D. (2008) Regenerative effect of neural-induced human mesenchymal stromal cells in rat models of Parkinson's disease. Cytotherapy. 10(4):340-52.
56. Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., Wolffe, A.P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19, 187-191.
57. Kitada M, Dezawa M. (2009) Induction system of neural and muscle lineage cells from bone marrow stromal cells; a new strategy for tissue reconstruction in degenerative diseases. Histol Histopathol. 24(5):631-42.
58. 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(5193):2011-5.
59. Kimura H, Shiota K. (2003) Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1. J Biol Chem. 278(7):4806-12.
60. Khanna KK, Lavin MF, Jackson SP, Mulhern TD. (2001) ATM, a central controller of cellular responses to DNA damage. Cell Death Differ. 8(11):1052-65.
61. Klose, R.J., Sarraf, S.A., Schmiedeberg, L., McDermott, S.M., Stancheva, I., Bird, A.P. (2005) DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. Mol Cell 19, 667-678
62. Kokura, K., Kaul, S.C., Wadhwa, R., Nomura, T., Khan, M.M., Shinagawa, T., Yasukawa, T., Colmenares, C., Ishii, S. (2001) The Ski protein family is required for MeCP2-mediated transcriptional repression. J Biol Chem 276, 34115-34121.
63. Kosztowski T, Zaidi HA, Quiñones-Hinojosa A. (2009) Applications of neural and mesenchymal stem cells in the treatment of gliomas. Expert Rev Anticancer Ther. 9(5):597-612. Review.
64. Kholodenko BN. (2000) Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades. Eur J Biochem. 267(6):1583-8.
65. Krause, DS. (2002) Plasticity of marrow-derived stem cells. Gene Therapy 9, 754-758.
66. Krepischi, A.C., Kok, F., Otto, P.G. (1998) X chromosome-inactivation patterns in patients with Rett syndrome. Hum Genet 102, 319-321.

67. Kriaucionus S, B.A. (2004) The major form of MeCP2 has a novel N-terminus generated by alternative splicing. Nucleic Acids Res. 32(35):1818-1823.
68. 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. 2000 Oct;113 (Pt 20):3613-22.
69. La Salle, S., Mertineit, C., Taketo, T., Moens, P.B., Bestor, T.H., Trasler, J.M. (2004) Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells. Dev Biol 268, 403-415
70. Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R., Li, E. (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122, 3195-3205
71. Levy YS, Merims D, Panet H, Barhum Y, Melamed E, Offen D. (2003) Induction of neuron-specific enolase promoter and neuronal markers in differentiated mouse bone marrow stromal cells. J Mol Neurosci. 21(2):121-32.
72. Li LC, Dahiya R. (2002) MethPrimer: designing primers for methylation PCRs. Bioinformatics. 18(11):1427-31.
73. Lois, C., Alvarez-Buylla, A. (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A 90, 2074-2077.
74. Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, Alt FW. (2005) DNA repair, genome stability, and aging. Cell. 2005 120(4):497-512.
75. Matijevic, T., Knezevic, J., Slavica, M., Pavelic, J. (2009) Rett syndrome: from the gene to the disease. Eur Neurol 61, 3-10
76. Mikkers H, Frisén J. (2005) Deconstructing stemness. EMBO J. 2005 ;24(15):2715-9.
77. Miranda, T.B., Jones, P.A. (2007) DNA methylation: the nuts and bolts of repression. J Cell Physiol 213, 384-390.
78. Molofsky AV, Pardal R, Morrison SJ. (2005) Diverse mechanisms regulate stem cell self-renewal. Curr Opin Cell Biol. 16(6):700-7
79. Mnatzakanian, G.N., Lohi, H., Munteanu, I., Alfred, S.E., Yamada, T., MacLeod, P.J., Jones, J.R., Scherer, S.W., Schanen, N.C., Friez, M.J., Vincent, J.B., Minassian, B.A. (2004) A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to Rett syndrome. Nat Genet 36, 339-341
80. Mullen RJ, Buck CR, Smith AM. (1992) NeuN, a neuronal specific nuclear protein in vertebrates. Development. 116(1):201-11.
81. Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., Bird, A. (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393, 386-389
82. Narita M, Nũnez S, Heard E, Narita M, 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(6):703-16.
83. Nilsson SK, Dooner MS, Weier HU, Frenkel B, Lian JB, Stein GS, Quesenberry PJ. (1999) Cells capable of bone production engraft from whole bone marrow transplants in nonablated mice. J Exp Med. Feb 15;189(4):729-34.
84. 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(1):241-9.
85. Park KS, Lee YS, Kang KS. (2006) In vitro neuronal and osteogenic differentiation of mesenchymal stem cells from human umbilical cord blood. J Vet Sci. 7(4):343-8.
86. 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(15):2247-56.
87. Oberdoerffer P, Sinclair DA. (2007) The role of nuclear architecture in genomic instability and ageing. Nat Rev Mol Cell Biol. 8(9):692-702.
88. Ohlstein B, Kai T, Decotto E, Spradling A. (2004) The stem cell niche: theme and variations. Curr Opin Cell Biol. 16(6):693-9.
89. Peddada, S., Yasui, D.H., LaSalle, J.M. (2006) Inhibitors of differentiation (ID1, ID2, ID3 and ID4) genes are neuronal targets of MeCP2 that are elevated in Rett syndrome. Hum Mol Genet 15, 2003-2014
90. Preston, S.L., Alison, M.R., Forbes, S.J., Direkze, N.C., Poulsom, R., Wright, N.A. (2003) The new stem cell biology: something for everyone. Mol Pathol 56, 86-96
91. Prockop DJ., (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. Apr 4;276(5309):71-4.
92. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. (2002) "Stemness": transcriptional profiling of embryonic and adult stem cells. Science. 298(5593):597-600.
93. Renieri, A., Meloni, I., Longo, I., Ariani, F., Mari, F., Pescucci, C., Cambi, F. (2003) Rett syndrome: the complex nature of a monogenic disease. J Mol Med 81, 346-354
94. Rett, A. (1966) On a unusual brain atrophy syndrome in hyperammonemia in childhood. Wien Med Wochenschr 116, 723-726.
95. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. (2004) Rational siRNA design for RNA interference. Nat Biotechnol. 22(3):326-30.
96. Rhee, I., Bachman, K.E., Park, B.H., Jair, K.W., Yen, R.W., Schuebel, K.E., Cui, H., Feinberg, A.P., Lengauer, C., Kinzler, K.W., Baylin, S.B., Vogelstein, B. (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416, 552-556
97. Robertson, K.D. (2005) DNA methylation and human disease. Nat Rev Genet 6, 597-610.
98. Ronen A, Glickman BW. (2001) Human DNA repair genes. Environ Mol Mutagen. 37(3):241-83.
99. Scadden DT. (2006) The stem-cell niche as an entity of action. Nature. 441(7097):1075-9.
100. Shahbazian, M.D., Zoghbi, H.Y. (2002) Rett syndrome and MeCP2: linking epigenetics and neuronal function. Am J Hum Genet 71, 1259-1272
101. Sharp, A., Kusz, K., Jaruzelska, J., Tapper, W., Szarras-Czapnik, M., Wolski, J., Jacobs, P. (2005) Variability of sexual phenotype in 46,XX(SRY+) patients: the

influence of spreading X inactivation versus position effects. J Med Genet 42, 420-427.
102. Singh J, Saxena A, Christodoulou J, Ravine D. (2008) MECP2 genomic structure and function: insights from ENCODE. Nucleic Acids Res. 36(19):6035-47.
103. Suzuki M, Yamada T, Kihara-Negishi F, Sakurai T, Hara E, Tenen DG, Hozumi N, Oikawa T. (2006) Site-specific DNA methylation by a complex of PU.1 and Dnmt3a/b. Oncogene. 25(17):2477-88.
104. Takahashi, N., Miura, I., Kobayashi, Y., Kume, M., Yoshioka, T., Otane, W., Ohtsubo, K., Takahashi, K., Kitabayashi, A., Kawabata, Y., Hirokawa, M., Nishijima, H., Ichinohasama, R., Decoteau, J., Miura, A.B., Sawada, K. (2005) Fluorescence in situ hybridization monitoring of BCR-ABL-positive neutrophils in chronic-phase chronic myeloid leukemia patients during the primary stage of imatinib mesylate therapy. Int J Hematol 81, 235-241.
105. Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell.; 126(4):663-76.
106. Tao H, Rao R, Ma DD. (2005) Cytokine-induced stable neuronal differentiation of human bone marrow mesenchymal stem cells in a serum/feeder cell-free condition. Dev Growth Differ. 200;47(6):423-33.
107. Tatematsu KI, Yamazaki T, Ishikawa F. (2000) MBD2-MBD3 complex binds to hemi-methylated DNA and forms a complex containing DNMT1 at the replication foci in late S phase. Genes Cells. 5(8):677-88.
108. Trevathan E, Naidu S. (1988) The clinical recognition and differential diagnosis of Rett syndrome. J Child Neurol, ;3 Suppl:S6-16. Review.
109. Vaissiere, T., Sawan, C., Herceg, Z. (2008) Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res 659, 40-48.
110. von Zglinicki T, Bürkle A, Kirkwood TB. (2001) Stress, DNA damage and ageing an integrative approach. Exp Gerontol. 36(7):1049-62.
111. Valk-Lingbeek ME, Bruggeman SW, van Lohuizen M. (2004) Cell. 118(4):409-18.
112. von Zglinicki T, Bürkle A, Kirkwood TB. (2001) Stress, DNA damage and ageing an integrative approach. Exp Gerontol. 36(7):1049-62.
113. Wagers, A.J., Weissman, I.L. (2004) Plasticity of adult stem cells. Cell 116, 639-648
114. Weaving, L.S., Ellaway, C.J., Gecz, J., Christodoulou, J. (2005) Rett syndrome: clinical review and genetic update. J Med Genet 42, 1-7
115. Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M., Schubeler, D. (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39, 457-466
116. Williamson, S.L., Christodoulou, J. (2006) Rett syndrome: new clinical and molecular insights. Eur J Hum Genet 14, 896-903
117. Wislet-Gendebien S, Leprince P, Moonen G, Rogister B. (2003) Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. J Cell Sci.; 116 (Pt 16): 3295-302.
118. Yasui DH, Peddada S, Bieda MC, Vallero RO, Hogart A, Nagarajan RP, Thatcher KN, Farnham PJ, Lasalle JM. (2007) Integrated epigenomic analyses of neuronal

MeCP2 reveal a role for long-range. Proc Natl Acad Sci U S A. 104(49):19416-21. Epub 2007 Nov 27.

119. Young, J.I., Hong, E.P., Castle, J.C., Crespo-Barreto, J., Bowman, A.B., Rose, M.F., Kang, D., Richman, R., Johnson, J.M., Berget, S., Zoghbi, H.Y. (2005) Regulation of RNA splicing by the methylation-dependent transcriptional repressor methyl-CpG binding protein 2. Proc Natl Acad Sci U S A 102, 17551-17558
120. Yu, J., Palmer, C., Alenghat, T., Li, Y., Kao, G., Lazar, M.A. (2006) The corepressor silencing mediator for retinoid and thyroid hormone receptor facilitates cellular recovery from DNA double-strand breaks. Cancer Res 66, 9316-9322.
121. Yu K, Ganesan K, Tan LK, Laban M, Wu J, Zhao XD, Li H, Leung CH, Zhu Y, Wei CL, Hooi SC, Miller L, Tan P. (2008) A precisely regulated gene expression cassette potently modulates metastasis and survival in multiple solid cancers. PLoS Genet. 4(7):e1000129.
122. Zappella, M., Meloni, I., Longo, I., Canitano, R., Hayek, G., Rosaia, L., Mari, F., Renieri, A. (2003) Study of MECP2 gene in Rett syndrome variants and autistic girls. Am J Med Genet B Neuropsychiatr Genet 119B, 102-107
123. Zappella, M., Meloni, I., Longo, I., Hayek, G., Renieri, A. (2001) Preserved speech variants of the Rett syndrome: molecular and clinical analysis. Am J Med Genet 104, 14-22
124. 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(5):1230-48.
125. Zysman L, Lotan M, Ben-Zeev B. (2006) Osteoporosis in Rett syndrome: A study on normal values. ScientificWorldJournal. 6:1619-30.
126. Kim NW, Wu F (1997), Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res.;25(13):2595-7.
127. Maniatis et al., (1983) "DNA Isolation and Sequencing" (Essential Techniques Series), Published by John Wiley & Sons.
128. Matarazzo V, Cohen D, Palmer AM, Simpson PJ, Khokhar B, Pan SJ, Ronnett GV (2004), The transcriptional repressor Mecp2 regulates terminal neuronal differentiation. Mol Cell Neurosci; 27(1):44-58.
129. Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH (2003) Visualizing superoxide production in normal and diabetic rat islets of Langerhans. J Biol Chem. 278(11):9796-801.
130. Diggle CP, Bentley J, Kiltie AE (2003) Development of a rapid, small-scale DNA repair assay for use on clinical samples. Nucleic Acids Res. 31(15):e83.
131. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science. 279(5356):1528-30.

List of publications

Frequency of the LRRK2 G2019S mutation in Italian patients affected by Parkinson's disease.
Squillaro T, Cambi F, Ciacci G, Rossi S, Ulivelli M, Malandrini A, Mencarelli MA, Mari F, Renieri A, Ariani F.
J Hum Genet. 2007;52(3):201-4. Epub 2007 Jan 18.

A case report: bone marrow mesenchymal stem cells from a Rett syndrome patient are prone to senescence and show a lower degree of apoptosis.
Squillaro T, Hayek G, Farina E, Cipollaro M, Renieri A, Galderisi U.
J Cell Biochem. 2008 Apr 15;103(6):1877-85.

Histone Deacetylase inhibitors promote apoptosis and senescence in human mesenchymal stem cells.
Di Bernardo G, **Squillaro T**, Dell'aversana C, Miceli M, Cipollaro M, Cascino A, Altucci L, Galderisi U.
Stem Cells Dev. 2009 May;18(4):573-81.

In Vitro Senescence of Rat Mesenchymal Stem Cells is Accompanied by Downregulation of Stemness-Related and DNA Damage Repair Genes.
Galderisi U, Helmbold H, **Squillaro T**, Alessio N, Komm N, Khadang B, Cipollaro M, Bohn W, Giordano A.
Stem Cells Dev. 2008 Dec 19. [Epub ahead of print].

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Mesenchymal stem cells effectively reduce surgically induced stenosis in rat carotid.
Forte A, Finicelli M, Mattia M, Berrino L, Rossi F, De Feo M, Cotrufo M, Cipollaro M, Cascino A, Galderisi U.
J Cell Physiol. 2008 Dec;217(3):789-99.

Genes involved in regulation of stem cell properties: Studies on their expression in a small cohort of neuroblastoma patients.
Melone MA, Giuliano M, **Squillaro T**, Alessio N, Casale F, Matteoli E, Cipollaro M, Giordano A, Galderisi U.
Cancer Biol Ther. 2009 Jul 30;8(13). [Epub ahead of print].

Partial silencing of Methyl Cytosine Protein Binding 2 (*MECP2*) in mesenchymal stem cells induces senescence along with increase of damaged DNA.
Tiziana Squillaro, Nicola Alessio, Marilena Cipollaro, Alessandra Renieri, Antonio Giordano and Umberto Galderisi [Submitted to FASEB Journal].

The BRG1 ATPase of chromatin remodelling complexes is involved in modulation of mesenchymal stem cell senescence through RB-P53 pathways.
Nicola Alessio, **Tiziana Squillaro**, Marilena Cipollaro, Luigi Bagella, Antonio Giordano and Umberto Galderisi [Submitted to Stem Cells].