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The Role of DNA Damage and Repair Proteins in Adipose-Derived Adult Stem Cell Differentiation in Neural- Like Cells

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Abstract

The development of a clinically translatable method of engineering with adipose-derived adult stem (ADAS) for reconstruction requires investigation of several components. The differentiation of ADAS cells into neuronal cells has been reported by several groups. The stringent maintenance of genomic stability in adult stem cells via anti-stress defenses and DNA repair mechanisms is particularly important because any genetic alteration can compromise the genomic stability and functionality of the cell. The main objective of this data was to examine some parameters related to DNA damage in cells submitted to the neural differentiation protocol and to understand if DNA damage can be associated to cell differentiation. The comet assay, micronucleus tests, and the cell viability assay were utilized to observed ADAS cells treated with neural induction medium. The results of our genotoxicity assays suggest that increased DNA damage observable by the comet assay was induced by neural differentiation. Emerging findings suggest that DNA damage; telomerase and DNA repair proteins play important roles in neurogenesis developing. Surprisingly we obtain evidence for an association between DNA damage and neuronal-like differentiation and hypothesize that during neural differentiation DNA damage will recruit telomerase TIP60 and MCM3, where they may function in DNA repair, chromatin remodeling and limiting DNA replication.

Keywords: Adipose-Derived adult stem cell (ADAS); DNA damage; Differentiation; Neural- like cells

Introduction

Recently, human adult stem cells have been developed as important tools for several cell-based therapies, some of which include tissue engineering approaches. Human mesenchymal stem cells (MSCs) are among the most promising candidates for such therapeutic approaches. Human adipose-derived stem (ADAS) cells display typical human MSC characteristics, including a common surface marker profile and differentiation potential *in vitro* and *in vivo* [1]. Murine and human ADAS cells undergo morphologic and phenotypic changes consistent with neuronal differentiation [2]; thus, adipose tissue may represent a source of cells that are capable of neuronal differentiation and might be used successfully in the treatment of various neurological diseases. The differentiation of ADAS cells into neural-like cells has been reported by several groups [2-5].

One important characteristic of ADAS cells is their maintenance of genomic stability during the differentiation process. The stringent maintenance of genomic stability in adult stem cells via anti-stress defenses and DNA repair mechanisms is particularly important because any genetic alteration can compromise the genomic stability and functionality of an entire cell lineage. It is well-documented that DNA is continuously subject to damage from endogenous and exogenous sources [6]; to prevent the deleterious consequences of DNA damage, cells have evolved several DNA repair pathways to remove DNA mismatches and lesions [6-10]. In cases of persistent unrepaired DNA damage, cells induce complex signaling pathways that culminate in senescence or apoptosis. Interestingly, defects in DNA repair, cellular senescence and/or apoptosis have been implicated in cancer and aging [11-13]. Some proteins implicated in DNA repair also act in diverse biological processes such as apoptosis and senescence. For example, the ataxia-telangiectasia mutated (ATM) and Rad3-related (ATR) proteins are stress-response kinases that respond to a variety of insults including

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ionizing radiation, replication arrest, ultraviolet radiation and hypoxia/re-oxygenation [14]. ATM is activated by DNA double-strand breaks (DSBs) caused by agents such as ionizing radiation or chemotherapeutic drugs; ATR is activated by stresses such as hydroxyurea treatment, ultraviolet light (UV) and hypoxia that induce replication-type insults [14].

The regulation of gene expression can also be modified by epigenetic alterations such as chromatin remodeling and DNA methylation [15]. Chromatin structure plays a key role in most processes involving DNA metabolism, including transcriptional regulation and DNA replication, and the epigenetic control of DNA metabolism relies on different histone proteins that comprise a histone code. This code, involving phosphorylation, ubiquitylation, sumoylation, acetylation and methylation, regulates chromatin accessibility either by disrupting chromatin contacts or by recruiting non-histone proteins to chromatin [16]. Recent evidence also suggests that a histone code is involved in DNA damage detection and repair. For example, formation of DSBs results in the phosphorylation of histone H2AX (the so-called gamma-H2AX) on the chromatin surrounding the DNA lesion [16]. In addition to H2AX, many other proteins such as TRRAP and TIP60 that affect chromatin structure also regulate the epigenetic control of DNA metabolism [17]. TRRAP is a component of several multiprotein histone acetyltransferases (HAT) complexes that have been implicated in both transcriptional regulation and DNA repair [18]. TIP60 was originally identified as a cellular HAT protein that interacts with the HIV-1-associated protein Tat; it affects the functions of many targets including transcriptional regulators, cell cycle and checkpoint machinery and DNA repair regulators [19]. The MCM2-7 complex acts as a replicative helicase during DNA synthesis and plays a central role in S-phase genome stability [20,21]. Another important structure related to genetic stability is the telomeric region at the extremities of chromosomes. Telomeres are maintained by telomerase, a specialized ribonucleo protein complex that includes an RNA template and a reverse transcriptase catalytic subunit. Telomerase expression is low or absent in most human somatic tissues, whereas it is robust in early proliferative progenitor germ and stem cells [22].

Considering the importance of some proteins for genomic stability during the differentiation process of adult stem cells, the main of this data is examine parameters related to DNA damage in cells submitted to the neural differentiation protocol and understand if the DNA damage is involved in this differentiation cellular. This study was also to observe the viability and integrity of ADAS cells treated with procedures that lead to neural induction. To characterize genomic and cellular integrity during *in vitro* neuronal differentiation, the genotoxic properties of neural inducing medium were analyzed using the comet and micronucleus tests and the MTT cell viability assay. Moreover, the expression of some proteins involved in DNA damage and repair was analyzed during the differentiation process.

Materials and Methods

Human ADAS cell source

Human adipose tissue was obtained from healthy patients who underwent liposuction surgery for aesthetic reasons at the Hospital São Lucas of Pontificia Universidade Católica do Rio Grande do Sul (PUCRS) (Porto Alegre, RS – Brazil). The samples were obtained from patients with informed consent and according to a protocol approved by the Ethics Committee of this University. No diabetes, hepatitis, metabolic diseases or other systemic complications were reported in these donors.

Liposuction tissue was digested with 0.015% type I collagenase, and the stromal cell pellet was obtained by centrifugation (400 x g). The stromal cells were washed three times with PBS and collected each time by centrifugation. The recovered cells were resuspended and plated in control medium containing DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin in tissue culture flasks. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2-4 passages.

Cell culture and neuronal induction procedures

ADAS cells were initially cultured in DMEM supplemented with 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin. After 2-4 passages, the cells were induced to become neuronal cells. In the last passage before the induction, the cells were plated at a density of approximately 8,000 cells/cm^2 and grown for 48 h. All subsequent assays were performed with induced and non-induced (control) cells, in triplicate independent experiments with 4 different donors' cells.

Subconfluent cultures of human ADAS cells were maintained in DMEM supplemented with 10% FBS. Twenty-four hours prior to neuronal induction, the culture medium was replaced with pre-induction medium consisting of DMEM supplemented with 20% FBS and 1 mM β-mercaptoethanol (BME). To initiate neuronal differentiation, the pre-induction medium was removed and the cells were washed with PBS and transferred to neuronal induction medium, which consisted of DMEM supplemented with 2% dimethylsulfoxide (DMSO) and 200 μM butylated hydroxyanisole (BHA). The experiments were performed after 24 h of induction.

Immunocytochemistry, quantification of morphological changes and immunolabeling

Chemically-induced ADAS cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 min. Nonspecific antibody reactions were blocked with 5% horse serum for 10 min at room temperature. The fixed cells were incubated overnight at 4°C with primary antibodies. After three washes, cells were incubated with biotinylated universal secondary antibodies for 10 min at room temperature, washed and incubated for 10 min at room temperature with a streptavidin/peroxidase complex. Diaminobenzidine (0.05%) with nickel chloride (0.04%) was used as chromogen, and the reactions were performed for 6-10 min at room temperature. Omission of primary antibodies served as a negative control and HeLa cells and K-562 cells as a positive control.

The percentage of cells showing specific immunolabeling was quantified in three-ten randomly selected fields at 200× magnification using a bright-field microscope. Approximately 50-100 cells were analyzed; the number of cells exhibiting positive immunolabeling was divided by the total number of cells in the same field as determined by phase optics to obtain the mean percentage of cells that were immunolabeled.

Single-cell gel electrophoresis (Comet assay)

After 24 h of neural induction, the cells were washed with ice-cold PBS and trypsinized with 100 μL trypsin (0.15%). The alkaline comet assay was performed as described elsewhere [23,24], with minor changes. Cells were visually divided into five classes according to tail length: (1) Class 0 cells were undamaged, with no tail; (2) Class 1 cells had a tail shorter than the diameter of the head (nucleus); (3) Class 2 cells had a tail as long as 1-2× the diameter of the head; (4) Class 3 cells had a tail longer than 2× the diameter of the head; and

(5) Class 4 cells had comets with no heads. International guidelines and recommendations for the comet assay consider visual scoring as a valid evaluation method [25,26]. A value (damage index) was assigned to each comet according to its class. Damage index (DI) and damage frequency (DF) were calculated according to Tice et al. [25]. Results are expressed as the mean and standard deviation of three independent experiments.

Micronucleus test

After treatment, the cells were separated from the plate by trypsinization, and the cellular suspension was centrifuged at 15,000 rpm for 5 min. Next, a micronucleus (MN) assay was performed accordingly to Matsuoka et al., with modifications [27,28]. Micronucleated cells were counted in 2000 cells with well-preserved cytoplasm. The identification of micronuclei was carried out according to Fenech [27].

Cell viability analysis using the MTT assay

Cell survival was evaluated by means of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay; this assay is based on the formation of a visible dark blue formazan product by the action of cellular mitochondrial dehydrogenases, which acts as a marker for living cells. Analysis of MTT was performed according to Mosmann [29].

RNA extraction and real-time polymerase chain reaction

Total RNA from a 60 cm^2 plate of ADAS cells induced or non-induced to neural tissue was extracted using the RNeasy mini kit (Qiagen, Valencia, CA) following the cell culture protocol of the manufacturer's instructions. RNA was dissolved in water and spectroscopically quantified at 260 nm using a BioPhotometer 6131 (Eppendorf, Hamburg, Deutschland.). The purity of the RNA was verified by absorbance (A) using the ratio $A_{260\ nm}/A_{280\ nm}$ between 1.80 and 2.06 (mean = 2.0). RNA was reverse transcribed into cDNA using the RT2 First Strand kit (Superarray, Frederick, MD). Primers for nestin (PPH02388A), GFAP (PPH02408E) and NSE (PPH02058A) were purchased from Superarray (available at www.superarray.com). Primers for telomerase were CAGCTTTTCCTCACCAGGAG (F) and GTACAGGGCACACCTTTGGT (R). qPCR reactions were carried out using SYBR Green polymerase chain reaction master mix (SuperArray, Frederick, MD) in an iCycler (BioRad, Hercules, CA). The PCR conditions were as follows: 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A final stage of 1 min at 95°C, 2 min at 65°C and 65°C to 95°C at 2°C/ sec was used to determine the dissociation curves of the amplified products. For each measurement, a threshold cycle value (Ct) was determined. This value was defined as the number of cycles necessary to reach the point at which the fluorescent signal was first recorded as statistically significant above background. $2^{-\Delta\Delta CT}$ values ≤0.5 or ≥1.5 were considered significant for down- or up- regulated levels, respectively. The mRNA level relative to the β2-microglobulin (PPH01094E) level was analyzed using the comparative critical threshold (ΔΔCt) method, in which the amount of target RNA is adjusted to a reference (internal target RNA). The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method as previously described [30].

Results

ADAS cells undergo neurogenic differentiation *in vitro*

Induction of ADAS cells using the Woodbury's protocol, which uses β-mercaptoethanol (BME), dimethylsulfoxide (DMSO) and butylated hydroxyanisole (BHA) caused responsive cells to assume neuronal morphological characteristics within 24 h of induction [31]. In induced ADAS cell cultures, we observed changes in morphology in most cells (90-95%) by optical microscopy (Figure 1). Overall, the cells developed neuronal morphological characteristics. To determine whether the morphological changes were related to neural induction, the expression of neuronal markers (nestin, NSE, GFAP, and S100) was measured by immunohistochemistry (Figure 2). Immunocytochemistry is the best methodology for this type of experiments, it was possible observed the morphology of the cells, the relative intensity of the protein expression in with cells, cell's localization of protein expression and the relative quantify of protein expression in the sample. Expression of NSE was observed in most (78%) of the cells with neuronal morphological characteristics, and nestin expression was observed in 8% of such cells. Expression of GFAP was found in less than 1% of differentiated cells, and S100 was not observed in these cells. The levels of nestin, GFAP and NSE mRNAs in these cells were quantified by qRT-PCR (Figure 3). GFAP and NSE expression was significantly upregulated in induced cells ($P< 0.02$); however, nestin mRNA expression was quite variable in the samples analyzed.

Genotoxicity assays

The comet assay is a versatile technique for detecting a wide variety of DNA-altering lesions. Electrophoresis at alkaline pH facilitates the detection of single- and double-strand breaks, incomplete excision repair sites and cross-links [23,32]. When the comet assay was performed with induced and control cells, it was possible to observe an increase in damage frequency and damage index, especially in neuronal-induced cells (Table 1). This observation indicates that the neuronal differentiation used in this work could lead to an increase in DNA damage of ADAS cells.

In the micronuclei test, differences between neuronal-induced and control cells were not observed; both showed a micronucleus frequency of 0.001% per 1,000 cells. In addition, the MTT assay did not reveal any differences between control and neural-induced cells (Figure 4), indicating that treatment with this induction medium does not interfere with cell viability.

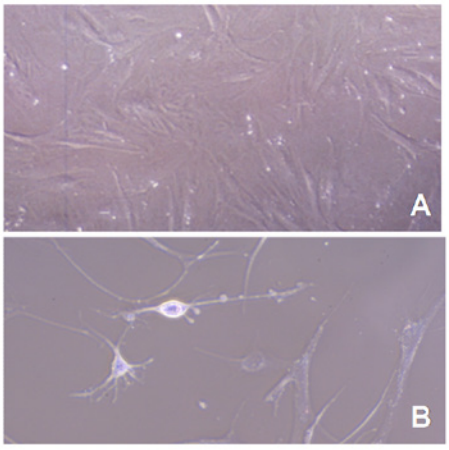
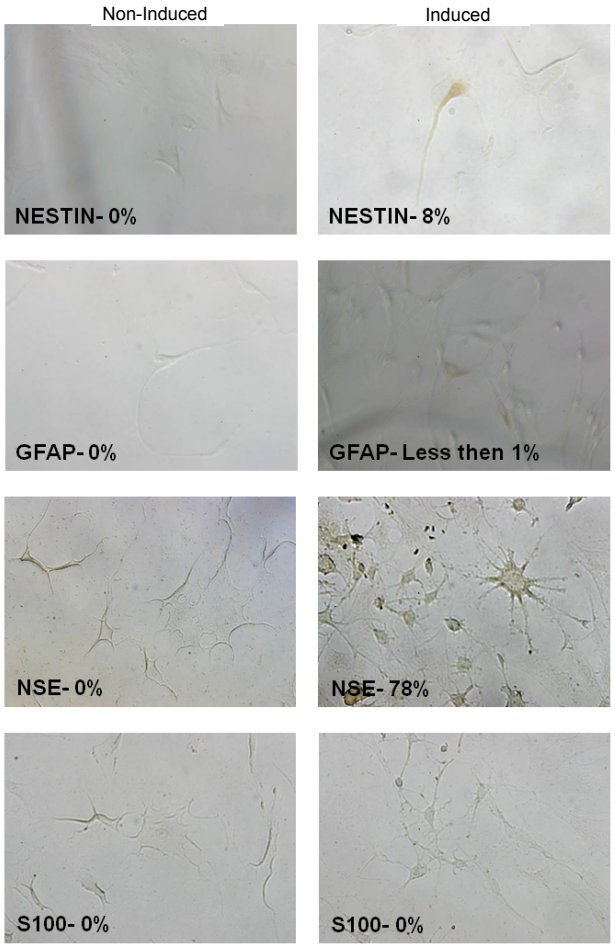


Figure 1: Morphological changes following neuronal induction of ADAS cells. **A:** Under control conditions (DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin), ADAS cells grow as a monolayer of large, flat cells. **B:** After 24 h of culture in neuronal induction medium, ADAS cells display cytoplasmic retraction and a spherical cell body appearance. All images were obtained at 200X magnification.

Expression of proteins related to DNA damage, chromatin remodeling and DNA replication

After 24 h of neural induction, the expression of ATM, ATR, TRRAP, TIP60, MCM2, MCM3, and gamma-H2AX was analyzed by immunocytochemistry (Figure 5). The expression of ATM, ATR, TRRAP, MCM2 and gamma-H2AX were not observed differences in non-induced and induced cells. However, though TIP60 expression showed cytoplasmic *foci* expression in both non-induced and induced cells, it was security more highly expressed in induced cells (Figure 5). MCM3 was expressed in both induced and non-induced cells, but the nuclear expression of MCM3 was more intense in induced cells (Figure 5). This experiment was conducted in triplicate independent experiments with 4 different donors cells.



* GFAP (glial fibrillary acidic protein), NSE (neuron-specific enolase)

Figure 2: Immunocytochemistry of ADAS cells to assess neuronal and glial markers. **Non-induced** - ADAS cells grown under control conditions in DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin. **Induced** - ADAS cells after 24 h culture in neuronal induction medium. Diaminobenzidine (DAB) served as a brown chromagen.The percentage of cells showing specific immunolabeling was quantified in three randomly selected fields at 200X with a bright field microscope. The number of cells positive for immunolabeling was divided by the total number of cells in the same field as determined by phase optics to obtain the percentage of cells that were immunolabeled. All images were obtained at 200X magnification. NESTIN (marker of immature neural cells), GFAP (mature glial cells - astrocyte), NSE (early neuronal) e S100 (nuclei of astrocytes and Schwann cells).

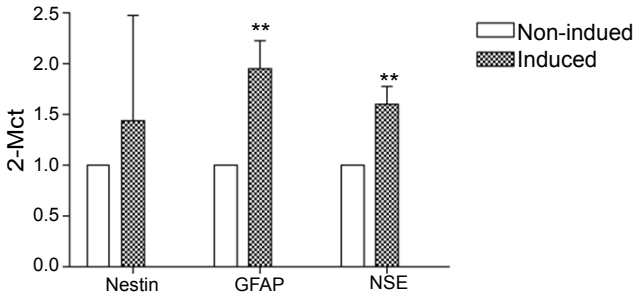


Figure 3: Expression of "neuronal marker" genes in non-induced and induced ADAS cells. Non-Induced - ADAS cells grown under control conditions in DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin. **Induced**- ADAS cells after 24 h culture in neuronal induction medium. **(P<0.003).

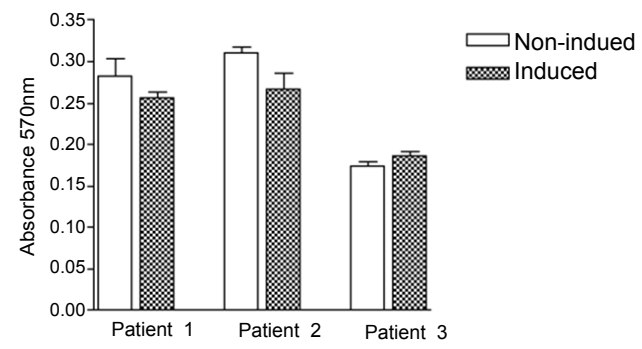


Figure 4: Cell viability after 24 h exposure to neural induction medium. The relative number of cells per well was determined using the MTT assay. The data presented are the mean percentage cell viability ± SD. **Non-induced** - ADAS cells grown under control conditions in DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin. **Induced-** ADAS cells after 24 h culture in neuronal induction medium. The comparative means of non-induced and induced patients resulted in non significative difference with $P < 0.464$ at *t*-student test.

	Damage frequency (DF)		Damage index (DI)	
Patients	**Non-induced**	**Induced**	**Non-induced**	**Induced**
1	13.0 ± 5.44	23.8 ± 6.61	17.5 ± 3.88	33.0 ± 3.74
2	25.0 ± 10.80	51.5 ± 9.74	26.8 ± 12.20	72.8 ± 26.40
3	22.3 ± 9.07	67.0 ± 14.40	24.0 ± 11.10	165.6 ± 66.90
Means	20.11 ± 8.44	47.43 ± 10.26*	22.77 ± 9.06	90.49 ±32.34*

*Significant difference as compared to non-induced control group at $P < 0.01$/*t*-student test

Table 1: Evaluation of DNA damage by the comet assay. **Non-induced** - ADAS cells grown under control conditions in DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin **Induced -** ADAS cells after 24 h culture in neuronal induction medium.

Expression of telomerase mRNA

Telomerase mRNA expression was analyzed by qRT-PCR, as shown in Figure 6. Telomerase expression was significantly upregulated in induced cells (P=0.0026). This result demonstrates that neural induction medium increases telomerase expression in ADAS cells. This

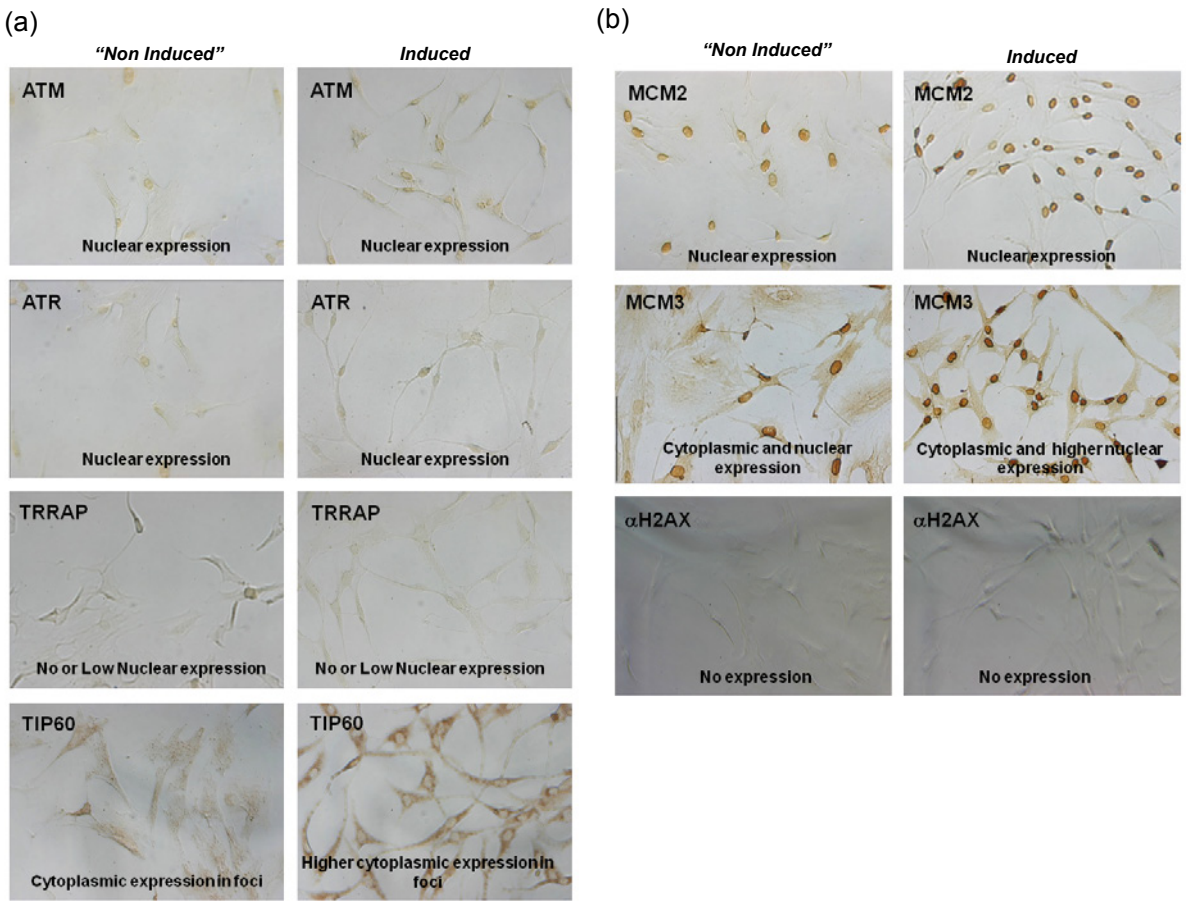


Figure 5: Expression of proteins involved in DNA damage signaling, chromatin remodeling and proliferation as measured by immunocytochemistry. Non-induced - ADAS cells grown under control conditions in DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin. **Induced** - ADAS cells after 24 h culture in neuronal induction medium. Diaminobenzidine (DAB) served as a brown chromagen. All images were obtained at 200X magnification.

experiment was conducted in triplicate independent experiments, with 4 different donors' cells.

Discussion

Our observations indicate that ADAS cells retain the capacity to express specific proteins of non-mesenchymal derivatives, specifically neurons, suggesting that intrinsic genomic mechanisms of commitment, lineage restriction, and cell fate can be reprogrammed using specific culture medium. After 24 hours of exposure to neuronal inducing medium, morphological changes in ADAS cells similar to those described by Woodbury et al. were observed [31]. Our experiments confirm that ADAS cells retain the capacity to express neuronal proteins, nestin and NSE as shown by immunocytochemistry and mRNA GFAP and NSE as shown by qRT-PCR. Several studies using ADAS have reported the simultaneous expression of multiple molecular markers [2-5]. As reviewed in Franco-Lambert et al., the simultaneous expression of nestin (a marker of immature neural cells) and NSE (a marker that indicates the presence of mature neural cells) in the same cell is considered unusual [33]. Nevertheless another important issue is the existence of a heterogeneous cell population in the culture; each cell population can express different types of marker and could stay in a different part of neural differentiation [33]. The presence of a pool of heterogeneous cells in the cultures used in our study can explain to the variability of nestin mRNA expression observed. The fact that human ADAS cells express neuron-specific proteins and show increased expression of several early neuronal and glial markers does not absolutely confirm that these cells will ultimately differentiate into mature neurons. However, the main of this data is examine parameters related to DNA damage in cells submitted to the differentiation protocol, not to generate functional neuron, and understand if the DNA damage and proteins related to DNA repair are involved in this differentiation cellular.

It should be noted that the efficacy of neural induction medium is dependent on the chemical compounds present in the medium; strong antioxidant substances like BME and BHA are especially effective inducers. The precise mechanisms by which BME induces neuronal differentiation are unclear. Its antioxidant properties, which enhance neuronal survival *in vitro*, may be partially responsible for neuronal induction [34]. Woodbury et al. (2000) treated bone marrow stem cells with DMSO, BHA, or butylated hydroxytoluene (BHT) alone and in combination. These treatments elicited neuronal morphologies with a time course similar to that of BME; treatment with 2% DMSO and 200 mM BHA (DMSO/BHA) was the most effective [31]. Lu et al. (2004) showed that exposure to BME results in progressive cell death over time. BHA has cytotoxic effects in different cell lines [35-38]. On the other hand, BME induces protection *in vitro* against DNA fragmentation

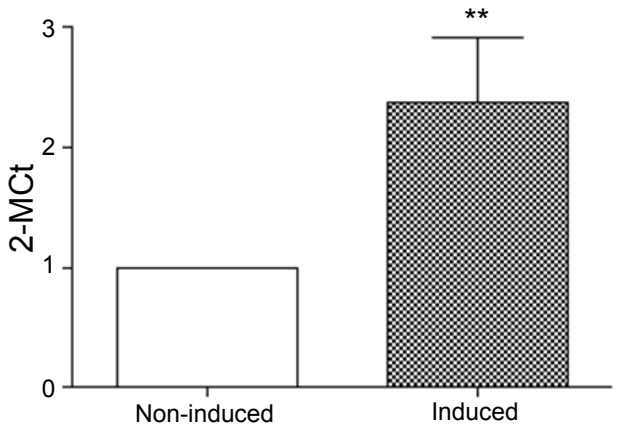


Figure 6: Expression of telomerase mRNA in non-induced and induced ADAS cells. Non-induced- ADAS cells grown under control conditions in DMEM, 10% FBS, 1% streptomycin-penicillin and 0.1% gentamycin. **Induced-** ADAS cells after 24 h of culture in neuronal induction medium. **(P<0.0026).

in stored embryos and can prevent some of the damage induced by oxidative stress [39,40]. BHA is an antimutagenic and antigenotoxic agent *in vitro* [41,42]. Thus, the cytotoxic effects of BME and DMSO/BHA are controversial.

The micronucleus assay has emerged as one of the preferred methods for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably [27]. The study of DNA damage at the chromosomal level is an essential part of genetic toxicology because chromosomal mutations are important events that occur during carcinogenesis. In the micronuclei test, differences were not observed between neuronal-induced and control cells showing a very low micronuclei frequency in both. The maintenance of genomic stability in stem cells is a major requirement for their use in regenerative medicine. Increased mutation rates or an absence of DNA repair may give rise to failures in stem cell differentiation or even cancer stem cell induction. Thus, study of the major proteins related to DNA repair and/or chromatin remodeling is necessary in order to verify the integrity of the stem cells' genome.

The comet assay is a versatile technique for detecting a wide variety of DNA altering lesions, such as single- and double-strand breaks. In the alkaline conditions used, additional DNA structures are detected, indicating DNA damage such as a basic sites (AP sites), missing either a pyrimidine or purine nucleotide, and sites where excision repair is taking place [23,32]. Our results for comet assay reveal that neuronal induction cells show an increase in damage frequency and damage index. Corroborate to this date some authors demonstrated that proliferating neural stem cells as well as newly generated post mitotic neurons may be particularly vulnerable to DNA damage in early development of nervous system [43,44]. The results of our genotoxicity assays suggest that increased DNA damage observable by the comet assay was induced by neural differentiation.

ATM normally exists in an inactive dimeric form; upon DNA damage, it is autophosphorylated and converted to an active, monomeric form that signals the existence of DNA lesions to downstream mediators. One well-characterized target of ATM is the histone variant H2AX, which becomes phosphorylated at its C-terminus almost immediately following DNA-damaging stimuli such as ionizing radiation. The phosphorylated form of H2AX, termed gamma-H2AX, acts as a landmark for DNA repair enzymes. Our results demonstrate that gamma-H2AX expression does not occur in either induced or non-induced cells and that ATM is similarly expressed in induced and non-induced cells. These results indicate that the DNA damage observed in the comet assay is likely not due to DSB. Moreover, these data corroborate the findings of Biton et al. (2007) and suggest that ATM may not play a critical role in neuronal differentiation under the conditions used here [45].

Acetylation of ATM by TIP60 is required for efficient ATM autophosphorylation and upregulation of ATM kinase activity. The catalytic activity of TIP60 is stimulated in response to DNA damage but does not appear to be regulated by ATM. This finding leads to the speculation that TIP60 functions upstream of ATM, sensing DNA damage-caused chromatin changes, which cause it to signal to ATM [19]. Interestingly, an RNAi screen in mouse embryonic stem cells revealed that Tip60 is required for pluripotency, and genome-wide expression analysis of Tip60 depleted embryonic stem cells suggests that Tip60 represses a large number of genes that are expressed during differentiation [46,47]. In our immunocytochemical experiments, TIP60 expression was evident in cytoplasmic *foci* in both non-induced and induced cells (Figure 5), with higher foci expression in induced cells (Figure 5), but there was little or no TIP60 expression in nuclei. It is known that TIP60 is a predominantly nuclear protein [48-51]; however, TIP60 has also been found in the cytoplasm, where it is associated with internalized membrane receptors thus regulating downstream kinase pathways or gene expression [19]. The highly cytoplasmic *foci* expression of TIP60 in induced cells could be related to represses a large number of genes that are expressed during differentiation how related in data of [46,47].

No differences in the expression of MCM2 in induced and non-induced cells were detected by immunocytochemistry (Figure 5). However, strong nuclear expression of MCM3 was visible in the induced cells. The MCM proteins are required for processive DNA replication and are a target of S-phase checkpoints, and their loss causes DNA damage and genome instability. One major mechanism by which MCM proteins promote genome stability is by limiting DNA replication to one round per cell cycle [52]. Because of their crucial role in limiting DNA replication, MCM proteins are potential targets for cellular oncogenes that inhibit or deregulate the replication process. Deregulation of MCM activity may therefore contribute to abnormal cell proliferation and genome instability [53-55]. MCM proteins are highly expressed not only in actively replicating cells but also in cells with the potential to proliferate.

It is generally thought that embryonic stem cells express a high level of telomerase, which maintains telomere length [56]. Because adult stem cells generally tend to give rise to a large number of daughter cells, they might also be expected to express high amounts of telomerase. However, for adult stem cells, the picture is less clear. Highly sensitive telomerase assays have demonstrated very low levels of telomerase in most adult stem cells, and in adult stem cells, telomeres are slowly shortened during life [57]. Like most somatic cells, fully differentiated neurons and astrocytes lack telomerase activity [58,59]. However, when subject to oxidative, hypoxic or excitotoxic stress the expression of telomerase reverse transcriptase (TERT) and telomerase activity increases in astrocytes [60], neurons [61] and/or microglia [59, 62]. The function of this stress-induced telomerase reactivation is not clear, a study suggests that it could be involved in DNA repair and chromatin remodeling [44,63]. Indeed, emerging evidence suggests

that telomerase has additional extratelomeric roles in mediating cell survival and anti-apoptotic functions against various cytotoxic stresses [64,65]. Corroborating to Zhang et al. [44] our results show the level of telomerase mRNA was higher in induced (neural like cells) than in non-induced cells.

Emerging findings suggest that DNA damage, telomerase and DNA repair proteins play important roles in neurogenesis developing, according to Zhang et al. [44] proliferating neural stem cells have high levels of telomerase to maintain telomeres and promote cell survival. Telomerase levels decrease precipitously in neurons soon after they differentiate from neural stem cells, which may contribute to the vulnerability of newly generated neurons to apoptosis [44].

In conclusion, the results obtained in this work suggest an increase in the DNA damage observed by comet assay is induced by neural-like differentiation process. We obtain evidence for an association between DNA damage and neuronal differentiation and hypothesize that during neural-like differentiation DNA damage will recruit telomerase, TIP60 and MCM3, where they may function in DNA repair, chromatin remodeling and limiting DNA replication. More studies of differentiation of stem cells and animal models, and of human disorders, could suggest the potential for targeting of telomere associated molecules, chromatin remodeling and DNA repair proteins as a therapeutic approach for neurological tissue engineering design.

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References

1. Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. Circ Res 100: 1249-1260.
2. Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, et al. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294: 371-379.
3. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, et al. (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13: 4279-4295.
4. Ashjian PH, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, et al. (2003) In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. Plast Reconstr Surg 111:1922-1931.
5. Krampera M, Marconi S, Pasini A, Galie M, Rigotti G, et al. (2007) Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone 40: 382-390.
6. Peterson CL, Cote J (2004) Cellular machineries for chromosomal DNA repair. Genes Dev 18: 602-616.
7. Bohr VA (2002) Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic Biol Med 32: 804-812.
8. Houtgraaf JH, Versmissen J, van der Giessen WJ (2006) A concise review of DNA damage checkpoints and repair in mammalian cells. Cardiovasc Revasc Med 7:165-172.
9. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73: 39-85.
10. Maynard S, Swistowska AM, Lee JW, Liu Y, Liu ST, et al. (2008) Human embryonic stem cells have enhanced repair of multiple forms of DNA damage. Stem Cells 26: 2266-2274.
11. Andressoo JO, Hoeijmakers JH, Mitchell JR (2006) Nucleotide excision repair disorders and the balance between cancer and aging. Cell Cycle 5: 2886-2888.
12. von Zglinicki T, Burkle A, Kirkwood TB (2001) Stress, DNA damage and ageing -- an integrative approach. Exp Gerontol 36:1049-1062.
13. Wilson DM 3rd, Bohr VA (2007) The mechanics of base excision repair, and its relationship to aging and disease. DNA Repair (Amst) 6: 544-559.
14. Hammond EM, Giaccia AJ (2004) The role of ATM and ATR in the cellular response to hypoxia and re-oxygenation. DNA Repair (Amst) 3: 1117-1122.
15. Mathews LA, Crea F, Farrar WL (2009) Epigenetic gene regulation in stem cells and correlation to cancer. Differentiation 78: 1-17.
16. Escargueil AE, Soares DG, Salvador M, Larsen AK, Henriques JA (2008) What histone code for DNA repair? Mutat Res 658: 259-270.
17. Fernandez-Capetillo O, Lee A, Nussenzweig M, Nussenzweig A (2004) H2AX: the histone guardian of the genome. DNA Repair (Amst) 3: 959-967.
18. Herceg Z, Li H, Cuenin C, Shukla V, Radolf M, et al. (2003) Genome-wide analysis of gene expression regulated by the HAT cofactor Trrap in conditional knockout cells. Nucleic Acids Res 31: 7011-7023.
19. Sapountzi V, Logan IR, Robson CN (2006) Cellular functions of TIP60. Int J Biochem Cell Biol 38: 1496-1509.
20. Maiorano D, Lutzmann M, Mechali M (2006) MCM proteins and DNA replication. Curr Opin Cell Biol 18: 130-136.
21. Labib K, Diffley JF (2001) Is the MCM2-7 complex the eukaryotic DNA replication fork helicase? Curr Opin Genet Dev 11: 64-70.
22. Deng Y, Chan SS, Chang S (2008) Telomere dysfunction and tumour suppression: the senescence connection. Nat Rev Cancer 8: 450-458.
23. Hartmann A, Speit G (1997) The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). Toxicol Lett 90: 183-188.
24. Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175: 184-191.
25. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, et al. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35: 206-221.
26. Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, et al. (2007) Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup. Mutat Res 627: 31-35.
27. Fenech M (2000) The in vitro micronucleus technique. Mutat Res 455: 81-95.
28. Matsuoka A, Yamazaki N, Suzuki T, Hayashi M, Sofuni T (1992) Evaluation of the micronucleus test using a Chinese hamster cell line as an alternative to the conventional in vitro chromosomal aberration test. Mutat Res 272: 223-236.
29. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.
30. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
31. Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61: 364-370.
32. Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 26: 249-261.
33. Franco Lambert AP, Fraga Zandonai A, Bonatto D, Cantarelli Machado D, Pegas Henriques JA (2009) Differentiation of human adipose-derived adult stem cells into neuronal tissue: does it work? Differentiation 77: 221-228.
34. Ishii K, Katayama M, Hori K, Yodoi J, Nakanishi T (1993) Effects of 2-mercaptoethanol on survival and differentiation of fetal mouse brain neurons cultured in vitro. Neurosci Lett 163: 159-162.
35. Kirlin WG, Cai J, DeLong MJ, Patten EJ, Jones DP (1999) Dietary compounds that induce cancer preventive phase 2 enzymes activate apoptosis at comparable doses in HT29 colon carcinoma cells. J Nutr 129: 1827-1835.
36. Yu R, Mandlekar S, Kong AN (2000) Molecular mechanisms of butylated hydroxylanisole-induced toxicity: induction of apoptosis through direct release of cytochrome c. Mol Pharmacol 58: 431-437.

37. Sarafian TA, Kouyoumjian S, Tashkin D, Roth MD (2002) Synergistic cytotoxicity of Delta(9)-tetrahydrocannabinol and butylated hydroxyanisole. Toxicol Lett 133: 171-179.

38. Lu P, Blesch A, Tuszynski MH (2004) Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J Neurosci Res 77: 174-191.

39. Takahashi J, Palmer TD, Gage FH (1999) Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. J Neurobiol 38: 65-81.

40. Caamano JN, Ryoo ZY, Thomas JA, Youngs CR (1996) beta-mercaptoethanol enhances blastocyst formation rate of bovine in vitro-matured/in vitro-fertilized embryos. Biol Reprod 55: 1179-1184.

41. Horvathova E, Slamenova D, Bonatti S, Abbondandolo A (1999) Reduction of genotoxic effects of MNNG by butylated hydroxyanisole. Neoplasma 46: 356-362.

42. Slamenova D, Horvathova E, Robichova S, Hrusovska L, Gabelova A, et al. (2003) Molecular and cellular influences of butylated hydroxyanisole on Chinese hamster V79 cells treated with N-methyl-N'-nitro-N-nitrosoguanidine: antimutagenicity of butylated hydroxyanisole. Environ Mol Mutagen 41: 28-36.

43. Gilmore EC, Nowakowski RS, Caviness VS Jr, Herrup K (2000) Cell birth, cell death, cell diversity and DNA breaks: how do they all fit together? Trends Neurosci 23: 100-105.

44. Zhang P, Dilley C, Mattson MP (2007) DNA damage responses in neural cells: Focus on the telomere. Neuroscience 145: 1439-1448.

45. Biton S, Gropp M, Itsykson P, Pereg Y, Mittelman L, et al. (2007) ATM-mediated response to DNA double strand breaks in human neurons derived from stem cells. DNA Repair (Amst) 6: 128-134.

46. Fazzio TG, Huff JT, Panning B (2008) An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. Cell 134: 162-174.

47. Voss AK, Thomas T (2009) MYST family histone acetyltransferases take center stage in stem cells and development. Bioessays 31: 1050-1061.

48. Yamamoto T, Horikoshi M (1997) Novel substrate specificity of the histone acetyltransferase activity of HIV-1-Tat interactive protein Tip60. J Biol Chem 272: 30595-30598.

49. Cao X, Sudhof TC (2001) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip 60. Science 293: 115-120.

50. Gavaravarapu S, Kamine J (2000) Tip60 inhibits activation of CREB protein by protein kinase A. Biochem Biophys Res Commun 269: 758-766.

51. Ran Q, Pereira-Smith OM (2000) Identification of an alternatively spliced form of the Tat interactive protein (Tip60), Tip60(beta). Gene 258: 141-146.

52. Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. Annu Rev Biochem 71: 333-374.

53. Hess GF, Drong RF, Weiland KL, Slightom JL, Sclafani RA, et al. (1998) A human homolog of the yeast CDC7 gene is overexpressed in some tumors and transformed cell lines. Gene 211: 133-140.

54. Freeman A, Morris LS, Mills AD, Stoeber K, Laskey RA, et al. (1999) Minichromosome maintenance proteins as biological markers of dysplasia and malignancy. Clin Cancer Res 5: 2121-2132.

55. Kumagai H, Sato N, Yamada M, Mahony D, Seghezzi W, et al. (1999) A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G1/S transition in mammalian cells. Mol Cell Biol 19: 5083-5095.

56. Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, et al. (2004) Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn 229: 243-258.

57. Serakinci N, Graakjaer J, Kolvraa S (2008) Telomere stability and telomerase in mesenchymal stem cells. Biochimie 90: 33-40.

58. Klapper W, Shin T, Mattson MP (2001) Differential regulation of telomerase activity and TERT expression during brain development in mice. J Neurosci Res 64: 252-260.

59. Flanary BE, Streit WJ (2004) Progressive telomere shortening occurs in cultured rat microglia, but not astrocytes. Glia 45: 75-88.

60. Baek S, Bu Y, Kim H, Kim H (2004) Telomerase induction in astrocytes of Sprague-Dawley rat after ischemic brain injury. Neurosci Lett 363: 94-96.

61. Kang HJ, Choi YS, Hong SB, Kim KW, Woo RS, et al. (2004) Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity. J Neurosci 24: 1280-1287.

62. Fu W, Lee J, Guo Z, Mattson MP (2002) Seizures and tissue injury induce telomerase in hippocampal microglial cells. Exp Neurol 178: 294-300.

63. Masutomi K, Possemato R, Wong JM, Currier JL, Tothova Z, et al. (2005) The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. Proc Natl Acad Sci U S A 102: 8222-8227.

64. Mattson MP, Fu W, Zhang P (2001) Emerging roles for telomerase in regulating cell differentiation and survival: a neuroscientist's perspective. Mech Ageing Dev 122: 659-671.

65. Chung HK, Cheong C, Song J, Lee HW (2005) Extratelomeric functions of telomerase. Curr Mol Med 5: 233-241.