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The DNA glycosylases OGG1 and NEIL3 influence differentiation potential, proliferation, and senescence-associated signs in neural stem cells

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ABSTRACT

Embryonic neural stem cells (NSCs) exhibit self-renewal and multipotency as intrinsic characteristics that are key parameters for proper brain development. When cells are challenged by oxidative stress agents the resulting DNA lesions are repaired by DNA glycosylases through the base excision repair (BER) pathway as a means to maintain the fidelity of the genome, and thus, proper cellular characteristics. The functional roles for DNA glycosylases in NSCs have however remained largely unexplored. Here we demonstrate that RNA knockdown of the DNA glycosylases OGG1 and NEIL3 decreased NSC differentiation ability and resulted in decreased expression of both neuronal and astrocytic genes after mitogen withdrawal, as well as the stem cell marker Musashi-1. Furthermore, while cell survival remained unaffected, NEIL3 deficient cells displayed decreased cell proliferation rates along with an increase in HP1γ immunoreactivity, a sign of premature senescence. Our results suggest that DNA glycosylases play multiple roles in governing essential neural stem cell characteristics.

1. Introduction

The use of stem cell therapy for treatment of many neurology and neurodegenerative-related diseases holds great potential, and many advances have been made towards trying understanding the mechanistic cues governing the ability of these cells to differentiate into several cell types [1–3]. DNA repair is an essential cellular function and it has been postulated that stem cells may display an increased ability to repair DNA damage [4–6]. Oxidative stress inflicted by reactive oxygen species (ROS) represents one of the major menaces to the integrity of the eukaryotic cell genome. Cells from the developing and adult central nervous system (CNS) are especially sensitive cell types as the cells in the adult CNS are post-mitotic to a large extent, and the actual size of the CNS and thus cell numbers must be kept under strict regulation. Thus NSCs have to possess an efficient tolerance to this threat to maintain differentiation potential and survival throughout the developmental process.

DNA repair is also crucial part of a major cellular process, the DNA damage response (DDR). The DDR is a very well orchestrated succession of cellular responses taking place when the cell senses damage to its genome [7–9]. Notably in this context is chromatin conformation change occurring as consequence of histone modifications such as methylation and acetylation of lysine residues [10,11]. Such epigenetic changes may facilitate the access of DNA repair factors to sites of damage and instruct the cell to reassume its normal cell cycle progression [12,13]. The base excision repair (BER) is the main repair pathway dealing with lesions resulting from oxidative stress. The most common base pair modification due to ROS is the 7,8-dihydro-8-oxoguanine (8-oxoG). This is a highly mutagenic DNA lesion that can lead to translation of defective proteins if left unrepaired [14,15]. This type of lesion is primarily removed by the DNA glycosylase OGG1. There are however other members of the DNA glycosylase family, namely NEIL1-3 among a few others, that can be found concomitantly in the cell nucleus and display overlapping functions with OGG1 despite having well defined substrate specificities [16]. Functions for the mammalian proteins NEIL1-2 homologous to *Escherichia coli* FPG and Nei proteins have been well characterized [17,18], while NEIL3 properties have at large remained unclear. Recent reports have demonstrated NEIL3 glycosylase activity [19] highlighting its putative function during embryonic development and early postnatal stages. Increased base excision activity and expression in brain sub-regions containing highly proliferative cells have been convincingly demonstrated strengthening this argument [20]. Paradoxically, oxidative stress is not always necessarily a negative event for the cell or organism [21,22]. When occurring in controlled fashion it can generate positive feedback and contribute to the recruitment of transcription factors and introduction of chromatin modifications

Abbreviations: NSCs, neural stem cells; BER, base excision repair; ROS, reactive oxygen species; DDR, DNA damage response; CNS, central nervous system; HDAC, histone deacetylase; FGF, fibroblast growth factor.

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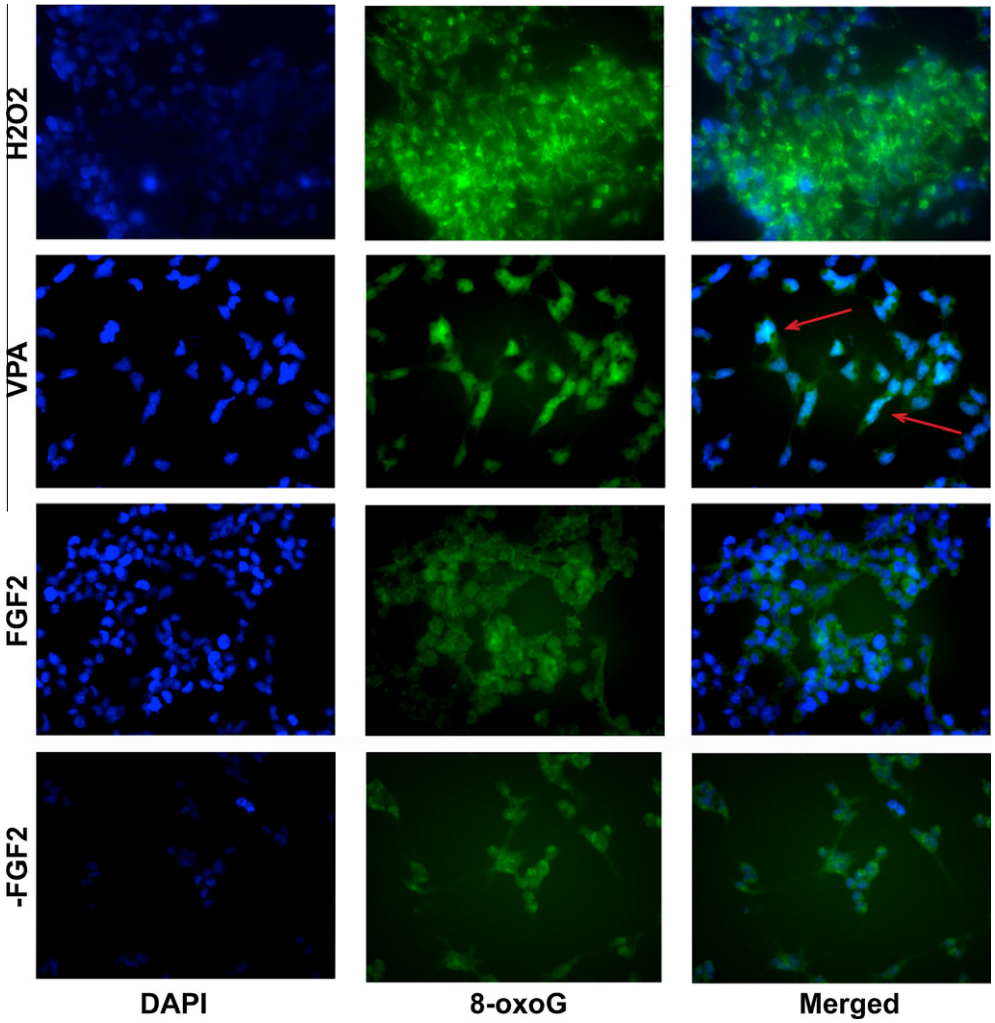

Fig. 1. Increased accumulation of nuclear oxidative damage in valproic acid (VPA) treated neural stem cells (NSCs). Fluorescent photomicrographs displaying results from experiments using FITC-labeled protein conjugate in VPA, FGF2, EtOH (–FGF2), and H_2O_2 (control) conditions. Blue labeling represents staining for the nuclear marker DAPI whereas green labeling represents 8-oxoG reactivity by the fluorescent-tagged 8-oxoG-binding protein.

that support the fine-tuning of cellular processes promoting survival and successful cell specialization [23].

Due to the predicted essential roles for DNA glycosylases in maintaining NSC characteristics and integrity, the aim of this study was to investigate the function of DNA glycosylases in cell survival, proliferation, and differentiation of embryonic NSCs. We found that RNA knockdown of the DNA glycosylases OGG1 and, in particular, NEIL3, resulted in aberrant proliferation as well as neuronal and astrocytic differentiation, along with an increase in certain signs of senescence. Our results suggest that DNA glycosylase activity is required for retaining NSC characteristics.

2. Materials and methods

2.1. Cell culture

Isolation and culture of multipotent neural progenitors, referred to as neural stem cells (NSCs) were performed as previously described [24,25]. Briefly, cortices from rats at embryonic day 15.5 were dissected and mechanically dissociated in a serum-free N2-supplemented DMEM/F12 medium (Invitrogen). The primary cells were plated on 60 mm dishes pre-coated with poly-L-ornithine and fibronectin (Sigma). The cells were treated with human recombinant basic FGF2 (R&D Systems) at 10 ng/ml every 24 h and the N2 medium was replaced every 48 h. All treatments were performed after the first passage when >90% of the cells displayed nestin expression and typical neural stem cell morphology and <1% of the cells expressed neuronal and glial differentiation markers. To achieve enhanced neuronal differentiation, cells were in some experiments treated with valproic acid (VPA) (Sigma) at 1 mM every 24 h for 2 or 3 days.

2.2. Oxy-DNA assay

NSCs were seeded in 6 well plates and treatments were performed according to desired differentiation protocol after achievement of 60% confluence in N2 medium and FGF2 conditions. Following the treatments, the cells were fixed with 10% formalin for 20 min at RT. The 6 well plates were then washed with PBS/Tween-20 and permeabilized by serial washes in methanol prior to washes with PBS/Tween-20. FITC-labeled protein (Biotrin Oxy-DNA test, Biotrin), which binds to 8-oxoG, was added to the fixed cells according to the manufacture's instructions.

2.3. Reverse transcription and quantitative real-time PCR

Total RNA was isolated from the rat NSCs, using RNeasy extraction kit (Qiagen). Samples were treated with RNase-free DNase

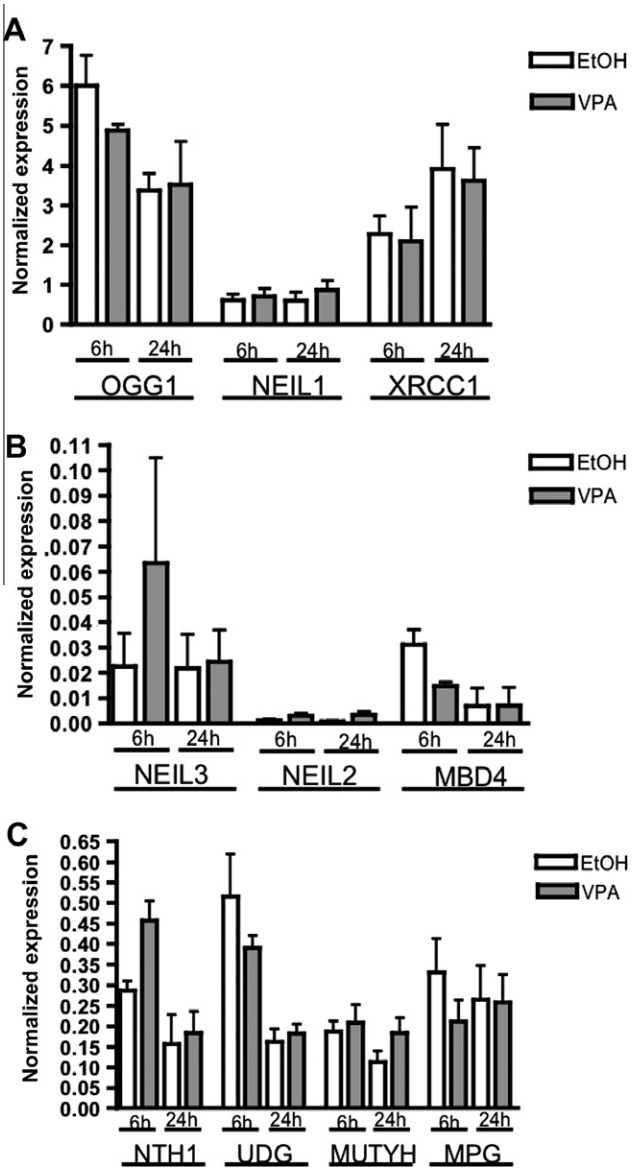

Fig. 2. The expressions of DNA glycosylases and base excision repair-associated genes are not significantly altered during differentiation-enhancing conditions. (A–C) Quantitative RT-PCR results showing effects of 6 h and 24 h VPA treatment in the gene expression of (A) the DNA glycosylases OGG1 and NEIL1, and the DNA repair protein XRCC1, (B) the DNA glycosylases NEIL3 and NEIL2 and the chromatin-binding protein MBD4, and (C) the DNA glycosylases NTH1, UDG, MUTYH, and MPG. Data are shown as mean +/− SEM.

(Qiagen) according to the protocol. cDNA synthesis was performed using superscript III transcriptase kit (Invitrogen). Real time PCR reactions were performed in triplicates each reaction containing a final volume of 25 μl containing a mix of diluted cDNA and platinum Quantitative PCR SuperMix (Invitrogen). Data was normalized to TATA binding protein (TBP). Primer sequences used for TBP, OGG1, NEIL1, NEIL2, NEIL3, NTH1, UDG, MUTYH, MPG, MBD4, XRCC1, DXC, GFAP, Musashi-1, Prominin-1, and Sox2 are all available upon request.

2.4. RNA interference

siRNA was delivered to NSCs by nucleofection using an Amaxa nucleofector according to the manufacture's instructions (Rat NSC kit, VPG-105 program A-33) as previously described [26]. Approximately 4–6 × 10^6 cells and 3 μg of siRNA per nucleofection were used. The nucleofected cells were allowed to recover for at least 12 h in the presence of mitogen (FGF2) prior to treatment and/or RNA extraction. siRNA against ECFP (control) was provided by Dharmacon, and siRNA against OGG1 and NEIL3 by Sigma (sequences available upon request).

2.5. Immunocytochemistry

Primary cultures from rat NSC cells were passaged once and supplemented with 10 μg/ml FGF2 for 2 days with one change of N2 media before stimulation. Cells were fixed in 10% formalin for 20 min at RT and incubated overnight with 1° Ab (HP1γ; AbCam) at 4 °C. Thereafter 2° Ab was incubated for 1 h at RT and one drop of mounting media was placed in each plate before visualizing in the fluorescent microscope (Zeiss Axioscope).

2.6. Cell death assay

NSCs were nucleofected, as previously described, with siRNAs (siECFP, siOGG1, siNEIL3) and thereafter seeded at equal cell densities in individual cell culture dishes. Cell viability was assessed 24 h post nucleofection using the Live/Dead kit (Sigma) according to the manufacturers standard protocol.

2.7. Cell proliferation

Briefly, NSCs were nucleofected using the afore mentioned procedures followed by 15 min cell fixation in 10% formalin and 30 min EdU (Click-iT EdU Kit; Invitrogen) incubation, according to the manufacturers recommendations. The cells were then permeabilized with 0.5% Triton-X-100 during 20 min and after two washes with 3% BSA in PBS, 0.5 ml of Click-iT reaction cocktail (1X Click-iT reaction buffer, $CuSO_4$, Alexa Fluor azide, reaction buffer additive) was added to each well followed by 30 min incubation at room temperature. Finally, cells were washed once with 3% BSA PBS and one drop of mounting media containing DAPI added to each well before visualizing under the fluorescent microscope.

3. Results

3.1. Treatment of neural stem cells with the differentiation-enhancing factor valproic acid leads to increased nuclear accumulation of 8-oxoG

We and others have previously found that treatment of neural stem cells (NSCs) with histone deacetylase inhibitors (HDACi) results in an enhanced neuronal differentiation [25,27]. To initiate the investigation of putative functional roles for DNA glycosylases and associated proteins in embryonic NSCs, we first treated the NSCs with the HDACi valproic acid (VPA; 0.5–1 mM) for 6–48 h and assessed the 8-oxoG formation. This experiment revealed that NSCs treated with VPA tended to accumulate 8-oxoG in the cell nucleus to a larger extent compared to control conditions treated with mitogen (FGF2) or vehicle, thus suggesting an increase in nuclear oxidative damage (Fig. 1).

The increased nuclear oxidative damage in differentiating cells prompted us to perform studies of the gene expression of base excision repair (BER) enzymes and related factors in NSCs treated with VPA for 6 or 24 h compared to withdrawal of mitogen (–FGF2) conditions (Fig. 2) as well as cilliary neurotrophic factor (CNTF) which induces astrocytic differentiation (data not shown), in order to clarify putative roles for DNA glycolylase repair activity in NSC fate decision. We did however not detect any significant differences in the expression of DNA glycoylases or related proteins involved in DNA repair in these differentiating conditions (Fig. 2).

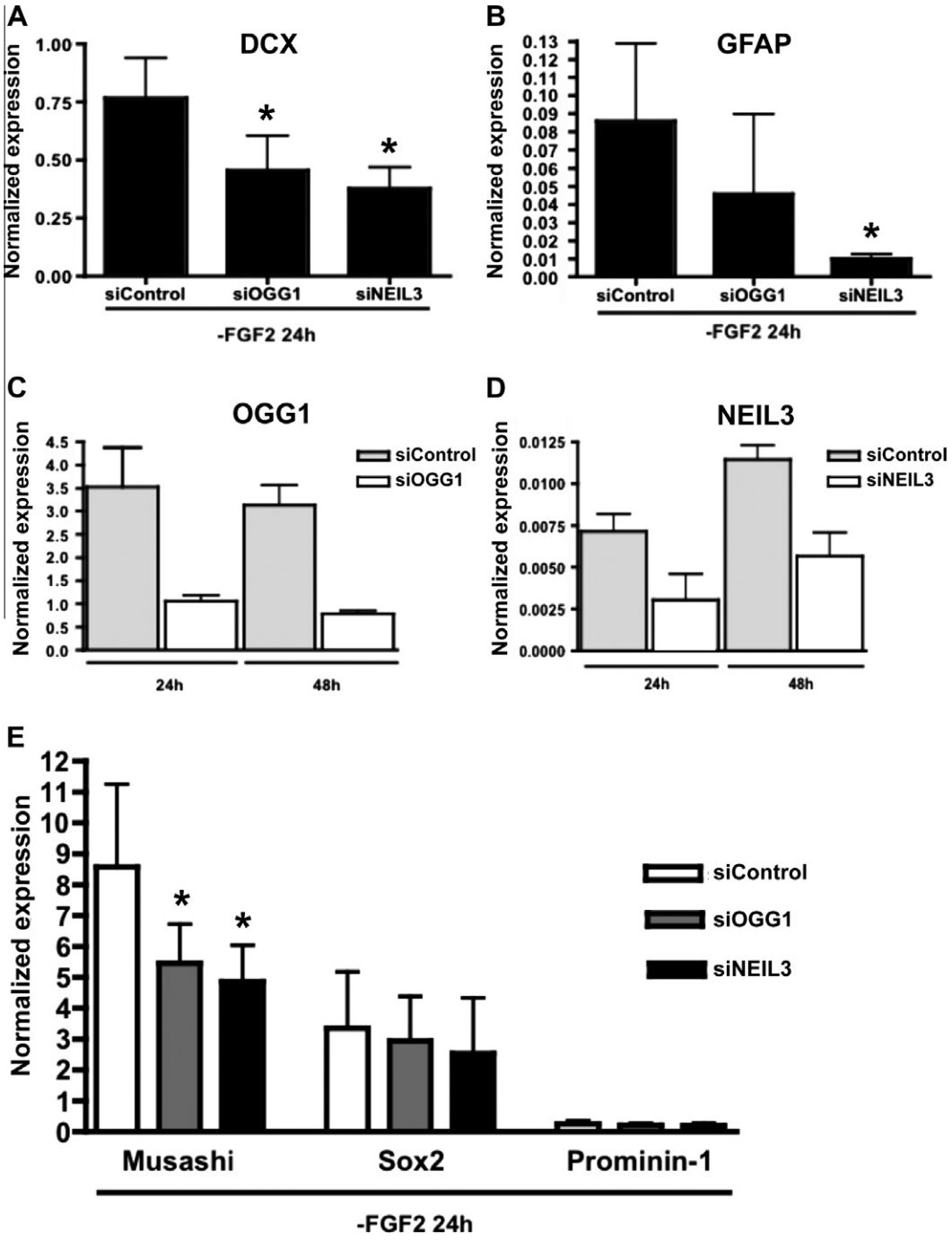

Fig. 3. DNA glycosylase RNA knockdown in NSCs leads to a decrease in the expression of neuronal and astrocytic genes in differentiating conditions and neural stem cell marker Musashi-1 in proliferating conditions. (A and B) Quantitative RT-PCR results showing expression of (A) the early neuronal marker DCX and (B) the astrocytic marker GFAP after 24 h of FGF2 withdrawal. (C–E) Quantitative RT-PCR results showing (C) knockdown efficiency of siOGG1 compared to control siRNA, (D) knockdown efficiency of siNEIL3 compared to control siRNA, and (E) the expression of multipotency genes Musashi-1, Sox2 and Prominin-1 after knockdown of OGG1 or NEIL3 in proliferating conditions. Data are shown as mean +/− SEM. $^{*}p < 0.05$.

3.2. RNA knockdown of the DNA glycosylases OGG1 and NEIL3 results in decreased differentiation potential of neural stem cells

As the gene expression studies failed to identify any strong candidate for increased BER or DNA repair activity in spite of the increased oxidative damage identified after VPA-enhanced neuronal differentiation, we went further to more in detail investigate the role in NSC differentiation progress of NEIL3, recently suggested to be implicated in early stages of neural differentiation [20], and OGG1, which is by default the main DNA glycosylase in charge of removing 8-oxoG and previously demonstrated to be closely linked to activity of the demethylase LSD1 [23]. RNA knockdown of NEIL3 and OGG1 under mitogen withdrawal (–FGF2) resulted in decrease of markers of both neuronal and astrocytic differentiation of NSCs. A significant decrease in the gene expression of doublecortin (DCX), an early marker for neuronal differentiation, and GFAP (glial fibrilary acidic protein), a marker for astrocytic differentiation compared to control siRNA was found after NEIL3 RNA knockdown (Fig. 3A and B). These results suggested a role for DNA glycosylase activity in differentiation potential rather than lineage decision of NSCs.

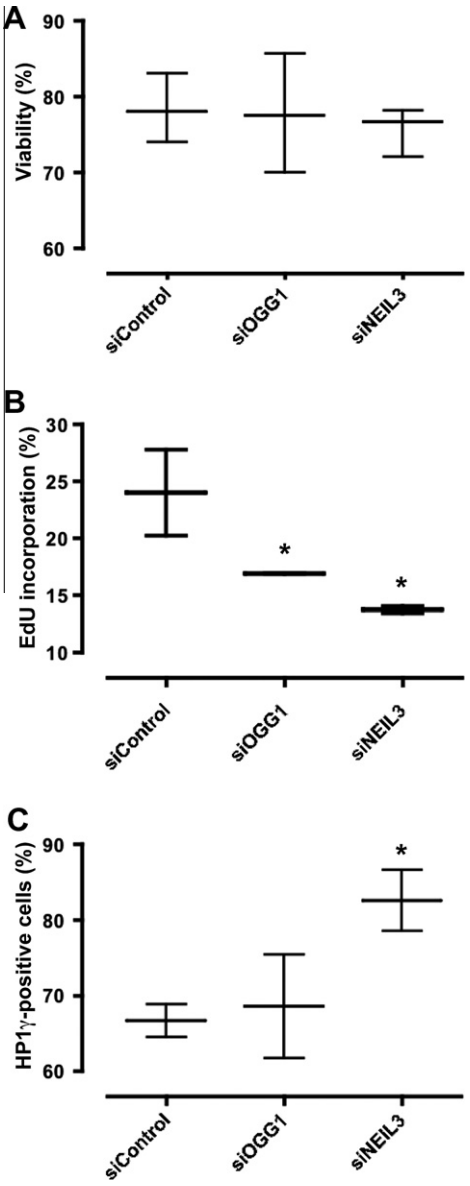

Fig. 4. RNA knockdown of DNA glycosylases OGG1 and NEIL3 influences proliferation but not the viability of NSCs, and NEIL3 knockdown results in increased HP1γ immunoreactivity in NSCs. (A) Percentage of cell viability of NSCs in "live/dead" assays following OGG1 or NEIL3 knockdown compared to control. (B) Percentage of EdU incorporation assessing the proliferation rate in NSCs after OGG1 or NEIL3 knockdown compared to control. (C) Percentage of HP1γ-positive cell nuclei after OGG1 or NEIL3 RNA knockdown in NSCs. Data are shown as mean +/− SEM. $^{*}p < 0.05$.

We therefore investigated the expression of markers of undifferentiated NSCs under proliferating conditions. Results from these experiments showed a significant down-regulation of the expression of the gene Musashi-1 [28] 24 h after administration of NEIL3 or OGG1 siRNA (Fig. 4). In contrast, no significant changes were seen in the expression pattern of the genes Sox2 and Prominin-1 (Fig. 3E).

3.3. RNA knockdown of the DNA glycosylases OGG1 and NEIL3 results in decreased proliferation but has no effect on viability of neural stem cells

Due to the general roles for DNA glycosylases in maintaining the integrity of DNA, we investigated putative effects of OGG1 and NEIL3 RNA knockdown on cell viability by live/dead assay. Notably, RNA knockdown of either OGG1 or NEIL3 at 24 h did not produce any significant changes in survival rate of NSCs (Live-positive cells: siControl: 78.4%, siOGG1: 77.7%, siNEIL3: 75.7%) (Fig. 4A) suggesting that the decrease in differentiation potential was not secondary to an increased cell death rate.

We next investigated the effects of DNA glycosylase knockdown on NSC proliferation by incorporation of 5-ethynyl-2′deoxyuridine (EdU). These experiments revealed that knockdown of OGG1 or NEIL3 indeed resulted in significantly decreased EdU incorporation (17.2% and 13.8%, respectively compared to control 24.0%) (Fig. 4B). This observation is in accordance with the idea that repair impairment influences cell cycle progression.

3.4. RNA knockdown of the DNA glycosylases OGG1 and NEIL3 results in increased immunoreactivity for senescence-associated HP1γ protein

As our experiments in mitogen withdrawal conditions revealed a lower proliferation rate but no signs of increased cell death or differentiation, we asked which state these NSCs were in. Whereas embryonic NSCs due to their immature phenotype rarely show signs of classical senescence, we and our collaborators have previously shown that signs of senescence-related alterations such as increased immunoreactivity for heterochromatin protein 1 gamma (HP1γ), a marker for increased formation of senescence-associated heterochromatin foci, may be induced in embryonic NSCs under certain conditions [29,30]. To further explore possible outcomes of RNA knockdown of OGG1 and NEIL3, we therefore used specific antibodies against HP1γ, and found a significantly increased labeling of HP1γ in NSCs with siRNA against NEIL3 (82.4%) compared to OGG1 (64.4%) and control siRNA (65.5%) (Fig. 4C). It should be noted that nucleofection of siRNA by itself yielded a larger number of HP1γ-positive NSCs than in naïve control cultures [29]. Altogether our results suggest that normal levels of DNA glycosylases, in particular NEIL3, are required for NSC characteristics and cellular integrity.

4. Discussion

Our results suggest that both NEIL3 and OGG1 are important regulators of differentiation potential and stem cell state in multipotent embryonic NSCs. NEIL3 deficiency in particular, affected negatively the expression of neuronal (DCX) and astrocytic (GFAP) genes in differentiating conditions, and notably the expression of the stem cell marker Musashi-1 decreased significantly by siRNA targeting OGG1 and NEIL3. We did however not detect any direct correlation between induced cell differentiation and regulation of DNA glycosylase gene expression. These results suggest that there may be compensatory mechanisms beyond the regulation of the levels of DNA glycosylases, and current investigations are aiming at characterizing the actual enzymatic activity associated with mitotic and differentiating conditions.

Our *in vitro* results suggest that NEIL3 has an important function in maintaining stem cell differentiation potential by being essential for proper expression of multipotency factors, neuronal, and glial genes. These findings are partly in agreement with recent reports that highlight the importance of NEIL3 in regeneration of progenitor abundant brain regions after oxidative stress inducing lesions *in vivo* [31]. Additionally we found that NEIL3 deficiency hampered astrocyte differentiation, cell cycle progression, and is likely to contribute to protection from cell senescence. Due to the general effect of ROS and implications for DNA glycosylases in BER, we expected a more pronounced effect on cell viability by RNA knockdown of OGG1 and NEIL3. However, we instead noticed nuclear signs of senescence by increased levels of HP1γ,

and it is tempting to speculate that the NSCs may use this cellular state to escape cell death and protect the integrity of the chromatin. Altogether, our results suggest unexpected roles for DNA glycosylases in regulation of neural stem cell differentiation and characteristics, and future studies may aim at elucidating the specific mechanisms underlying these functional roles.

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