SNEV<sup>hPrp19/hPso4</sup> Regulates Adipogenesis of Human Adipose Stromal Cells

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SUMMARY

Aging is accompanied by loss of subcutaneous adipose tissue. This may be due to reduced differentiation capacity or deficiency in DNA damage repair (DDR) factors. Here we investigated the role of SNEV<sup>hPrp19/hPso4</sup>, which was implicated in DDR and senescence evasion, in adipogenic differentiation of human adipose stromal cells (hASCs). We showed that SNEV is induced during adipogenesis and localized both in the nucleus and in the cytoplasm. Knockdown of SNEV perturbed adipogenic differentiation and led to accumulation of DNA damage in hASCs upon oxidative stress. In addition, we demonstrated that SNEV is required for fat deposition in <i>Caenorhabditis elegans</i>. Consequently, we tested other DDR factors and found that WRN is also required for adipogenesis in both models. These results demonstrate that SNEV regulates adipogenesis in hASCs and indicate that DDR capacity in general might be a pre-requisite for this process.

INTRODUCTION

Adipose tissue is formed at specific locations as a major energy storage compartment and is an important source of signaling activity. The distribution of adipose reservoirs within the body undergoes major changes during normal aging (Caso et al., 2013), while excess or dysfunctional fat tissue leads to reduced lifespan and accelerates the onset of age-related diseases (Ahima, 2009; Muzumdar et al., 2008). Moreover, loss of subcutaneous fat and increased visceral adiposity is observed in patients with segmental progeroid syndromes such as Werner syndrome (Mori et al., 2001), Cockayne syndrome, or trichothiodystrophy. These diseases, mirroring certain aspects of accelerated aging, are characterized by mutations in DNA damage repair (DDR) factors, leading to accumulation of DNA damage over time and hence potentially to reduced proliferation and differentiation or to senescence of pre-adipocytes (Tchkonia et al., 2010). Mouse models deficient in DNA repair also show adipose tissue degeneration (Karakašiolioti et al., 2013). However, it remains unclear whether DNA repair factors themselves have an impact on adipogenic differentiation of human adipose stromal cells (hASCs).

WRN and SNEV<sup>hPrp19/hPso4</sup> are members of a DDR protein complex (Zhang et al., 2005) and are involved in adipogenesis of mouse 3T3-L1 cells (Cho et al., 2007; Turaga et al., 2009). WRN is a helicase required for DNA recombination and repair and interacts with the SNEV complex during repair of interstrand crosslinks (Zhang et al., 2005). However, due to differences in the murine and human adipogenic differentiation processes (Mikkelsen et al., 2010), the impact of the human homologs on adipogenesis is still unclear.

SNEV<sup>hPrp19/hPso4</sup>, termed SNEV in the following, is highly conserved from yeast to humans and plays a role in several cellular pathways. It is an essential splicing factor (Grillari et al., 2005), possesses E3 ubiquitin ligase activity (Song et al., 2010), and interacts with the proteasome (Löschler et al., 2005). In addition, SNEV is involved in various types of DDR, such as DNA double-strand break repair (Mahajan and Mitchell, 2003) and homologous recombination (Abbas et al., 2014). It also interacts with two major DDR regulators: ataxia-telangiectasia mutated regulator phosphorylates SNEV after exposure to oxidative stress (Dellago et al., 2012), and SNEV contributes to the activation Rad3-related (ATR) regulator (Wan and Huang, 2014). SNEV is also linked to cellular senescence (Voglauer et al., 2006) and skin aging (Monteforte et al., 2016).

Here we show that SNEV indeed regulates adipogenesis in human cells and that these findings can be extended...
SNEV Expression Is Induced during Adipogenesis
We analyzed SNEV expression on mRNA and protein level during adipogenic differentiation of hASCs at various time points. Adipogenic differentiation was confirmed by oil red O staining after 10 days. Indeed, SNEV mRNA (Figure 1A) as well as protein levels (Figure 1B) increased in a time-dependent manner over 9 days, in line with adipogenic markers PPARγ and FASN (Figures 1C and 1D). In addition, we observed changes in the cellular localization of SNEV from mainly nuclear in undifferentiated controls to also cytoplasmic in differentiated cells (Figure 1E).

SNEV Regulates Adipogenic Differentiation of hASCs by Modulating PPARγ and Insulin Signaling
To determine whether SNEV is not only regulated, but also necessary for adipogenic differentiation, hASCs were transfected with a small interfering RNA (siRNA) pool against SNEV (siSNEV) or a non-targeting control siRNA pool (siControl), and differentiation was induced 48 hr post-transfection (Figure 2A). siSNEV transfection resulted in a 90% knockdown of SNEV mRNA over the entire period of differentiation (Figure 2B). Indeed, this knockdown resulted in formation of fewer lipid droplets in comparison with control, as shown by oil red O staining (Figure 2C) and intracellular triglyceride content (Figure 2D).

To investigate how SNEV might inhibit adipogenesis, we performed microarray analysis at day 3 of adipogenic differentiation in response to SNEV knockdown (Figure 2E). Thereby, 163 genes with at least 2-fold differential expression in siSNEV versus siControl were identified. Then, we performed gene-set-enrichment analysis and found that genes involved in the pro-adipogenic PPARγ (Figures 2F and S1A) and insulin signaling (Figures 2F and S1B) pathways were downregulated, whereas genes involved in the anti-adipogenic transforming growth factor β pathway (Figures 2F and S1C) were upregulated, among others (see also Table S1). We further confirmed the microarray data by qPCR of PPARγ (Figure 2G) and FASN (Figure 2H). Collectively, these data suggest that SNEV is necessary at an early step of adipogenesis, as it inhibits global changes of gene transcription that are usually induced by the differentiation process.

SNEV Is Required for Functional DDR in hASCs
To prove that SNEV is involved also in DNA repair in ASCs, we knocked down SNEV (Figure 3A) and exposed ASCs to reactive oxygen species (ROS) which arise during adipogenesis. DNA damage was assessed by comet assay. Representative images of comet assays are shown in Figure 3B. The resulting comets were quantified by comparing the fluorescence intensity in the head versus the tail as a measure of DNA damage. On days 8 and 11, we observed elevated ROS levels in differentiating cells, independent of ROS levels in non-differentiated cells (Figures 3D and 3E). siSNEV-transfected cells showed increased DNA damage compared to control cells (Figure 3F). This finding strongly depended on the donor-specific differentiation propensity and on the construct used, hence on the precise degree of overexpression. Notably, SNEV overexpression had no inhibitory effect on adipogenesis in any case, while knockdown resulted in reduced lipid droplet accumulation in all cases. In addition, we did not observe obvious effects on osteogenic differentiation (Figures S1D–S1F), which is often seen to increase at the expense of adipogenic differentiation (James, 2013).

RESULTS

SNEV expression was analyzed on mRNA and protein level during adipogenic differentiation of hASCs at various time points. Adipogenic differentiation was confirmed by oil red O staining after 10 days. Indeed, SNEV mRNA and protein levels increased in a time-dependent manner over 9 days, in line with adipogenic markers PPARγ and FASN. In addition, we observed changes in the cellular localization of SNEV from mainly nuclear in undifferentiated controls to also cytoplasmic in differentiated cells.

SNEV was necessary for adipogenic differentiation, as it inhibited global changes of gene transcription that are usually induced by the differentiation process. Our findings are corroborated by the observation that overexpression of SNEV in hASCs resulted in accelerated adipogenic differentiation. However, this finding strongly depended on the donor-specific differentiation propensity and on the construct used, hence on the precise degree of overexpression. Notably, SNEV overexpression had no inhibitory effect on adipogenesis in any case, while knockdown resulted in reduced lipid droplet accumulation in all cases. In addition, we did not observe obvious effects on osteogenic differentiation.

SNEV was required for DNA repair in ASCs. To prove that SNEV is involved also in DNA repair in ASCs, we knocked down SNEV and exposed ASCs to reactive oxygen species. DNA damage was assessed by comet assay. Representative images of comet assays are shown in Figure 3B. The resulting comets were quantified by comparing the fluorescence intensity in the head versus the tail as a measure of nuclear DNA damage. On days 8 and 11, we observed elevated ROS levels in differentiating cells, independent of ROS levels in non-differentiated cells. This finding strongly depended on the donor-specific differentiation propensity and on the construct used, hence on the precise degree of overexpression. Notably, SNEV overexpression had no inhibitory effect on adipogenesis, while knockdown resulted in reduced lipid droplet accumulation in all cases. In addition, we did not observe obvious effects on osteogenic differentiation.

To test if the ability to modulate adipogenic differentiation is restricted to SNEV or a general property of DDR factors, we tested if genes mutated in segmental progeroid syndromes influence adipogenesis as well. First, we specifically visualized the expression of genes involved in DDR using existing transcriptomic data of an adipogenic
differentiation time-course experiment (available at Gene Expression Omnibus, accession number GEO: GSE64845). From these, we selected WRN, CSA, and XPE for further analysis (Figures S4A–S4D). Mutations of these genes mirror aspects of accelerated aging and represent different DDR pathways, CSA being specific for transcription-coupled nucleotide excision repair (TC-NER), while XPE is involved in global genome- and TC-NER, as well as in homologous recombination. We knocked down WRN, CSA, and XPE in hASCs by siRNA transfection and induced adipogenesis. Knockdown was confirmed by qPCR (Figure S4E), and adipogenic differentiation was assessed by intracellular triglyceride accumulation at day 10 of differentiation. CSA and XPE knockdown reduced intracellular triglycerides slightly, but not statistically significantly, whereas WRN knockdown resulted in a significant reduction by 50% (Figure S4F).

Loss of SNEV and WRN Lead to Reduced Fat Deposition in C. elegans
To test the functional conservation of the DDR factors SNEV, WRN, CSA, and XPE in adipogenesis, we assessed...
their role in fat deposition in *C. elegans*. For this purpose, we selected *prp-19, wrn-1, M18.5*, and *xpa-1* as orthologs of the human DDR factors *SNEV, WRN, XPE*, and *XPA*. Since we did not detect major differences in developmental timing between RNAi-treated and control animals (data not shown), RNAi treatment was performed already upon hatching and led to a downregulation of the target mRNA by 80%–100% (Figure 4A). Young adult hermaphrodites (6 days after hatching) were subjected to oil red O staining to assess neutral lipid storage (Soukas et al., 2009). Indeed, *prp-19* RNAi animals exhibited reduced fat mass compared with the RNAi control, while *wrn-1, M18.5*, and *xpa-1* RNAi did not yield significant differences (Figures 4B and 4C).

We expected to further enhance the observed fat storage phenotype by using the CF1814 strain, which is mutated in *rrf-3* and *daf-2* and exhibits increased RNAi efficiency (Simmer et al., 2002), as well as elevated fat mass (Soukas et al.,...
While the RNAi control group stained positive for oil red O throughout the whole body and especially surrounding intestine and pharynx, prp-19 and wrn-1 RNAi worms stained only weakly positive in close proximity to the pharynx (Figures 4D and 4E), now also detecting differences induced by WRN deficiency. These findings indicate that loss of the conserved DDR factors prp-19 and wrn-1 reduces the accumulation of neutral lipids in C. elegans.

**DISCUSSION**

SNEV regulates diverse cellular processes, such as mRNA splicing (Grillari et al., 2005), transcription (Chanarat et al., 2012), mitosis (Watrin et al., 2014), apoptosis (Lu et al., 2014), and multiple branches of DNA repair (reviewed in Mahajan, 2016). On the one hand, SNEV catalyzes the formation of polyubiquitin chains on replication protein A associated with single-stranded DNA arising at donor 812 and 1× donor 803). Error bars indicate SD. Unpaired two-sided Student's t tests were performed to compare control and siSNEV treated samples and did not reveal statistical differences for any condition.

(G) Upper panel, 48 hr post-transduction, before adipogenic differentiation is induced, Comet assays reveal that DNA damage levels are slightly higher in siControl than siSNEV transfected ASCs. Lower panel, after adipogenic differentiation, SNEV knockdown leads to significant increase of DNA damage. Numbers indicate percentage of cells in the respective category. Pooled data from three independent differentiation experiments are shown (donors 803, 812, and 851). A minimum of 200 cells per condition and replicate were analyzed. Chi-square test was performed to compare results of control and SNEV knockdown: \(* * p < 0.001, \ast p < 0.05\). See also Figures S3 and S4.

2009). While the RNAi control group stained positive for oil red O throughout the whole body and especially surrounding intestine and pharynx, prp-19 and wrn-1 RNAi worms stained only weakly positive in close proximity to the pharynx (Figures 4D and 4E), now also detecting differences induced by WRN deficiency.

These findings indicate that loss of the conserved DDR factors prp-19 and wrn-1 reduces the accumulation of neutral lipids in C. elegans.

**Figure 3. SNEV Is Required for Repair of Oxidative DNA Damage during Adipogenic Differentiation of hASCs**

hASCs were transfected with siSNEV or si-Control, treated with 500 μM H₂O₂ for 90 and 60 min recovery, and submitted to comet assay.

(A) Transfection with siSNEV results in 90% reduction of SNEV mRNA expression. The average of four technical replicates of cells from donor 812 is shown. Error bars indicate SD.

(B) Representative images of comet assays. Scale bar, 100 μm.

(C) In hASCs transfected with siSNEV and treated with H₂O₂, the percentage of cells with high levels of DNA damage triples, while the percentage of cells with low DNA damage drops to one-third compared with control cells after H₂O₂ treatment. Numbers indicate the percentage of cells in the respective category. Pooled data from three biological replicates are shown (donors 803, 812, and 851). A minimum of 150 cells per condition and replicate were analyzed. Chi-square test was performed to compare results from control and SNEV knockdown: \(* * * p < 0.001\).

(D) Schematic representation of experimental design. hASC were transfected with SNEV or control siRNAs and submitted to adipogenic differentiation. RNA samples to monitor knockdown and ROS measurements were taken on days 0, 8, and 11 of differentiation. Comet assays were performed on days 0 and 11.

(E) RT-qPCR shows stable knockdown over the course of differentiation.

(F) ROS formation was quantified by H₂DCFDA staining. Mean values of three independent differentiation experiments are shown (2× donor 812 and 1× donor 803). Error bars indicate SD. Unpaired two-sided Student’s t tests were performed to compare control and siSNEV treated samples and did not reveal statistical differences for any condition.

(G) Upper panel, 48 hr post-transduction, before adipogenic differentiation is induced, Comet assays reveal that DNA damage levels are slightly higher in siControl than siSNEV transfected ASCs. Lower panel, after adipogenic differentiation, SNEV knockdown leads to significant increase of DNA damage. Numbers indicate percentage of cells in the respective category. Pooled data from three independent differentiation experiments are shown (donors 803, 812, and 851). A minimum of 200 cells per condition and replicate were analyzed. Chi-square test was performed to compare results of control and SNEV knockdown: \(* * * p < 0.001, \ast p < 0.05\). See also Figures S3 and S4.
sites of DNA damage, which enhances ATR-ATRIP recruitment and consequently downstream DDR signaling (Wan and Huang, 2014). On the other hand, SNEV is recruited to RNA polymerase II via U2AF65, and this interaction stimulates co-transcriptional splicing in vitro (David et al., 2011). It is possible that SNEV senses the slowing of RNA polymerase II processivity when it encounters a lesion and acts as signal transducer to attract other DNA repair factors.

Albeit we cannot completely rule out off-target effects of the single siRNA pool we used in this study to deplete SNEV in hASCs, the requirement of SNEV for adipogenesis seems to be evolutionary well conserved, as we observed reduced fat deposition in the nematode C. elegans upon depletion of prp-19 by RNAi.

In addition to our data, several other lines of evidence point to a possible role for the DNA repair function of SNEV in adipogenesis. The loss of multiple stem cell functions with increasing age can be attributed to an accumulation of DNA damage (reviewed in Behrens et al., 2014), and hematopoietic stem cell self-renewal was already shown to be diminished after DNA damage (Wang et al., 2012), supporting the idea that the capacity of repairing DNA damage is a general checkpoint before differentiation.

But why does reduced DNA repair upon SNEV knockdown specifically block adipogenic, but not osteogenic differentiation? One hypothesis is based on the fact that ROS are specifically formed during adipogenic differentiation and are thought to reinforce pro-adipogenic signaling pathways (Kanda et al., 2011), while osteogenic differentiation is accompanied by a reduction of intracellular ROS (Atashi et al., 2015). Indeed, we observed accumulation of DNA damage in hASCs after SNEV knockdown, both after acute hydrogen peroxide treatment and after endogenous ROS accumulation resulting from adipogenic differentiation. Therefore, we suggest that only cells with sufficient DDR capacity might be allowed to enter adipogenic differentiation, as DDR is induced during early adipogenesis (Meulle et al., 2008). This lack of adipogenic differentiation together with adipose tissue degeneration induced by senescent adipocytes might contribute to the subcutaneous fat loss in segmental progeroid syndrome patients (Martin and Oshima, 2000) and mouse models of premature aging, such as ERCC1-, Cxb-, or Xpa-deficient mice (Jaarsma et al., 2013; Kamenisch et al., 2010).

However, we cannot rule out the possibility that SNEV might play a more direct role during early adipogenic commitment of hASCs, e.g., via differential pre-mRNA splicing. Also, a scenario involving p21 regulation by SNEV is possible. After inducing adipogenesis in preadipocytes, cells undergo a transient increase in DNA synthesis, followed by an arrest in the G1 phase, which is characterized by an increase in p21 protein levels (Reichert and Eick, 1999). Interestingly, the SNEV/Cdc5L complex is recruited to the p21 gene and mRNA and is specifically required for protein expression of p21, but not of pro-apoptotic p53-targets (Chen et al., 2011).
To summarize, we suggest that availability of DDR might represent a checkpoint for cellular differentiation programs, which is of special importance for differentiation processes that involve high levels of ROS, or for long-lived cells such as adipocytes.

**EXPERIMENTAL PROCEDURES**

Experimental details can be found in the Supplemental Experimental Procedures.

**hASC Cultivation**

Human subcutaneous adipose tissues were obtained from three different donors by liposuction (Table S2). Informed consent of the donors was obtained and therefore this study was performed according to the Declaration of Helsinki and approved by the local ethics commission (Ethikkommission des Landes Oberösterreich). hASCs were isolated and cultivated as described by Wolbank et al. (2009) and characterized for surface markers (Table S3). Adipogenic and osteogenic differentiation, as well as oil red O and alizarin red staining were performed as described by Schosserer et al. (2015). Triglycerides were quantified by an Infinity Triglyceride Quantification Kit (Thermo Scientific) and normalized to total protein concentration as measured using the BCA Kit (Thermo Scientific).

siRNA against SNEV (ON-TARGETplus Human PRPF19 [27339] siRNA, SMARTpool) and control siRNA (ON-TARGETplus Non-targeting Pool) were purchased from Thermo Scientific. siRNAs against WRN, CSA, and XPE and a non-targeting control were purchased from Riboxx Pharmaceuticals.

A total of 14,000 hASCs was seeded into 1.9 cm² plates. Forty-eight hours post-seeding, cells were transfected with siRNA using 50 nM of the respective siRNAs and DharmaFECT1 Transfection Reagent (Thermo Scientific). Protein concentration as measured using the BCA Kit (Thermo Scientific).

Total RNA was extracted using TRIZol Reagent (Life Technologies). RNA concentration was on a NanoDrop ND-1000 spectrophotometer. cDNA was synthesized using 500 ng total RNA with the DyNAmo cDNA Synthesis Kit (Thermo Scientific). qPCR was performed using HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne). Primers for qPCR are listed in Table S4. mRNA expression was normalized to GAPDH.

SNEV cDNA was amplified by PCR and cloned into the retroviral plasmid pLenti6. The negative control vector contained only the bacterial resistance gene. Retroviral particles were generated according to the manufacturer’s protocol (Life Technologies). A total of 14,000/1.9 cm² hASCs were seeded in growth medium and, after 48 hr, infected with retroviral particles at an MOI of 2 in DMEM (4.5 g/L glucose), supplemented with 4 mM L-glutamine, 10% fetal calf serum, 1 ng/mL basic fibroblast growth factor, and 8 μg/mL polybrene. The medium was replaced with the same medium without polybrene 24 hr post-transduction and with adipogenesis-inducing medium 48 hr post-transduction.

**Microarray Analysis**

Global gene expression analysis was performed by two-color microarrays for hASCs upon SNEV knockdown (donors 803 and 812 as independent biological replicates), as well as for human multipotent adipose-derived stem cells at various stages of adipocyte differentiation. Metadata (experimental parameters and detailed procedures), raw data files, and final (filtered and normalized) data are accessible via Gene Expression Omnibus (GEO: GSE64937 and GSE64845).

**Western Blotting and Immunofluorescence**

hASCs were harvested in radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 1× protease and phosphatase inhibitor [Roche]), sonicated for 30 cycles (30 s on and 30 s off) at 4°C using a Bioruptor sonicator (Diagenode) and centrifuged for 30 min at 10,000 rpm. Protein concentration in the supernatant was determined using the BCA Kit (Thermo Scientific). Protein (30 μg) was mixed with 4× SDS loading dye (240 mM Tris-Cl [pH 6.8], 8% SDS, 40% glycerol, 0.05% bromphenol blue, and 5% β-mercaptoethanol), heated to 95°C for 10 min and submitted to SDS-PAGE and western blotting. SDS-PAGE and western blotting were carried out as described previously (Dellago et al., 2012).

At day 10, cells were fixed in paraformaldehyde and processed for immunofluorescence as described previously (Schosserer et al., 2015); antibody details can be found in the Supplemental Information.

**Quantification of ROS**

Adipogenic differentiation was induced 48 hr post-transfection as described above. On days 0, 8, and 11 of adipogenic differentiation, cells were harvested, resuspended in PBS containing 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Life Technologies) and incubated for 30 min at room temperature in the dark. After incubation, cells were put on ice and fluorescence was measured on a Gallios Flow Cytometer (Beckman Coulter).

** Comet Assay**

Undifferentiated hASCs were transfected with siSNEV and siControl as described above. Two days after transfection, cells were treated with 500 μM hydrogen peroxide (Sigma) in growth medium for 90 min. After 60 min recovery in growth medium, cells were harvested and processed for comet assays (Wojewódzka et al., 2002) and quantified as described previously (Guo et al., 2013).

**C. elegans**

**C. elegans** strains were cultured at 20°C under standard laboratory conditions on nematode growth medium agar as described previously (Brenner, 1974). Worms were synchronized by timed egg-lay on fresh RNAi plates and transferred to FUdR (Sigma)-containing plates upon adulthood. For knockdown, worms were fed double-stranded RNA expressed in bacteria as described previously (Timmons et al., 2001). RNAi constructs against prp-19, wnn-1, xpe-1, and M18.5 (xpa), derived from J. Ahringer’s RNAi library, were obtained from Source BioScience. Oil red O staining was performed 6 days after hatching as described previously (Soukas et al., 2009). Stained worms were embedded in Mowiol and pictures were taken on a Leica DM IL LED inverted microscope with a 10× dry objective and staining was quantified as described previously (Yen et al., 2010). For qPCR, 30–40 worms were rinsed off
plates and washed with S Basal, precipitated by gravity, and homogenized in Trizol using a pellet pestle. Samples were then processed as described for hASCs.

**Statistical Analysis**

Differences between datasets were tested for statistical significance using multiple-comparison adjusted Student's t tests, one-way ANOVA, or two-way ANOVA implemented in Prism QuickCalcs (GraphPad), and p < 0.05 was considered statistically significant. All error bars represent SDs of the mean if not indicated otherwise. For comet assays, counts from three donors were pooled, classified into categories, and analyzed by chi-square test.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.001.

**AUTHOR CONTRIBUTIONS**

A.K., H.D., R.G.V., M.Scho., and J.G. designed the experiments; A.K., H.D., M.Scho., V.S., and L.T. performed the experiments with ASCs and analyzed the data; M.K. and M.Sche. designed the experiments; A.K., H.D., R.G.V., M.Scho., and J.G. designed the experiments; A.K., H.D., M.Scho., and J.G. designed the figures and wrote the manuscript. All authors read and corrected the manuscript.

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