Down-regulation of MicroRNA-21 Is Involved in the Propofol-induced Neurotoxicity Observed in Human Stem Cell–derived Neurons

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ABSTRACT

Background: Recent studies in various animal models have suggested that anesthetics such as propofol, when administered early in life, can lead to neurotoxicity. These studies have raised significant safety concerns regarding the use of anesthetics in the pediatric population and highlight the need for a better model to study anesthetic-induced neurotoxicity in humans. Human embryonic stem cells are capable of differentiating into any cell type and represent a promising model to study mechanisms governing anesthetic-induced neurotoxicity.

Methods: Cell death in human embryonic stem cell–derived neurons was assessed using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling staining, and microRNA expression was assessed using quantitative reverse transcription polymerase chain reaction. miR-21 was overexpressed and knocked down using an miR-21 mimic and antagonor, respectively. Sprouty 2 was knocked down using a small interfering RNA, and the expression of the miR-21 targets of interest was assessed by Western blot.

Results: Propofol dose and exposure time dependently induced significant cell death (n = 3) in the neurons and down-regulated several microRNAs, including miR-21. Overexpression of miR-21 and knockdown of Sprouty 2 attenuated the increase in terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling–positive cells following propofol exposure. In addition, miR-21 knockdown increased the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling–positive cells by 30% (n = 5). Finally, activated signal transducer and activator of transcription 3 and protein kinase B (Akt) were down-regulated, and Sprouty 2 was up-regulated following propofol exposure (n = 3).

Conclusions: These data suggest that (1) human embryonic stem cell–derived neurons represent a promising in vitro human model for studying anesthetic-induced neurotoxicity, (2) propofol induces cell death in human embryonic stem cell–derived neurons, and (3) the propofol-induced cell death may occur via a signal transducer and activator of transcription 3/miR-21/Sprouty 2–dependent mechanism. 

What We Already Know about This Topic

• Anesthetic neurotoxicity has been repeatedly demonstrated in rodent and primate models. A model for evaluation of toxicity in human tissue is not currently available.
• Neurons were derived from human embryonic stem cells and were grown in culture. The effect of propofol exposure on cell death was examined. In addition, the role of miR-21, a non-coding microRNA that inhibits target mRNA expression, was evaluated.

What This Article Tells Us That Is New

• Propofol induced apoptosis of human embryonic stem cell–derived neurons and reduced expression of miR-21. Overexpression of miR-21 reduced this toxicity.
• Neurons derived from human embryonic stem cell represent a useful model for the study of anesthetic neurotoxicity in humans. miR-21 plays a role in propofol-induced toxicity, and manipulation of miR-21 may serve as a therapeutic approach for prevention of toxicity.
was performed in animal models with no direct evidence available in a human model. In addition, for ethical reasons, it is not feasible to perform these studies on young children and the only human data available come from a limited number of epidemiological studies. Moreover, these human studies are often limited by many confounding variables and have produced widely mutable results. Therefore, the neurological effects of anesthetics on young children remain uncertain. Human embryonic stem cells (hESCs) are pluripotent cells that are derived from the inner cell mass of a human blastocyst. The benefit of using hESCs lies in their ability to differentiate into any cell type, making them a potentially powerful model of human physiology and pathophysiology. Therefore, neurons derived from hESCs are a valuable model to directly study the effects of anesthetics on immature, human-derived neurons.

MicroRNAs (miRs) are endogenous, noncoding RNA molecules that act to regulate nearly every cellular process through inhibition of target messenger RNA expression. MicroRNAs are produced through the processing of long stem–loop transcripts by the nucleases Drosha and Dicer. The mature microRNA then combines with the RNA-induced silencing complex and interacts with its target to induce gene silencing through target mRNA degradation or translational repression. MicroRNAs have been implicated to play important roles in many different disease processes, including neurological diseases. Neurotoxicities conferred by ethanol, cocaine, Huntington disease, and brain injuries have all been linked to microRNA dysregulation. However, the role of microRNAs in anesthetic-induced neurotoxicity has yet to be studied.

One microRNA, miR-21, has been shown to decrease apoptosis and can protect neurons from ischemic injury. Exposure of fetal cerebral cortical derived neuroepithelial cells to ethanol was shown to suppress miR-21. miR-21 has been shown to decrease apoptosis in varying cell types by directly targeting and suppressing Sprouty 2 which, in turn, negatively regulates protein kinase B (Akt) activation. Additionally, signal transducer and activator of transcription 3 (STAT3) is a known regulator of miR-21. After screening microRNAs and finding that miR-21 was down-regulated following exposure to propofol, we hypothesized that the miR-21 signaling pathway (STAT3/Sprouty2/Akt) plays a role in the increased cell death observed in the hESC-derived neurons following propofol administration.

**Materials and Methods**

**Culturing of hESCs**

hESCs (H1 cell line, WiCell Research Institute Inc., Madison, WI) were cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) that were mitotically inactivated using mitomycin C (Sigma-Aldrich, St. Louis, MO) as previously described by our laboratory. Briefly, culture dishes were coated with 0.1% gelatin overnight in a humidified incubator. MEFs were then plated onto the gelatin and cultured in MEF media consisting of Dulbecco’s modified Eagle media supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 1% nonessential amino acids (Invitrogen, Carlsbad, CA). The MEFs were cultured overnight in a humidified, hypoxic incubator (4% O2/5% CO2, 37°C). hESCs were then plated onto the MEFs and cultured in the hypoxic incubator in hESC media containing Dulbecco’s modified Eagle media/F12 supplemented with 20% knockout serum (Gibco), 1% nonessential amino acids, 1 mM L-glutamine, 1% penicillin–streptomycin (Invitrogen), 4 ng/ml human recombinant basic fibroblast growth factor (Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma-Aldrich). The media was changed every day, and the cells were passaged mechanically every 5 days. hESCs at passage 55–70 were used for these studies.

**Differentiation of Neurons from hESCs**

To generate neurons from the hESCs, the cells were taken through a four-step differentiation protocol as previously described. The timeline for the differentiation protocol is shown in figure 1A and was performed as follows: (1) embryoid body (EB) culture: hESCs cultured on MEFs were dissociated for 40 min using the protease dispase (1.5 U/ml, Invitrogen). The hESCs were then spun down, resuspended in hESC medium without basic fibroblast growth factor, and cultured in ultralow attachment 6-well plates (Corning Inc., Corning, NY) in a normoxic incubator (20% O2/5% CO2, 37°C). The media was changed daily, and spherical EBs were present 24 h after dispase digestion. Five days after digestion, EBs were switched to neural induction media containing Dulbecco’s modified Eagle media/F12 supplemented with 1% N2 (Invitrogen), 1% nonessential amino acids, 1 mg/ml heparin (Sigma), and 5 ng/ml basic fibroblast growth factor. The media was changed daily for an additional 4 days. (2) Rosette formation: On day 9, the EBs were plated to matrigel-coated 35-mm dishes and cultured in neural induction media. The media was changed every other day, and rosette-like structures were present within 5 days of the EBs plating down. (3) Expansion of neural stem cells (NSCs): Two days after the rosette morphology was clearly visible, the rosettes were manually separated from the surrounding cells using a 5-ml serological pipette. The rosette cells were then transferred to matrigel-coated culture dishes and cultured in neural expansion media containing Dulbecco’s modified Eagle media/F12 supplemented with 2% B27 without vitamin A, 1% N2 (Invitrogen), 1% nonessential amino acids, 20 ng/ml basic fibroblast growth factor, and 1 mg/ml heparin. The media was changed every other day, and the NSCs were passaged enzymatically every 5 days with Accutase (Innovative Cell Technologies, San Diego, CA). (4) Neuron differentiation: NSCs were cultured in 60-mm matrigel-coated dishes (500,000 cells/dish) for 2 weeks in neuron differentiation media containing...
neurobasal media (Gibco) supplemented with 2% B27, 0.1 μM cyclic adenosine monophosphate, 100 ng/ml ascorbic acid (Sigma-Aldrich), 10 ng/ml brain-derived neurotrophic factor, 10 ng/ml glial cell-derived neurotrophic factor, and 10 ng/ml insulin-like growth factor 1 (PeproTech Inc., Rocky Hill, NJ). The media was changed every other day, and after 2 weeks of culture, the cells displayed clear neuronal morphology and were used for the studies.

**Immunofluorescence Staining, Confocal Microscopy, and Electron Microscopy**

Two-week-old hESC-derived neurons cultured on matrigel-coated, glass cover slips were fixed for 30 min at room temperature in 1% paraformaldehyde. Cells were then washed with phosphate-buffered saline (PBS) three times followed by a 15-min incubation in 0.5% Triton X-100 (Sigma-Aldrich) in PBS. The cells were then washed with

![Image of differentiation and characterization of human embryonic stem cell (hESC)-derived neurons.](image-url)

**Fig. 1.** Differentiation and characterization of human embryonic stem cell (hESC)-derived neurons. (A) A four-step differentiation protocol was used to generate neurons from the hESCs, which included embryoid body culture, rosette formation, expansion of neural stem cells, and neuron differentiation. (B) The differentiated cells displayed a morphology characteristic of neurons with small cell bodies and projections (a) and stained positive for the neuron-specific markers, microtubule-associated protein 2 (MAP2, b, green), and β-tubulin III (c, green). The blue dots are the cell nuclei stained with Hoechst 33342. (C) The neurons also stained positive for doublecortin (a, green), a marker of immature neurons and again for β-tubulin III (b, red). The doublecortin staining colocalized with the β-tubulin III staining (c, orange). (D) Finally, synapse-like structures were visible in the differentiated neurons (indicated by the yellow arrow) upon electron microscopy imaging.
PBS and blocked for 20 min at room temperature with 10% donkey serum. Following the blocking, the cells were incubated with the primary antibodies (microtubule-associated protein 2, β-tubulin III, or doublecortin [Abcam, Cambridge, MA]) for 1 h in a humidified, 37°C incubator. The cells were washed three times with PBS and incubated for 45 min at 37°C with Alexa Fluor 488 or 594 donkey anti-mouse or -rabbit immunoglobulin G (Invitrogen) secondary antibodies. The cells were washed with PBS, and the cell nuclei were stained with Hoechst 33342 (Invitrogen). Finally, the cover slips were mounted onto glass slides and imaged using a laser-scanning confocal microscope (Nikon Eclipse TE2000-U, Nikon Inc., Melville, NY). To assess the differentiation efficiency, the ratio of microtubule-associated protein 2–positive cells over the total cell nuclei was calculated. Electron microscopy imaging was performed as previously described.29,30

**Propofol Exposure**

While assessment of brain concentrations of propofol in humans during the induction and maintenance of anesthesia is difficult, studies have shown and estimated that it ranges from 4 to 20 μg/ml.31–34 The dose of propofol used clinically in children varies widely but typically ranges from about 1 to 10 μg/ml (blood concentration) with higher doses used for the induction of anesthesia and lower doses used for maintenance.35–37 In addition, cell culture and whole-animal studies have shown that propofol can induce toxicity at high doses or prolonged exposure times after a single exposure.5,31,38,39 Thus, 2-week-old hESC-derived neurons were treated with 0, 5, 10, and 20 μg/ml of research-grade propofol (0–112 μM, Sigma-Aldrich) or equal volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich) as the vehicle control in 60-mm culture dishes (500,000 cells/dish) or 12-mm glass cover slips (100,000 cells/cover slip). A stock solution (40 mg/ml) of propofol was prepared in DMSO, and serial dilutions to the desired doses were prepared from the stock. Cells were exposed to propofol for 6 h either one time or three times (once per day for 3 consecutive days). In the multiple exposure groups, following the 6 h of propofol exposure each day, the cells were rinsed with PBS and placed in fresh media overnight. Following exposure to the propofol, the cells were lysed for microRNA analysis and Western blot or fixed for immunostaining and TUNEL staining. The mechanistic experiments in this study were performed in hESC-derived neurons following a single exposure to 6 h of 20 μg/ml propofol.

**TUNEL Staining**

Apoptosis, and to some extent necrosis, was analyzed using a cell death detection kit (Roche Applied Bio Sciences, Indianapolis, IN) based on terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling (TUNEL) following instructions provided by the manufacturer. TUNEL identifies single- and double-stranded DNA breaks by labeling the free 3′-OH termini with modified nucleotides in an enzymatic reaction with terminal deoxynucleotidyl transferase. Cells were cultured on glass cover slips in 24-well plates and exposed for 6 h to 20 μg/ml propofol or DMSO. Following an 18-h washout in media, the cells were then rinsed and fixed with 1% paraformaldehyde, and DNA fragmentation was assessed using the polymerase terminal deoxynucleotidyl transferase, which incorporates into areas of DNA breaks. Hoechst 33342 was used to stain the nuclei, and the cells were imaged using the confocal microscope. Apoptosis/necrosis was quantified by determining the ratio of TUNEL-positive nuclei to total cell nuclei. To eliminate the possibility of different wells of the 24-well plate receiving preferential conditions and affecting the outcome of the studies, the cover slips were assigned randomly to the experimental groups across the entire plate. In addition, to minimize the introduction of bias into the cell imaging and counting studies, the experimenter was blinded to the group being analyzed.

**Total RNA Extraction**

Following exposure to propofol or DMSO, total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA). Briefly, the cells were rinsed with PBS and lysed with QIAzol lysis reagent (Qiagen). Chloroform was added, and the lysates were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new Eppendorf tube. RNA was precipitated from the upper phase with 100% ethanol and collected using the RNeasy spin columns (Qiagen). Samples were rinsed twice with buffer RPE, and the RNA was eluted from the column by adding 30 μl of RNase-free water (Qiagen). The quantity and quality of the RNA were assessed using an Epoch nanodrop spectrophotometer (BioTek Instruments Inc., Winooski, VT). Each RNA sample was then diluted to 100 ng/μl in RNase-free water.

**cDNA Preparation and MicroRNA Analysis by Quantitative Reverse Transcription-PCR (qRT-PCR)**

The RNA was reverse transcribed to cDNA using the miScript II RT kit (Qiagen) following the manufacturer’s instructions. Briefly, a mixture containing 1 μg of RNA, 10× miScript nucleic mix, RNase-free water, reverse transcriptase mix, and 5× HiSpec buffer (Qiagen) was prepared. The RT reaction mixture had a final volume of 20 μl and was incubated at 37°C for 1 h and 95°C for 5 min to stop the reaction. The RT product was diluted in 200 μl of RNase-free water to give a final RNA concentration of 4.5 ng/μl. MicroRNA expression levels were assessed using qRT-PCR. To screen for potential microRNAs contributing to the propofol-induced neurotoxicity, we used human miFinder miRNA PCR arrays (Qiagen). These arrays allow for rapid screening of 84 of the most abundantly expressed microRNAs as characterized in miRBase*. For these arrays, a master

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mix (25 μl/well) containing the template cDNA (4.5 ng/well), universal primer, RNase-free water, and miScript SYBR Green (Qiagen) was prepared according to the manufacturer's directions. The primers for each of the 84 microRNAs to be analyzed were lyophilized in the 96-well plates of the arrays. To confirm the array results for the expression of miR-21, a validation assay was performed in which the three cDNA samples used for the arrays were each run in triplicate on the same PCR run. To prepare the samples for the triplicate assays, a similar master mix to that used for the arrays was prepared with the addition of the primers (miR-21 and the housekeeping gene, Rnu-6). The PCR was run using a Bio-Rad iCycler for 15 min at 95°C followed by 40 cycles of a three-step denaturation (15 s at 94°C), annealing (30 s at 55°C), and extension (30 s at 70°C). Reverse transcriptase and melt curve controls were run to ensure primer specificity and sample purity, respectively.

**cDNA Preparation and mRNA Analysis by qRT-PCR**

Following total RNA extraction, cDNA was prepared using an RT2 First Strand Kit (Qiagen) following the manufacturer's instructions. Briefly, 500 ng of RNA was combined with the genomic DNA elimination buffer and water and incubated for 5 min at 42°C to purify the RNA samples. The samples were then placed on ice for 1 min. A mixture of 5× buffer BC3, RT mix, water, and an external control was prepared and combined with the genomic DNA elimination mix. The samples were then incubated for 15 min at 42°C, and the reaction was stopped by incubating the samples at 95°C for 5 min. The cDNA was then diluted in 91 μl of RNase-free water and combined with RT2 SYBR Green ROX FAST Mastermix, primers (Sprouty 2 or the housekeeping gene, B2M), and water (Qiagen). The samples were loaded into the 96-well plates (25 μl/well), and the PCR was run using the Bio-Rad iCycler Real-Time PCR detection system for 10 min at 95°C followed by 40 cycles of a denaturation step (15 s at 95°C) and a combined annealing/extension step (30 s at 60°C). The Ct values of the mRNAs in each sample were collected, and the expression data was normalized to the housekeeping gene, B2M. Melt curve controls were also run.

**miR-21 Overexpression and Knockdown**

To manipulate the level of miR-21, 2-week-old hESC-derived neurons were transfected in 6-well plates with 25 nM locked nucleic acid anti-miR-21 (Exiqon, Vedbaek, Denmark) or 1 nM miR-21 mimic (pre-miR-21) (Qiagen) to knock down miR-21 or increase miR-21 abundance, respectively, using lipofectamine (Invitrogen). These concentrations were chosen based on dose studies implicating the specified concentrations as sufficient to increase abundance or knockdown of miR-21 in our cells. Scrambled locked nucleic acid anti-miR control (Exiqon) and pre-miR precursor negative control (Qiagen) were used as controls. Twenty-four hours after transfection, a subset of cells was used to confirm miR-21 overexpression and knockdown in the neurons by qRT-PCR. Following confirmation of overexpression/knockdown of miR-21, the remaining cells were exposed to 20 μg/ml propofol for 6 h. The effect of miR-21 overexpression and knockdown on propofol-induced neurotoxicity in the hESC-derived neurons was analyzed by TUNEL staining. To eliminate possible bias, the wells were assigned randomly to the various experimental and control conditions.

**Sprouty 2 Knockdown**

To knock down Sprouty 2 in the hESC-derived neurons, the cells were transfected in 6-well plates with 20 nM Sprouty 2 siRNA or scramble control siRNA (Qiagen) following instructions provided by the manufacturer. To eliminate any possible bias introduced by well position in each plate, the wells were assigned randomly to the various experimental groups. Briefly, a mix of opti-MEM and siRNA was prepared, and 12 μl of HiPerFect Transfection Reagent (Qiagen) was added to the mixture. The mixture was incubated at room temperature for 10 min. Fresh culture media was added to each well, and the siRNA mixture was added dropwise to the appropriate wells. The cells were then incubated at 37°C for 48 h. These doses and times produced the largest knockdown as implicated by dose and time studies performed in these cells (data not shown). Following the transfection period, a subset of cells was used to confirm the Sprouty 2 knockdown by qRT-PCR. The remaining cells were exposed for 6 h to 20 μg/ml propofol or DMSO. The effect of Sprouty 2 knockdown on propofol-induced toxicity was assessed by TUNEL staining.

**Western Blot**

Following exposure to propofol or DMSO (control), the cells were rinsed with PBS and were lysed and sonicated in RIPA lysis buffer (Cell Signaling, Danvers, MA) with phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were centrifuged at 10,000 g for 10 min at 4°C. Pellets were discarded, and the total protein concentration of the supernatants was determined using a DC Protein Assay Reagents Package kit (Bio-Rad, Hercules, CA). The samples were boiled for 5 min at 97°C. Twenty-five micrograms of protein was loaded per lane for sodium dodecyl sulfate polyacrylamide gel electrophoresis gel separation and then transferred to nitrocellulose membrane. Proteins were blocked with Blocking Buffer (Thermo Fisher Scientific, Waltham, MA) and then incubated overnight at 4°C with primary antibodies against phosphorylated STAT3 (pSTAT3tyr705), STAT3, Sprouty 2, Akt, phosphorylated Akt (pAktser473), and tubulin (Cell Signaling). The membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling) for 1 h at room temperature, and labeled proteins were detected with chemiluminescence detection reagent (Cell Signaling) and obtained on x-ray film. Optical densities were quantified using Imagej software (National Institutes of Health, Bethesda, Maryland), and the data were reported as % of control.

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Results
Characterization of hESC-derived Neurons
To generate neurons from the hESCs, the cells were taken through a four-step differentiation protocol as outlined in figure 1A. Differentiated neurons displayed characteristic neuronal morphology with small cell bodies and projections, and over time, the neurons formed extensive, interconnected networks (fig. 1B-a). The cells displayed a very distinct morphology at each stage of the differentiation protocol. The hESCs formed tight colonies while cultured on the feeder layer of MEFs, while the EBs formed three-dimensional aggregates when suspended in culture. At the neural rosette stage, the NSCs formed into tightly packed, circular arrangements bordered closely by various additional cell types. Once mechanically separated from the surrounding cells and digested, the NSCs spread on the matrigel-coated dishes and extensively proliferated.

Following 14 days in neuronal differentiation media, the cells displayed a very characteristic neuronal morphology with small cell bodies and interconnected projections (fig. 1B-a). The neurons were immunostained after 2 weeks in differentiation media and expressed the neuron-specific markers microtubule-associated protein 2 and β-tubulin III (fig. 1B-b, c). Based on the immunostaining, the differentiation protocol was about 90–95% efficient in the generation of neurons.

In an attempt to better gauge the maturity level of the hESC-derived neurons, the cells were also immunostained 2 weeks after the initiation of differentiation media for doublecortin, a marker of immature/migrating neurons. Based on the results of this staining, most of the neurons in culture (90–95%) were positive for this marker of immature neurons (fig. 1C), suggesting that this is a valuable model of developing human neurons. It has been shown that the period in which developing mammalian neurons are the most vulnerable to anesthetic administration is the period of rapid synaptogenesis or brain growth spurt.40–42 To assess the synaptogenic capacity of the 2-week-old hESC-derived neurons, we used electron microscopy, and we were able to visualize synapse-like structures as indicated by the yellow arrow in figure 1D. It is extremely difficult to identify the exact stage in development that these cells represent. Therefore, further work will be needed to better define the maturation stage of these cells.

Propofol Induces Cell Death in hESC-derived Neurons
TUNEL staining was used to assess cell death in hESC-derived neurons following propofol exposure by labeling breaks in the DNA. The cells were exposed one and three times to 6 h of 5, 10, and 20 μg/ml propofol. The number of TUNEL-positive cells was significantly increased when compared with DMSO-treated cells following one exposure to 20 μg/ml propofol but not after a single exposure to 5 or 10 μg/ml propofol (fig. 2A-a). In the group exposed one time to 20 μg/ml propofol, 9.42 ± 1.48% of the total cells were TUNEL positive while only 1.7 ± 0.4% of cells treated with an equal volume of the vehicle control (DMSO) were TUNEL positive (fig. 2A-b). Following three exposures to propofol, the number of TUNEL-positive cells was significantly increased in both the 10 μg/ml and the 20 μg/ml propofol-treated groups but not the 5 μg/ml propofol-treated group (fig. 2B-a). The number of TUNEL-positive cells was increased to 19.9 ± 1.1% following three exposures to 20 μg/ml propofol compared with the control in which 1.52 ± 0.32% of the cells were TUNEL positive. However, after three exposures to 10 μg/ml propofol, the number of TUNEL-positive cells was only slightly increased to 4.48 ± 1.15%, although this was a statistically significant difference when compared with the control-treated cells (fig. 2B-b). For all mechanistic studies, the cells were exposed one time to 20 μg/ml propofol for 6 h.

Propofol Exposure Down-regulates 20 MicroRNAs
As shown in figure 3A, with the use of the human miFinder miRNA PCR arrays, we identified 20 microRNAs that were significantly down-regulated following exposure to 6 h of 20 μg/ml propofol when compared with vehicle-treated cells (P < 0.05, n = 4/group). Of these 20 microRNAs, several were of interest to us based on their established roles in other diseases or models. For example, the let-7 family has been shown to be highly expressed in the brain and is important in stem cell differentiation and apoptosis.43 In addition, miR-9 and 124 have been shown to play a role in neuronal differentiation.44 However, miR-21 was of particular interest to us due to the fact that it is a well-established antiapoptotic factor.45,46 Therefore, we hypothesized that the down-regulation of miR-21 conferred by propofol exposure would play a role in the increased cell death observed in the hESC-derived neurons following propofol administration. However, the potential role of additional microRNAs in propofol-induced developmental neurotoxicity cannot be excluded. To confirm the PCR array results for the expression of miR-21, an assay was performed which again showed that miR-21 was significantly down-regulated following exposure to propofol (fig. 3B).

Overexpression of miR-21 Significantly Attenuates the Propofol-induced Cell Death
To evaluate the role of miR-21 in the propofol-induced neurotoxicity observed, a mimic was used to artificially overexpress miR-21 in the hESC-derived neurons. Transfection
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using lipofectamine in a cell culture system is a well-documented strategy for investigating the functions of microRNAs.\textsuperscript{47,48} Following 20 h of transfection with the miR-21 mimic (pre-miR-21), the overexpression of miR-21 was confirmed using qRT-PCR. The levels of miR-21 were significantly higher in the cells transfected with the miR-21 mimic when compared with scramble-treated cells (fig. 4A).

TUNEL staining was used to assess cell death following exposure to propofol with and without overexpression of miR-21. Exposure to 6 h of 20 μg/ml propofol significantly increased the number of TUNEL-positive cells (10.2 ± 1.9%) when compared with vehicle-treated cells (3.3 ± 0.4%). Overexpression of miR-21 significantly reduced the number of TUNEL-positive cells following exposure to propofol.

Fig. 2. Propofol dose dependently and exposure time dependently increases the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling (TUNEL)-positive cells. (A) TUNEL staining was used to identify damaged DNA and assess cell death following a single exposure to 6 h of various doses of propofol. Hoechst 33342 was used to stain the nuclei that are shown in blue. Most of the TUNEL-positive staining (red) was localized to the DNA-stained nuclei, and the number of TUNEL-positive cells observed was considerably higher in the 20 μg/ml propofol-treated cells when compared with control cells (a). The TUNEL-positive cells were counted to quantify the data, and cell death was significantly increased after exposure to 20 μg/ml propofol but not after exposure to either 5 or 10 μg/ml propofol (b). (B) TUNEL staining was also used to assess cell death following three, 6-h exposures to various doses of propofol. The number of TUNEL-positive cells appeared to be much greater in the 10 and 20 μg/ml propofol-treated cells when compared with control (a). To quantify the results, the number of TUNEL-positive cells was manually counted and we found that cell death was significantly increased in both the 10 μg/ml and 20 μg/ml propofol-treated groups when compared with control but not in the 5 μg/ml propofol-treated group (b). \textsuperscript{*}P < 0.05 and \textsuperscript{**}P < 0.01 versus respective control, n = 3/group.
(5.3 ± 0.7%), suggesting that miR-21 plays an important role in the propofol-induced neurotoxicity. Moreover, scramble transfection did not have an effect on the number of TUNEL-positive cells following propofol exposure (10.4 ± 1.1%), indicating that the transfection process itself is not having an effect on the cell death detected (fig. 4B).

**Knockdown of miR-21 Exacerbates the Propofol-induced Neuronal Cell Death**

To further assess the role that miR-21 plays in propofol-induced neurotoxicity, a miR-21 antagonim was used to knock down miR-21 in the hESC-derived neurons using lipofectamine. The cells were transfected for 20 h with the anti-miR-21 or a scramble control, and the knockdown was assessed by qRT-PCR. miR-21 expression was significantly reduced in the cells treated with the antagonim (0.03 ± 0.01%) when compared with the scramble-treated cells (100 ± 28.1%), confirming that the knockdown was successful (fig. 5A).

To assess the effects of miR-21 knockdown on propofol-induced cell death, TUNEL staining was used. A single exposure to 6 h of 20 μg/ml propofol significantly increased the number of TUNEL-positive cells (10.2 ± 1.9%) when compared with vehicle-treated, control cells (3.3 ± 0.4%). Knockdown of miR-21 exacerbated the effects of the propofol and led to an increase in the number of TUNEL-positive cells (13.0 ± 1.7%), further confirming that miR-21 plays a role in the propofol-induced toxicity observed in the hESC-derived neurons. Scramble transfection did not have an effect on the number of TUNEL-positive cells following propofol exposure (9.3 ± 2.1%), indicating that the lipofectamine and transfection itself do not affect the measured cell death (fig. 5B).

**Propofol Induces Alterations in the Expression of Several Members of the miR-21 Pathway**

There are several well-established regulators and targets of miR-21. We investigated the targets of miR-21 that have been shown to play important roles in apoptotic processes. STAT3 is a known regulator of miR-21 that has been shown to have antiapoptotic properties.49 STAT3 is activated when phosphorylated at the tyrosine 705 position. A single exposure to 6 h of 20 μg/ml propofol significantly decreased the expression of pSTAT3, which was consistent with the miR-21 expression data (fig. 6A-a). Sprouty 2 is known to be a direct target of miR-21. Exposure to propofol significantly increased the expression of Sprouty 2, which is consistent with...
with the miR-21 expression data since microRNAs act as negative regulators of their target genes (fig. 6A-b). Finally, Sprouty 2 is known to act on Akt, specifically to reduce the levels of activated/phosphorylated Akt. The expression of pAkt (Ser 473) was significantly reduced following exposure to 6 h of 20 μg/ml propofol (fig. 6A-c). Since Akt is known to play an important role in survival pathways, this represents a possible pathway by which propofol induces toxicity in the hESC-derived neurons.

**Manipulation of miR-21 Expression Alters the Expression of Sprouty 2**

To confirm that miR-21 targets Sprouty 2 in the hESC-derived neurons, we overexpressed and knocked down miR-21 and assessed the expression of Sprouty 2 by Western blot. We found that knockdown of miR-21 significantly increased the expression of Sprouty 2 (171.6 ± 23.2) when compared with scramble-treated cells (100 ± 17.4%) (fig. 6A-c). In addition, overexpression of miR-21 led to a significant reduction in the expression of Sprouty 2 (83 ± 7.5%) when compared with scramble-transfected cells (100 ± 6.6%) (fig. 6B-b). These data suggest that miR-21 targets Sprouty 2 in these cells.

**Knockdown of Sprouty 2 Partially Attenuates the Propofol-induced Cell Death and Alters the Expression of pAkt**

To further assess the role of Sprouty 2 in the propofol-induced toxicity, we used an siRNA-mediated approach to knock down Sprouty 2 in the hESC-derived neurons. The cells were transfected for 48 h using Sprouty 2 or scramble siRNAs and transfection reagents provided by Qiagen. Following the transfection, the knockdown efficiency was assessed by qRT-PCR. Sprouty 2 expression was significantly decreased in the group treated with the Sprouty 2 siRNA (34.5 ± 2.4%) when compared with the scramble-treated group (100 ± 1.5%) (fig. 7A-a).

To evaluate the role of Sprouty 2 in the propofol-induced toxicity, 2-week-old hESC-derived neurons were transfected with Sprouty 2 or scramble siRNA for 48 h followed by...
exposure to 6 h of 20 μg/ml propofol or control. TUNEL staining was then performed on all groups. Exposure to 6 h of propofol increased the number of TUNEL-positive cells in the control group (9.3 ± 0.5%) when compared with control, vehicle-treated cells (2.4 ± 0.5%). This increase in TUNEL-positive cells was partially attenuated by Sprouty 2 knockdown (3.9 ± 0.6%), suggesting that Sprouty 2 plays an important role in the propofol-induced toxicity. Scramble transfection did not have a significant effect on the number of TUNEL-positive cells observed in the control or propofol-treated groups, indicating that the transfection process itself does not play a role in the cell death (fig. 7A-b).

To confirm that Sprouty 2 was targeting and suppressing pAkt in our model, we transfected 2-week-old hESC-derived neurons with Sprouty 2 or scramble siRNA for 48 h and assessed the expression of pAkt and Akt by Western blot. We found that the expression of pAkt/Akt was significantly elevated following Sprouty 2 knockdown (181.7 ± 17.8%) when compared with scramble-transfected cells (100 ± 7.7%), confirming that Sprouty 2 acts to suppress activated Akt (fig. 7B).

**Discussion**

In this study, we examined, for the first time, the effects of propofol on human stem cell–derived neurons and the role of the miR-21 pathway in the observed toxicity. We found that (1) exposure to propofol induced significant cell death in the hESC-derived neurons; (2) propofol altered the expression level of several microRNAs in the neurons and down-regulated miR-21; (3) overexpression of miR-21 and knockdown of Sprouty 2 significantly attenuated the increase in TUNEL-positive cells following propofol administration, while miR-21 knockdown exacerbated the effects; and (4) the expression of activated STAT3 and Akt was significantly down-regulated, and Sprouty 2 was up-regulated following propofol exposure.

Fig. 5. MicroRNA-21 (miR-21) knockdown exacerbates the propofol-induced toxicity in human embryonic stem cell (hESC)–derived neurons. (A) hESC-derived neurons were transfected for 20 h with an miR-21 antagonor (anti-miR-21) or scrambled control (anti-scramble) microRNA (25 nM) to knockdown miR-21. To confirm the knockdown of miR-21, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used. miR-21 expression was substantially reduced in the group treated with the miR-21 antagonor when compared with the scramble-treated group, confirming the successful knockdown. (B) miR-21 knockdown exacerbated the increase in terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling (TUNEL)–positive cells following exposure to 6 h of 20 μg/ml propofol. **P < 0.01 versus anti-scramble, †P < 0.01 versus all control-treated groups, #P < 0.05 versus all other groups; (A) n = 3, (B) n = 5.
Fig. 6. Propofol exposure induces alterations in the expression of the microRNA-21 (miR-21) regulator, pSTAT3, and the direct and indirect targets of miR-21, Sprouty 2 and pAkt, respectively, and manipulation of miR-21 expression alters the expression of Sprouty 2. (A) Exposure to 6 h of 20 μg/ml propofol significantly decreased the expression of pSTAT3 (a). Propofol exposure significantly increased the expression of Sprouty 2 (b) and decreased the expression of pAkt (c). (B) Human embryonic stem cell (hESC)-derived neurons were transfected for 20 h with an miR-21 antagonir (anti-miR-21, 25 nM) or scrambled control miRNA. Following the transfection, the expression of Sprouty 2 was assessed by Western blot. Sprouty 2 expression was significantly elevated following miR-21 knockdown (a). In addition, miR-21 abundance was artificially increased by transfecting hESC-derived neurons with 1 nM miR-21 mimic (pre-miR-21). Following the transfection, Sprouty 2 expression was again assessed by Western blot. The expression of Sprouty 2 was significantly reduced following miR-21 overexpression, suggesting that Sprouty 2 is a direct target of miR-21 in our model (b). *P < 0.05 versus control or scramble, n = 3. pAkt = phosphorylated (Ser 473) protein kinase B; pSTAT3 = phosphorylated (Tyr 705) signal transducer and activator of transcription 3.

Human neurons are thought to be the most vulnerable to anesthetic administration between the third trimester in utero and the second or third year of life.50 The exact neuronal markers in humans during this time period are not well understood, making it difficult to define this stage of development. Although it is difficult to assess the exact maturity level of the hESC-derived neurons, we have shown that most of the cells in culture are doublecortin positive, a marker of migrating/immature neurons (fig. 1C). In addition, it is the period of rapid synaptogenesis in which developing neurons are most vulnerable to anesthetic administration.40-42 Thus, it is important to understand the synaptogenic capacity of the cells. We showed previously that 2-week-old hESC-derived neurons express the pre- and postsynaptic markers synapsin I and homer I, respectively.29,30 Additionally, we showed in this study that these cells display synapse-like structures upon electron microscopy imaging (fig. 1D), suggesting that these cells undergo synaptogenesis and are likely representative of this critical stage of development. However, further electrophysiological studies will be needed to better understand the developmental stage of these cells.

Studies have shown that anesthetics, when administered early in life, can lead to learning disabilities later in life in animal models.51,52 In addition, studies performed in rodent and primate models have shown that propofol induces neuroapoptosis when administered for 5–6 h.4,53 For example, a 5-h exposure to an amount of propofol sufficient to maintain a surgical plane of anesthesia induced significant neuroapoptosis in both fetal and neonatal rhesus macaques.4 Additionally, exposure of cultured neonatal rat hippocampal neurons to 5 h of 5 and 50 μM propofol was sufficient to induce cell death when compared with control-treated cells.54 In 7-day-old rat pups administered six bolus injections, at 1-h intervals of 20 mg/kg propofol, there was a significant increase in activated caspase-3 levels immediately following the exposure.55 Despite the numerous findings in animal models, the effects of propofol on a human model of neurons have yet to be studied. We found that a high, but...
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Clinically relevant dose of propofol, when administered one time for 6 h, can induce significant cell death in 2-week-old hESC-derived neurons (fig. 2A).

The mechanisms by which anesthetics induce neurotoxicity are not well understood, and although microRNAs have been shown to play crucial roles in gene regulation and disease processes, their role in anesthetic-induced neurotoxicity has yet to be examined. We found that the expression of several microRNAs, many of which have established roles in differentiation and cell death, was significantly altered by propofol administration. In particular, miR-21 was down-regulated following exposure to propofol. miR-21 was of particular interest to us due to its well-established antiapoptotic effects and its role in neuronal protection against various insults. Suppression of miR-21 in fetal rat neural progenitor cells and cultured rat cortical neurons significantly increased cell death, suggesting that miR-21 is an important antiapoptotic factor in the brain. We found that overexpression of miR-21 could attenuate the propofol-induced cell death seen in the hESC-derived neurons (fig. 2B). Additionally, we found that miR-21 knockdown exacerbated the toxic effects of propofol (fig. 5B). These data indicate that miR-21 plays an important role in the neuronal cell death. There were no differences in the number of TUNEL-positive cells among the vehicle-treated groups when miR-21 was overexpressed or knocked down. This is expected since miR-21 is a stress–response microRNA.

STAT3 is a well-established, positive regulator of miR-21. It has been shown that siRNA-induced down-regulation of STAT3 completely reverses the overexpression of miR-21 typically observed in prostate cancer cells. Moreover, down-regulation of STAT3 in ER-Src cells using both

Fig. 7. Sprouty 2 knockdown attenuates the propofol-induced cell death in the human embryonic stem cell (hESC)–derived neurons and increases the expression of pAkt. (A) hESC-derived neurons were transfected with a Sprouty 2 small interfering RNA (siRNA, 20 nM) or a control siRNA for 48 h to knockdown Sprouty 2. Knockdown of Sprouty 2 was assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Sprouty 2 expression was significantly reduced in the group treated with the Sprouty 2 siRNA when compared with scramble-transfected cells confirming successful knockdown of Sprouty 2 (a). Knockdown of Sprouty 2 partially attenuated the increase in terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling (TUNEL)–positive cells following exposure of hESC-derived neurons to 6 h of 20 μg/ml propofol (b). (B) hESC-derived neurons were transfected for 48 h with a Sprouty 2 siRNA or a control/scramble siRNA (20 nM). To confirm that Sprouty 2 was indeed acting to suppress the levels of activated Akt, we assessed the expression of pAkt and total Akt after Sprouty 2 knockdown. We found that the expression of pAkt was significantly increased in the group treated with the Sprouty 2 siRNA when compared with the scramble-transfected cells. *P < 0.01 versus Scramble. #P < 0.01 versus all other groups, †P < 0.01 versus all vehicle-control–treated groups and Sprouty 2 siRNA/propofol-treated group, (A-a) and (B): n = 3, A-b: n = 5. pAkt = phosphorylated (Ser 473) protein kinase B.
an siRNA and a pharmacological inhibitor (JSI-124) induced a significant reduction in the basal expression of miR-21.28 In addition, 2 conserved STAT3-binding sites have been identified in the miR-21 enhancer sequence, and several studies have shown, through the use of chromatin immunoprecipitation studies, that STAT3 directly binds to miR-21.27,29 STAT3 is activated when phosphorylated at the tyrosine 705 position. In addition, STAT3 has been shown to have anti-apoptotic effects on its own and in combination with alterations in miR-21 expression.69,70 We found that STAT3 and miR-21 were significantly down-regulated following a single exposure to 20 μg/ml propofol, suggesting that this pathway is contributing to the propofol-induced cell death.

Although miR-21 has many well-established targets, we decided to focus our initial studies on miR-21 targets that had recognized roles in survival pathways. Protein kinase B (Akt) plays a crucial role in cell survival and apoptosis.61,62 miR-21 can indirectly regulate Akt through several targets, including Sprouty 2. Sprouty 2 is a direct target of miR-21 that can act to negatively regulate Akt expression.19–21 We found that Sprouty 2 expression was increased following exposure to propofol with a concomitant decrease in the expression of pAkt. To confirm that miR-21 was directly targeting Sprouty 2 in our model, we overexpressed and knocked down miR-21 and assessed the expression of Sprouty 2 by Western blot. We found that Sprouty 2 was significantly up-regulated following miR-21 knockdown and significantly down-regulated following miR-21 overexpression (fig. 6B). These data suggest that miR-21 targets Sprouty 2 in our cells and altering its expression. To further elucidate the role of Sprouty 2 in the observed toxicity, we used an siRNA-mediated approach to knock down Sprouty 2. To assess the role of Sprouty 2 in the observed toxicity, we exposed hESC-derived neurons to propofol following Sprouty 2 or scramble siRNA transfection. Sprouty 2 knockdown partially attenuated the increase in TUNEL-positive cells following propofol exposure, suggesting an important role of Sprouty 2 in this process (fig. 7A). We believe that the partial return to control conditions following Sprouty 2 knockdown is likely due to the incomplete knockdown of Sprouty 2 in these cells. However, the role of additional miR-21 targets in the propofol-induced toxicity cannot be excluded and may also explain the lack of complete attenuation of the toxicity following Sprouty 2 knockdown. Nevertheless, our findings suggest a crucial role of the STAT3/miR-21/Sprouty 2/Akt pathway in the propofol-induced neurotoxicity observed.

One of the caveats of this study lies in the relevance of our in vitro model to an in vivo system. In the human brain, there are many different cell types including astrocytes, neurons, and various glial cells. These cells all interact and affect one another including responsiveness to agents such as propofol. Our model consists of a culture of pure neurons, which may prevent us from observing the true effects of anesthetics on neurons. However, we are interested in teasing out the direct effects of propofol on neurons which would not be possible with other cell types present. A second limitation of our study is that we evaluated cell death as the final endpoint. However, there may be detrimental effects on the surviving neurons that could possibly influence the long-term outcome such as increased intracellular calcium levels and aberrant cell signaling.54,63 Finally, our current study focused on the role of the miR-21 pathway in the toxicity conferred by propofol exposure. Based on our findings, it appears that miR-21 plays a crucial role in this toxicity. However, the possible role of additional microRNAs or additional miR-21 targets in developmental anesthetic-induced neurotoxicity cannot be excluded.

In conclusion, this is the first time that a role of microRNAs in the mechanism of anesthetic-induced neurotoxicity has been established. Our data suggest that (1) hESC-derived neurons represent a promising in vitro human model for studying anesthetic-induced neurotoxicity, (2) propofol induces cell death in hESC-derived neurons that can be attenuated by overexpression of miR-21 or Sprouty 2 knockdown, and (3) propofol induces neuronal cell death possibly through the STAT3-miR-21-Sprouty 2-Akt pathway. This study has a high degree of clinical relevance as many human infants and children are exposed to propofol either alone or in combination with other anesthetic agents for imaging or surgical purposes. Additionally, the incidence of propofol abuse among pregnant women is on the rise, which has the potential to affect the developing neurons of the fetus. These findings raise safety concerns regarding the use of anesthetics in children and pregnant women. The increases observed in neuronal death following propofol exposure could possibly translate to future learning disabilities as seen in the animal studies. Finally, establishing the role of microRNAs in propofol-induced neurotoxicity could possibly pave the way for new research into possible neuroprotective strategies.

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Competing Interests
The authors declare no competing interests.

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