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# Exposure to 1800 MHz radiofrequency radiation impairs neurite outgrowth of embryonic neural stem cells

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A radiofrequency electromagnetic field (RF-EMF) of 1800 MHz is widely used in mobile communications. However, the effects of RF-EMFs on cell biology are unclear. Embryonic neural stem cells (eNSCs) play a critical role in brain development. Thus, detecting the effects of RF-EMF on eNSCs is important for exploring the effects of RF-EMF on brain development. Here, we exposed eNSCs to 1800 MHz RF-EMF at specific absorption rate (SAR) values of 1, 2, and 4 W/kg for 1, 2, and 3 days. We found that 1800 MHz RF-EMF exposure did not influence eNSC apoptosis, proliferation, cell cycle or the mRNA expressions of related genes. RF-EMF exposure also did not alter the ratio of eNSC differentiated neurons and astrocytes. However, neurite outgrowth of eNSC differentiated neurons was inhibited after 4 W/kg RF-EMF exposure for 3 days. Additionally, the mRNA and protein expression of the proneural genes *Ngn1* and *NeuroD*, which are crucial for neurite outgrowth, were decreased after RF-EMF exposure. The expression of their inhibitor *Hes1* was upregulated by RF-EMF exposure. These results together suggested that 1800 MHz RF-EMF exposure impairs neurite outgrowth of eNSCs. More attention should be given to the potential adverse effects of RF-EMF exposure on brain development.

The worldwide use of mobile phones has rapidly increased over the past decade, which has raised concerns about possible adverse health effects. The electromagnetic fields (EMFs) emitted from cellular phones ranges from 800 to 2000 MHz, which falls in the radiofrequency (RF) spectrum. The Global System for Mobile Communications (GSM) 1800 MHz RF-EMF is one of the most widely used frequencies. However, the biological effects of 1800 MHz RF-EMF on mammalian cells are largely unknown. The brain is a main concern with regards to the effects of RF-EMF because the brain is particularly sensitive to toxic chemicals and physical stimuli, especially during development. Previous studies have reported the potential effects of RF-EMF exposure on cell proliferation, apoptosis, and neuronal loss in the brain<sup>1–3</sup>. The underlying mechanisms may be due to increased ROS production, impaired mitochondrial functions, disruption of intracellular calcium homeostasis, upregulated heat shock protein expression, and finally, specific gene expression changes in the brain<sup>4–8</sup>. However, the available evidence is not sufficient to draw any definite conclusions, and future investigations still need to fully explore the detailed mechanisms. Epidemiologic and laboratory animal studies have also reported that irradiation of mobile phone frequency EMFs causes neurobehavioral dysfunction, impairs hippocampal neuronal plasticity, and increases the permeability of the blood-brain barrier and the risk of neurodegenerative diseases and brain tumours<sup>9–12</sup>. However, the effects of RF-EMF exposure on brain development remain largely unknown.

To date, the vast majority of mechanistic information regarding the effects of EMFs on brains have been derived from *in vitro* studies using primary neural cultures or immortalised or tumour-derived cell lines. Extrapolation of these results to the *in vivo* situation is not always feasible. Stem cell technology does, however, provide a new tool for better understanding the adverse reactions induced by environmental hazards<sup>13</sup>. Stem cell technology is especially useful for developmental neurotoxicology research because stem cells are widely represented in developing systems<sup>14</sup>. Embryonic neural stem cells (eNSCs) are considered to be multipotent because they can give rise to the three major cell types of the brain<sup>15</sup>. These cells can be derived from foetal nervous system tissues. The fate decision of eNSCs is critical for brain development<sup>16</sup>. This includes processes such as proliferation of eNSCs, neuronal and glial cell differentiation, cell death, and the development of neurites. For these reasons, eNSCs represent the developing brain in most situations. Embryonic NSCs have been widely used for the analysis



of developmental neurotoxicology properties of chemical agents but may also be applied to analyse the effects of physical factors<sup>17</sup>. Thus, the identification of the potential effects of RF-EMF exposure on eNSCs is of special importance for understanding the developmental neurotoxicology effects of RF-EMFs.

Previous studies have revealed that exposure of developing rodents to RF-EMFs may lead to an impairment of brain cells, which depends on variables such as the target-specific absorption rate (SAR) and the duration and frequency of EMF exposure. SAR defines the tissue-absorbed energy per unit of mass, which is expressed in W/kg, and is widely used to measure the doses of RF-EMF exposure. The loss of neurons was detected in the rat brain as a consequence of repeated *in utero* exposure to GSM 900 MHz at a SAR value of 2 W/kg<sup>18</sup>. An upregulation of the transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells was observed after GSM 1710 MHz RF-EMF exposure at a SAR value of 1.5 W/kg<sup>19</sup>. Alterations in neuronal functions were observed after 900–1800 MHz RF-EMF exposure with a SAR value of 1.6 W/kg during phases of development<sup>20</sup>. In addition, previous studies examined the effects of extremely low frequency (ELF)-EMFs on adult NSCs, and ELF-EMF exposure promoted adult NSC proliferation and neural differentiation<sup>21–24</sup>. These results strongly indicate that RF-EMFs may influence various processes of eNSC development. However, the effects of RF-EMF exposure on eNSCs and the underlying mechanisms are unknown.

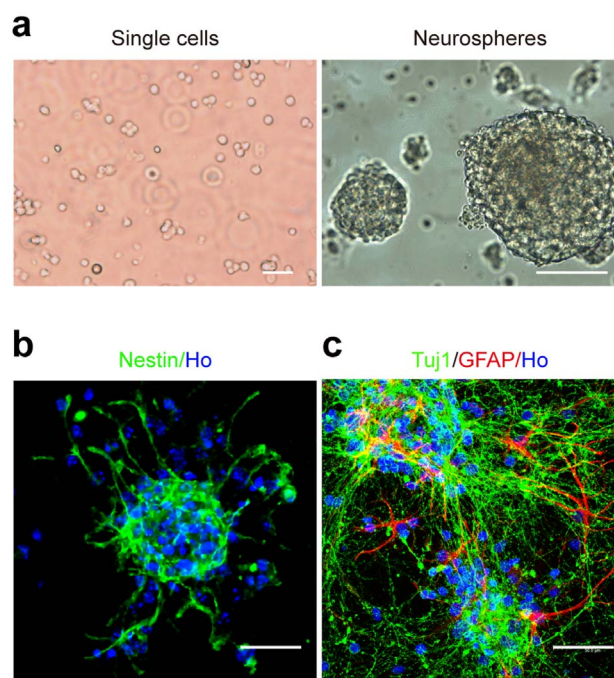
The present study was conducted to determine whether RF-EMF exposure influences the development of eNSCs and, if so, to identify the molecular mechanisms underlying this effect. Specifically, these experiments used NSCs from embryonic mouse brains as a cell model and focused on proliferation, neuronal and glial differentiation, cell death, and neurite outgrowth of differentiated neurons. The results revealed an impairment of 1800 MHz RF-EMF exposure on neurite outgrowth of eNSCs and raised a potential adverse effect of RF-EMF exposure on brain development.

## Results

### Exposure to 1800 MHz RF-EMF does not cause cell death in eNSCs.

To explore the effects of 1800 MHz RF-EMF exposure on eNSCs, we cultured eNSCs from embryonic day (E) 13.5 mouse cortex. Cell identification was performed. The isolated cells formed neurospheres after 6 days cultured *in vitro* (Fig. 1a). In addition, the neurospheres were Nestin positive, which is a specific marker of NSCs (Fig. 1b). After differentiating for 7 days *in vitro*, the cells were stained with the neuron specific marker beta-III Tubulin (Tuj1) and the astrocyte specific marker glial fibrillary acidic protein (GFAP). We found that the differentiated cells contained both Tuj1+ and GFAP+ cells (Fig. 1c). These results demonstrated that the cells cultured were NSCs.

We first detected whether 1800 MHz RF-EMF exposure caused cell death in eNSCs using terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) and Hoechst33342 staining. These two methods are sensitive to detect DNA damage during cell death and are widely used to detect cell apoptosis<sup>25,26</sup>. SAR value was used to define the exposure unit, according to previous reports<sup>27,28</sup>. The cells were exposed to a SAR value of 4 W/kg RF-EMF for 3 days. No change in the percentage of TUNEL positive cells was found (Fig. 2a, b). In addition, the results from the Hoechst33342 staining also showed no change in cell death after RF-EMF exposure (Fig. 2c, d). To further confirm these findings, we detected the protein expression and activity of Caspase-3, which have been previously demonstrated as apoptosis indicators and are also widely used for apoptosis detection<sup>2,29</sup>. The protein expression of total and cleaved Caspase-3, the active form of Caspase-3, was not changed after a 4 W/kg RF-EMF exposure for 3 days (Fig. 2e, f). Previous studies have indicated that RF-EMF exposure may cause transcriptional changes in apoptosis related genes, such as the pro-apoptotic gene Bax and anti-apoptotic gene



**Figure 1 | eNSC culture and identification.** (a) The isolated single cells from E13.5 mouse telencephalons. (b) The cultured cells formed neurospheres at 6 days *in vitro*. (c) The cultured cells were Nestin positive. (d) The cultured cells can differentiated into Tuj1+ and GFAP+ cells *in vitro*. Scale bar: 50  $\mu$ m.

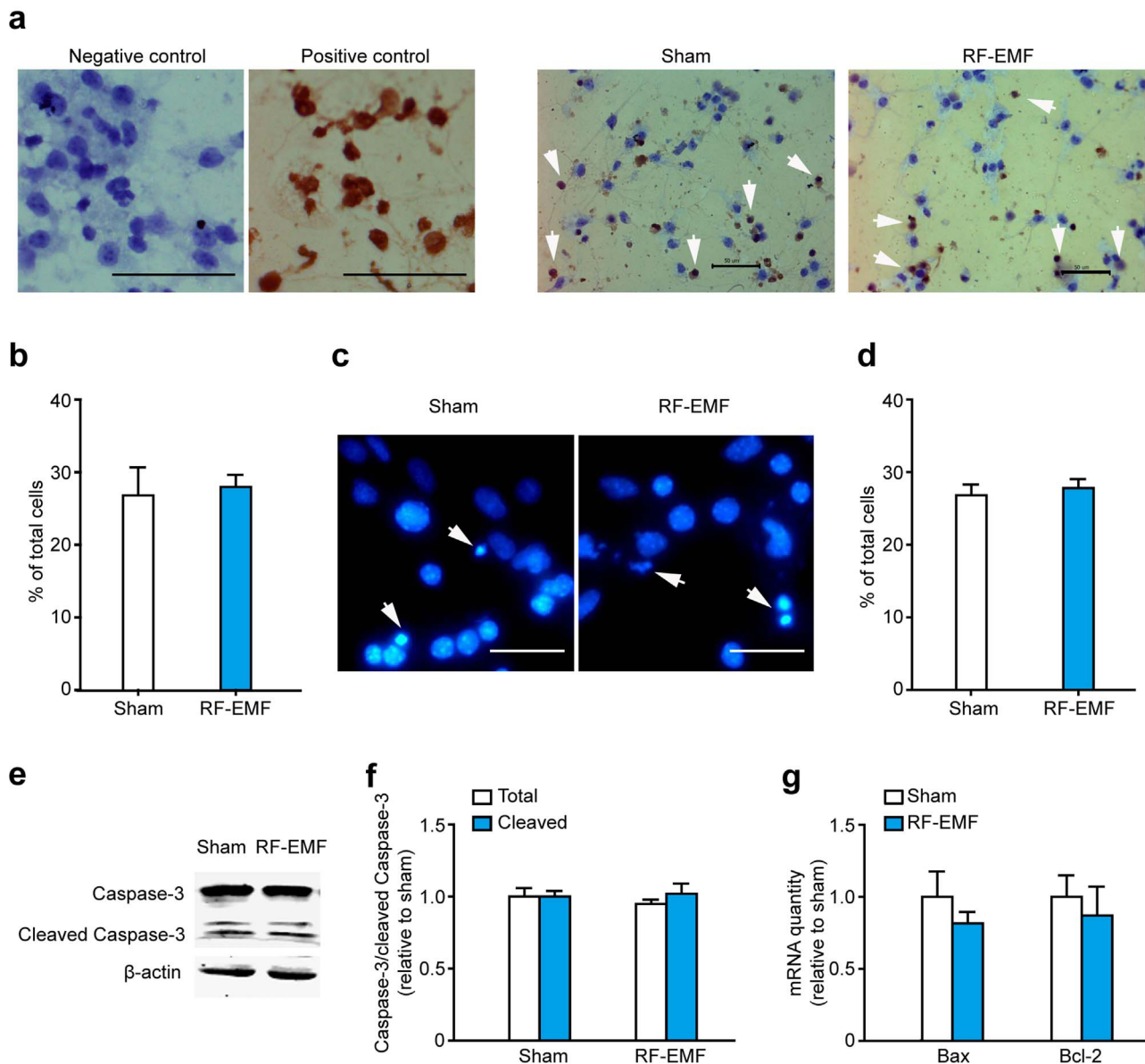
Bcl-2<sup>2</sup>. To reveal whether 1800 MHz RF-EMF exposure causes this effect in eNSCs, we detected the mRNA expression of Bax and Bcl-2 after RF-EMF exposure. No change in either gene was found (Fig. 2g). These results suggested that there is no effect of RF-EMF exposure on the cell death of eNSCs.

### Exposure to 1800 MHz RF-EMF does not influence eNSC proliferation or the cell cycle.

To further explore the effects of 1800 MHz RF-EMF exposure on eNSCs, cell viability was detected. Cultured eNSCs were exposed to a 4 W/kg RF-EMF for 1, 2, and 3 days. Cell viability was detected using a Colorimetric Cell-counting Kit-8 (CCK-8) assay. No change was found in any of the three time points after RF-EMF exposure (Fig. 3a). Then, eNSCs were exposed to 1, 2, and 4 W/kg RF-EMF for 3 days. The results suggested that cell viability was not impaired in any of the three doses (Fig. 3b). Cell proliferation was detected using Bromodeoxyuridine (BrdU) incorporation methods after RF-EMF exposure. There was no alteration in the percentage of BrdU+ cells between the sham group and cells exposed to a 4 W/kg RF-EMF for 1, 2, and 3 days or to 1, 2, and 4 W/kg exposure for 3 days (Fig. 3c–e).

The cell cycle is an important characteristic related to proliferation. To further confirm the effects of 1800 MHz RF-EMF exposure on eNSC proliferation, the changes in the cell cycle after RF-EMF exposure were detected. No significant difference was found for the percentages of G1, S, and G2 phase cells between the sham group and the cells exposed to a 4 W/kg RF-EMF for 3 days (Fig. 4a–c). To investigate whether there were any changes in gene expression related to the cell cycle, the mRNA expression of P21, P53 and GADD45 were detected by real-time PCR. The results showed that there was no shift in the expression of any of the three genes after RF-EMF exposure (Fig. 4d). These findings suggested that RF-EMF exposure exerts no effect on the proliferation of eNSCs.

### 1800 MHz RF-EMF exposure does not alter the ratio of eNSC-differentiated neurons and astrocytes, but inhibits neurite outgrowth of



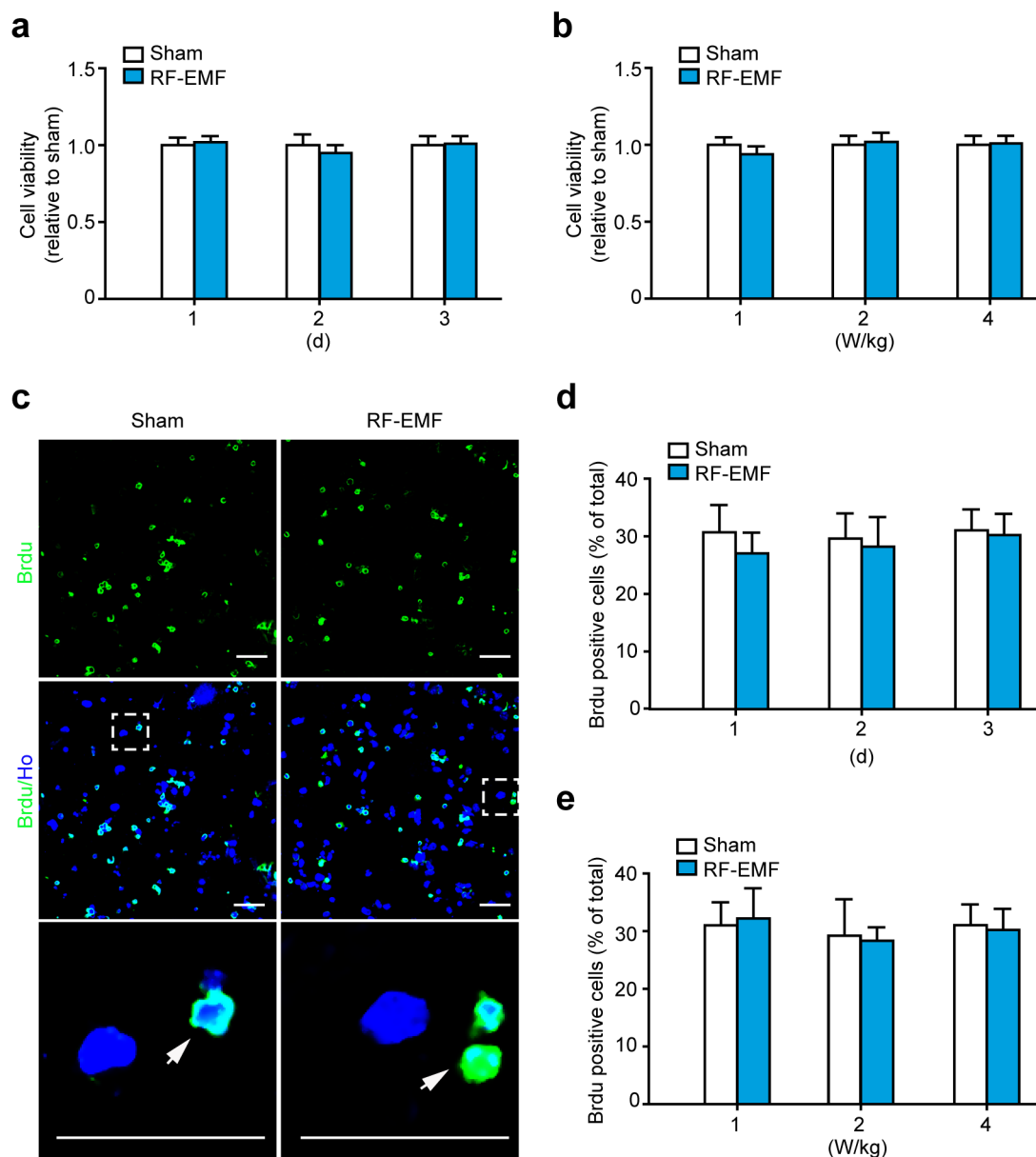
**Figure 2 | Effects of 1800 MHz RF-EMF exposure on eNSC apoptosis.** (a, b) Cell apoptosis detected by TUNEL assay after exposure to 4 W/kg RF-EMF for 3 days. No change in cell apoptosis was found after RF-EMF exposure. Images in (a) are the representative images from four independent experiments. The negative control was treated without reaction buffer TdT, and all the cells are TUNEL-. The positive control was treated with DNase I recombinant to break DNA before adding the reaction buffer, and all the cells are TUNEL+. Scale bar: 50  $\mu$ m. Arrow shows the representative TUNEL+ cells. (b) The statistical results of the percentage of TUNEL+ cells. (c, d) Cell apoptosis detected by Hoechst33342 staining. (c) The representative images from four independent experiments. Scale bar: 25  $\mu$ m. Arrows show representative dead cells. (d) The statistical results. (e, f) Caspase-3 activity detected by western blot analysis. No alteration in Caspase-3 activity was found after RF-EMF exposure. (e) The total and cleaved Caspase-3 western blotting bands from four independent experiments. Full-length blots are presented in Supplementary Figure S1a. (f) The statistical results. (g) No shift in Bax and Bcl-2 mRNA expression after RF-EMF exposure as detected by real-time PCR. Data are represented as the fold changes relative to the controls from six independent experiments. For all experiments, data are presented as the mean  $\pm$  s.e.m.

**eNSC-derived neurons.** The ability to differentiate into neurons and astrocytes is a critical function of eNSCs. To analyse the effects of 1800 MHz RF-EMF exposure on eNSC differentiation, the cultured cells were exposed to a 4 W/kg RF-EMF for 3 days in the differentiation medium. Next, the cells were then cultured for another 3 days to allow the full differentiation of neurons and astrocytes. Then, the percentages of differentiated neurons and astrocytes were detected by Tuj1 and GFAP staining. No shift in the percentage of differentiated Tuj1+ and GFAP+ cells was found (Fig. 5a, b).

To further detect the effects of 1800 MHz RF-EMF exposure on the neuronal differentiation of eNSCs, we measured the neurite

outgrowth of differentiated neurons. Three parameters were used to analyse the outgrowth of neurites: the number of primary neurites, the number of branch points, and the total length of neurites per cell. All trans-retinoic acid (RA) was used as a positive control for the significant effects on the neuronal differentiation of eNSCs, and PD98059 was used as a negative control for the notable role of the Erk1/2 signalling pathway in regulating neurite outgrowth. We found that a 4 W/kg RF-EMF exposure inhibited the number of primary neurites, the number of branch points, and the total length of neurites per cell. However, 1 and 2 W/kg RF-EMF exposure had no significant effect on neurite outgrowth (Fig. 6a–d). The robust stimulatory action of RA and the remarkable inhibitory effects of



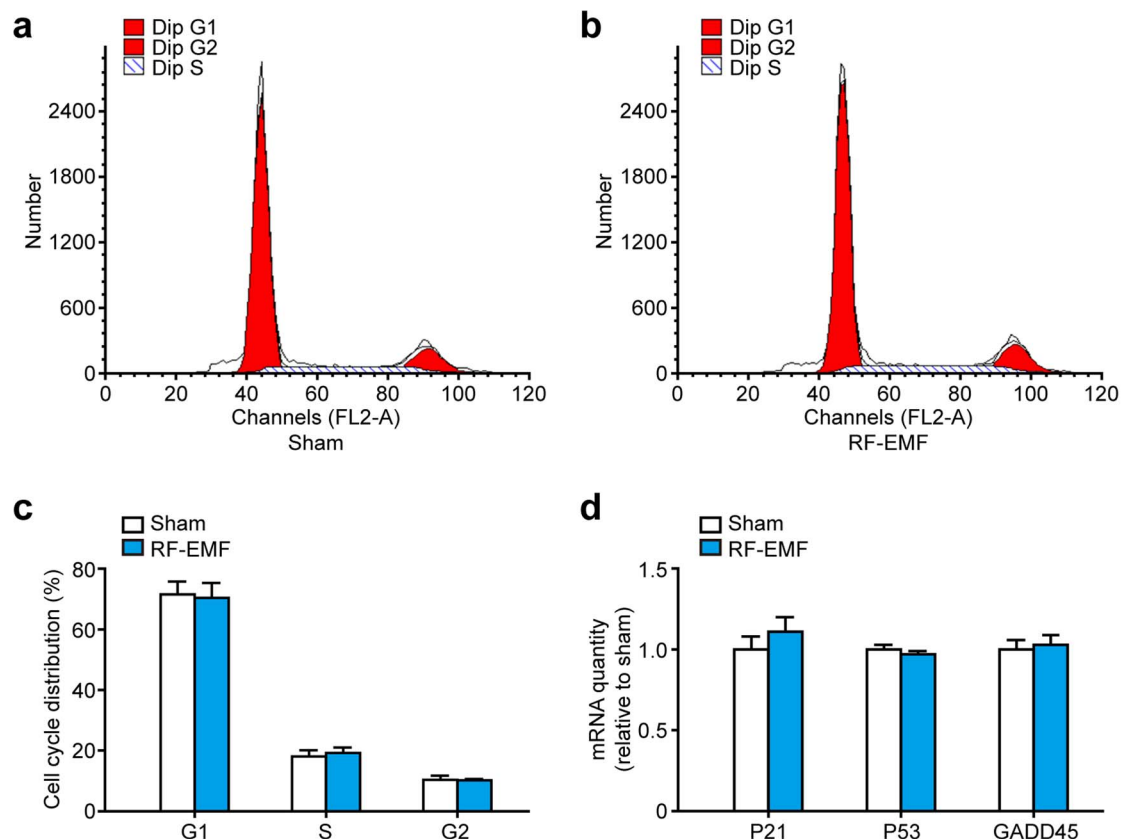


**Figure 3** | Effects of 1800 MHz RF-EMF exposure on eNSC cell viability and proliferation. (a, b) Cell viability was not influenced by RF-EMF exposure. (a) eNSCs were exposed to 4 W/kg RF-EMF for 1, 2, and 3 days. Cell viability was detected by a CCK-8 assay. (b) Cell viability of the eNSCs detected after the cells were exposed to 1, 2, and 4 W/kg RF-EMF for 3 days. (c–e) Cell proliferation detected by BrdU incorporation after exposure to 4 W/kg RF-EMF for the indicated number of days or exposure to the indicated SARs for 3 days. No difference in cell proliferation was found between the sham and RF-EMF exposure groups. (c) The representative images of BrdU+ cells from Sham and RF-EMF exposure groups. Arrows show the representative BrdU+ cells. Scale bar: 50  $\mu$ m. (d) and (e) show the statistical results of the percentage of BrdU+ cells in each condition. For all experiments, data are from four independent experiments and are presented as the mean  $\pm$  s.e.m.

PD98059 on neurite outgrowth observed in our experiments further confirmed the reliability of our results (Fig. 6a–d). Together, these results revealed that RF-EMF exposure impairs neurite outgrowth of eNSCs differentiated neurons.

**1800 MHz RF-EMF exposure disrupts the expression of basic helix-loop-helix gene (bHLH), which is critical for neuron development.** The bHLHs are critical for controlling eNSC differentiation and neurite outgrowth as previously reported<sup>30,31</sup>. To explore the underlying mechanism of the effects of RF-EMF on neurite outgrowth, we detected the effects of 1800 MHz RF-EMF exposure on the mRNA expression of bHLHs using real-time PCR. First, the mRNA expression of the proneural genes Neurogenin 1 (Ngn1), Neurogenin 2 (Ngn2), Mash1, NeuroD, Math1, and Math3 were

detected after a 4 W/kg RF-EMF exposure for 3 days in the differentiation medium. Ngn1 and NeuroD mRNA expression were remarkably decreased, while no changes were observed in the other genes (Fig. 7a). We then detect the time-dependent effects of RF-EMF exposure on the mRNA expression of Ngn1 and NeuroD, and no changes were found after a 4 W/kg RF-EMF exposure for 1 or 2 days (Fig. 7b, c). Second, the Hes genes, most of which are inhibitors of neuronal development, were detected. We found that Hes1 expression was significantly upregulated after a 4 W/kg RF-EMF exposure, while no change in Hes5 or Hes6 was found (Fig. 7d). Then, the Hes1 mRNA expression was detected after a 4 W/kg RF-EMF exposure for 1 and 2 days, and we found that RF-EMF exposure upregulated Hes1 expression significantly after a 2 day RF-EMF exposure (Fig. 7e). Furthermore, we found that the protein



**Figure 4 | Effects of 1800 MHz RF-EMF exposure on cell cycle changes in eNSCs.** (a–c) eNSCs were exposed to 4 W/kg RF-EMF for 3 days; the cell cycle was detected with a flow cytometer. No shift in the cell cycle was found after RF-EMF exposure. (a) and (b) The representative images of the sham and RF-EMF groups from four independent groups. (c) The statistical results of the cell cycle distributions. (d) mRNA expression of the cell cycle related genes detected by real-time PCR. None of the three genes were influenced by RF-EMF exposure. Data are from six independent experiments. For all experiments, data are presented as the mean  $\pm$  s.e.m.

expression of Ngn1 and NeuroD was decreased, while the Hes1 protein expression was increased significantly after a 4 W/kg RF-EMF exposure for 3 days (Fig.7f–i). In addition, the mRNA expression of Tuj1, which is a downstream factor of the proneural genes and is also critical for neuronal differentiation and neurite outgrowth, was significantly downregulated after RF-EMF exposure for 3 days (Fig. 7j). These results indicate that the disruption of bHLH expression may be part of the underlying mechanisms of the effects of 1800 MHz RF-EMF exposure on neurite outgrowth in eNSC-derived neurons.

## Discussion

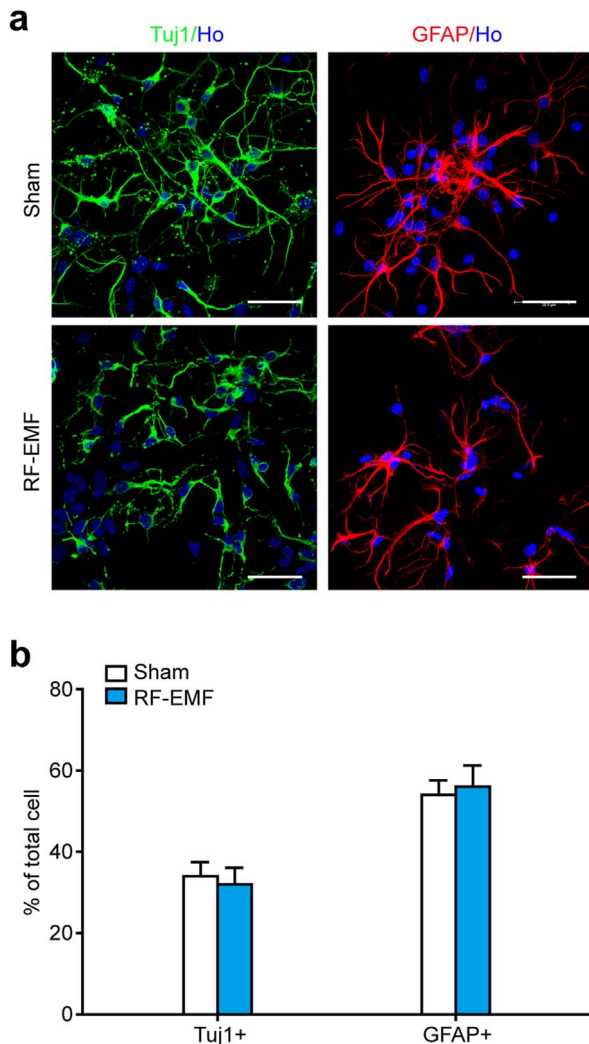
Neuronal development from eNSCs is a critical process of brain development. Here, we found that an 1800 MHz RF-EMF, which is widely used in mobile phone communication, has no effect on the proliferation, apoptosis, or differentiation of neuron and astrocyte ratios of eNSCs. However, RF-EMF exposure inhibits neurite outgrowth in eNSC differentiated neurons. We also found that 1800 MHz RF-EMF exposure disrupts the mRNA and protein expression of bHLHs, which are crucial factors for neural differentiation and neurite outgrowth. These results improve our understanding of the effects of RF-EMF exposure on eNSC development and the potential adverse effects and mechanisms of RF-EMF exposure on the developing brain.

Given the wide use of mobile communications in everyday life, whether RF-EMF exposure affects brain development is a major concern. During brain development, any environmental stimuli that influence eNSC fate decisions may disturb brain development. In this study, we found even a SAR of 4 W/kg RF-EMF exposure has no

effect on the cell viability and apoptosis of eNSCs or on the expression of the apoptosis related genes Caspase-3, Bax, and Bcl-2. Most previous studies in different cell lines and animal models obtained the same conclusions<sup>32</sup>. However, there are reports that indicate the loss of neurons in mouse brains after a 900 MHz RF-EMF exposure<sup>3,33</sup>. The upregulation of apoptosis related genes have also been previously observed in embryonic stem cell derived neural progenitor cells *in vitro*<sup>19</sup>. These conflicts may be due to the different cell models, the exposure SARs, and the durations and frequencies of exposure.

The proliferation of eNSCs is related to the self-renewal ability of the cells, which is of great importance in maintaining the total number of eNSCs for generating various types of neurons and glia during brain development. The effects of EMF exposure on adult NSC proliferation were extensively studied in the ELF frequencies, which is generated by power lines and consumer devices. A facilitative effect on cell proliferation was found in NSCs from adult brain<sup>21,24</sup>. However, the effects of RF-EMF exposure on NSC proliferation are largely unknown, especially during the embryonic phase. Our studies address this issue in a timely manner using eNSCs. From our data, we cannot conclude that RF-EMF exposure affects eNSC proliferation or the cell cycle, or influences the mRNA expression of the cell cycle related genes P21, P53, and GADD45. It has been previously reported that the GADD45 mRNA level is upregulated by RF-EMF exposure in neural progenitor cells. However, in that study, there was still no change in the cell cycle<sup>19</sup>. We presume that the exposure durations and SARs are the primary reasons for these differences. However, to fully explore the details, further studies are needed.

The neuronal differentiation and maturation of eNSCs are critical for embryonic neurogenesis in the brain. To date, there is little



**Figure 5 | Effects of 1800 MHz RF-EMF exposure on eNSC differentiation.** eNSCs were cultured in a differentiation medium and exposed to 4 W/kg RF-EMF for 3 days. After RF-EMF exposure, the cells were cultured for another 3 days. Then, the cells were fixed and stained with Tuj1 and GFAP antibodies. No differences in the percentages of Tuj1+ and GFAP+ cells were observed between the sham and RF-EMF exposure groups. (a) The representative images of Tuj1+ and GFAP+ cells. Scale bar: 50  $\mu$ m. (b) Statistical results of the percentage of Tuj1+ and GFAP+ cells in each condition. For all experiments, data are from four independent experiments and are presented as the mean  $\pm$  s.e.m.

evidence regarding the influence of RF-EMF exposure on embryonic neurogenesis in the brain. Here, we found that RF-EMF has no effect on the ratio of eNSC differentiated neurons and astrocytes but does impair the neurite outgrowth of eNSC-derived neurons. The inhibitory action of RF-EMF exposure on neurite outgrowth was also observed in a murine SN56 cholinergic cell line and rat primary cortical neurons<sup>34</sup>. The effects of EMF exposure on neurite outgrowth were also studied in other frequency band EMFs, and the results suggested that neurite outgrowth is sensitive to EMF exposure<sup>35,36</sup>. These results indicate that neurite outgrowth may be a major target by which RF-EMF affects eNSC development and suggest a potential mechanism by which RF-EMF influences brain development. The safety limit for mobile-phone microwave-radiation emission set by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) is 2 W/kg<sup>37</sup>. In our experiments, the inhibitory effect on neurite outgrowth was found at SAR of 4 W/kg after RF-EMF

exposure for 3 days. Although this SAR is two times higher than the limit, the results also implicate a potentially hazardous effect of RF-EMF exposure on brain development for longer exposure times.

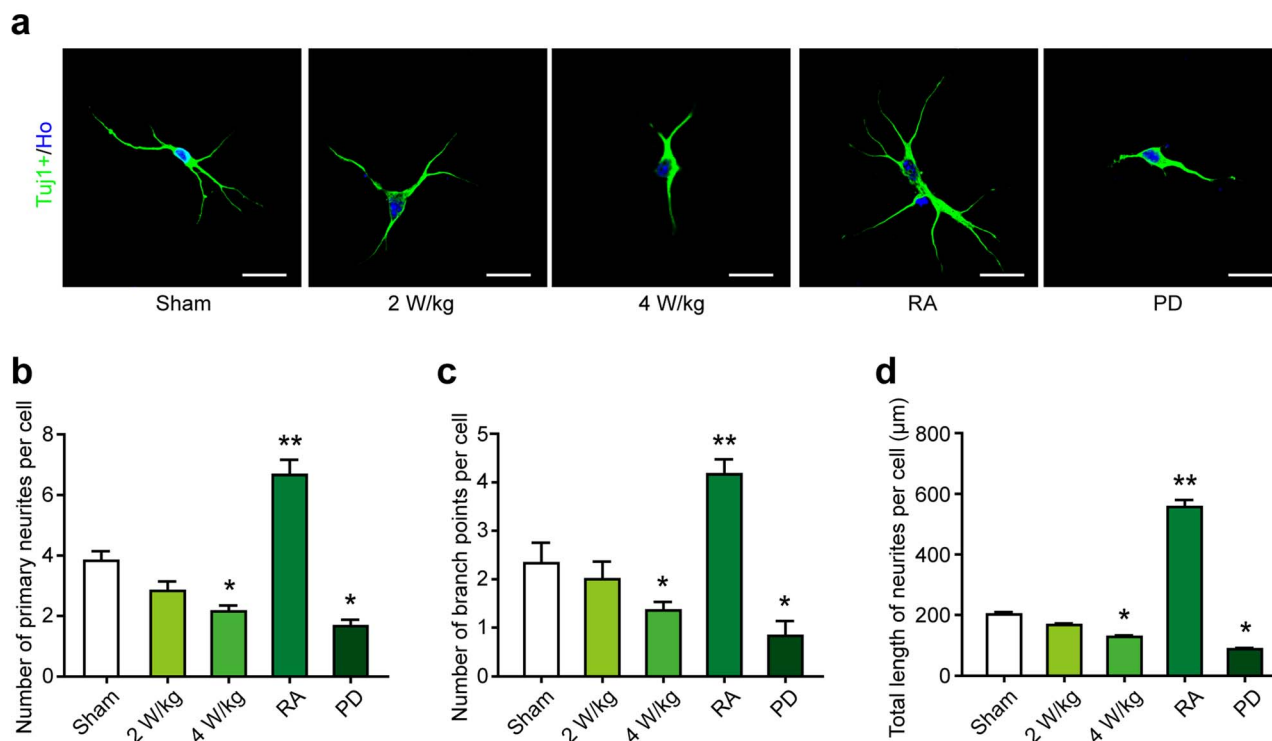
The molecular mechanisms controlling neurite outgrowth have been extensively studied. The bHLH gene family encode a group of transcriptional factors, which play a crucial role in controlling neuronal differentiation from eNSCs and neurite outgrowth<sup>30</sup>. This family includes various gene members that play different roles in regulating the differentiation and maturation of neurons. In general, the Hes genes, including Hes1 and Hes5, are inhibitory factors of neural development, whereas Hes6 is an inhibitory factor of these two genes and acts as a promoter of neural differentiation<sup>38</sup>. Ngn1, Ngn2, Mash1, NeuroD, Math1, and Math3 are proneural genes, which play diverse roles in promoting neural development from eNSCs<sup>31,39</sup>. We found that RF-EMF exposure significantly upregulates Hes1 expression and downregulates Ngn1 and NeuroD expression. All three genes are closely related to the control of neurite outgrowth, according to previous reports. Repressing Hes1 expression promotes neurite outgrowth, while overexpression of the Hes1 gene has been shown to have the opposite effect<sup>40,41</sup>. The effects of Ngn1 and NeuroD on neurite outgrowth have also been extensively studied, and the two genes are critical to the promotion of neurite outgrowth<sup>42–46</sup>. These results revealed that the potential mechanism by which RF-EMF exposure influences neurite outgrowth is the disruption of bHLH expression. However, how RF-EMF exposure disrupts the expression of bHLHs still requires further investigation. In addition, bHLHs are not only involved in neurite outgrowth but also play a critical role in regulating the neuronal differentiation of eNSCs. In our experiments, no change in neuronal differentiation was found, and this finding might be because the disruption of bHLHs occurred in the late phase of our *in vitro* differentiation cell model while the fate commitment of eNSCs had already completed. However, these findings also suggest that a higher dose or a much longer period of RF-EMF exposure on undifferentiated eNSCs may change the ratio of differentiated neurons from eNSCs. The inhibitory effects of RF-EMF exposure on Tuj1 gene expression further confirmed this possibility. Tuj1 is generally considered to be a specific neuronal marker. It is expressed very early in new-born neurons and is essential for neuronal differentiation and neurite outgrowth<sup>47,48</sup>. However, further *in vivo* and *in vitro* investigation is required to confirm whether RF-EMF exposure changes the ratio of differentiated neurons from eNSCs.

Previous studies have revealed the potential adverse effects of RF-EMF exposure on brain development. However, the exact underlying cellular and molecular mechanisms are unknown. Here, we found that RF-EMF exposure impairs the neurite outgrowth of eNSC-derived neurons by disrupting bHLH expression. This may be a potential target by which RF-EMFs act on brain development. Our studies also emphasise that many more studies are urgently required to address the potentially hazardous effects of RF-EMF exposure on brain development.

## Methods

**eNSC culture and differentiation.** Timed pregnant Balb/c mice were used for eNSC preparation. All experiments were approved by the Ethics Committee of the Third Military Medical University. Animals were housed and handled in accordance with the guidelines of the National Institutes of Health. The telencephalons of E13.5 mice were isolated for eNSC culture as previously described<sup>49,50</sup>. Briefly, eNSCs were cultured in a complete medium comprised of a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 medium (Gibco, USA) supplemented with bFGF (20 ng/ml, Sigma-Aldrich, USA), EGF (20 ng/ml, Sigma-Aldrich, USA), and B27 (1 $\times$ ) supplements (Gibco, USA). The medium was half changed every 3 days. The cultured cells were passaged every 6 days, and the second to third passages were used in the experiments. To induce differentiation, the growth medium was replaced with a differentiation medium, DMEM/F-12 containing 1% FBS and B27 (1 $\times$ ) in the absence of bFGF and EGF.

**RF-EMF exposure.** For all experiments, eNSCs were irradiated in the sXC-1800 exposure system, which was developed and provided by the Foundation for Research



**Figure 6 | Effects of 1800 MHz RF-EMF exposure on neurite outgrowth in eNSC-differentiated neurons.** eNSCs were cultured in a differentiation medium and exposed to 2 and 4 W/kg RF-EMF for 3 days. RA (1 nM) and PD98059 (10 μM) treatments at the same time were used as the positive and negative controls, respectively. Neurites were visualised by Tuj1 staining after exposure. Characteristics of the neurites were quantified as described in the methods section. (a) Representative images of Tuj1 staining. Scale bar: 25 μm. (b) Exposure to 4 W/kg RF-EMF decreased the number of primary neurites. (c) Exposure to 4 W/kg RF-EMF reduced the number of branch points. (d) Exposure to 4 W/kg RF-EMF decreased the total length of the neurites. For all experiments, data are from four independent experiments and are presented as the mean ± s.e.m. \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared with the sham group, one-way ANOVA followed by a Fisher's post hoc test.

and Information Technologies in Society (IT<sup>2</sup>S Foundation, Zurich, Switzerland). A detailed description of the system and its dosimetry has been described previously<sup>27,28</sup>. Briefly, the system consists of two identical exposure chambers mounted inside the same cell culture incubator (HERAcell® 150 i, Thermo Scientific, USA). One of the chambers was a sham control (no radiation) and the other chamber was an experimental condition (with radiation); the chambers were randomly assigned by the computer program. The exposure set-up was controlled and monitored by a computer through specific sensors. During exposure, the temperature in the chambers was monitored and maintained at  $37.0 \pm 0.5^\circ\text{C}$ . The temperature variance between the chambers did not exceed  $0.3^\circ\text{C}$ .

All experiments were performed at a carrier frequency of 1800 MHz with intermittent exposure (5 min field on, 10 min field off) in a GSM Talk-signal mode. The monolayers of eNSCs were located in two 6-dish holders and placed inside the exposure chambers. An average SAR value of 1, 2, or 4 W/kg was selected. The exposure time varied between 1 and 3 days.

**Cell apoptosis analysis.** Cell apoptosis was analysed by TUNEL and Hoechst 33342 staining. The cultured eNSCs were exposed to a 4 W/kg RF-EMF for 3 days. The cells were then dissociated and plated onto poly-L-lysine-coated round coverslips (12 mm in diameter) at a concentration of  $1.0 \times 10^5$  cells/ml. Six hours after attachment, the eNSCs were harvested and fixed with 4% paraformaldehyde for 20 min. Then, the cells were rinsed in PBS and were stained with TUNEL or Hoechst 33342.

The TUNEL assay was performed using an in situ cell death detection POD kit (Roche Diagnostics Corp., USA) following the manufacturer's instructions. TUNEL-positive cells were counted in four non-overlapping fields per coverslip using a  $40\times$  objective. For each condition, more than 1000 cells in 12 coverslips from four independent experiments were counted. Data were converted to percentages of the total cell numbers.

For nuclear morphology staining, Hoechst 33342 (Sigma-Aldrich, USA) was applied to the cells at a concentration of 5 μg/ml for 30 min at  $37^\circ\text{C}$ . Then, the cells were washed with PBS and examined under a Leica fluorescence microscopy (Leica CTR6000, Germany). Apoptotic nuclei were identified by morphological changes, such as chromatin condensation and nuclear fragmentation. Data were obtained as described in the TUNEL assay section.

**Cell viability assay.** Cell viability was measured using a CCK-8 (Dojindo, Japan) assay, following the manufacturer's instructions. Briefly, eNSCs ( $1.0 \times 10^5$  cells/ml)

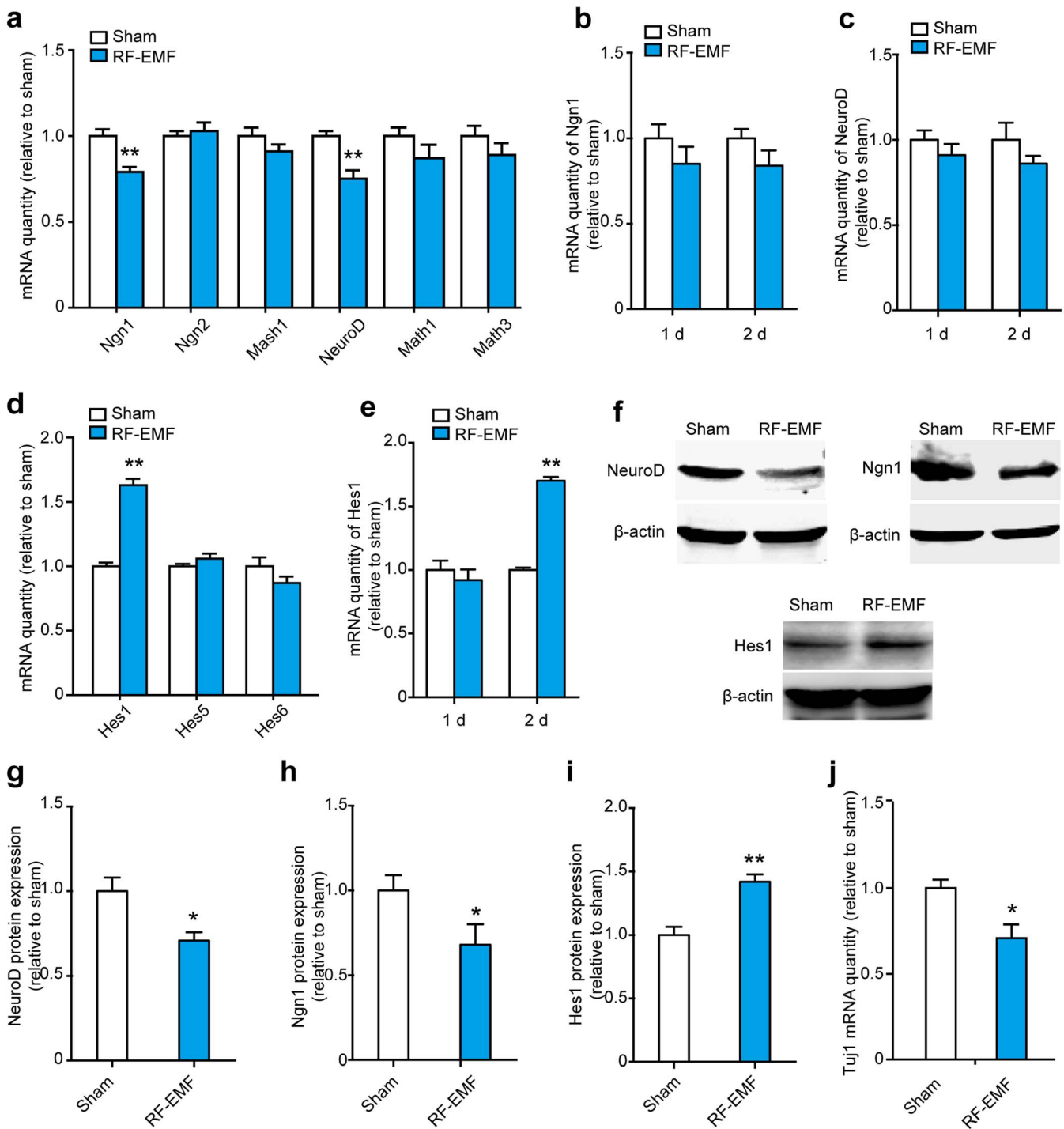
were cultured in the culture dishes and exposed to a 1, 2, or 4 W/kg RF-EMF for the indicated number of days. After exposure, a CCK-8 solution was added to the medium at a ratio of 1 : 10 and incubated at  $37^\circ\text{C}$  for 3 h. The OD value was measured by the absorbance at 450 nm with a microplate reader (TECAN, Austria).

**BrdU incorporation.** BrdU incorporation was used to detect cell proliferation as previously described<sup>51</sup>. Briefly, eNSCs ( $1.0 \times 10^5$  cells/ml) were exposed to RF-EMF for the indicated number of days. BrdU was added to the culture medium in the last 24 h. After exposure, the cells were dissociated and plated onto poly-L-lysine-coated coverslips for attachment and then fixed with 4% paraformaldehyde. Then, the sections were treated with 2 N HCl for 30 min at  $37^\circ\text{C}$  followed with 0.1 M sodium tetraborate for 15 min to denature DNA and expose BrdU. The sections were then processed for immunohistochemistry for BrdU with the following antibodies: mouse anti-BrdU (1 : 200, Sigma-Aldrich, USA) primary antibodies and Alexa Fluor 488-labelled anti-mouse secondary antibodies (1 : 200, Invitrogen, USA). Cell nuclei were visualised by staining with Hoechst33342 (5 μg/ml). The percentage of BrdU+ cells was quantified as described in the TUNEL assay section.

**Real-time PCR.** Total RNA was isolated from proliferating and differentiating eNSCs after RF-EMF exposure using the TRIzol reagent (Takara, Japan). The cDNAs were obtained by reverse transcription-PCR. Real-time PCR was performed on a CFX96™ real-time system (Bio-Rad) using SYBR® Master Mix (Life Technologies). The gene specific primers used are described in Table 1. The fold-change in gene expression was calculated using the  $2^{-\Delta\Delta\text{CT}}$  method as previously described and was normalised to endogenous GAPDH<sup>52</sup>. Subsequently, the relative gene expression levels were calculated in reference to the control.

**Immunocytochemistry and cell counts.** Cells were allowed to adhere to poly-L-lysine-coated round dishes (35 mm in diameter) and were exposed to RF-EMF for 3 days in the differentiation medium. The cells were cultured for another 3 days to allow full differentiation and were then fixed with  $4^\circ\text{C}$  4% PFA for 20 min. Immunocytochemistry staining was carried out as previously described<sup>53</sup>. The primary antibodies used were rabbit anti-mouse Tuj1 (1 : 100, GeneTex) and mouse anti-mouse GFAP (1 : 100, Abcam). Alexa Fluor 488- and 647-labelled goat anti-mouse and goat anti-rabbit secondary antibodies (1 : 200, Invitrogen) were used for visualisation. Cell nuclei were stained with Hoechst33342 (5 μg/ml). Tuj1+ and GFAP+ cells were counted in four different fields of each coverslip using a  $40\times$





**Figure 7 | Effects of 1800 MHz RF-EMF exposure on bHLH gene expression.** (a) mRNA expression of the proneural genes. eNSCs were cultured in a differentiation medium and exposed to 4 W/kg RF-EMF for 3 days. The mRNA expression was analysed using real-time PCR. Ngn1 and NeuroD mRNA expression was inhibited after RF-EMF exposure. \*\*  $p < 0.01$ ,  $n = 6$ , compared with the sham group, Student's *t*-test. (b, c) mRNA expression of Ngn1 and NeuroD after 4 W/kg RF-EMF exposed for 1 and 2 days ( $n = 4$ ). (d) mRNA expression of the Hes genes after 4 W/kg RF-EMF exposed for 3 days. Hes1 is upregulated after RF-EMF exposure. \*\*  $p < 0.01$ ,  $n = 6$ , compared with the sham group, Student's *t*-test. (e) mRNA expression of the Hes1 after 4 W/kg RF-EMF exposed for 1 and 2 days. \*\*  $p < 0.01$ ,  $n = 4$ , compared with the sham group, one-way ANOVA followed by a Fisher's post hoc test. (f–i) Protein expression of Ngn1, NeuroD, and Hes1 after 4 W/kg RF-EMF exposed for 3 days. (f) The representative western blotting bands from four independent experiments. Full-length blots are presented in Supplementary Figure S1b–d. (g–i) The statistical results. \*  $p < 0.05$ , \*\*  $p < 0.01$  compared with the sham group,  $n = 4$ , Student's *t*-test. (j) mRNA expression of TuJ1 was decreased by 4 W/kg RF-EMF exposure for 3 days. \*  $p < 0.05$ ,  $n = 6$ , compared with the sham group, Student's *t*-test. For all experiments, data are presented as the mean  $\pm$  s.e.m.

objective under a Leica fluorescence microscope. For each condition, more than 1000 cells in 12 coverslips from four independent experiments were counted. The TuJ1+ and GFAP+ cells were expressed as a percentage of the total cells. The images were collected with a Leica TCS SP5 confocal microscope.

**Neurite outgrowth analysis.** The neurite outgrowth analysis was performed as previously described<sup>34,55</sup>. Briefly, eNSCs were cultured in 35 mm dishes exposed to RF-EMF for 3 days and then were fixed with 4% paraformaldehyde for immunofluorescence staining for TuJ1. Images were acquired with a Leica





Table 1 | Primers used in real-time PCR analyses

Gene	Forward primer	Reverse primer
Bcl-2	5'-tgg gat gcc ttg gta gaa ct-3'	5'-gag aca gcc agg aga aat caa ac-3'
Bax	5'-gca cgt cca cga tca gtc ac-3'	5'-cct gga tga aac cct gta gca-3'
P21	5'-cca atc ctg gta atg tcc ga-3'	5'-agt caa agt tcc acc gtt ctg g-3'
P53	5'-gag gcc ggc tct gag tat acc a-3'	5'-ggc agg cac aaa cac gaa cc-3'
GADD45	5'-gct ggc tgc tga cga aga c-3'	5'-cgg atg agg gta aaa tgg at-3'
Hes1	5'-gaa gag gcg aag ggc aag aa-3'	5'-gag gta ctt cac agt cat ttc ca-3'
Hes5	5'-gac ggc atc aac agc agc at-3'	5'-ggc gaa ggc ttg gct gta t-3'
Hes6	5'-cct ggt gga gaa gaa ggc ac-3'	5'-ttg gcc tgc acc tgc gta-3'
Ngn1	5'-cca gcg aca ctg agt cct g-3'	5'-cgg gcc ata ggt gaa gtc tt-3'
Ngn2	5'-gtc atc ctg caa ctg cac gtc-3'	5'-agg cgc ata acg atg ctt ct-3'
Mash1	5'-act tga act cta tgg cgg gtt-3'	5'-cca gtt ggt aaa gtc cag cag-3'
NeuroD	5'-aca aca gga agt gga aac atg acc-3'	5'-cac tca tct gtc cag ct ggg-3'
Math1	5'-gag tgg gct gag gta aaa gag t-3'	5'-ggt cgg tgc tat cca gga g-3'
Math3	5'-ctc tta tgg aat gct cgg aac c-3'	5'-aat ctt tca agg cga gct tta gtc-3'
Tuj1	5'-cgc cat gtt cag acg caa g-3'	5'-ctc gga cac cag gtc gtt ca-3'
GAPDH	5'-ata cgg cta cag caa cag gg-3'	5'-gcc tct ctt gct cag tgt cc-3'

microscope (20×) by investigators blind to the experimental condition. After taking images of Tuj1+ neurons, three parameters of neurite growth were analysed using the Image J software: the total length of the neurites, the number of primary neurites and the number of branch points per cell. Neurite length was assessed by measuring the total length from one cell body to the end of all neurites, and the final length was considered the sum of all neurites measured from this one cell body. If neurons in these fluorescence images overlapped with neighbouring neurons, they were excluded from this analysis. Images of at least 20 neurons per condition were captured for each experiment, and four independent experiments were performed.

**Cell cycle analysis.** For the cell cycle analysis, the eNSCs were harvested and digested into single cells with Accutase (eBioscience, USA) after RF-EMF exposure. The cells were washed with 0.01 M cold PBS and then fixed in 75% ethanol overnight at 4°C. Then, the cells were stained at room temperature for 30 min with a solution containing 50 µg/ml propidium iodide (PI) and 50 µg/ml RNase A (DNase free). The cell cycle characteristics of the eNSCs were analysed in a flow cytometer and quantified using FlowJo software (BD Biosciences, USA).

**Western blot analysis.** After RF-EMF exposure, eNSCs were harvested and lysed in RIPA buffer (Thermo, USA), which contained a cocktail of protease inhibitors (Roche, USA). The Western blot analysis was performed as previously described and quantified with an Odyssey infrared imaging system (LI-COR; USA)<sup>51</sup>. The primary antibodies used were rabbit anti-mouse Caspase-3 (1 : 1000, CST, USA), rabbit anti-mouse Hes1 (1 : 500, Santa Cruz, USA), rabbit anti-mouse NeuroD (1 : 500, Proteintech, USA), goat anti-mouse Ngn1 (1 : 500, Santa Cruz, USA), and mouse anti-mouse β-actin (1 : 5000, Sigma-Aldrich, USA). The special Odyssey secondary antibodies were IRDye® 800 donkey anti-mouse, and IRDye® 680 donkey anti-rabbit and donkey anti-goat antibodies.

**Statistics.** Data analysis was performed using the SPSS13.0 software package. All data were collected from at least four independent duplicate experiments and are expressed as the mean ± standard error of the mean (s.e.m.). One-way ANOVA, followed by Fisher's post hoc tests or Student's t-tests, were used to determine significance. A P-value less than 0.05 was considered significant.

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## Author contributions

Conceived and designed the experiments: C.C. and Z.Z. Performed the experiments: C.C., Q.M., C.L., P.D., G.Z., L.Z., M.H., Y.L., W.D., L.P. and M.L.; Analysed the data and performed the statistical analysis: C.C. and Q.M. Helped analysed the data and provided critical strategic advice Z.Y. and Z.Z. Wrote the paper: C.C.

## Additional information

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