

RESEARCH ARTICLE

Electromagnetic field-induced adaptive response in Schwann cells through DNA methylation, histone deacetylation, and oxidative stress

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Abstract

Schwannomas are benign tumors of the peripheral nervous system arising from the transformation of Schwann cells (SCs). On the whole, these tumors are related to alterations of the neurofibromin type 2 gene, coding for the oncosuppressor merlin, a cytoskeleton-associated protein belonging to the ezrin–radixin–moesin family. However, the underlying mechanisms of schwannoma onset and progression are not fully elucidated, whereas one of the challenges might be the environment. In this light, the exposure to electromagnetic field (EMF), generated by the use of common electrical devices, has been defiantly suggested as the cause of SCs transformation even if the evidence was mostly epidemiologic. Indeed, insubstantial mechanisms have been so far identified to explain SCs oncotransformation. Recently, some in vitro evidence pointed out alterations in proliferation and migration abilities in SCs exposed to EMF (0.1 T, 50 Hz, 10 min). Here, we used the same experimental paradigm to discuss the involvement of putative epigenetic mechanisms in SCs adaptation to EMF and to explain the occurrence of hypoxic alterations after the exposure. Our findings indicate a set of environmental-induced changes in SCs, toward a less-physiological state, which may be pathologically relevant for the SCs differentiation and the schwannoma development.

KEYWORDS

electromagnetic field, epigenome, hypoxia, mitochondria, peripheral nervous system, schwannoma

1 | INTRODUCTION

Schwann cells (SCs) are the main glial cells of the peripheral nervous system, primarily committed to form the myelin sheath. SCs are highly plastic cells moving along many differentiation stages, as immature, pre-myelinating, mature myelinating, and/or non-myelinating cells

(Castelnovo et al., 2017; Monk et al., 2015). Also peculiar of SCs is their participation in peripheral nerve regeneration. The mature myelinating or nonmyelinating SCs, indeed, dedifferentiate into repair SCs, a process governed by the activation of a number of transcription factors (Jessen & Mirsky, 2016; Parkinson et al., 2008). Generally, all steps of SCs differentiation are controlled by a number of intracellular

Abbreviations: Bcl2a1, B-cell lymphoma 2-related protein A1; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DNMT, DNA methyl transferase; EMF, electromagnetic field; ERM, ezrin–radixin–moesin; FCS, fetal calf serum; HDAC, histone deacetylase; Hes-5, hairy enhancer of split-5; HMOX-1, heme-oxygenase 1; IFL, immunofluorescence; Nf2, neurofibromin type 2 gene; P0, myelin protein zero; SC, Schwann cell; Sox10, SRY-box transcription factor 10; VS, vestibular schwannoma.

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pathways and factors that maintain the acquisition/loss of SCs properties (Castelnovo et al., 2017), thus giving relevance to proliferation, migration, adhesion, and transformation as crucial events in defining the SCs fate.

Schwannomas are benign, slow-growing tumors of peripheral nerves, arising from the oncotransformation of SCs (Carroll, 2012). The most frequent form is the vestibular schwannoma (VS), a tumor affecting the vestibulocochlear nerve, whose etiology may be sporadic, unilateral, or associated with neurofibromin type 2 gene (*Nf2*)-related schwannomatosis (Evans et al., 2005; Plotkin et al., 2022). *Nf2* is the gene coding for the oncosuppressor merlin, an ezrin-radixin-moesin (ERM)-like protein, participating in the control (CTRL) of cell adhesion, migration, and proliferation (Carroll, 2012). Merlin is always inactivated in schwannoma, due to gene mutation (in *Nf2*-related schwannomatosis) or to loss of function (in sporadic VS). However, the mechanisms through which SCs participate in the induction and/or progression of schwannoma might not be exclusively related to merlin, and thus are not fully elucidated. In addition, only very limited epigenetic studies of schwannomas have been performed to date. In this regard, great importance has been recently attributed to some environmental challenges, such as the inflammatory milieu, mainly constituted by infiltrating polarized macrophages (Gregory et al., 2023), as well as to the exposure to electromagnetic field (EMF), like those generated by appliances and electronic devices commonly used (Mohamed et al., 2023). Data obtained from human epidemiological studies, namely case-CTRL and case-case studies, suggested a correlation between the mobile phone use and tumor growth (Hardell & Carlberg, 2013; Hardell et al., 2013; Hephzibah et al., 2021; Moon et al., 2014). Indeed, in vitro data indicated that rat SCs, acutely exposed to EMF modify their morphology, proliferation, and migration toward a tumoral phenotype, likely through a merlin/Hippo-dependent mechanism (Melfi et al., 2015). Importantly, prolonged EMF exposure may alter the transcriptomic profile of human SCs, giving changes in cell translation, mitochondrial activities, and expression in hearing-loss-related genes (Colciago et al., 2021). However, the complete cellular mechanisms correlating EMF exposure to the schwannoma onset are so far unraveled and need further elucidation.

Here, we deepen the molecular mechanisms underlying SC changes, coming behind EMF exposure, which might be crucial in determining SCs ability to acquire a tumoral phenotype. We found a different cell distribution along the cell cycle and changes in an anchorage-independent growth of the exposed SCs, which might be correlated with epigenetic markers and transcriptional signaling related to hypoxia and oxidative stress.

2 | MATERIALS AND METHODS

2.1 | Animals

Newborn Sprague–Dawley rats (Charles River) were used for all the experiments, according to current European rules concerning care and use of animals (Council Directive 2010/63/EU of the European

parliament and the Council of September 22, 2010 on the protection of animal used for scientific purposes) and to 3R's (replacement, reduction, and refinement) guidelines. Ethical Committee of the University of Milan approved the use of animals for cell culture.

2.2 | SCs primary cultures

As previously described (Magnaghi et al., 2006), SC cultures were obtained from rat sciatic nerves, digested with 1% collagenase and 0.25% trypsin (Merk Life Science), mechanically dissociated, then filtered through a 100 μ m filter (BD Biosciences) and centrifuged 5 min at 900 rpm. Pellets were suspended in Dulbecco's modified Eagle's medium (DMEM; Serotec) plus 10% fetal calf serum (FCS; Thermo Fisher Scientific) and plated on Petri dishes. After 24 h, 10 μ M Ara-C (Merk Life Science) was added to the culture medium. The medium was then changed with DMEM-FCS 10% plus 10 μ M forskolin (Merk Life Science) and 200 μ g/mL bovine pituitary extract (Thermo Fisher Scientific). Cells became confluent in 10 days. Immunopanning for final purification was carried out by using mouse anti-rat Thy1.1 antibody (Bio-Rad Laboratories), and 500 μ L of baby rabbit complement (Cedarlane). Cell suspension (6×10^4 cells) was seeded on 35 mm Petri dishes, in the presence of 2 μ M forskolin. At the third in vitro passage, SCs were treated for 48 h with 4 μ M forskolin and then used for different assays. SCs purity (more than 98%) was tested with a specific antibody against glycoprotein myelin protein zero (P0) (Mauro et al., 2013).

2.3 | EMF treatments

Cells were acutely exposed once to 10 min, 50 Hz, 0.1 T EMF, at 37°C. The EMF was generated by a specific magnetic field generator (Ugo Basile). The cells were then analyzed at different time points according to the specific assays. Cells used as CTRLs were plated in the same culture conditions without EMF exposure.

2.4 | DNA isolation

DNA was isolated from SCs using the DNeasy Blood & Tissue Kit from Qiagen, which provides a fast and easy silica-based DNA extraction of tissue and cell samples, lysed with proteinase K, in spin-columns. Purity and quantification of the extracted DNA were obtained by NanoDrop2000 (Thermo Fisher Scientific).

2.5 | Global DNA methylation analysis

The Global DNA methylation levels were detected by means of the colorimetric MethylFlash™ Global DNA Methylation (5-methylcytosine [5-mC]) ELISA Easy Kit (EpigenTek), on DNA

isolated from CTRL and EMF-exposed SCs. The assay was done according to the manufacturer's protocol. It measures the amount of 5-mC formed at the 5-carbon of the cytosine ring by DNA methyltransferases. The % of 5-mC in each sample is calculated by referring to a standard curve.

2.6 | CpG island methylation analysis

The methylation degree of the CpG islands in the *Nf2* promoter was analyzed by bisulfite conversion of genomic DNA. Treating DNA with bisulfite converts unmethylated cytosines into uracil, while methylated cytosines remain unchanged. Once converted, the methylation profile of the DNA can be determined by polymerase chain reaction (PCR) amplification followed by DNA sequencing. In detail, 1 µg of total DNA was bisulfite-converted using the EZ DNA Methylation-Gold Kit (Zymo Research). After quantification using NanoDrop2000 (Thermo Fisher Scientific), converted DNA was amplified by methylation-specific PCR, using primers specifically designed on the *Nf2* promoter. To verify the sequence of PCR products, they were purified, sequenced, and then analyzed by the Multiple Sequence Alignment Program Clustal W, on PRABI (https://npsa-pbil.ibcp.fr/cgi-in/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html).

2.7 | Gene expression

Gene expression was evaluated by real-time PCR (quantitative reverse transcription PCR [qRT-PCR]) on total RNA isolated from CTRL and EMF-exposed SCs.

2.8 | RNA isolation

RNA samples from SCs cultures were extracted using TRIzol™ (Thermo Fisher Scientific) according to the manufacturer's protocol; DNA was digested by DNase treatment using a specific digestion kit (Merk Life Science) and pure RNA quantified with NanoDrop2000 (Thermo Fisher Scientific).

TABLE 1 Oligoprimers used in real-time PCR.

	Forward	Reverse
DNMT3a	TGAACGCACAAGAGAACGGCT	AAGAGTGGGTGCTCCAGGGTAA
DNMT3b	ACAAGGGCAATCTGGAAGACCG	TCAAAGAGGGGTGGAAGGACA
HDAC1	GATCGGCTAGGTTGCTTCAA	CAGCACCGAGCGACATTAC
HDAC2	GCTGTCTCGAGCTACTGAAA	GTCATCACGCGATCTGTTGT
α-tubulin	TCGCGCTGTAAGAAGCAACACC	GGAGATACACTCACGCATGGTTGC
β2-microglobulin	TGCTTGACAGAGTTAAACACGTCAC	TTACATGTCTCGGTCCCAGGTG

Abbreviations: DNMT, DNA methyl transferase; HDAC, histone deacetylase; PCR, polymerase chain reaction.

2.9 | qRT-PCR

One microgram of RNA was reverse-transcribed to complementary DNA (cDNA) using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). Primers were designed by PrimerBlast software (NIH) and the efficiencies set up for each couple of primers (primer sequences are in Table 1). Ten nanograms of cDNA/sample were amplified by real-time PCR, using SsoFast™ EvaGreen® dye (Bio-Rad Laboratories) in a CFX 96 Real Time System-C1000 touch thermal cycler (Bio-Rad). Data analysis was performed using the CFX Manager 2.0 software (Bio-Rad Laboratories). Data were normalized for the housekeeping genes α-tubulin and β2-microglobulin and expressed as $2^{-\Delta\Delta C_t}$. RNA obtained from CTRL samples was used as a reference.

2.10 | Soft agar assay

Soft agar colony formation assay is a well-established method for characterizing the anchorage-independent growth of cells in vitro. Cells were grown on a layer of soft agar, thus preventing them from adhering to the culture plate and exposed to EMF as described above. SCs were acutely exposed to EMF (10 min, 50 Hz, 0.1 T) immediately after being seeded in the soft agar and then analyzed after 25 days in culture. Following 25 days in culture, all the formed colonies were stained with crystal violet 0.005% and photographed (three independent experiments, CTRL vs. EMF-exposed, each group $n = 4$). A total of about 70 colonies in each experiment were analyzed (about 20 CTRLs vs. 50 in EMF-exposed). From each image, the colony's area (µm²) was calculated by means of the software ImageJ to calculate media and SEM for CTRL and EMF-exposed groups. Data of EMF-exposed samples were expressed as % versus the mean of CTRL.

2.11 | Cell cycle analysis

Cell cycle analysis was performed by flow cytometry using the Novocyte3000 Cytofluorimeter (ACEA Biosciences).

6 and 24 h after EMF exposure, SCs were harvested, washed in phosphate-buffered saline (PBS), fixed in methanol, and resuspended in Invitrogen™ FxCycle™ PI/Rnase Staining Solution for 30 min.

Data were analyzed by the Novoexpress software. The number of cells in G0/G1, S, and G2/M and superG2 phases were expressed as percentages of total events (50,000 cells).

2.12 | Immunofluorescence (IFL)

IFL for the specific SCs proteins P0 and S100 was performed in CTRL and EMF exposed cells, seeded and grown on slides, and fixed in 4% paraformaldehyde. Cells were incubated overnight (4°C in PBS, 0.25% bovine serum albumin, 0.1% Triton X-100) with the primary antibodies for rabbit anti-S100 1:150 (DAKO Agilent) and rabbit anti-glycoprotein P0 1:150 (Proteintech; DBA Italia); then, slides were incubated with Alexa-488 (green) or Alexa-594 (red) specific secondary antibody (1:300; Gibco-Life Technologies). SCs purity was more than 98%. Slides were mounted using Vectashield (Vector Laboratories) and nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). Cells stained with no primary antibody were included as CTRL for antibody specificity. CLSM was carried out using a Zeiss LSM 510 System (Gottingen and Zen software analysis (Zeiss), and images were processed with Image Pro-Plus 6.0 (Media Cybernetics).

2.13 | Expression profile of RT² profiler PCR array

The Rat Signal Transduction PathwayFinder (RT² profiler PCR Array; Qiagen) was used to profile the expression of 84 key genes related to 10 commonly studied signal transduction pathways important for developmental, immunological, metabolic, and stress-activated processes. Two hours after EMF exposure, total RNA was isolated from CTRL and exposed SCs, as described above; 2 µg of total RNA was retro-transcribed using iScript[™] Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). cDNAs were then mixed with SuperArray RT2 Real-time SYBR Green/ROX PCR master mix and real-time PCR was performed in accordance with the manufacturer's instructions. Thermal cycling and fluorescence detection were performed using a CFX 96 Real Time System-C1000 touch thermal cycler (Bio-Rad). For data analysis, the expression of the investigated genes was analyzed using the Qiagen's online web analysis tool. It produces comparative heat maps and fold change was calculated by determining the ratio of messenger RNA (mRNA) levels of exposed cells versus CTRL using the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$). All data were normalized to the average of five housekeeping genes, Gusb, Hpvt, Hsp90ab1, Gapdh, and Actb. PCR conditions were: 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C.

2.14 | MitoTracker staining

MitoTracker Probes were applied to detect mitochondrial function of CTRL and EMF-exposed cells. In details, MitoTracker Orange CMTMRos (Thermo Fisher Scientific) is taken up by negatively charged, polarized mitochondria, thus depending on mitochondrial potential. As mitochondrial potential changes upon specific stimuli,

Mito Orange can be used to determine the gain or loss of mitochondria functionality.

MitoTracker Green FM (Thermo Fisher Scientific) is a mitochondrial selective probe covalently binding mitochondrial proteins, by cysteine residues. It accumulates in the mitochondrial matrix with a reaction that does not depend by mitochondrial potential but is representative of mitochondrial mass. In the mitochondrial lipid environment, it will fluoresce regardless of the membrane potential and serves as a dye for mitochondrial mass quantification.

Control and EMF-exposed SCs were incubated with prewarmed MitoTracker staining solutions 1:10,000 (10 nmol/L) for 30 min at 37°C, then were harvested, washed in PBS, and finally resuspended in PBS. Cells were then analyzed by flow cytometry using Novocyt3000 (Agilent) and Novoexpress software. Results are the mean of 3 different experiments: data are expressed as the % of the mean fluorescence intensity. As a measure of the relative mitochondrial function, the ratio of MytoOrange (MTO) intensity/MytoGreen (MTG) intensity has been calculated.

2.15 | Statistical analysis

Data were statistically evaluated by GraphPad Prism 4.00. Statistical significance between groups was determined by means of an unpaired Student's *t* test, one-way analysis of variance (ANOVA) with Tukey's posttest, or by two-way ANOVA analysis using Bonferroni's post hoc test. *p* < 0.05 were considered significant.

3 | RESULTS

3.1 | EMF exposure affects SCs growth

SC primary cultures were obtained from 3-day-old rats and characterized by IFL for specific SC markers. Cells show the characteristic spindle-shaped morphology in vitro; cell purity, more than 98%, was assessed by the immunopositivity for myelin protein zero (P0, red) and protein S100 (green) (Figure 1a). Nuclei were stained with DAPI (blue).

We previously demonstrated that EMF exposure (50 Hz, 0.1 T, 10 min) increased rat SC proliferation (Colciago et al., 2015). Here, we showed that the increased proliferation relates to the changes in SCs distribution among the different phases of the cell cycle and to an increase in three-dimensional (3D) nonlinear cell growth. Flow cytometry analysis (Figure 1b) showed that 24 h after EMF exposure, the number of cells in the G2 phase was significantly increased (**p* < 0.05) compared to CTRLs, together with a significant decrease (***p* < 0.01) in the number of cells in the S phase. No differences either in G1 or in the S phase were observed 6 h after the exposure between exposed and CTRL SCs. Colonies formation was assessed by the focus forming assay: EMF exposure significantly increases (*****p* < 0.0001) SCs 3D growth (25 days in culture) in soft agar

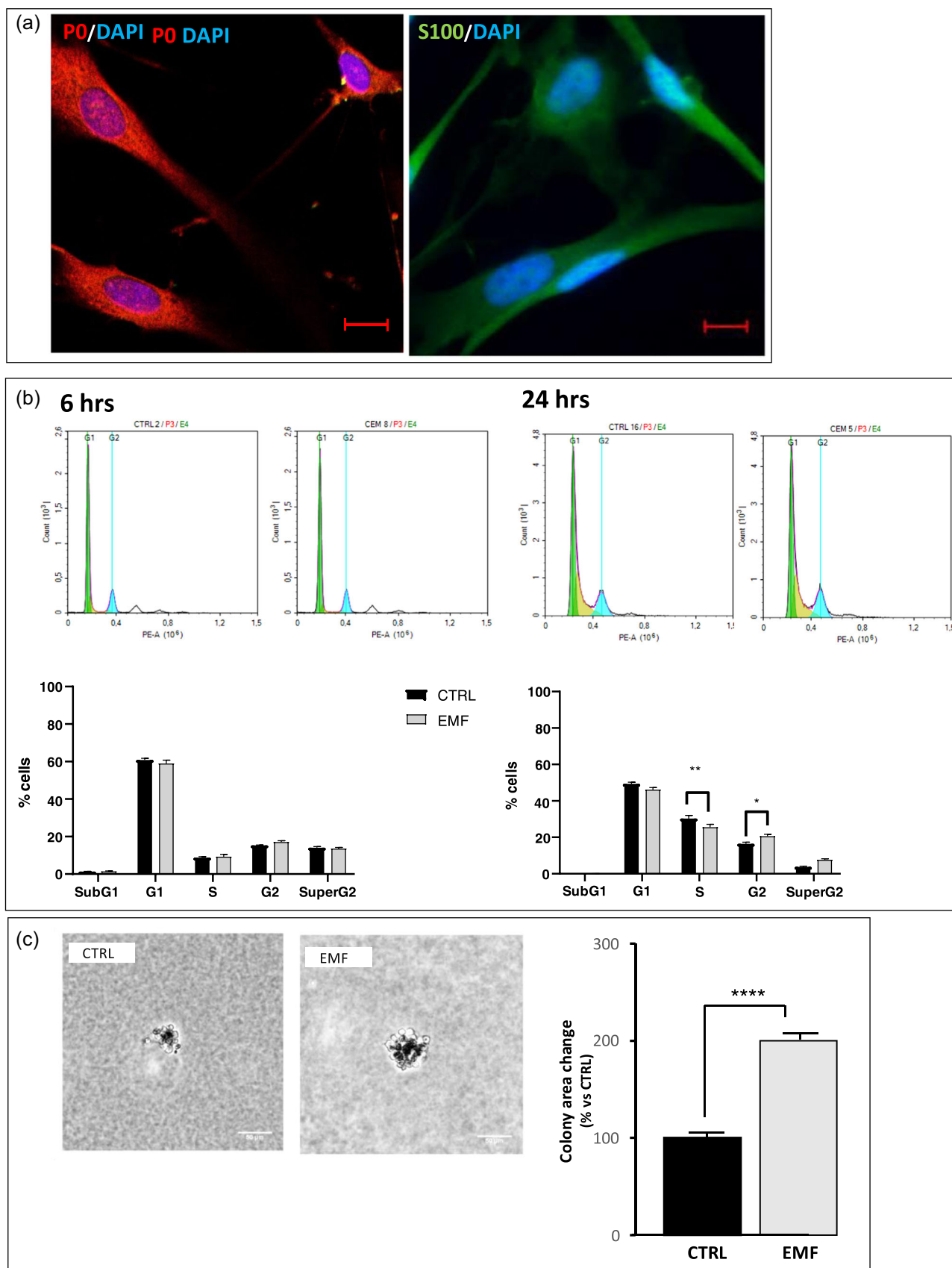


FIGURE 1 (See caption on next page).

(Figure 1c), suggesting the induction of an anchorage-independent process (see also Figure S1).

3.2 | EMF exposure modifies the epigenetic profile of SCs

Acute EMF exposure influences global DNA methylation in SCs: the percentage of 5-methyl-cytosine was significantly ($*p < 0.05$) reduced 2 and 24 h after EMF exposure (Figure 2a). Since we previously demonstrated (Colciago et al., 2015) that *Nf2*, the gene coding for the protein merlin, was downregulated by EMF exposure, we wonder whether this might be related to an alteration of the methylation degree of CpG islands in the *Nf2* gene. For this reason, *Nf2* gene promoter was analyzed by means of the DNA bisulfite conversion method: no variation of *Nf2* promoter methylation has been evidenced (Figure 2b), suggesting that merlin is not a direct epigenetic target of EMF.

As possible epigenetic targets regulating DNA methylation degree, we sought to investigate the expression levels of the inducible methylating enzymes DNA methyltransferases type 3a (DNMT3a) and (DNMT3b). DNMT3a and 3b (Figure 2c) expression was significantly reduced 6 ($*p < 0.05$) and 24 h ($**p < 0.01$) after EMF exposure, respectively. Moreover, given the important role of histone deacetylases (HDAC) in SCs (reviewed in Castelnovo et al., 2017; Jacob et al., 2011), the expression of HDAC1 and HDAC2 (Figure 2d) was evaluated in CTRL and EMF-exposed SCs. The mRNA level of both isoforms is significantly reduced by EMF exposure, even if with a different temporal profile: HDAC1 and HDAC2 levels significantly ($*p < 0.05$) diminished after 6 h from exposure, but the reduction of the expression of HDAC2 was sustained after 24hrs ($***p < 0.001$) (Figure 2d).

3.3 | EMF exposure dysregulates rat signal transduction pathways

The RT² profiler PCR array was performed to profile the expression of many genes related to 10 commonly studied signal transduction pathways, including pathways important for developmental, immunological, metabolic, and stress-activated processes. The expression of these genes was analyzed in SCs exposed to EMF compared to

CTRLs and calculated by the $2^{-\Delta\Delta C_t}$ method: data analysis revealed that some of these genes were upregulated. In Figure 3a, the scatter plot for the normalized expression of every gene analyzed between the two selected groups, showed that all data were clustered. Data points lying on the central diagonal line represent unchanged genes. The outer diagonal lines indicated the selected fold regulation threshold (selected threshold 2). Data points beyond the outer lines, in the upper left, represent the upregulated genes (yellow dots). We focused our attention on three genes that are upregulated in EMF-exposed SCs versus CTRLs (Figure 3b): B-cell leukemia/lymphoma 2-related protein A1d (Bcl2a1), hairy and enhancer of split 5 (Hes5), and heme oxygenase (decycling) 1 (Hmox1). The log2 fold changes were 3.38, 2.68, and 2.35 for Hes5, Bcl2a1, and Hmox1, respectively. A fourth gene, Gata3, is positioned in the lower limit of the threshold (pale orange in the heat map, log2 fold change 1.98), and it is not considered here.

The total clustergram (Figure 3c), the clustergram of the only genes with log2 fold change >2 (Figure 3d), and the heat map (Figure 3e, visualization of log2 fold change) are three different representations of the changes in gene expression in EMF-treated cells versus CTRLs (Figure 3e—red/orange squares).

A qRT-PCR analysis (Figure 3f) was then performed to further validate the genes that resulted upregulated in the RT-profiler array. Data have been calculated by the $2^{-\Delta\Delta C_t}$ method on three samples/group. The analysis confirmed a significant upregulation ($*p < 0.05$) only for the expression of Hmox1 following EMF exposure. The other genes, Bcl2a1, Hes5, and Gata3, did not change significantly.

3.4 | EMF exposure affects mitochondrial function

In view of the results of the RT² profiler array, evidencing that EMF upregulates genes related to hypoxic conditions and oxidative stress, we focused our attention on mitochondrial functionality (Figure 4). We used flow cytometry and fluorescent probes (MitoTracker) to determine the relative mitochondrial function, based on mitochondrial mass and membrane potential. As stated above, we used two different probes: MTG (Thermo Fisher Scientific) which binds mitochondria proteins independently of mitochondria membrane potential, giving a measure of mitochondrial mass, and MTO, which accumulates in polarized mitochondria, thus measuring membrane potential.

FIGURE 1 EMF exposure alters SCs growth. Primary cultures of rat SCs were exposed to acute EMF (50 Hz, 0.1 T, 10 min).

(a) Characterization of SCs. IFL for P0 (anti-P0-594, red) and S100 (anti-s100-488, green); nuclei are stained with DAPI (blue). Cell purity was more than 98%. Scale bar = 10 μ m; (b) Cell cycle distribution after EMF exposure. SCs flow cytometry profile is changed by EMF exposure. After 24 h, the proportion of SCs in the S phase is significantly reduced ($*p < 0.05$), whereas the % of SCs in the G2 phase is significantly increased ($**p < 0.01$). Data are mean $\pm$ SEM of three independent experiments; ($n = 4$). (c) 3D anchorage-independent growth in SCs. Two representative images of the colonies (left panels), respectively, CTRL versus EMF-exposed SCs, were grown in soft agar after 25 days in culture. EMF exposure promotes colony formation inducing a significant SCs 3D growth (25 days in culture) in soft agar ($****p < 0.0001$). This accomplished an anchorage-independent process. Data (% vs. CTRL) are mean $\pm$ SEM of three independent experiments; ($n = 4$). Scale bar 10 μ m. 3D, three dimensional; CTRL, control; DAPI, 4',6-diamidino-2-phenylindole; EMF, electromagnetic fields; IFL, immunofluorescence; P0, myelin protein zero; SC, Schwann cell.

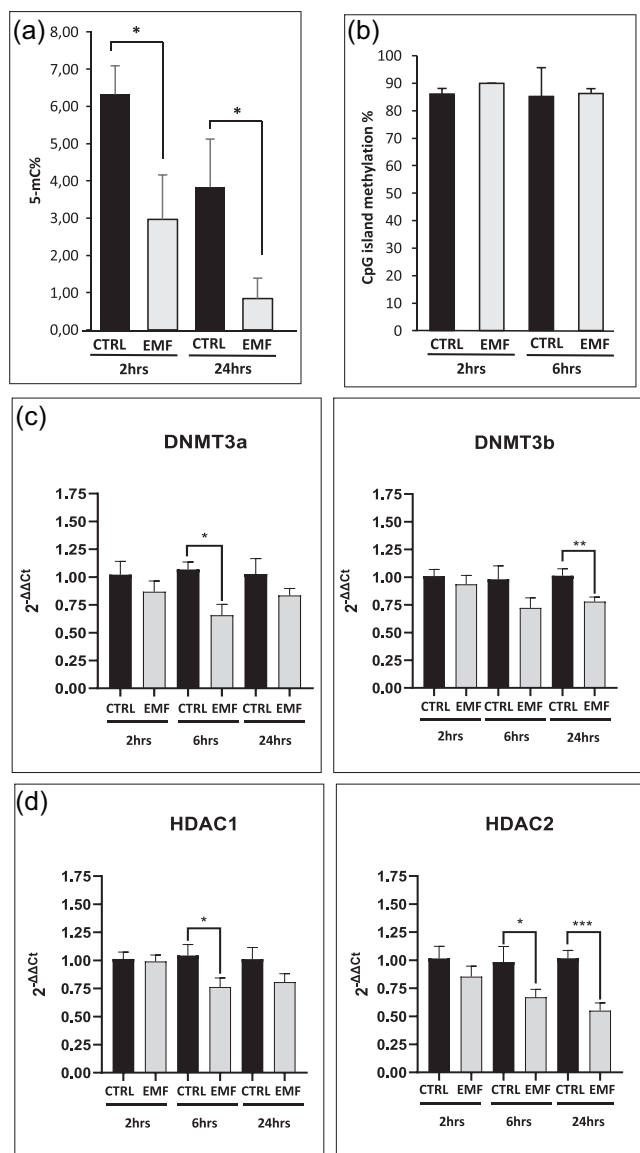


FIGURE 2 EMF exposure modifies the epigenetic profile of SCs. Primary cultures of rat SCs were exposed to the same experimental protocol as before (50 Hz, 0.1 T, 10 min). (a) The % of 5-mC was evaluated by the colorimetric ELISA Easy Kit on total DNA from CTRL and EMF exposed SCs, 2 and 24 hrs after EMF exposure. Data show that 2 and 24 h after the exposure, the total methylation degree is significantly ($*p < 0.05$) reduced. Data are mean $\pm$ SEM of three independent experiments; ($n = 5$). (b) As a potential target of the epigenetic alteration induced by EMF in SCs, the % of methylation of the CpG island in the Nf2 promoter has been analyzed by the DNA bisulfite-conversion method. No variations have been evidenced after 2 and 6 h from EMF exposure. (c, d) Expression of DNA methylating enzymes. mRNA levels of DNMT3a and DNMT3b (c) were assessed by real-time PCR. Data are expressed by the $2^{-\Delta\Delta C_t}$ method. EMF exposure significantly ($*p < 0.05$) reduced DNMT3a expression after 6 h versus CTRLs (vehicle), while DNMT3b level is significantly reduced ($**p < 0.01$) after 24 h. (d) Expression of HDAC1 and 2, evaluated by real-time PCR. Data are expressed by the $2^{-\Delta\Delta C_t}$ method. HDAC1 and HDAC2 mRNA levels significantly ($*p < 0.05$) diminished after 6 h from EMF exposure; HDAC2 expression is even more affected after 24 h ($***p < 0.001$). Data are mean $\pm$ SEM of three independent experiments; ($n = 4$). 5-mC, 5-methylcytosine; CTRL,

Figure 4 shows the flow cytometer analysis of CTRL and EMF-exposed SCs after staining with the two Mito probes. The mitochondrial membrane potential was significantly decreased in rat SCs by the exposure to EMF, 10 min, 0.1 T (Figure 4a,b), immediately after the exposure, as indicated by the fluorescence intensity. Data were confirmed by IFL of mitochondria stained with MTO, as shown in Figure 4d: rat SCs were stained with S100 (protein S100 is a specific SC marker; in green) and nuclei were stained with DAPI (blue). In the merge panel, MitOrange staining bleached in EMF-treated cells than in CTRL, confirming Figure 4b. The mitochondria membrane potential was rapidly restored after 24 h (Figure 4f). No differences in mitochondrial mass have been evidenced in the CTRL and EMF-exposed sample, either at $t = 0$, that is immediately after EMF exposure, or 24 h later ($t = 24$, Figure 4g). The ratio of MTO intensity/MTG intensity was calculated to give a measure of relative mitochondrial function (Soon et al., 2017): EMF-exposed SCs showed a significantly reduced mitochondrial function ($**p < 0.01$) versus CTRL.

4 | DISCUSSION

Previous data showed that rat SCs are modified by acute EMF exposure, changing their morphology, and reversing their myelinating program, thus acquiring a more proliferating, rather than myelinating, phenotype (Colciago et al., 2015). This resembles some characteristics of schwannoma growth. Here, we further investigated the effect of acute EMF, showing that EMF alters the cell cycle distribution of the EMF-exposed SCs, reducing their DNA synthesis and increasing the number of cells in the G2 phase, 24 h after the exposure. Interestingly, even this finding seems to be contradictory with our previous proliferative data (Colciago et al., 2015), we highlight that the rise in the G2 phase is a physiological response of SC to the EMF damage, namely a mechanism of cell repair in the attempt to overcome the insult.

As a consequence, the exposed cells acquire the ability of 3D growth during 25 days in culture, suggesting the induction of an anchorage-independent process. The mechanism involved in such an EMF response might be related to the alteration of some epigenetic markers, that is total DNA methylation and HDAC function. Moreover, a number of transcriptional pathways are modified by EMF, and, among them, genes related to hypoxia and oxidative stress.

First, we highlight that the morpho/functional shift of EMF-exposed rat SCs in culture, previously observed (Colciago et al., 2015), is related to some epigenetic modification induced by environmental adaptation. The reduction of total DNA methylation and the concurrent decrease of DNMT3a and DNMT3b and HDAC1 and HDAC2 expressions suggest that rat SCs respond to the environmental challenge increasing their transcriptional ability, thus enabling

control; DNMT, DNA methyl transferase; EMF, electromagnetic fields; HDAC, histone deacetylase; mRNA, messenger RNA; PCR, polymerase chain reaction; SC, Schwann cell.

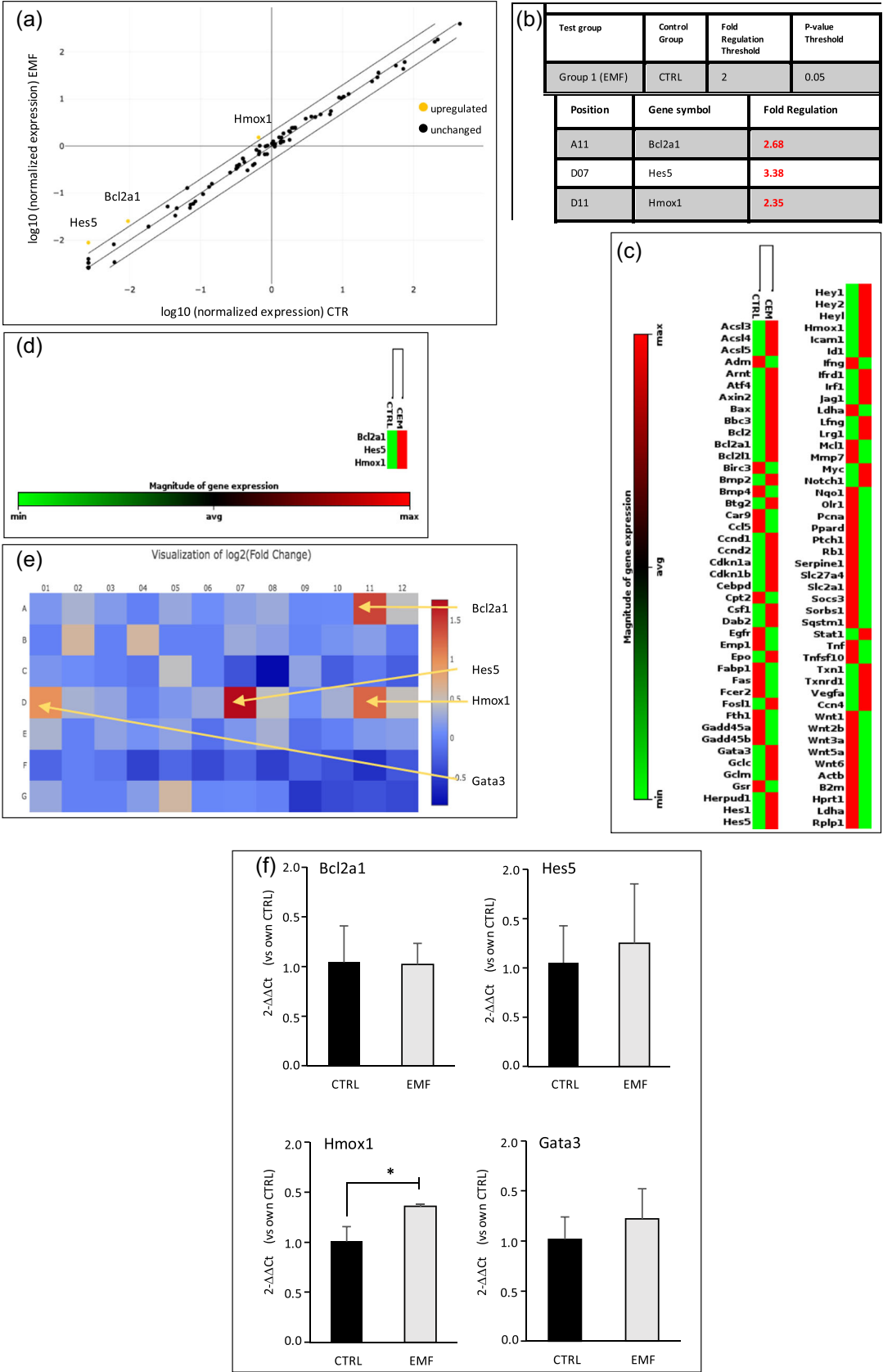


FIGURE 3 (See caption on next page).

more genes to be transcribed and to overcome the insult. A general reduction of total DNA methylation is one of the molecular signatures of cancer onset and progression (Esteller, 2007), favouring a less condensed chromatin that becomes more accessible to transcription factors. In addition, the aberrant methylation of unmethylated CpG islands on the promoter region of some oncosuppressor genes is often associated with their decreased expression (Esteller, 2007), and, accordingly, to the cancer progression. However, the EMF did not modify merlin expression through alteration in CpG island methylation. This is not surprising, since it was reported that in human schwannoma, in which SCs acquire a tumoral phenotype and merlin was generally downregulated (Plotkin et al., 2022), the aberrant methylation of the *Nf2* gene is rare (Gonzalez-Gomez et al., 2003; Lee et al., 2012). HDACs strictly participate in regulating SCs myelinating abilities with the removal of acetyl groups from histone tails, favouring chromatin remodeling, but also nonepigenetically, deacetylating nonhistone targets as transcription factors and associated proteins (Yadav et al., 2021). In particular, HDAC2 participates in the remyelination process after injury, activating the transcription of specific promyelinating factors and proteins, such as SRY-box transcription factor 10 (Sox10) and P0. Indeed, a downregulation of HDAC2 was linked to a decreased expression of some SC myelination markers, as Sox10, Krox20, P0, and myelin-associated glycoprotein (Brügger et al., 2017; Jacob et al., 2011). On this basis, we can infer that the decreased expression of P0 at 6 and 24h following rat SCs exposure to EMF, previously observed (Colciago et al., 2015), might be related to the HDAC downregulation here reported. Accordingly, the expression of some markers of immature/nonmyelinating SCs, such as p75 and GFAP (Mirsky et al., 2008), was found to be increased following EMF exposure (see Figure S2), further confirming the reversion to an immature and more proliferating phenotype.

We highlighted that the oncosuppressor merlin is the main protein involved in schwannoma development. It is known that merlin loss of function is present in almost all the schwannoma, either due to sporadic mutations or due to the concurrent mutations on other genes (i.e., SW1/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1 and/or

leucine zipper-like transcriptional regulator 1), which appear as a typical biallelic inactivation of *Nf2* in tumors (Chiasson-MacKenzie et al., 2023; Plotkin et al., 2022). Merlin is a cytoskeleton-associated protein belonging to the ERM family, which acts as a tumor suppressor (by negatively regulating cyclin D) arresting cell cycle progression (Xiao et al., 2005). As a protein associated with the cytoskeleton, merlin operates also as a metastasis suppressor by controlling cadherin-mediated cell-to-cell interaction (Lallemand et al., 2003). Indeed, one of the consequences of merlin loss of function is the inability of cells to undergo contact-dependent growth arrest and to form stable cadherin-containing cell-to-cell junctions (Lallemand et al., 2003). This underlines the ability of functional merlin to ensure contact inhibition of cell growth, thus repressing the invasion of transforming cells. The 3D growth of the SCs exposed to EMF, observed after some days in culture, is a peculiar characteristic of the spatial expansion of neoplastic cells, as in schwannoma, and might be strictly related to the merlin decrease, as previously observed (Colciago et al., 2015).

To date, a very limited number of epigenetic studies on schwannomas pathogenesis have been performed. In this light, the exposure to EMF might be relevant. Although recent observations tried to explain the possible effects of EMF exposure on cells and tissues (Franco-Obregón, 2023; Lai & Levitt, 2023; Ma et al., 2023; Sun et al., 2022), indeed, the molecular mechanisms behind those outcomes are still unresolved issues. Therefore, we focused our efforts on unveiling the transcriptional mechanisms and pathways at the base of the EMF exposure of SCs and their oncotransformation.

Our data indicate that EMF modifies a few genes related to hypoxia and oxidative stress. In particular, we found that EMF deregulates *HMOX1*, the gene coding for the protein HMOX-1. HMOX-1 is the inducible isoform of two HMOX isozymes, whose role in cells is to catalyze the rate-limiting step of the degradation of the heme group, releasing iron and carbon monoxide (Platt & Nath, 1998). In the promoter region of the *HMOX1* gene, the presence of a heat-shock element was demonstrated, which is normally strongly upregulated by several environmental stress factors, such as hypoxia, hyperthermia, heavy metals, inflammatory cytokines, and radiations (Platt & Nath, 1998). Thus, it is not

FIGURE 3 EMF exposure dysregulates rat signal transduction pathways. Rat signal transduction pathway finder RT² profiler PCR array: (a and b) Overview of the scatter plot and fold-change values for the normalized expression of 84 genes, related to 10 commonly studied rat signal transduction pathways, in CTRL and EMF-exposed SCs. Data were calculated by the $2^{-\Delta\Delta C_t}$ method. Data points around the central diagonal line represent unchanged genes. The outer diagonal lines indicate the selected fold regulation threshold of 2. Data points beyond the outer lines, in the upper left, represent the upregulated genes (yellow dots). Three genes were found to be upregulated in EMF-exposed SCs versus CTRL: Bcl2a1 (an antiapoptotic protein of the Bcl-2 family), Hes5 (related to the CTRL of myelination), and Hmox1 (the inducible isoform of the heme-oxygenase isozymes). Experiments were repeated at least three times, and normalized for β -actin (Actb), lactate dehydrogenase A (Ldha), and the 60S acidic ribosomal protein P1 (Rplp1). (c) Clustering of all normalized genes examined by RT² profiler PCR array and (d) of the only genes with log2 fold change >2. (e) Heat map of the analyzed genes (visualization of log2 fold change, red/orange squares). A fourth gene, Gata3 (GATA binding protein 3, a dual-zinc finger transcription factor), is in the lower limit of the threshold (pale orange in the heat map, log2 fold change 1.98). (f) qRT-PCR validation of the target-modified genes. Data are expressed as $2^{-\Delta\Delta C_t}$ versus their own CTRL. Only Hmox-1 gene expression confirmed the previously observed significant increase (* $p < 0.05$) in EMF-exposed SCs versus CTRL. Bcl2a1, B-cell lymphoma 2-related protein A1; CTRL, control; EMF, electromagnetic fields; Hes-5, hairy enhancer of split-5; HMOX-1, heme-oxygenase1; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SC, Schwann cell.

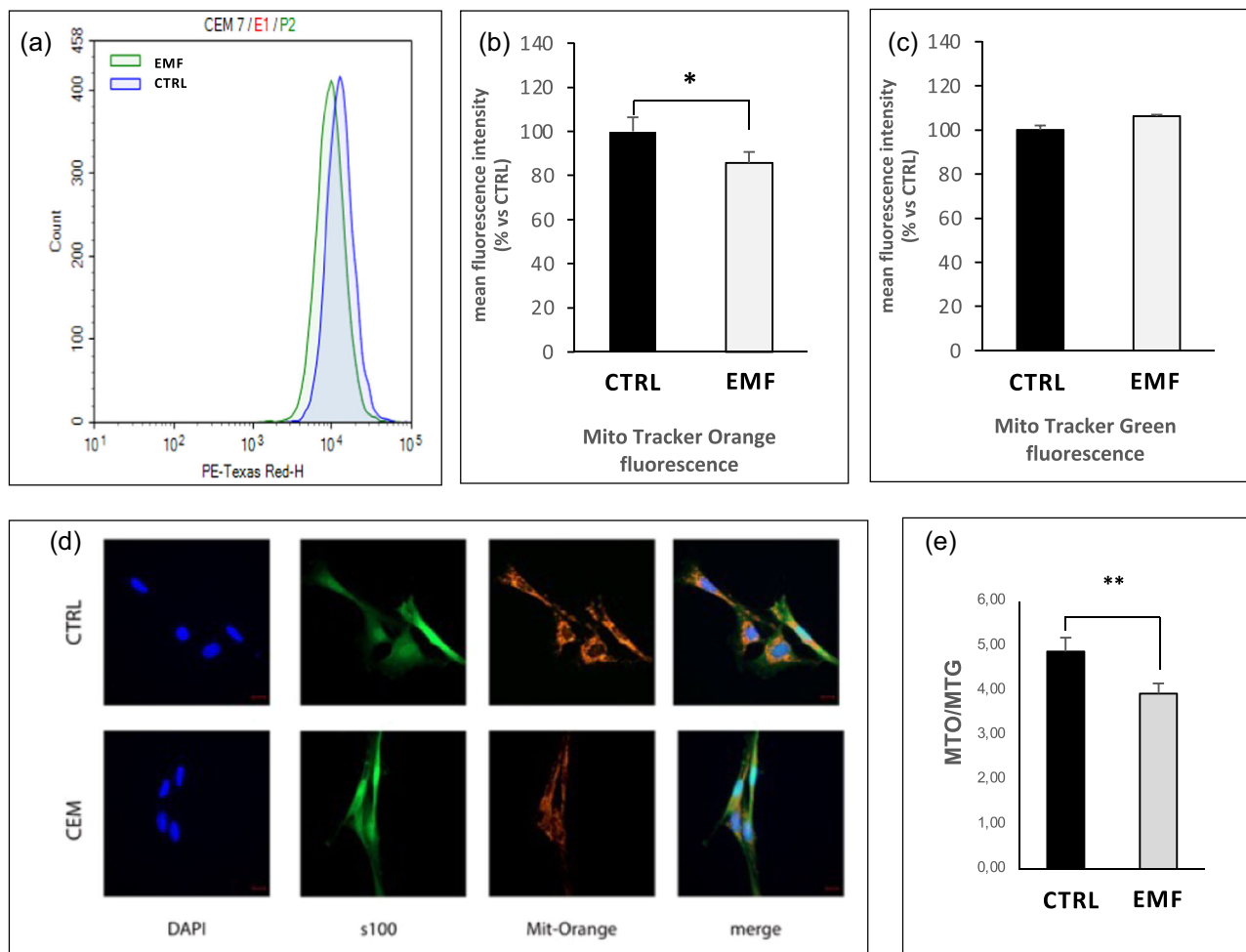
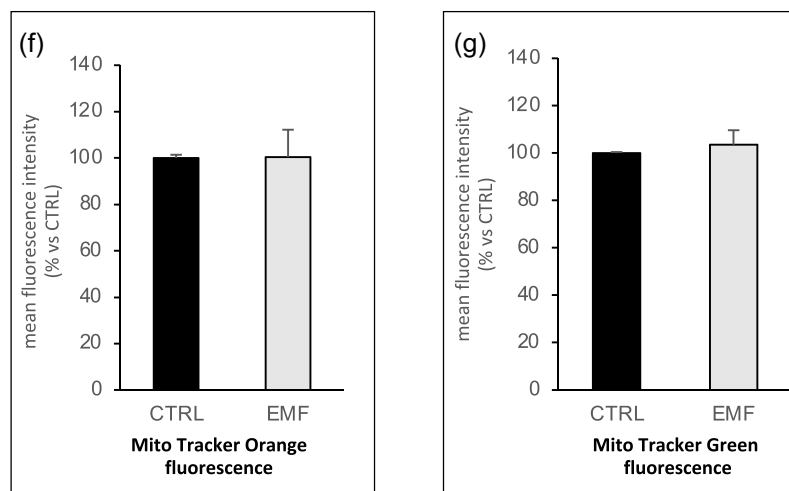
t=0hrs**t=24hrs**

FIGURE 4 EMF exposure affects mitochondrial function. (a and b) Flow cytometer analysis of mitochondrial function in CTRL and EMF-exposed SCs, assessed by MitoTracker Orange (MTO). The MMP is measured by fluorescence mean intensity immediately after EMF exposure. Compared to CTRL, EMF-exposed SCs showed a shift on the left of the relative pick of fluorescence (a) and a significantly lower MMP (b; data are expressed as % vs. own CTRL; * $p < 0.05$). (c) flow cytometer analysis of the mitochondrial mass evaluated by MitoTracker Green (MTG) immediately after EMF exposure. (d) IFL of SCs mitochondria stained with MTO (orange) immediately after EMF exposure; SCs were stained with S100 (green), and nuclei were stained with DAPI (blue). (e) Relative mitochondrial function is expressed as the ratio of MTO to MTG (** $p < 0.01$). (f and g) flow cytometer analysis of MMP (f) and mitochondrial mass (g) in CTRL and EMF-exposed SCs, 24 h following EMF exposure. Data were expressed as % versus own CTRL. CTRL, control; DAPI, 4',6-diamidino-2-phenylindole; EMF, electromagnetic fields; IFL, immunofluorescence; MMP, mitochondrial membrane potential; MTG, MitoGreen; MTO, MitoOrange; SC, Schwann cell.

surprising that also EMF might activate the expression of this gene. Indeed, in a human model of VS, the HEI193 cells, the HMOX1 gene is upregulated by chronic exposure to EMF (Colciago et al., 2021). In SCs, it was reported that HMOX-1 activation is related to a protective effect against oxidative stress and the consequent ROS (reactive oxygen species) accumulation (Jeong et al., 2019), so the transient HMOX-1 induction might be one of the adaptive strategies used by SCs to react against a hazardous environment (Hirata et al., 2000). Therefore, also in our paradigm, the transient change in HMOX1 levels might be a sort of protective response of SCs against EMF challenge. Notably, in different cell types, HMOX-1 is strongly induced by oxidative stress (Abraham & Kappas, 2008) assuming a protective role in a sort of ischemic preconditioning, promoting cell survival (Cai et al., 2019). Nevertheless, HMOX-1 overexpression has been observed in injured neurons and glia cells in many pathological conditions of the CNS (Schipper et al., 2009), suggesting a “Janus face” for this enzyme. HMOX-1 expression, indeed, correlates with tumor growth, angiogenic and metastatic potential, chemoresistance, and, in general, with poor prognosis in different types of human cancer (Nitti et al., 2017). Accordingly, it can be speculated that HMOX-1 is not exclusively cytoprotective or cytotoxic, but it assumes a different role according to cell needs and environment.

Hypoxia seems to be the thread linking the modified genes in EMF-exposed SCs. In experimental models of cerebral (Xu et al., 2018) and pulmonary (Morris et al., 2023) hypoxia/ischemia, the Hes5 levels sharply increased after the damage, suggesting that its expression is under the CTRL of hypoxia-inducible factors (Xu et al., 2018). Hes-5 expression is elevated during the development of oligodendrocytes precursors and in central demyelinating disorders (John et al., 2002). During oligodendrocytes maturation and differentiation, Hes-5 downregulates myelin gene expression (Liu et al., 2006), directly recruiting repressive complexes containing HDACs at the myelin genes promoter. Also in SCs, the transcription factor Hes-5 (Ohtsuka, 1999) participates in the regulation of the differentiation/maturation program, playing a key function at many stages of SCs differentiation, promoting the transition from SCs precursors to mature SCs, controlling SCs proliferation and acting as a brake for myelination (Mirsky et al., 2008). Additionally, Bcl2a1 (a member of Bcl2 family), is an inhibitor of the mitochondria-mediated apoptosis, which expression is induced by hypoxic conditions, mainly in the core of solid tumors (Liang et al., 2021). In our experimental conditions, we speculated that the slight overexpression of Bcl2a1 observed might be related to the increased number of colonies found in EMF-exposed cells; in agreement, it has been described that, in cancer cells, Bcl2a1a enhances the foci formation capacity in soft agar (Liang et al., 2021). Thus, the relationship between EMF-induced Bcl2a1 overexpression and cancer development cannot be excluded.

Mitochondria behave as cell sensor in a number of cellular stressing events, regulating energy metabolism and the production of ROS species. In general, when cells are EMF-exposed likely all the cellular components (i.e., membranes, nuclei, cytoplasm, and organelles)

could be targeted, therefore is expected that also mitochondria are affected. In line, recent evidence (Santini et al., 2020) suggests that, at least in the reproductive system, mitochondria integrity and function are impaired by EMF exposure, likely as the result of the increased oxidative stress. Here we found that EMF induces a derangement of mitochondria membrane potential, without affecting mitochondrial mass, but confirming mitochondria damage. Mitochondria disarrangements are also evidenced by our recent data showing that in HEI193 cells (a model of human VS) a chronic EMF exposure deregulates some signaling pathways related to mitochondria translational, elongation, and termination (Colciago et al., 2021). Altogether, these findings are indicative of an EMF effect on crucial biological mechanisms basic for cell energy support and metabolism.

In conclusion, here we further demonstrated that EMF exposure induces SCs damage, likely promoting a hypoxic cell environment and mitochondria disarrangement. These changes, in turn, activate a number of joined mechanisms. On one hand, EMF switch-on the SCs repair phenotype, likely softening the ability of these cells to make myelin; on the other hand, as a consequence of the oxidative stress induction, EMF compromises the cell growth CTRL, stimulating cell survival and the anchorage-independent proliferation. Even if further SCs studies are deserving, we cannot exclude that the induction of hypoxia and oxidative stress might be crucial for inducing tumor cell progression toward a malignant phenotype, likely characterized by invasion, angiogenesis, and metastasis.

AUTHOR CONTRIBUTIONS

Alessandra Colciago: Writing—original draft preparation; writing—review and editing. **Tasnim Mohamed:** Investigation; data curation. **Deborah Colleoni:** Investigation. **Valentina Melfi:** Investigation. **Valerio Magnaghi:** Conceptualization; data curation; writing—original draft preparation; writing—review and editing; supervision. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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