

High Frequency Electromagnetic Fields (GSM Signals) Affect Gene Expression Levels in Tumor Suppressor p53-Deficient Embryonic Stem Cells

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Effects of electromagnetic fields (EMF) simulating exposure to the Global System for Mobile Communications (GSM) signals were studied using pluripotent embryonic stem (ES) cells in vitro. Wild-type ES cells and ES cells deficient for the tumor suppressor p53 were exposed to pulse modulated EMF at 1.71 GHz, lower end of the uplink band of GSM 1800, under standardized and controlled conditions, and transcripts of regulatory genes were analyzed during in vitro differentiation. Two dominant GSM modulation schemes (GSM-217 and GSM-Talk), which generate temporal changes between GSM-Basic (active during talking phases) and GSM-DTX (active during listening phases thus simulating a typical conversation), were applied to the cells at and below the basic safety limits for local exposures as defined for the general public by the International Commission on Nonionizing Radiation Protection (ICNIRP). GSM-217 EMF induced a significant upregulation of mRNA levels of the heat shock protein, hsp70 of p53-deficient ES cells differentiating in vitro, paralleled by a low and transient increase of c-jun, c-myc, and p21 levels in p53-deficient, but not in wild-type cells. No responses were observed in either cell type after EMF exposure to GSM-Talk applied at similar slot-averaged specific absorption rates (SAR), but at lower time-averaged SAR values. Cardiac differentiation and cell cycle characteristics were not affected in embryonic stem and embryonic carcinoma cells after exposure to GSM-217 EMF signals. Our data indicate that the genetic background determines cellular responses to GSM modulated EMF. Bioelectromagnetics 25:296–307, 2004. © 2004 Wiley-Liss, Inc.

Key words: mouse embryonic stem cells; differentiation; hsp70; p53; GSM signals

INTRODUCTION

Due to increasing incidence in human environment, electromagnetic fields (EMF) radiated by Global System for Mobile Communications (GSM) equip-

ment have attracted common attention as a potentially hazardous environmental factor. Numerous in vivo and in vitro studies have been performed to investigate the biological consequences and to assess health risks of high frequency EMF, including use of animal models

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[Szmigielski et al., 1982; Sarkar et al., 1994; Wu et al., 1994; Klug et al., 1997; Repacholi et al., 1997; Adey et al., 2000; de Pomerai et al., 2000] as well as human volunteers [Eulitz et al., 1998; Mann et al., 1998].

Recently, a possible role of the genetic constitution of animal cells in their response to EMF has been proposed [Repacholi, 1998]. Repacholi and coworkers [Repacholi et al., 1997] reported that *Eμ-Pim1* transgenic mice showed an increased tumor rate after EMF exposure. The data were confronted by other reports, which did not detect any responses of transgenic animals to extremely low frequency EMF of 50 Hz [Harris et al., 1998], indicating that specific EMF parameters influence biological activities. Several factors may be responsible for the apparent discrepancies in EMF effects, such as EMF specifications, that is, carrier frequencies, modulation schemes and field intensities, and different and incomparable biological systems. Finally, also thermal effects during EMF exposure may have been the reason of controversial data.

For our studies, an experimental setup was designed for a highly controlled exposure of cultured cells to pulse modulated radio frequency EMF, enabling the simulation of different GSM exposure schemes at levels at or below the basic safety limits for local exposures as defined for the general public by the International Commission on Nonionizing Radiation Protection (ICNIRP). The limit for spatial peak SAR is defined as 2 W/kg, and exposures of larger than 1 W/kg were found to be induced during usage of mobile phones [Kuster et al., 1997].

In our experiments, the effects of EMF exposure were comparatively analyzed in pluripotent embryonic stem (ES) and embryonic carcinoma (EC) cells, including ES cells deficient for p53, a tumor suppressor protein, to determine the cellular susceptibility to develop spontaneous tumors [Sigal and Rotter, 2000]. ES and EC cells have the capacity to differentiate into cell types of all three primary germ layers in vitro [Wobus, 2001]. Undifferentiated and differentiating wild-type (wt) and p53 deficient ($p53^{-/-}$) ES cells were exposed to different EMF modulation schemes, followed by analyses of transcript levels of regulatory genes. We found an increased responsiveness of $p53^{-/-}$ ES cells to EMF, represented by continuously upregulated hsp70 mRNA levels, and a transient upregulation of other regulatory early genes after exposure to GSM-217, but not to GSM-Talk signals. On the other hand, GSM-217 signals exerted no effects on cell cycle, gene expression, and cardiac differentiation of wild-type cells. The data underline the role of the genetic background in the modulation of cellular responses to GSM modulated EMF.

MATERIALS AND METHODS

EMF Exposure Apparatus

A setup that enabled EMF exposure of cells under defined conditions with respect to field strengths, polarization, modulation, and temperature [Kuster and Schönborn, 2000] was configured to operate within the uplink band of the Global System for Mobile Communications (GSM)—GSM1800 or the digital cellular system (DCS—1.71 GHz). The original setup enabling GSM-217 exposure consists of four R14 wave guides (two for EMF exposure, two for sham exposure) enclosed in a conventional humidified incubator (5% CO₂, 37 °C). Petri dishes (60 mm diameter) were positioned in the H field maximum of a standing wave and exposed in E polarization. To guarantee atmospheric and temperature exchange, sensor monitored fans were mounted that enforced air flow through the wave guide chamber [Schönborn et al., 2000].

Later, the signal and monitoring unit as well as the software were updated, whereby two of the previous four wave guides were used and operated at the same carrier frequency. The wave guides were enhanced by field and temperature sensors. The modified signal unit consisted of a RF generator, arbitrary function generator, frame generator, and 5 W narrow band amplifier. The RF output was directed by the computer controlled RF switch to one of the wave guides (blinded). The entire setup was computer controlled, enabling the automated setting of the modulation scheme as well as the exposure strength for the different media and culture applications. The exposure strength and temperature, as well as all commands were logged to an encrypted file, which was evaluated after the experiments in order to ensure 'blind' conditions. The temperature difference between sham and EMF exposed cultures never exceeded 0.1 °C.

GSM-217 Hz. Signals were amplitude modulated by rectangular pulses with a repetition frequency of 217 Hz and a duty cycle of 1:8 (Fig. 1, pulse width 0.576 ms). Signals were applied to the cells at time-averaged SAR values of 1.5 W/kg (corresponding to slot SAR = 12 W/kg) for hanging drops and 2 W/kg (corresponding to slot SAR = 16 W/kg) for suspension and monolayer cultures (Table 1).

GSM-Talk. GSM-Talk generates temporal changes between GSM-Basic (active during talking phases) and GSM-DTX (active during listening phases) and simulates a typical conversation with average durations of 50 and 97 s for Basic and DTX, respectively. GSM-Basic is similar to GSM-217, except that every 26th frame is idle. The frame structure of the DTX signal results in 2,

GSM Signals

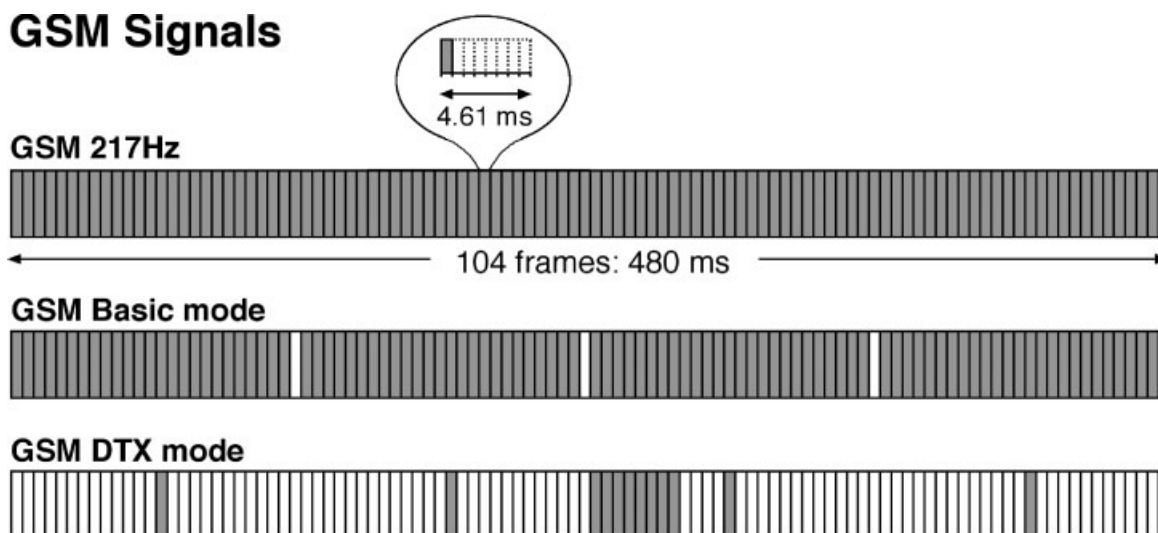


Fig. 1. Pulse structure of the applied GSM signals. The basic frame has a period of 4.61 ms and contains a 570 μ s slot including 15 μ s rising and falling edges. Twenty-six frames make up a GSM multiframe and 104 frames a GSM intermediate multiframe (IMF). GSM-217 is composed of a repetition of basic frames (104 bursts per IMF), whereby every 26th frame is idle in the case of the GSM-Basic signal (100 bursts per IMF). The GSM-DTX mode becomes active during a conversation, when the user is not speaking. In this period only control signals and comfort noise is transmitted using 12 bursts per IMF.

8, and 217 Hz components. Signals were applied to the cells at time-averaged SAR values of 0.4 W/kg (corresponding to slot SAR = 8 W/kg, Table 1). The maximum exposure level, that is, the slot averaged exposure, was kept nearly constant, whereas the long time-averaged SAR varied corresponding to the number of active slots in an intermediate multiframe.

Cell Culture

Pluripotent R1 ES cells [Nagy et al., 1993], wild-type (wt) D3 [Doetschman et al., 1985], and p53-

deficient [p53^{-/-}, Jacks et al., 1994] ES cells derived from D3 cells were cultured as described by Wobus et al. [2002], except that p53^{-/-} ES cells were maintained on neomycin resistant SNL feeder cells in the presence of 300 μ g/ml G418. EC cells of line P19 [Edwards and McBurney, 1983] were cultured without feeder cells [Wobus et al., 1994]. For differentiation, P19, R1, and wt or p53^{-/-} D3 cells were cultivated as embryoid bodies (EBs) in hanging drops in Dulbecco's modified minimal essential medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 20% FCS,

TABLE 1. Exposure Parameters for the Applied GSM Modulation Schemes; SAR Values have been Determined with Simulations and Measurements and Represent Voxel Average Values Over the Area of the Cells

Exposure parameters	GSM-217 Hz	GSM-Talk (33% GSM-Basic 67% GSM-DTX)	GSM-DTX
Cultivation in 'hanging drops'			
Slot SAR	12 W/kg	8 W/kg	8 W/kg
Time-averaged SAR	1.5 W/kg	0.4 W/kg	0.11 W/kg
Exposure duration	6 hr 48 hr	6 hr 48 hr	6 hr
ON/OFF cycles	5/30 min	5/30 min	5/30 min Continuous
Cultivation in suspension			
Slot SAR	16 W/kg		
Time-averaged SAR	2 W/kg		
Exposure duration	72 hr (22 hr, 40 hr ¹)		
ON/OFF cycles	5/30 min		

The uncertainty for the average values was assessed to be $\pm 50\%$ for hanging drops and $\pm 20\%$ for suspension cultivation.

¹Monolayer.

L-glutamine, nonessential amino acids (NEAA) and β -mercapthoethanol (β -ME) as described by Wobus et al. [2002]. Briefly, cells ($n = 400$) in 20 μ l of differentiation medium were cultivated in hanging drops on the lids of 10 cm petri dishes for 2 days, followed by suspension culture for 3 days. The cultivation as EBs results in the formation of early differentiated cell types of all three primary germ layers. For further differentiation, 5d EBs were plated separately onto gelatin coated, 24 well microwell or onto 6 cm tissue culture plates.

Cells were analyzed morphologically and samples were collected for reverse transcriptase polymerase chain reaction (RT-PCR) analysis, at indicated time points. For the induction of P19 cell differentiation, EBs were cultivated in the presence of 1% DMSO (Sigma) during the first 2 days of EB development [Wobus et al., 1994]. For further details of methods and materials used for cultivation and in vitro differentiation, see Wobus et al. [2002].

Experimental Protocols

EMF exposure of p53^{-/-} and wt D3 ES cells. ES cells derived from p53^{-/-} and wt D3 ES cells were EMF or sham exposed during differentiation in hanging drops for 6 or 48 h (Fig. 2). For 6 h experiments, samples were collected immediately after exposure and used for RT-PCR analysis. After 48 h exposure, EBs were further differentiated and samples were collected for RT-PCR analysis at indicated time points during in vitro differentiation. During EMF exposure and further incubation, p53^{-/-} and wt cells were cultivated in Dulbecco's modified minimal essential medium (DMEM) supplemented with 20% FCS, L-glutamine, nonessential amino acids (NEAA), and β -mercapthoethanol (β -ME) as described by Wobus et al. [2002].

Exposure of R1 ES and P19 EC cells. EBs derived from R1 cells were EMF (GSM-217) or sham exposed in hanging drops (SAR: 1.5 W/kg) for 2 days and in suspension (SAR: 2.0 W/kg) for 3 days. P19 cells ($n = 200\,000$) were seeded into 0.1% gelatin coated tissue culture dishes, cultured in DMEM (see above), and after 2 h preincubation at 37 °C, were placed into the apparatus for EMF and sham exposure. As controls, nonexposed cells were cultured in a separate humidified 5% CO₂ incubator at 37 °C. P19 cells were exposed to EMF at SAR value of 2.0 W/kg for 22 or 40 h. After exposure, cells were immediately processed for flow cytometric analysis (P19), and in parallel, R1 and P19 derived EBs were prepared for differentiation and RT-PCR analysis.

RT-PCR analysis. The expression of early response and growth regulatory genes in ES and EC cells after

differentiation was analyzed by semiquantitative RT-PCR as described by Wobus et al. [2002]. Total RNA was isolated using the single step extraction method [Chomczynski and Sacchi, 1987]. mRNA was reverse transcribed using Poly dT tail primer Oligo d(T)₁₆ (Perkin-Elmer, Überlingen, Germany), and cDNA was amplified using oligonucleotide primers complementary and identical to the analyzed genes [c-fos, c-jun, c-myc, early growth response-1 (egr-1), hsp70, p21, p53, bcl-2, α -myosin heavy chain (α -MHC), β -tubulin; primer sequences available on request]. PCR products were densitometrically analyzed with the E.A.S.Y system (Herolab GmbH, Wiesloch, Germany) and TINA2.08e software (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany). Samples from three to six independent experiments with two to six parallels were statistically analyzed with the nonparametric Mann–Whitney test. Data on analyzed genes were plotted as percentage in relation to the expression of the housekeeping gene β -tubulin.

Analysis of cardiac differentiation. Cardiac differentiation of EC or ES cells was used as a parameter of differentiation according to the embryonic stem cell test, EST [Spielmann et al., 1997], established for in vitro analysis of embryotoxic agents. Spontaneously beating cardiomyocytes were estimated at various stages after EB plating. The percentage of EBs containing beating cardiomyocytes and mRNA levels of α -MHC were used as degree of cardiac differentiation.

Flow cytometric analysis of cell cycle phases. EMF and sham exposed P19 cells were processed according to the two step procedure of DNA staining [Sehlmeyer et al., 1996]. Cells were analyzed with a FACStar^{PLUS} flow cytometer (Becton Dickinson, Heidelberg, Germany). Data from three (22 h) and four (40 h) independent experiments with three to six parallels were subjected to statistical analysis performed with the 'ModFitLT' software (Verity Software House, Inc., Topsham, ME).

RESULTS

Loss of p53 Function Increased ES Cell Sensitivity to GSM-217 Signals

To elucidate the influence of the genetic background on the sensitivity of ES cells to GSM generated EMF signals, undifferentiated and differentiating wt and p53^{-/-} ES cells were exposed to signals modulated according to the dominant modulation component of GSM (GSM-217). Short term cell responses were analyzed immediately after the end of 6 h exposure of

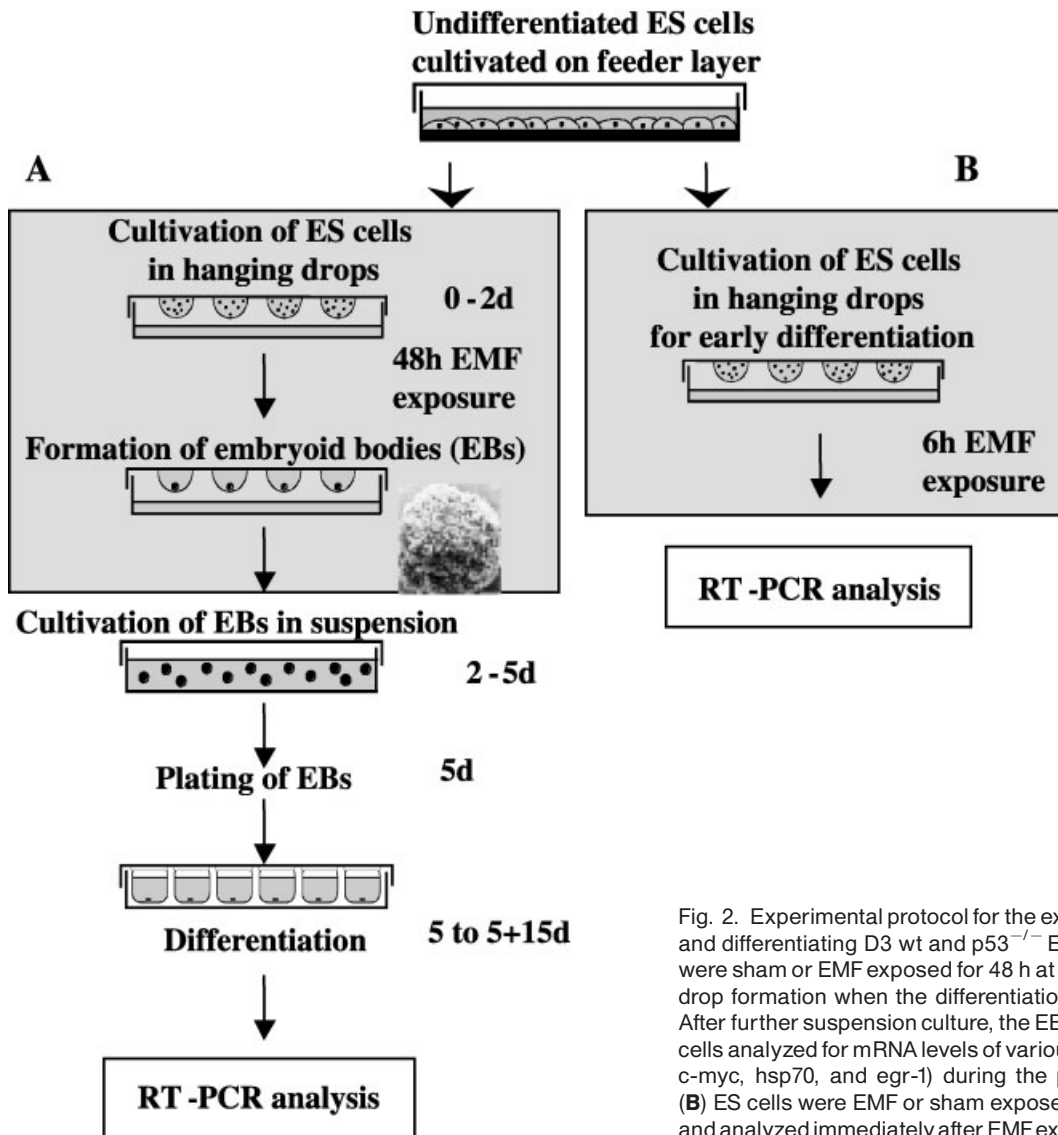


Fig. 2. Experimental protocol for the exposure of undifferentiated and differentiating D3 wt and p53^{-/-} ES cells to EMF. **A:** ES cells were sham or EMF exposed for 48 h at the initial stage of hanging drop formation when the differentiation processes are initiated. After further suspension culture, the EBs were plated at day 5 and cells analyzed for mRNA levels of various genes (bcl-2, p21, c-jun, c-myc, hsp70, and egr-1) during the process of differentiation; **(B)** ES cells were EMF or sham exposed in hanging drops for 6 h and analyzed immediately after EMF exposure.

undifferentiated ES cells, whereas long term shifts in gene expression were monitored after 48 h exposure of differentiating ES cells throughout the time of differentiation (see Fig. 2). Genes involved in immediate early and stress response (c-fos, c-jun, c-myc, egr-1, hsp70) and known to regulate cell cycle and apoptosis (p21, bcl-2) were studied.

Exposure of undifferentiated cells to GSM-217 signals for 6 h did not evoke any short term modification of gene expression patterns neither in wt nor in p53^{-/-} ES cells (Fig. 3A). On the other hand, long term (48 h) GSM-217 exposure of p53^{-/-} cells during EB development resulted in the upregulation of transcript levels of four out of six analyzed genes. Whereas c-jun, p21, and c-myc mRNA levels were only transiently upregulated at early stages (day 2, 5, and 5 + 2 of EB differentiation, Fig. 3B), a prominent induction of

hsp70 levels in p53^{-/-} cells was observed throughout the differentiation period (Fig. 3B,C).

The same experimental protocol was applied to analyze the influence of GSM signals simulating talking and listening phases during a typical conversation (GSM-Talk). However, no changes of gene expression patterns in both wt and p53^{-/-} cells were observed upon the exposure to GSM-Talk signals regardless of the protocol used (Fig. 4).

GSM-217 signals did not influence cardiac differentiation and gene expression levels in R1 ES cells. For the evaluation of embryotoxic effects of chemical compounds in vitro, the mouse embryonic stem cell test, EST [Spielmann et al., 1997], studying cardiac differentiation of ES cells as endpoint, has been established. Therefore, we further analyzed the effects

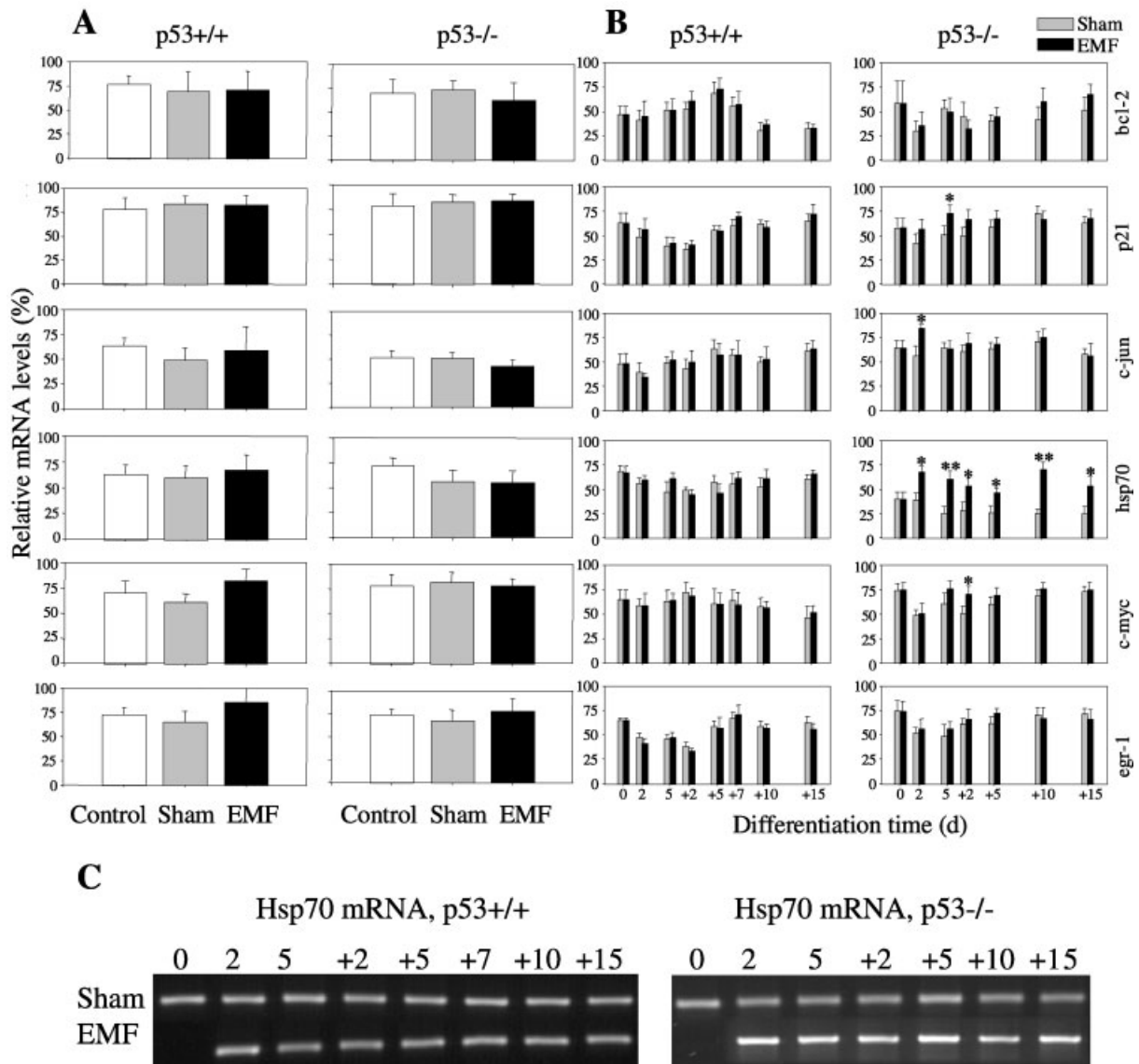


Fig. 3. Relative mRNA levels of genes encoding bcl-2, p21, c-jun, hsp70, c-myc, and egr-1 in p53-deficient ($p53^{-/-}$) and wild-type ($p53^{+/+}$) D3 ES cell-derived EBs after 6 (A) and 48 h (B) GSM-217 Hz exposure. GSM-217 Hz exposure resulted in a significant and long lasting upregulation of hsp70 transcripts (C), paralleled by a temporary upregulation of c-myc, c-jun, and p21 levels in differentiating $p53^{-/-}$ ES cells after 48 h exposure (B), but not after 6 h (A) exposure. Data were plotted (in %) in relation to β -tubulin mRNA levels. Similar data were obtained in six independent experiments with two parallels. Error bars represent standard deviations. Statistical significance was tested by the nonparametric Mann–Whitney test for significance levels of 1% and 5% (** $P \leq 0.01$, * $P \leq 0.05$).

of EMF on cardiac differentiation in R1 ES and P19 EC cells. R1-derived EBs were exposed to EMF for 5 days, and the cells were analyzed after further differentiation. Similar to D3 wt cells, no effect of GSM-217 signals on the expression of the regulatory genes including p53 was observed in R1 wt cells (Fig. 5A). Moreover, no significant differences in cardiac differentiation were found between sham and EMF exposed variants during EB differentiation (Fig. 5B). The results of morpholo-

gical investigations were confirmed by RT-PCR analyses, where no differences in cardiac α -MHC mRNA levels between sham and EMF exposed variants were observed (Fig. 5C).

GSM-217 signals did not induce cardiac differentiation and gene expression and the proliferation of P19 EC cells. Unlike ES (R1 or D3) cells, which spontaneously differentiate into the cardiac lineage,

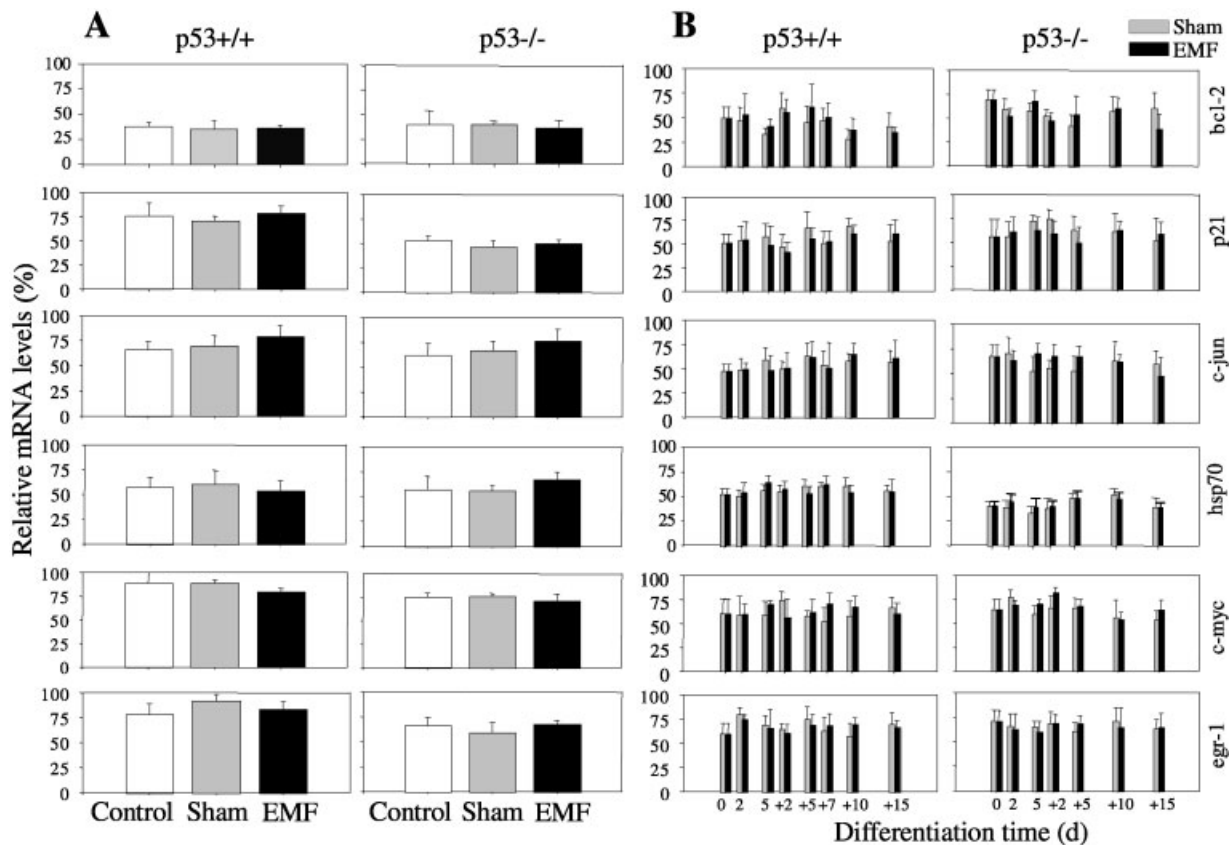


Fig. 4. Relative mRNA levels analyzed by semiquantitative RT-PCR of genes encoding bcl-2, p21, c-jun, hsp70, c-myc, and egr-1 in p53-deficient ($p53^{-/-}$) and wild-type ($p53^{+/+}$) D3 ES cell-derived EBs and EB outgrowths after 6 (A) and 48 h (B) exposure to GSM-Talk signals. No effects of GSM-Talk on gene expression levels in ES cells were observed. Data were plotted (in %) in relation to β -tubulin mRNA levels. Similar data were obtained in six independent experiments with two parallels.

differentiation of P19 EC cells has to be stimulated by external differentiation factors, that is, DMSO or retinoic acid. To elucidate, whether EMF signals interfere with DMSO induced cardiac differentiation, undifferentiated P19 cells were exposed to GSM-217 signals for 22 and 40 h, respectively, and differentiated in the absence or presence of 1% DMSO. As seen in Figure 6A, the mRNA levels of regulatory genes were not affected by EMF exposition, and no significant differences were found among EMF, sham exposed, and control variants of undifferentiated P19 cells, respectively (Fig. 6A).

Without DMSO induction, only low levels (5–10%) of spontaneous cardiac differentiation were found (Fig. 6B). By differentiation induction with DMSO during the first 48 h of EB development, the differentiation of spontaneously beating cardiac cells was increased to a maximum level of 90% in both variants (Fig. 6B). Exposure of P19 cells to GSM-217 signals for 22 (Fig. 6B) and 40 (Fig. 6B) h did not result in significant changes of cardiac differentiation, suggest-

ing that EMF had no effects on spontaneous or DMSO induced cardiogenesis.

Furthermore, undifferentiated EC cells were EMF- (GSM-217) (Fig. 6C) and sham-exposed (Fig. 6C) at a SAR value of 2.0 W/kg for 22 (Fig. 6C) or 40 h (Fig. 6C), and the lengths of cell cycle phases were analyzed by flow cytometry. In both cases, no differences in the distribution of cells in G1, S, or G2/M phases were observed between sham and EMF exposed variants.

DISCUSSION

Here, we analyzed the influence of the genetic constitution of pluripotent embryonic stem cells on their responsiveness to differently modulated GSM generated EMF. In ES cells deficient for the tumor suppressor p53, we found a permanent upregulation of mRNA levels of the stress response gene hsp70 paralleled by a slight and temporary increase of p21, c-jun, and c-myc expression in response to GSM-217 but not GSM-Talk modulated signals characterized by the

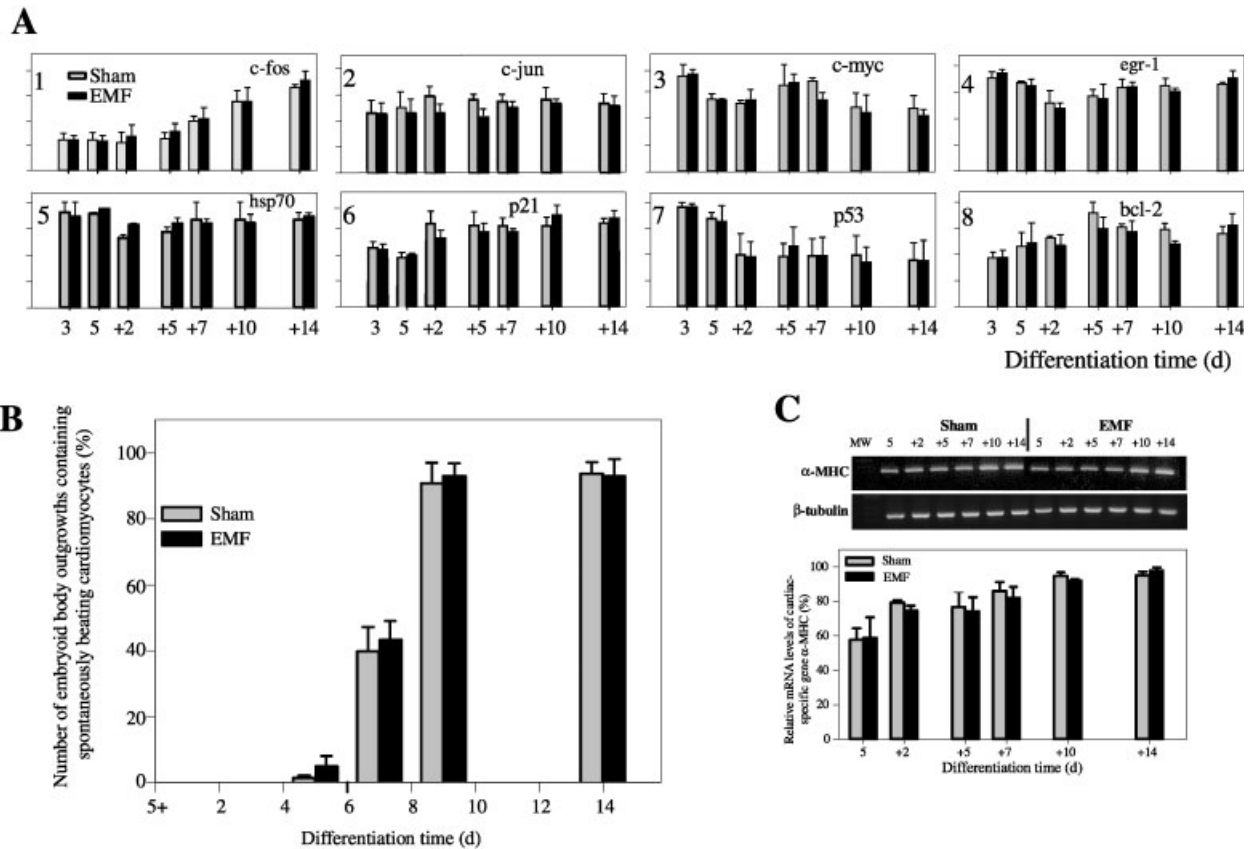


Fig. 5. Effects of GSM-217 Hz signals on mRNA levels of genes involved in immediate early response and regulation of cell cycle and apoptosis, and in cardiac differentiation after 5 days of EMF- and sham-exposition to R1 ES cell-derived EBs. **A:** The abundance of mRNA levels of regulatory genes was analyzed by computer-assisted densitometry and the data were plotted (in %) in relation to β -tubulin mRNA levels (1–8). **B:** Cardiac differentiation of R1 cells. EBs were EMF- or sham-exposed for 5 days and the number of EB outgrowths (in %) containing spontaneously beating cardiomyocytes was determined. Independent experiments ($n = 3$, with 48 EBs each) were analyzed. **C:** Relative mRNA levels of cardiac α -myosin heavy chain (α -MHC) of EMF or sham exposed R1-derived EBs (5d) and EB outgrowths (5 + 2 to 5 + 14d) analyzed by semiquantitative RT-PCR ($n = 6$ experiments, with 2 parallels each). Data were plotted (in %) in relation to β -tubulin mRNA levels. Error bars represent standard deviations.

presence of low frequency components. On the other hand, GSM-217 exposure of wt ES and EC cells corresponding to EST protocols [see Spielmann et al., 1997], revealed no effects on gene expression, cardiac differentiation, and proliferation. This would indicate that the genetic background of stem cells, determined by the p53 function, may potentially influence the response of early differentiating cells to GSM signals, depending on the modulation schemes, whereas wt cells analyzed in this study remained insensitive to GSM-modulated EMF.

p53^{-/-} mice are highly susceptible to the development of spontaneous tumors, in particular, of malignant lymphomas at early age [Attardi and Jacks, 1999; Sigal and Rotter, 2000]. p53 is required for G₁ arrest in response to DNA damage and is involved in apoptosis

[Attardi and Jacks, 1999] via modulating versatile regulatory genes. In our studies, using mouse p53^{-/-} ES cells, hsp70 mRNA levels were continuously elevated after 48 h EMF exposition. Heat shock proteins act as chaperons whose expression is activated or upregulated in response to external stress [Beere and Green, 2001]. Hsp70 has also been defined to regulate homeostasis in response to external stress during early embryo development [Luft and Dix, 1999], while upregulated hsp70 levels were observed in tumor cells and are correlated with metastases and poor prognosis [Zylicz et al., 2001]. Recently, a potential involvement of heat shock proteins in cell responses to EMF was reported. Increased hsp27 protein levels and a transient upregulation of hsp27 phosphorylation were found in human endothelial cells [Leszczynski et al., 2002].

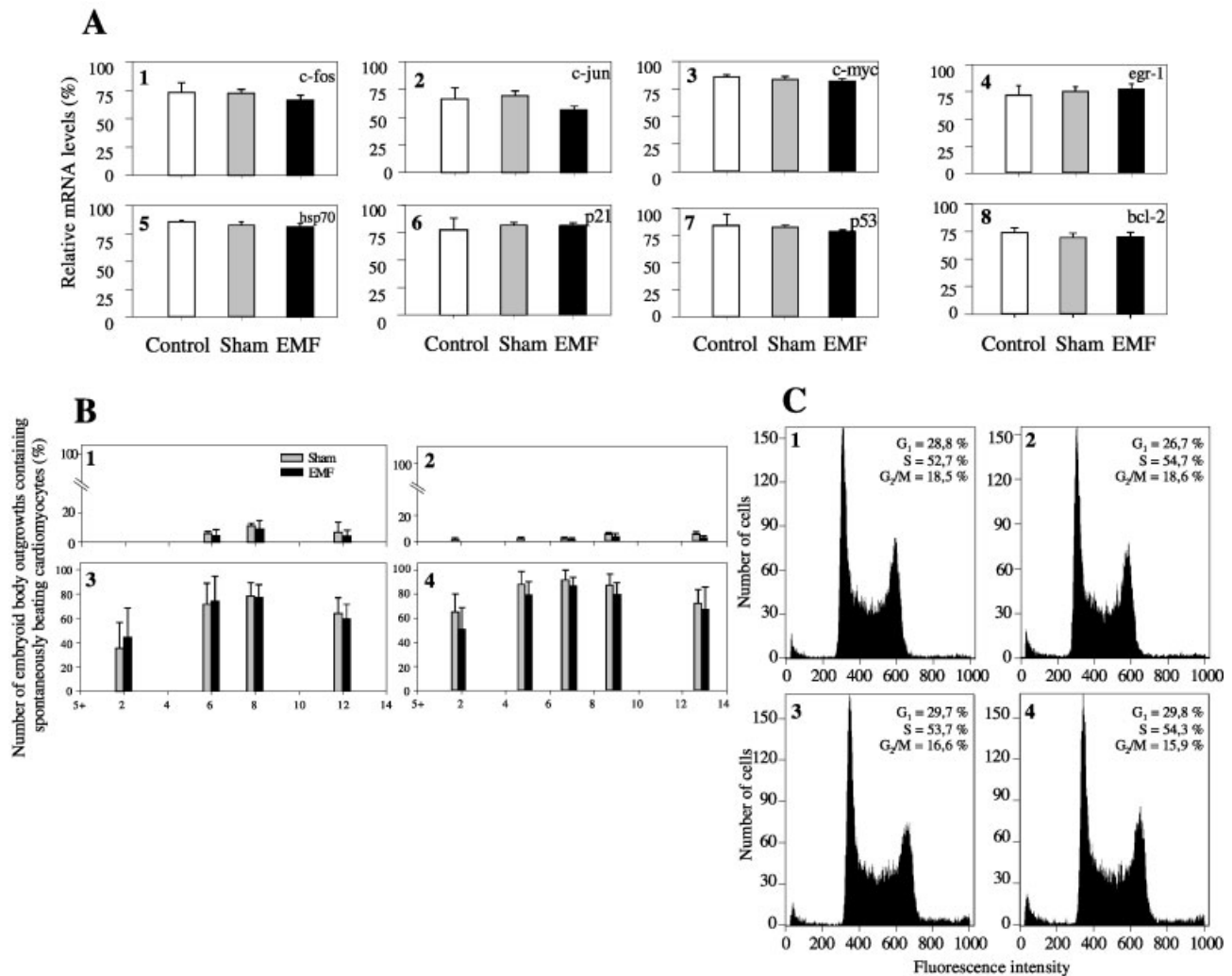


Fig. 6. Effects of GSM-217 Hz signals on mRNA levels of genes involved in immediate early response, regulation of cell cycle and apoptosis, and cardiac differentiation in P19 EC cells and P19 derived EB outgrowths after EMF and sham exposition. **A:** The abundance of PCR products of regulatory genes of (22 h) EMF exposed P19 cells. Data were plotted (in %) in relation to β -tubulin mRNA levels (1–8). **B:** Cardiac differentiation, determined as number of EBs (in %) containing spontaneously beating cardiomyocytes derived from P19 cells, exposed to EMF for 22 (1, 3) or 40 h (2, 4) without (1, 2) and with 1% DMSO treatment (3, 4). Independent experiments ($n = 3$) with 48 EBs each were analyzed. **C:** Distribution of P19 cells in G₀/G₁, S, and G₂/M cell cycle phases in sham (1, 3) and EMF exposed (2, 4) P19 cells (for 22 h, 1, 2 and 40 h, 3, 4, respectively). Calculation of cell cycle distribution (in %) demonstrated a similar distribution of cells in the three cell cycle phases for EMF and sham exposed P19 cells ($n = 12$ for 22 h, and $n = 22$ for 40 h of EMF or sham exposure). Similar data were obtained in 3 (22 h) and 4 (40 h) independent experiments.

However, if EMF act as inductor of cellular transformation processes, effects on expression levels of other early response genes should be expected. Indeed, we observed an upregulation of c-myc, c-jun, and p21 mRNA levels in p53^{-/-} cells upon GSM-217 exposure. The same genes were previously shown to be affected by environmental factors, such as UV or X radiation [Angel et al., 1988; Amati et al., 1993; Jean et al., 2001]. Because in our model, the shifts in gene expression were low and transient, it is conceivable that

EMF signals, while affecting gene expression patterns in p53^{-/-} cells, do not induce permanent cellular transformations in wt cells.

Contrary to GSM-217 signals, which elicited cellular responses in p53^{-/-} ES cells, GSM-Talk modulation exerted no effects on gene expression in our model. This indicates that low frequency components generated by GSM-Talk (2 and 8 Hz) do not promote the action of EMF signals in our cell system. The 217 Hz component is present in both modulation

schemes; however, the effects were observed only with GSM-217. Therefore, time-averaged SAR values, which differed for GSM-217 and GSM-Talk (1.5 W/kg and 0.4 W/kg, respectively), may be the key reason that determined the biological activity of EMF. Furthermore, it can not be excluded that the modulation scheme (time distribution of high SAR pulses) may also play a role in evoking biological responses, because slot averaged SAR values remained similar between the analyzed modulation schemes.

We show, however, that the duration of exposure may influence the biological responses to EMF. In our *in vitro* studies, upregulated transcript levels of regulatory genes in p53^{-/-} ES cells were observed after 2 day exposure to GSM-217 signals, whereas no response could be seen after short term 6 h exposure. This observation might result from the existence of genetic compensatory mechanisms and/or negative feedback loops.

The parameters of genetic constitution, carrier frequency, and exposure duration in determining the response of biological systems to EMF have been proposed by *in vivo* studies [Repacholi et al., 1997; Di Carlo and Litovitz, 1999]. Using *Eμ-Pim1*-transgenic mice predisposed to develop spontaneous lymphomas, an increase in tumor formation after long term (18 months) exposure of *Eμ-Pim1*-transgenic mice to 900 MHz EMF has been observed [Repacholi et al., 1997]. On the other hand, exposure of these animals to extremely low frequency 50 Hz EMF [Harris et al., 1998] did not evoke any detectable biological responses. These apparent discrepancies may indicate a role of carrier frequencies in determining the biological activity of EMF; however, the positive data obtained after long term exposure to 900 MHz EMF [see Repacholi et al., 1997] suggest that mobile phone radiation induced events may be hazardous to cells deficient in cell repair when occurring frequently over long periods.

For the evaluation of embryotoxic effects of chemical compounds *in vitro*, the mouse embryonic stem cell test [EST, Spielmann et al., 1997] using cardiac differentiation of ES cells as endpoint has been established. Therefore, for a further specification of the effects of GSM-217 signals, we analysed EMF exposure during the process of cardiac differentiation. GSM-217 EMF exerted no influence on ES derived cardiogenesis and did not affect DMSO induced cardiac differentiation, proliferation, and expression of regulatory genes in P19 EC cells. These data confirmed that, in contrast to p53^{-/-} cells, wild-type ES or EC cells are not sensitive to EMF with respect to the parameters analyzed.

Our observations on wild-type ES/EC cells are in agreement with other *in vitro* studies concerning nega-

tive effects of high frequency EMF on cell cycle, gene expression, and differentiation [Cain et al., 1997; Fritze et al., 1997; Ivaschuk et al., 1997; Goswami et al., 1999], DNA and chromatin structure [ICNIRP, 1996; Repacholi, 1998], and rat embryo development [Klug et al., 1997]. In contrast, several reports described positive effects by high frequency EMF exposure on the length of cell cycle phases, proliferation, and gene expression levels in mammalian cells [Czerska et al., 1992; Sarkar et al., 1994; Cleary et al., 1996; Lai and Singh, 1996; Goswami et al., 1999]. These studies, however, were performed with different experimental models, carrier frequencies (835 MHz to 2.45 GHz versus 1.71 GHz used in our study), and modulation schemes, and, therefore, are not comparable. Moreover, positive EMF effects were often observed at relatively high average SAR values [Czerska et al., 1992; Cleary et al., 1996; Fritze et al., 1997] and could arise from EMF evoked thermal effects. In our studies, GSM signals were applied under conditions of the ICNIRP safety limit using experimental apparatus that enabled precise temperature control [Schönborn et al., 2000], and any temperature increase as a consequence of EMF exposure [Laurence et al., 2000] can be excluded.

In summary, the present data suggest that the currently applied GSM radiation levels under certain circumstances might induce biological effects. Our results indicate that the genetic constitution of cells determined by loss of p53 function can influence EMF related cellular responses, whereas wild-type cells were insensitive. It remains to be elucidated, whether EMF induced changes of expression levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects *in vivo*. Further studies are needed to analyze the whole transcriptome of EMF exposed cells by genomics technologies, such as cDNA microchips or serial analysis of gene expression (SAGE). Because of conflicting epidemiological data on human EMF exposure (see Repacholi [1998]), the present data together with others indicate the need for further coordinated research on cellular responses to EMF.

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