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Proteomics analysis of human endothelial cells after short-term exposure to mobile phone radiation

Reetta Nylund

Säteilyturvakeskus
Strålsäkerhetscentralen
Radiation and Nuclear Safety Authority

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Reetta Nylund

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Aalto University School of Science

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Osoite / Address • Laippatie 4 , 00880 Helsinki
Postiosoite / Postal address • PL / P.O. Box 14, FI-00881 Helsinki, FINLAND
Puh. / Tel. +358 9 759 881 • Fax +358 9 759 88 500 • www.stuk.fi

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Radiation and Nuclear Safety Authority
Aalto University School of Science
Department of Biomedical Engineering and Computational Science

Proteomics analysis of human endothelial cells after short-term exposure to mobile phone radiation

Author: Reetta Nylund
Radiation Biology Laboratory
STUK – Radiation and Nuclear Safety Authority
Helsinki, Finland

Supervisor: Research Professor Dariusz Leszczynski
Radiation Biology Laboratory
STUK – Radiation and Nuclear Safety Authority
Helsinki, Finland

Supervising professor: Professor Risto Ilmoniemi
Department of Biomedical Engineering
and Computational Science
Aalto University School of Science
Espoo, Finland

Reviewers: Docent Tuula Nyman
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

Affiliate Professor Ceon Ramon
Department of Electrical Engineering
University of Washington
Seattle, WA, USA

Opponent: Professor Jukka Juutilainen
Department of Environmental Science
University of Eastern Finland
Kuopio, Finland

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Abstract

Mobile phones have been a part of our everyday life in the developed world since the late 1990s. This has raised concerns over the potential health risks of mobile phone use. Biological and health effects potentially caused by mobile phone radiation have been extensively studied and several biological and medical endpoints have been examined. So far, results have not been conclusive on the potential effects of mobile phone radiation.

Mobile phones generate a modulated radio frequency electromagnetic field (RF-EMF), which is a form of non-ionizing radiation. This means that mobile phone radiation does not have enough energy to ionize atoms and it cannot break chemical bonds directly (e.g., in DNA strands). There could, however, be other mechanisms by which mobile phone radiation may affect cellular and physiological functions. Whether these mechanisms exist is unknown.

In this thesis, large-scale screening techniques, such as proteomics, were applied to examine changes on the proteome level after exposure to mobile phone radiation. Proteomics techniques allow the screening of several hundreds, and even thousands, of proteins simultaneously, and are thus more efficient than single endpoint techniques.

Four different types of human endothelial cells (two cell lines, two types of primary cells) were exposed to two types of mobile phone radiation (900 and 1800 MHz GSM). The proteome of these cells was examined immediately after short-term exposure using two-dimensional gel electrophoresis (2DE). Two protein detection/analysis techniques were used: silver staining for the cell line samples and difference gel electrophoresis (DIGE) for the primary cells. 2DE-DIGE technology is currently a state-of-the-art technique in 2DE studies.

Several changes were found in the proteome of the human endothelial cell line EA.hy926 after exposure to 900 MHz GSM mobile phone radiation. In addition, the proteome of a variant of the same cell line, the EA.hy926v1, was affected after 900 MHz GSM mobile phone radiation exposure, but the altered proteins were different from those in the EA.hy926 cells. The changes in the

proteome of the EA.hy926 cells were weaker after 1800 MHz GSM exposure compared to those after 900 MHz GSM exposure. Furthermore, certain proteins affected earlier after 900 MHz GSM exposure were unaffected after 1800 MHz GSM exposure.

The proteome of the primary human endothelial cells was not affected after 1800 MHz GSM exposure when examined using 2DE-DIGE technology. 2DE-DIGE technology is more reliable than the technology used with the EA.hy926 cell line, and these results should therefore be highly relevant when assessing the potential immediate effects of mobile phone radiation.

The results presented in this thesis on the proteome-level effects of mobile phone radiation exposure are contradictory. The results with EA.hy926 cells suggest that minor effects do occur, whereas no effects were observed when using the more reliable 2DE-DIGE technology and primary cells. The responses with EA.hy926 cells varied according to the cell variant and exposure conditions, and consistent responses at the cellular level could not therefore be identified. Further research is recommended to understand the variation in responses and whether consistent cellular-level responses exist.

NYLUND Reetta. Ihmisen endoteelisolujen proteomiikka-analyysi lyhytaikaisen matkapuhelinsäteilyaltistuksen jälkeen. STUK-A250. Helsinki 2011, 100 s. + liitteet 73 s.

Avainsanat: Radiotaajuiset sähkömagneettiset kentät, matkapuhelinsäteily, ihmisen endoteelisolut, proteomiikka, kaksisuuntainen geelielektroforeesi, proteiinien ilmentyminen

Tiivistelmä

Matkapuhelimet ovat olleet osa jokapäiväistä elämäämme 1990-luvun loppupuolelta lähtien, mikä on aiheuttanut huolta niiden mahdollista terveysvaikutuksista. Matkapuhelinsäteilyn mahdollisia biologisia ja terveysvaikutuksia on tutkittu laajalti ja tarkasteltavana on ollut useita erilaisia biologisia ja lääketieteellisiä ilmiöitä. Toistaiseksi tutkimustulosten perusteella ei ole saatu varmuutta, onko matkapuhelinsäteilyllä mahdollisia vaikutuksia.

Matkapuhelimet lähettävät radiotaajuisia aaltoja, jotka ovat ionisoimatonta säteilyä. Matkapuhelinsäteilyn energia ei riitä atomien ionisoimiseen ja se ei pysty suoraan rikkomaan kemiallisia sidoksia (esimerkiksi DNA-ketjussa). Matkapuhelinsäteily saattaa kuitenkin vaikuttaa solutason fysiologisiin toimintoihin muiden mekanismien välityksellä. Tällaisten mekanismien olemassa olosta ei ole toistaiseksi saatu varmuutta.

Tässä väitöskirjassa sovellettiin laaja-alaisia seulontatekniikoita, kuten proteomiikkaa, proteiinitason muutosten tutkimiseen matkapuhelinsäteilyaltistuksen jälkeen. Proteomiikka-menetelmien avulla voidaan yhtäaikaisesti tutkia satoja tai jopa tuhansia proteiineja ja ne ovat näin ollen tehokkaampia kuin yksittäistä ilmiötä tutkivat menetelmät.

Tutkimuksessa käytettiin solumallina ihmisen endoteelisoluja. Solumalleja oli yhteensä neljä (kaksi solulinjaa ja kahdenlaisia primäärisoluja) ja niitä altistettiin kahdella eri taajuudella (900 ja 1800 MHz GSM). Muutoksia solujen proteomissa tutkittiin välittömästi lyhytkestoisen altistuksen jälkeen kaksisuuntaista geelielektroforeesia (2DE) käyttäen. Tutkimuksissa käytettiin kahta erilaista proteiinien värjäys-/analysointitekniikkaa: solulinjanäytteille hopeavärjäystä ja primäärisoluille fluoresoivia leimoja (DIGE-tekniikka). 2DE-DIGE-tekniikka edustaa parasta mahdollista teknologiaa 2DE-tutkimuksissa.

Tutkimuksissa löydettiin useita muutoksia ihmisen endoteelisolulinjan EA.hy926:n proteomissa 900 MHz GSM -altistuksen jälkeen. Lisäksi muutoksia

havaittiin saman endoteelisolulinjan muunnoksen, EA.hy926v1:n, proteomissa 900 MHz GSM -altistuksen jälkeen, mutta nämä muutokset olivat erilaisia kuin EA.hy926-soluissa. EA.hy926-solujen proteomissa löydettiin muutoksia myös 1800 MHz GSM -altistuksen jälkeen, mutta nämä muutokset olivat heikompia kuin 900 MHz GSM -altistuksen jälkeen. Lisäksi tiettyjen proteiinien ilmentyminen, joka muuttui aiemmin 900 MHz GSM -altistuksen jälkeen, ei muuttunut 1800 MHz GSM -altistuksen jälkeen.

Primääristen ihmisen endoteelisolujen proteomissa ei havaittu muutoksia 1800 MHz GSM -altistuksen jälkeen, kun tutkimuksissa käytettiin 2DE-DIGE-tekniikaa. 2DE-DIGE-tekniikka on luotettavampi kuin menetelmä, jota käytettiin EA.hy926-solujen tutkimiseen. Näin ollen näillä tuloksilla tulisi olla paljon painoarvoa arvioitaessa lyhytkestoisen matkapuhelinsäteilyaltistuksen mahdollisia välittömiä biologisia vaikutuksia.

Tässä väitöskirjassa esitetyt tulokset matkapuhelinsäteilyn vaikutuksista solujen proteomin tasolla ovat ristiriitaisia. EA.hy926-soluilla saadut tulokset näyttävät, että joitakin muutoksia voi esiintyä altistuksen jälkeen. Luotettavammalla 2DE-DIGE-tekniikalla ja primäärisoluilla ei puolestaan havaittu muutoksia altistuksen jälkeen. Vaikutukset EA.hy926-soluissa vaihtelivat solumuunnoksen ja altistusolosuhteiden perusteella ja näin ollen ei ole mahdollista havaita yhdenmukaista solutason vastetta matkapuhelinsäteilylle. Jatkotutkimuksilla tulisi selvittää, miksi vaikutukset vaihtelevat ja onko yhdenmukaista solutason vastetta olemassa.

Contents

Original Publications

I Leszczynski D., Nylund R., Joenväärä S., and Reivinen J.:
Applicability of discovery science approach to determine biological effects of mobile phone radiation
Proteomics 2004, 4: 426–431

II Nylund R. and Leszczynski D.:
Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation
Proteomics 2004, 4: 1359–1365

III Nylund R. and Leszczynski D.:
Mobile phone radiation causes changes in gene and protein expression in human endothelial cell lines and the response seems to be genome- and proteome-dependent
Proteomics 2006, 6: 4769–4780

IV Nylund R., Tammio H., Kuster N., and Leszczynski D.:
Proteomic analysis of the response of human endothelial cell line EA.hy926 to 1800 GSM mobile phone radiation
J Proteomics Bioinform 2009, 2: 455–462

V Nylund R., Kuster N., and Leszczynski D.:
Analysis of proteome response to the mobile phone radiation in two types of human primary endothelial cells
Proteome Science 2010, 8:52 (pages 1–7)

Additionally, some unpublished data are discussed.

Author's Contribution

I Dariusz Leszczynski had the main responsibility for the designing and preparing this publication. The author (RN) participated in the design and provided data for the manuscript.

II, IV, V RN had the main responsibility for the execution of this study: the development of the experimental platform, performing of the experiments, and data analysis. The study was designed and the manuscript prepared in co-operation with Dariusz Leszczynski.

III RN had the main responsibility for the proteomics experiments presented in this study: the execution of the experiments and data analysis. The study was designed and the manuscript prepared in co-operation with Dariusz Leszczynski.

The author has the main responsibility for the unpublished data discussed here: the execution of the experiments and data analysis. The studies were designed in co-operation with Dariusz Leszczynski.

List of Abbreviations

2DE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
ACTH	Adrenocorticotropic hormone
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BVA	Biological variation analysis module in DeCyder analysis software
CDMA	Code division multiple access
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CW	Continuous wave
ddH_2O	Double distilled water
DIGE	Difference gel electrophoresis
DTT	Dithioreitol
EA.hy926	Human endothelial cell line
ECL	Enhanced chemiluminescence
EDA	Extended data analysis module in DeCyder analysis software
EDTA	Ethylene diamine tetraacetic acid
EEG	Electroencephalography
EMF	Electromagnetic field
FDR	False discovery rate
FDTD	Finite-difference time domain
GSM	Global system for mobile communications
HBMEC	Human brain microvascular endothelial cell
HSC	Heat shock cognate
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cell
IAA	Iodoacetamide
IARC	International Agency for Research on Cancer
ICNIRP	International Commission on Non-Ionizing Radiation Protection
IEEE	Institute of Electrical and Electronics Engineers
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
LR	Linear reflectron
LTE	Long-term evolution
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MS	Mass spectrometry/ mass spectrometer
MW	Molecular weight
NEPHGE	Non-equilibrium pH gradient electrophoresis
NH_4HCO_3	Ammonium bicarbonate

NMT	Nordic mobile telephone
ODC	Ornithine decarboxylase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
pI	Isoelectric point
PMF	Peptide mass fingerprint
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
PW	Pulsed wave
RF	Radio frequency
ROS	Reactive oxygen species
RT	Real time
SAGE	Serial analysis of gene expression
SAR	Specific absorption rate
SDS	Sodium dodecyl sulphate
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UMTS	Universal mobile telecommunications system
WB	Western blot/blotting
WCDMA	Wideband code division multiple access
WHO	World Health Organization

1. Introduction

Mobile phones have been a part of everyday life for most people in the developed world since the late 1990s. At the end of 2007 there were over six million mobile phone subscriber connections in Finland, corresponding to 115 subscriber connections per 100 inhabitants (Tilastokeskus 2008). Worldwide, the number of mobile phone subscribers has been estimated to have reached five billion (International Telecommunication Union (ITU) 2011). Simultaneously with the increase in the use of mobile phones, concern over the potential health risks due to mobile phone radiation has also arisen. Mobile phone technology has improved substantially since the first generation of cell phones, but the way they are used has also changed over time and is still changing. At first, mobile phone calls were expensive and the use of mobile phones was rare. However, mobile phones have developed and nowadays they serve people more as entertainment centers that are used for listening to music, photographing, surfing the Internet, and other purposes. In addition, land-line networks are no longer maintained in certain areas, causing phone calls to be transferred to wireless networks in these areas, thus further increasing the use of mobile phones.

Studies on the potential health and biological effects of mobile phone radiation have been extensively conducted over the years. Epidemiological as well as *in vivo* and *in vitro* approaches have been applied to examine the potential health and biological effects of mobile phone radiation. A few large epidemiological studies have been performed, mainly related to the incidence of cancer due to mobile phone use. Furthermore, human studies have been carried out to examine the effects of mobile phone radiation on sleep, different cognitive functions and behavioral aspects. There have also been several studies related to cancer incidence, genotoxic effects, cellular behavior and gene and protein expression *in vivo* and *in vitro*. So far, a number of these studies have focused on a single medical/biological endpoint (e.g., cancer incidence, apoptosis, or single protein expression). However, the results of the various studies have been contradictory. There is currently no consensus on whether mobile phones might have health or even biological effects, and in particular, no plausible mechanism for the effects of mobile phone radiation has been suggested.

Mobile phones generate a modulated radio frequency electromagnetic field (RF-EMF), which is a form of non-ionizing radiation. Mobile phone radiation is unable to cause ionizations in atoms or molecules and it does not have enough energy to directly break chemical bonds (e.g., in DNA strands). However, it is unknown whether mobile phone radiation could affect cellular and physiological functions by other mechanisms. A few hypotheses of these mechanisms have been presented, but to date there has been no generally accepted mechanism for

the potential non-thermal effects of mobile phone radiation.

Since the mid-1990s, new genome-wide screening techniques ('omics-techniques') have greatly developed and have become increasingly popular in research. These large-scale screening techniques allow the simultaneous examination of several endpoints, e.g., at the gene (transcriptomics) or protein (proteomics) expression level. These techniques have been successfully applied in several research fields, from clinical biomarker discovery to various systems biology approaches.

In this thesis, non-thermal biological and health effects related to mobile phone-based radiation are discussed, with a special focus on protein expression *in vitro*. An application of large-scale screening techniques in mobile phone radiation research is presented, and proteomics methods were used in the presented research to investigate the effects of the mobile phone radiation. Human endothelial cells were used as an *in vitro* model and the cellular proteome was examined immediately after short-term exposure to mobile phone radiation.

2. Review of the Literature

2.1. Biological and health effects of mobile phone radiation

2.1.1. Mobile phone radiation

In everyday life, we are constantly surrounded by electromagnetic fields (EMF). There are natural sources of EMF, such as the earth's magnetic field, as well as man-made sources, such as power cables, domestic appliances, radio stations, mobile phones, wireless networks, and radars. Many technical systems, such as mobile phones, use modulated radio frequency electromagnetic fields (RF-EMF) to transfer information. Typically, RF-EMF refers to the frequency range from 100 kHz up to 300 GHz (Nyberg, Jokela 2006).

Currently, there are two public mobile phone systems in large-scale use in Finland: the second generation GSM (Global System for Mobile Communications) system operating at 900/1800 MHz and the third generation UMTS/WCDMA system (Universal Mobile Telecommunications System/Wideband Code Division Multiple Access) operating at around 2000 MHz. The first generation analogue NMT (Nordic Mobile Telephone, 450/900 MHz) system was shut down by the end of the last millennium. The fourth generation LTE (Long Term Evolution) network, operating at around 2600 MHz, was launched on a pilot scale in late 2010.

Mobile phones emit RF-EMF in specified frequency bands during the transmission phase (i.e., when speaking during a phone call). GSM phones emit a digital pulse-modulated signal. The maximum transmission power of GSM phones is 0.25 W at 900 MHz frequency and 0.125 W at 1800 MHz frequency. The maximum transmission power during one pulse, however, is 8-fold higher (i.e. 2 W at 900 MHz and 1 W at 1800 MHz), because the signal is emitted in pulses with a duration of 0.577 milliseconds in 4.615 millisecond frames (that is, the signal is only emitted during one eight of the time). The signal in UMTS systems is more irregular than in GSM systems. In WCDMA systems, the signal is emitted on 5-Hz-wide radio channels. The maximum transmission power is 0.125 W and the peak transmission power below 1 W (Nyberg, Jokela 2006). Mobile phone systems continuously adapt the transmission power output level, and the maximum transmission power is only used when the field is weak, e.g., because of a long distance between the phone and the receiving base station.

The level of exposure to mobile phone radiation is generally measured as a specific absorption rate (SAR), which describes the power absorption per

unit mass and is expressed as W/kg. Basically, SAR represents the thermal load directed to the tissue from the electromagnetic field. A strong electric field might warm up tissues, and thermal effects of this kind are nowadays well understood (Adair, Black 2003). Based on these well-established biological effects of EMF, guidelines have been established to limit human exposure to RF-EMF. These guidelines have been published, for example, by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 1998) and the Institute of Electrical and Electronics Engineers (IEEE) (The Institute of Electrical and Electronics Engineers (IEEE) 2006). In the EU area, RF-EMF exposure limits are based on the ICNIRP guidelines, and in Finland, for example human exposures are regulated by a decree of the Ministry of Social Affairs and Health (294/2002). These basic restrictions contain 10–100-fold safety margins, and for exposure of the general public SAR levels have been set to 0.08 W/kg concerning the whole body, 2 W/kg for local exposure to the head and torso, and 4 W/kg for local exposure to the limbs. The SAR levels for local exposures are calculated as an average in 10 g of tissue. Based on these basic restrictions, a maximum SAR of 2 W/kg is permitted for mobile phones. Mobile phones commonly operate close to the exposure limits, around SAR levels of 1 W/kg. Thus, mobile phones are the only RF sources that operate close to basic restriction limits. In addition, local exposures in certain small areas might exceed the basic restrictions. (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 1998, Nyberg, Jokela 2006)

2.1.2. Potential mechanism

Electromagnetic fields induce an electric field and a current into the body. A strong electric field, depending on its frequency, might warm up tissues or disturb the neuronal functions. Thermal effects are based on energy absorption from the field to the tissue, which causes the oscillation of molecules. These types of effects are nowadays well known (Adair, Black 2003, Nyberg, Jokela 2006), and guidelines to limit human exposure to EMF are based on them. However, it is unclear whether RF-EMF might cause other non-thermal effects at low exposure levels.

Mobile phone radiation is non-ionizing, i.e. it is not able to cause ionizations in atoms or molecules. The minimum energy required for ionization of a ground state hydrogen atom is 13.6 eV, and the ionization energy for other atoms and molecules is also in the eV order of magnitude. The order of magnitude of the photon energy of RF-EMF, e.g., mobile phone radiation, is one millionth of the ionization energy and one thousandth of the thermal energy at room

temperature or the energy required to break weak non-covalent chemical bonds. Thus, the photon energy of mobile phone radiation is too weak to induce direct chemical changes and break chemical bonds directly (e.g., in DNA strands), and the mechanism for mobile phone radiation-based effects is therefore likely to be indirect, if such a mechanism exists at all. Several hypotheses for these potential mechanisms have been suggested (for a review, see e.g., Foster 2000, Nyberg, Jokela 2006, Sheppard, Swicord & Balzano 2008, Adair 2003), including for instance a reactive oxygen species (ROS)-mediated mechanism (Brocklehurst, McLauchlan 1996), oscillating resonances, and induced dipole moments. However, based on current knowledge, it seems that weak fields are quite unlikely to generate significant effects in their interactions (Adair 2003, Sheppard, Swicord & Balzano 2008). Thus, no generally accepted mechanism for the potential non-thermal mobile phone radiation-based effects has so far been established.

2.1.3. Overview of potential effects

2.1.3.1. Research approaches

Biological research regarding the potential biological and health effects of EMF has been conducted for decades and it has related to several different biological and health aspects. The studies have been conducted using different frequencies, exposure levels and durations as well as modulation types of RF-EMF. Several research strategies have been applied using epidemiology as well as *in vivo* and *in vitro* methods, since each of these approaches has its own strengths and weaknesses. Specifically, exposure assessment is one of the common challenges concerning all the study approaches in mobile phone radiation research.

Epidemiological studies aim to demonstrate a direct impact on humans and are thus usually considered to be the most suitable for human health risk assessment, which is an important issue in mobile phone radiation research. However, epidemiological studies in mobile phone radiation research are often limited by the assessment of exposure. In the studies executed so far, exposure assessment has often been carried out using questionnaires on mobile phone use completed by study participants. This might potentially cause bias in exposure assessment, e.g., due to recall bias. Human volunteer studies also provide direct evidence of the actual human response, but due to ethical reasons, these studies are limited to transient physiological phenomena. Studies *in vivo* can be used to mimic human studies to obtain physiological information in experimental situations where it is not possible to use human volunteers. Studies *in vitro* are extremely useful for determining basic biological effects and potential

mechanisms behind the effects, while they cannot be directly applied in health risk assessment.

In some of the early studies *in vivo* and *in vitro*, estimation of the exposure has been challenging, as the samples might have been heated to excess due to the RF-EMF exposure. Thus, it is unclear whether the effects potentially observed in these studies have been caused by heating or RF-EMF exposure (i.e., whether those are non-thermal effects). Furthermore, in certain studies the exposures have been executed by placing a regular mobile phone close to an animal cage or cell culture dishes. Such exposures do not allow reliable exposure assessment. Nowadays, specific exposure set-ups have been designed for studies both *in vivo* and *in vitro*. In the most optimized set-ups the study subjects are exposed to RF-EMF in a highly controlled environment and several factors such as temperature and field parameters can be monitored continuously during the exposure.

2.1.3.2. Cancer-related studies

One of the most common fears regarding mobile phone radiation exposure has been its potential ability to cause cancer. The cancer incidence due to mobile phone radiation exposure has been examined with epidemiological studies that examine the direct influence on humans. Additionally, effects on the incidence of cancer have been examined using animal studies.

Epidemiology provides the most direct evidence of the carcinogenic potential of specific agents in humans. Therefore, several epidemiological studies have examined the effects of mobile phone use on tumor formation. By far the most common area of focus has been on tumors of the head and neck area (e.g., gliomas, meningiomas, acoustic neuromas, and salivary gland tumors). Most of the completed epidemiological studies regarding mobile phone use and cancer incidence have been case-control studies, whereas only a few cohort studies have been performed. In case-control studies a patient with a diagnosed disease is asked to participate in the study, and after the permission is granted, he/she is often interviewed. Additionally a respective control person is sought for the study. Exposure assessment is based on either interviews or records from mobile phone network operators. If the exposure assessment is based on interviews, recall bias (i.e., how well the study person recalls the duration of phone calls, the location where the phone was held, etc.) may significantly affect the findings. The most recent large epidemiological study on mobile phone use and tumor risk has been the INTERPHONE study, which included over 5000 brain tumor cases with respective controls in 13 different countries (INTERPHONE Study Group 2010). The research group concluded that no overall increased risk of glioma or meningioma was observed due to mobile phone use. However, the

research group observed some indications of an increased risk of glioma at the highest exposure levels (long-term heavy usage), but biases, such as recall bias, and error prevented a causal interpretation. Several expert groups currently conclude that overall the studies published so far do not demonstrate a raised risk within approximately ten years of mobile phone use for brain tumors or any other head tumors. However, for slow-growing tumors the latency period is still too short to draw conclusions (e.g., International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009). Recently, a new cohort study (COSMOS) was launched in five countries to examine health risks related to long-term mobile phone use (Schüz et al. 2011) according to high priority research needs identified by the World Health Organization (WHO) (World Health Organization (WHO) 2010).

Animal studies have also been used to examine the incidence of cancer in relation to mobile phone radiation exposure. For instance, Repacholi et al. exposed transgenic mice to 900 MHz pulsed wave (PW) SAR values ranging from 0.008 to 4.2 W/kg and examined incidence of lymphoma (Repacholi et al. 1997). In this study, the lymphoma incidence increased after exposure and the authors reported that PW could enhance lymphoma formation in genetically cancer-prone mice. Subsequently, the study of Repacholi et al. was replicated by Utteridge et al. (Utteridge et al. 2002) and Oberto et al. (Oberto et al. 2007), but no increased lymphoma incidence was found. In several other studies, in which genetically wild type mouse strains have been exposed to mobile phone radiation, no increased tumor incidence has been detected (e.g., La Regina et al. 2003, Tillmann et al. 2007). Animal studies have also been used to examine whether mobile phone radiation could enhance the carcinogenicity of other agents. For example, Tillmann et al. recently exposed mice lifelong to the UMTS signal in the presence of known carcinogen and reported a doubled rate of lung cancers in the treated group when compared to the controls (Tillmann et al. 2010). The authors of this pilot study suggested that the UMTS signal might be potentially cocarcinogenic. Nonetheless, most published studies have not reported potential epigenetic carcinogenicity of mobile phone radiation (e.g., Heikkinen et al. 2006).

2.1.3.3. Human volunteer studies

Human volunteer studies can provide direct evidence of the actual human response, and several human volunteer studies have therefore been performed regarding mobile phone radiation exposure. However, due to ethical reasons, the endpoints in human volunteer studies are limited to transient physiological phenomena, such as nervous and endocrine system function or thermoregulation. Other endpoints include effects on sleep quality and symptoms of illness such

as headaches.

Nervous system studies have included, for instance, behavioral and neurophysiological measurements. These have recently been reviewed, among others, by van Rongen et al. and Kwon and Hämäläinen (van Rongen et al. 2009, Kwon, Hämäläinen 2011). A few positive findings have been reported in early behavioral studies, e.g., improved learning after mobile phone radiation exposure. Nevertheless, these findings have not been reproduced in later larger studies, even though these studies might have been performed by the same research groups (e.g., Koivisto et al. 2000a, 2000b vs. Haarala et al. 2003a, 2003b, 2004). Potential reasons for the diverse results could be the better experimental design in later studies, i.e., more study subjects, double blinding, and better control for false positives in statistical analysis.

Sleep quality has been examined in relation to mobile phone radiation exposure. Certain results suggest that mobile phone exposure has an effect on sleep electroencephalography (EEG) by increasing the EEG alpha range in the sleep EEG (e.g., Lowden et al. 2011). However, several other studies have found no effects on other sleep quality parameters (e.g., Fritzer et al. 2007, Mohler et al. 2010).

Volkow et al. exposed human volunteers to mobile phone radiation and investigated brain glucose metabolism using positron emission tomography (Volkow et al. 2011). A minor, but statistically significant, increase in brain glucose metabolism in the brain regions closest to mobile phone was reported after the exposure. Recently, Kwon et al. applied similar type of research approach as Volkow et al. (Kwon et al. 2011a, 2011b). No effects were found on cerebral blood flow, while brain glucose metabolism was suppressed after mobile phone radiation exposure. However, it is unknown whether changes in brain glucose metabolism have any clinical significance.

Some human studies *in vivo* on genotoxicity have also been performed. For example, Gandhi and Anita reported an increase in chromosomal damage when comparing mobile phone users and never-users, but the results were indicated as preliminary (Gandhi, Anita 2005). Yadav et al. reported an increased frequency of micronuclei in cells exfoliated from the human oral cavity of mobile phone users in comparison to controls (Yadav, Sharma 2008). The authors also reported a correlation between the years of exposure. Hintzsche and Stopper used a similar type of study set-up to Yadav et al. but found no differences related to mobile phone use (Hintzsche, Stopper 2010). Thus, no conclusions can yet be drawn on human genotoxicity studies. Additionally, Karinen et al. have examined molecular responses in human skin *in vivo* (Karinen et al. 2008). This study is presented in section 2.2.3.

2.1.3.4. Physiological endpoints in animal studies

In addition to cancer incidence (section 2.1.3.2), other biological and physiological endpoints have also been studied *in vivo* after mobile phone radiation exposure. Either rats or mice have most commonly been used in these experiments, but some studies have also used rabbits or flies (*Drosophila melanogaster*) as study subjects.

One of the interesting endpoints related to mobile phone radiation exposure has been the blood-brain barrier (BBB) and potential leakages in it. Possible leakages in the BBB may allow molecules of the blood circulation to enter the cerebrospinal fluid, causing potentially harmful effects. Some studies have indicated that mobile phone radiation might have an effect on BBB permeability. For example, Salford et al. reported an increase in the permeability of the blood-brain barrier to albumin after a two-hour exposure to a GSM signal (SARs 2 mW/kg, 20 mW/kg, and 200 mW/kg) (Salford et al. 2003). It was also suggested to cause neuronal damage throughout the brain, especially in the cortex, hippocampus, and basal ganglia. However, no effects on the BBB have been observed in replications of the Salford et al. study (de Gannes et al. 2009, Masuda et al. 2009). In addition, several other studies have reported no effects on the BBB (e.g., Finnie et al. 2006a). Therefore, based on the current scientific evidence, the effects on the BBB following mobile phone radiation exposure remain controversial, but seem very improbable.

Other brain areas and functions have also been examined after mobile phone radiation exposure. Recently, for example, Finnie et al. found no evidence of microglial activation (Finnie et al. 2010). In their study, they observed no perturbation of the neural tissue after acute (60 min) or long-term (2 years) exposure of mice using 900 MHz GSM with a whole body SAR of 4.0 W/kg. However, Maskey et al. reported hippocampus damage in rodents after a few months of exposure to an 835 MHz CDMA signal with an SAR range of 1.6 to 4.0 W/kg (Maskey et al. 2010a, 2010b).

Reproduction, fertility, and postnatal juveniles have recently been a concern related to RF-EMF exposure, as juveniles are subjected to a long-term exposure over their life time. At present, it is known that exposure to thermal levels of RF-EMF has a harmful impact on pregnancies and fertility (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009). Meanwhile, the number of studies on the non-thermal level of exposure is limited, and no consistent non-thermal effects have yet been reported. For instance, Lee HJ et al. recently exposed rats to a CDMA signal at a SAR of 2 W/kg and found no effects on spermatogenesis in rats after a subchronic exposure (Lee et al. 2010). However, a few human studies *in vitro* have presented contradictory evidence (Falzone et al. 2008, 2011). Fragopoulou et al. recently detected cranial and postcranial

skeletal variations induced in mouse embryos after exposure to a commercial mobile phone (Fragopoulou, Koussoulakos & Margaritis 2010), whereas Kumlin et al., for instance, observed no morphological changes in juvenile rats (Kumlin et al. 2007). In the study by Kumlin et al., animals exposed to mobile phone radiation showed significantly improved performance in a water maze task when compared to sham-exposed animals, indicating improved learning and memory (Kumlin et al. 2007). In general, results related to reproduction and postnatal development after mobile phone radiation exposure are still sparse and, for instance, WHO has recommended studies in this field as a high priority research need (World Health Organization (WHO) 2010).

Several other physiological endpoints related to, for example, nervous, auditory, endocrine, and cardiovascular systems, and different organs have also been investigated (for a review, see e.g., International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009). Recently, for instance, Bartsch et al. examined health effects at the general level in several rat studies *in vivo* after chronic exposure to a GSM-like signal (Bartsch et al. 2010). They suggested that the chronic exposure may incur negative health effects and shorten the life span of the animals if the treatment time is sufficiently long and the observational period covers the full life span of the animals. Meanwhile, Jin et al. reported that a one-year simultaneous CDMA/WCDMA chronic exposure at a SAR of 2.0 W/kg did not increase chronic illnesses in rats, although there were some altered parameters in the complete blood count and serum chemistry (Jin et al. 2011).

2.1.3.5. Genotoxicity

Genetic effects after mobile phone radiation exposure have been widely studied. The potential presence of genotoxic effects might lead to tumor formation in the future. Several techniques have been used, such as the detection of chromosomal aberrations, sister chromatid exchanges, and micronuclei, as well as the comet assay and γ-H2AX phosphorylated histone assays. Studies both *in vivo* and *in vitro* have on this topic been published. Most of the published genotoxicity results have not reported effects. Nevertheless, some results remain contradictory (for a review, see e.g., Verschaeve 2009, Verschaeve et al. 2010).

The potential genotoxicity of RF-EMF has been studied with animals, mainly with mice or rats. For instance, in the mid-1990s, Lai and Singh reported that a pulsed 2450 MHz RF-EMF has genotoxic potential. The authors found that the number of DNA single- and double-strand breaks increased in rat brain cells after 2 hours of exposure. The effects were reported immediately after the exposure and four hours after 0.6 and 1.2 W/kg exposures *in vivo* (Lai, Singh 1995, 1996, 1997). However, neither replication studies (Malyapa et al. 1998,

Lagroye et al. 2004) nor studies using GSM exposures (Belyaev et al. 2006) have reported similar results. Furthermore, long-term exposures of mice to GSM signals have not caused increased micronuclei frequencies (e.g., Juutilainen et al. 2007, Ziemann et al. 2009). In summary, most of the animal studies have not reported direct genotoxic effects after RF-EMF exposure at non-thermal levels (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009).

Genotoxicity has been examined *in vitro* using several different techniques. The majority of studies have not reported effects after mobile phone radiation exposure, while a few positive results concerning rather severe chromosomal effects after mobile phone radiation exposure have also been reported. For example, Tice et al. exposed human leukocytes and lymphocytes to several different mobile phone radiofrequency signals at various SAR levels (Tice et al. 2002). They reported micronuclear changes after a 24-hour exposure with all applied RF technologies at SAR averages of 5 or 10 W/kg in human lymphocytes, but not in leukocytes. After a shorter exposure time of 3 hours, no effects were observed. Using a continuous wave (CW) exposure, Mashevich et al. and Mazor et al. reported increased chromosomal aneuploidy (Mashevich et al. 2003, Mazor et al. 2008). These studies were recently repeated in part by Bourthoumieu et al. (Bourthoumieu et al. 2010, 2011). They examined the cytogenetic effects of 900 MHz mobile phone radiation on cultured amniotic cells and found no significant change in the rate of aneuploidy of chromosomes 11 and 17 or other direct cytogenetic effects. However, the exposure conditions (duration of exposure and SAR levels) were not identical. Several other studies *in vitro* have also reported no genotoxic effects (e.g., Vijayalaxmi et al. 2001a, 2001b, McNamee et al. 2002, 2003, Zeni et al. 2003, 2005, 2008, Scarfi et al. 2006, for review, see e.g., Verschaeve et al. 2010).

Many comet assay studies examining DNA damage and repair *in vitro* have also been published. Recently, an Italian research group reported that modulated GSM signals induced a significant increase in comet parameters in trophoblast cells after a 16- and 24-hour exposure at an SAR level of 2 W/kg (Franzellitti et al. 2010), while CW exposure did not. The changes were reversible after 2 hours of recovery. After shorter exposure times, no effects were observed (Valbonesi et al. 2008). Changes in the comet assay after mobile phone exposure have also been published by an Austrian research group (Diem et al. 2005, Schwarz et al. 2008). DNA strand breaks were already reported at low SAR levels in human fibroblasts. However, results of these studies have been criticized, and using the same study design the results have been negative elsewhere (Speit, Schutz & Hoffmann 2007). Most studies using the comet assay to assess DNA damage and repair have not reported any alterations in these

after mobile phone radiation exposure (e.g., McNamee et al. 2002, 2003, Hook et al. 2004, Zeni et al. 2005, 2008, for review, see e.g., Verschaeve et al. 2010).

DNA strand breaks can also be examined with a γ-H2AX phosphorylated histone assay, which is currently considered to be the most sensitive method for detecting DNA damages. So far, the method has not been widely used in mobile phone radiation research. Recently, for example, Belyaev et al. applied the γ-H2AX technique to study effects on human lymphocytes using exposures at different frequencies, signal modulations, and at an average SAR of 0.4 W/kg (Belyaev et al. 2009). The results suggested a long-lasting inhibition in the formation of DNA double-strand breaks co-localizing the 53BP1/γ-H2AX DNA repair foci. The effect was suggested to depend on the carrier frequency, with the UMTS signal being more effective than the GSM signal.

Additionally, the genotoxicity of the mobile phone radiation has been studied in the presence of known mutagens. Recently, for example, Luukkonen et al. (Luukkonen, Juutilainen & Naarala 2010) and Sannino et al. (Sannino et al. 2009a) examined the combined effects of mobile phone radiation and a known chemical mutagen using the comet assay. Chemical treatment with the mutagen led to the induction of DNA damage, but no additional DNA damage was observed when mobile phone radiation exposure was also applied. However, another micronuclei assay study by Sannino et al. (Sannino et al. 2009b) reported an adaptive response in human lymphocytes caused by pre-exposure to 900 MHz mobile phone radiation before chemical mutagen treatment. The lymphocytes for the study were collected from different donors, and lymphocytes from only some donors responded adaptively.

2.1.3.6. Cellular effects

Different cellular effects have also been examined after mobile phone radiation exposure. The potential differences in cellular behavior might play a role, for instance, in later tumor development. These cellular effects include proliferation, differentiation, apoptosis, and transformation, as well as the expression of specific genes and proteins (section 2.1.4).

Cellular growth has been examined with several methods. Recently, for instance, Lee KY et al. found no differences in cell cycle distribution after exposure *in vitro* to the CDMA/WCDMA signal for one hour at an SAR level of 4 W/kg (Lee et al. 2011). Sekijama et al. exposed three different cell types to the WCDMA signal with different durations up to 96 hours and the SAR averages up to 0.8 W/kg and found no differences in cell growth (Sekijima et al. 2010). The ornithine decarboxylase (ODC) expression levels have also been assessed, since the enzyme is involved in cell growth and its overexpression might regulate, for instance, cancer invasiveness. In the mid-1990s, increased ODC activity

was reported after exposure *in vitro* to various frequencies and modulations of RF-EMF (Litovitz et al. 1993, Penafiel et al. 1997). The increased activity was suggested to be exposure time-, frequency- and modulation-specific, peaking at certain points (the so-called 'window effect'). These studies have since been replicated, but no increase in ODC activity has been observed (Desta, Owen & Cress 2003, Höytö, Juutilainen & Naarala 2007). However, it has been reported that ODC activity is sensitive even to small temperature changes (Höytö et al. 2006), which could have been caused by mobile phone radiation exposure in the original study set-up. A few studies have also reported effects on cell proliferation after mobile phone radiation exposure (e.g., Velizarov, Raskmark & Kwee 1999), whereas several others have not shown effects on cell proliferation or viability (e.g., Nikolova et al. 2005, Gurisik et al. 2006, Merola et al. 2006, Sanchez et al. 2006, Chauhan et al. 2007a, Huang et al. 2008a, 2008b).

Cellular apoptosis is an important process in which mutated or otherwise damaged cells are guided to 'commit suicide' and thus be eliminated from the tissue. The effects of mobile phone radiation exposure on cellular apoptosis have been examined in several studies. For instance, Buttiglione et al. exposed a human neuroblastoma cell line to a 900 MHz PW at a mean SAR level of 1 W/kg. After 24 hours the authors reported an increase in apoptosis and also a decrease in known apoptosis inhibitor genes BCL-2 and survivin at the mRNA level (Buttiglione et al. 2007). Caraglia et al. also reported an increase in cellular apoptosis as well as changes in apoptosis-related genes after a 3-hour exposure to the 1950 MHz RF-EMF at an SAR level of 3.6 W/kg (Caraglia et al. 2005). However, most studies examining apoptosis after mobile phone exposure have not reported any changes (e.g., Gurisik et al. 2006, Joubert et al. 2006, 2007, Lantow et al. 2006c, Merola et al. 2006, Falzone et al. 2010).

Cellular transformation has been examined to determine whether the RF-EMF exposure could act as an inducer or a promoter of tumor formation or as a potential cocarcinogen. In the 1980s, Balcer-Kubiczek and Harrison reported that cellular transformation was increased in the presence of a known carcinogen after RF-EMF exposure (Balcer-Kubiczek, Harrison 1985, 1989, 1991). However, several newer studies have failed to support this observation. For example, Hirose et al. found no evidence of cellular transformation using the same cells as Balcer-Kubiczek and Harrison and a 2142.5 MHz WCDMA exposure (Hirose et al. 2008).

One potential mechanism that has been proposed for RF-EMF-associated effects is the ROS-mediated mechanism. ROS formation after the mobile phone exposure has consequently been examined in a few studies. Most of these have not reported any effects on ROS production after mobile phone radiation exposure alone or in combination with known chemical agents (e.g., Lantow et al. 2006a,

Brescia et al. 2009, Falzone et al. 2010, Luukkonen, Juutilainen & Naarala 2010). Thus, the current data suggest that mobile phone radiation exposure has no effect on ROS production in several different cell lines (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009).

2.1.4. Protein and gene expression

Gene expression on the transcriptional and translation level has been widely examined following mobile phone radiation exposure. Early studies focused on examining the expression of specific genes and proteins, such as heat shock proteins, proto-oncogenes and proteins of different signal transduction pathways. Furthermore, some proteins relating to cellular structures have been examined. Subsequently, new high-throughput screening techniques, such as transcriptomics and proteomics (section 2.2.3), have also been applied in mobile phone radiation research. There have been several studies reporting both effects and no effects on protein and gene expression after mobile phone radiation exposure. These studies have recently often been reviewed (e.g., Vanderstraeten, Verschaeve 2008, International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009, McNamee, Chauhan 2009, Gaestel 2010).

2.1.4.1. Heat shock proteins

Stress proteins, i.e., heat shock proteins (HSPs), are a large group of proteins that are highly conserved and found in all cell types among different species. They function as molecular chaperones and are expressed both constitutively and in response to several different types of environmental stresses, such as heat, cold, and chemical agents. For instance, the HSP70 protein family is commonly known to respond readily to different stressors (for a review, see e.g., Lindquist, Craig 1988, Kregel 2002). Because of their nature as stress responding proteins, these proteins have been suggested to be affected by mobile phone exposure (e.g., French et al. 2001, Leszczynski et al. 2002). Thus, the expression of HSP genes has been examined in several studies *in vivo* and *in vitro* both on transcriptional and translational levels after mobile phone radiation exposure. Most of the studies carried out to date have detected no effects after mobile phone exposure on HSP expression (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009), although some exceptions exist. The HSP studies are summarized in the text and Table 3 in Appendix 1. Additionally, a few early studies using CW exposure have been included.

Fritze et al. published one of the first studies *in vivo* examining the response of HSPs to mobile phone radiation (Fritze et al. 1997). In this study, rats were exposed to GSM and a respective CW signal. Immediately after

CW exposure of 7.5 W/kg, a slight induction of *Hsp70* mRNA was recorded in certain brain areas, but this was not observed at lower SAR values or 24 hours after the exposure. No other changes were observed, and the authors therefore suggested that acute high intensity microwave exposure may induce a minor stress response, but does not lead to lasting adaptive or reactive changes in the brain. In 2000, De Pomerai et al. reported an increase in the activity of the *Hsp16* reporter gene in *C. Elegans* after a low-level CW exposure of 750 MHz (de Pomerai et al. 2000). However, this study was later retracted, as the authors observed that the elevation of gene expression was probably due to the temperature rise during the exposure (Dawe et al. 2006). Subsequently, the same authors reported observing no increase in *Hsp16* reporter gene activity at higher exposure levels with either CW or GSM signals (Dawe et al. 2008). Weisbrot et al. examined *Drosophila melanogaster* after mobile phone radiation exposure and reported elevated HSP70 protein expression levels (Weisbrot et al. 2003). However, the exposure assessment for their study was inadequate, as the SAR values were not measured for the experimental set-up. Lee JS et al. exposed *Hsp70.1*-deficient mice for weeks to a CDMA signal at the SAR level of 0.4 W/kg (Lee et al. 2005). The expression levels of HSP25, HSP70, and HSP90 were not affected after the exposure. Sanchez et al. exposed rats to the GSM signals of 900 MHz and 1800 MHz and found no alterations in the expression of HSP25, HSC70, or HSP70 in rat skin (Sanchez et al. 2008). Finnie et al. exposed pregnant mice for a several days and examined the HSP expression in their pups (Finnie et al. 2009). HSP32 and HSP70 protein expression levels were not inducible in any mouse brains, while HSP25 protein expression showed no alterations after the exposure. Recently, Watilliaux et al. exposed developing rats for 2 hours to a GSM signal of 1800 MHz with the SAR ranging from 1.7 to 2.5 W/kg (Watilliaux et al. 2010). No effects were found on the expression of HSP60, HSC70, HSP70, or HSP90, or several glial markers after the exposure. In summary, the majority of recent studies *in vivo* examining HSP expression have not reported any effects of mobile phone radiation exposure.

Several studies *in vitro* on HSP expression have also been published. These studies have reported both effects and no effects on HSP expression levels. For instance, the following publications have described alterations in HSP expression levels. Leszczynski et al. reported a transient increase in the expression and phosphorylation of HSP27 in the human endothelial cell line EA.hy926 after a one-hour exposure to 900 MHz GSM at an SAR of 2.4 W/kg (Leszczynski et al. 2002). All changes were reversible in a few hours after the exposure. Czyz et al. observed an increase in the *Hsp70* mRNA level in mouse p53-deficient embryonic stem cells after exposure to a 1710 MHz GSM signal at SAR levels of 1.5 and 2.0 W/kg for 6 or 48 hours (Czyz et al. 2004). However,

no similar effects were found using other modulation schemes, e.g., GSM Talk modulation, or using wild type mouse embryonic stem cells. Caraglia et al. reported several changes in HSP27, HSP70, and HSP90 protein expression after exposing a human carcinoma cancer cell line to 1950 MHz RF-EMF (possible modulation of the signal was not specified) (Caraglia et al. 2005). Lixia et al. exposed human eye lens epithelial cells to a GSM signal of 1800 MHz for 2 hours at different SAR levels (Lixia et al. 2006). The authors reported an increase in HSP70 protein expression after 2 and 3 W/kg exposures, but no differences were detected in the *HSP70* mRNA level using RT-PCR. Sanchez et al. examined effects on human skin and exposed different cells and human reconstructed epidermis to a GSM signal of 900 MHz or 1800 MHz for 48 hours at an SAR level of 2 W/kg (Sanchez et al. 2006, 2007). A significant decrease in HSC70 protein expression was observed in fibroblasts after the 900 MHz exposure, but not after the 1800 MHz exposure. Additionally, a slight but significant increase in HSP70 protein expression was reported in the reconstructed epidermis after 3 and 5 weeks of culture. However, no effects on HSP27, HSC70, or HSP70 protein expression were found in keratinocytes with either of the exposures in these studies. Franzellitti et al. exposed a human trophoblast cell line to GSM and CW signals of 1800 MHz for 4 to 24 hours at an SAR of 2 W/kg and examined the *HSP70* gene and protein expression (Franzellitti et al. 2008). The authors found no differences in several members of the *HSP70* family, but inducible *HSP70C* transcript levels were altered (up or down) after certain exposure types. However, the same research group found no evidence of changes in either HSC70 or HSP70 protein or gene expression after a shorter exposure (Valbonesi et al. 2008). Yu et al. exposed human lens epithelial cells to 1800 MHz RF-EMF at various SAR levels and durations and reported a significant increase in HSP27 and HSP70 protein expression with an SAR above 2 W/kg after two hours of exposure (Yu et al. 2008). Unfortunately, the exposure assessment of this study was not reported and is thus inadequate.

However, several studies *in vitro* have reported no effects on HSP expression. Capri et al. exposed human mononuclear cells to three different modulation schemes of a 1800 MHz GSM signal with different SAR levels for 44 hours and found no changes in HSP70 protein expression (Capri et al. 2004). Lim et al. investigated the effects of mobile phone radiation on HSP expression, exposing human peripheral blood to the GSM and CW signals of 900 MHz at different SARs and durations (Lim et al. 2005). No changes were observed in HSP27 or HSP70 protein expression in human leukocytes, while a response to the heat shock exposure was observed. Vanderwaal et al. exposed two different cell lines, including the human endothelial cell line EA.hy926, to a TDMA signal of 847 MHz or a GSM signal of 1900 MHz at various SAR values and durations

(Vanderwaal et al. 2006). No alteration in HSP27 phosphorylation was observed in any of the exposure conditions, while the heat shock exposure of 41 °C or 45 °C increased HSP27 phosphorylation. Lee JS et al. exposed human and rat cells to a CDMA signal of 1763 MHz for 30 min or 1 hour at SARs of 2 or 20 W/kg (Lee et al. 2006) and reported no differences in the protein expression levels of HSP27, HSP70, or HSP90. Simko et al. exposed a human monocyte cell line to GSM and CW signals of 1800 MHz for one hour at an SAR of 2 W/kg and reported no changes in HSP70 protein expression (Simko et al. 2006). Later, the same research group also exposed several different cell types to a GSM signal at an SAR of 2 W/kg for one hour with different post-incubation times and found no changes in HSP70 protein expression (Lantow et al. 2006a, 2006b). Chauhan et al. exposed three human cell types to an intermittent 1900 MHz PW signal at average SARs of 1 and 10 W/kg for 6 hours (Chauhan et al. 2006a, 2006b). RT-PCR did not reveal any differences in the mRNA levels of *HSP27* or *HSP70* after the exposures, but changes were detected after heat shock treatment. Furthermore, the same authors found no changes in the mRNA levels of several *HSPs* after a longer lasting exposure with another cell type (Chauhan et al. 2007b). Hirose et al. examined HSP27 phosphorylation after exposing two types of human cells to a WCDMA and CW signal of 2142.5 MHz for different durations and SAR levels of up to 0.8 W/kg (Hirose et al. 2007). No differences were reported in HSP27 expression, phosphorylation or translocation. Neither were differences observed in the expression of other *HSPs* using DNA microchip analysis. Huang et al. exposed mouse auditory hair cells to a 1763 MHz CDMA signal at an SAR of 20 W/kg for various durations (Huang et al. 2008b). The authors found no effects on HSP27, HSP70, or HSP90 protein expression.

2.1.4.2. Proto-oncogenes

In addition to the HSPs, proto-oncogenes such as C-FOS, C-JUN, and C-MYC have been widely investigated regarding their response to mobile phone radiation exposure. These proteins function in cellular growth regulation. A mutation of these proto-oncogenes might lead to cell divisions occurring in an unregulated manner. Based on the current data, it appears that mobile phone radiation may not activate proto-oncogene expression, although some inconsistency in results still exists (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009). The studies carried out on proto-oncogenes are summarized in the text and Table 4 in Appendix 1.

A few studies *in vivo* have examined proto-oncogene expression. Fritze et al. investigated the expression of several FOS and JUN proteins in rats after GSM exposure, with no alterations in these proteins being found (Fritze et al. 1997). In a series of studies, Finnie et al. exposed mice to a GSM signal of

900 MHz at an SAR of 4 W/kg for either a short or long period and examined C-FOS protein expression in the mouse brains (Finnie 2005, 2006b, 2007). No changes were observed in C-FOS protein expression after any of the exposure conditions. Yilmaz et al. reported no changes in BCL-2 protein expression in rat brains or testes after a one-month exposure to a 900 MHz commercial mobile phone (Yilmaz et al. 2008). Meanwhile, in two separate studies, Lopez-Martin et al. exposed rats to a GSM signal of 900 MHz for 2 hours at rather low SAR values in the presence of a chemical agent to make the rats more seizure-prone (Lopez-Martin et al. 2006, 2009). The authors reported an increase in C-FOS protein expression in different areas of the rat brain after mobile phone radiation exposure. In summary, the number of studies *in vivo* on proto-oncogene expression is currently still limited, but the majority of existing studies do not report effects on proto-oncogene expression.

A few more studies *in vitro* on proto-oncogene expression have been published. Ivaschuk et al. exposed rat pheochromocytoma cells to a TDMA signal of 836.55 MHz for 20–100 minutes with an intermittent protocol (Ivaschuk et al. 1997). The mRNA levels of *C-Fos* and *C-Jun* were examined, and *C-Jun* transcript levels were observed to have decreased after the 20-min exposure at 9 mW/cm^{2,} while the other exposure conditions had no effect on either of the genes. Goswami et al. exposed mouse embryo fibroblasts in two growth phases to an 836 MHz CW or 848 MHz CDMA signal at an average SAR of 0.6 W/kg for 24 hours or 4 days (Goswami et al. 1999). No effects were reported in proto-oncogene expression of serum-deprived cells, but the *Fos* mRNA levels increased in exponential growth phase cells during the transit to the plateau phase and in plateau-phase cells. This study was replicated by Whitehead et al., with the same cells and similar types of exposures being used with higher SARs of 5 and 10 W/kg (Whitehead et al. 2005). No effect on Fos expression was observed using RT-PCR, and the results of Goswami et al. could not therefore be confirmed. Czyz et al. investigated *C-Jun* and *C-Myc* expression simultaneously with HSP expression (Czyz et al. 2004). *C-Jun* and *C-Myc* expression were reported to be transiently up-regulated in p53-deficient cells, but not in wild type cells after exposure to 1710 MHz GSM. No effects on *Bcl-2* mRNA levels were observed. Chauhan et al. also examined *C-FOS, C-JUN*, and *C-MYC* expression simultaneously with *HSP* expression and found no differences in the mRNA level of these in three different cell types after an intermittent 1900 MHz PW exposure for 6 hours at SARs of 1 and 10 W/kg (Chauhan et al. 2006a, 2006b). Merola et al. detected no changes in B-MYB or N-MYC protein expression after 48 or 72 hours exposure to a GSM signal of 900 MHz at an SAR of 1.0 W/kg (Merola et al. 2006). Buttiglione et al. reported a significant decrease in the mRNA levels of *BCL-2* and survivin genes in parallel with impaired cell cycle progression in human neuroblastoma

cells after 24 hours of exposure to a GSM signal of 900 MHz at an SAR of 1 W/kg (Buttiglione et al. 2007). Del Vecchio et al. exposed rat primary neurons to a 900 MHz GSM signal at an SAR of 1 W/kg for several days and found no differences in *C-Fos* or *C-Jun* mRNA levels (Del Vecchio et al. 2009).

2.1.4.3. Signal transduction pathways and structural proteins

While HSP and proto-oncogene expression have been the most frequently examined targets after mobile phone radiation exposure, the expression of certain other proteins has also been assessed. Most of these proteins are somehow related to signal transduction pathways and regulate different cellular functions. In addition, a few studies regarding certain structural proteins and some other protein targets have been published. Several of these studies have simultaneously examined HSP or proto-oncogene expression. Currently, there are several contradictory reports on this topic and further research should be conducted, particularly addressing protein activity rather than total expression. The existing studies are summarized in the text and Table 5 in Appendix 1.

Only a few studies carried out *in vivo* on this topic have been published, including the following. Weisbrot et al. reported an increase in ELK1 phosphorylation along with increased HSP70 expression in *Drosophila melanogaster* after mobile phone radiation exposure (Weisbrot et al. 2003). The SAR levels for this study are unknown and the exposure assessment is therefore inadequate. Lee JS et al. found no changes in the expression or phosphorylation of MAPK, ERK1/2, JNK1/2, or p38MAPK in *Hsp70.1*-deficient mice after CDMA exposure for several weeks (Lee et al. 2005). Dasdag et al. exposed rats to a GSM signal of 900 MHz for months and examined brain tissues for p53 and active caspase-3 protein expression (Dasdag et al. 2009). No effect on p53 was observed, while a decrease in apoptosis was reported. Yan et al. used a commercial mobile phone to expose rats to 800/1900 MHz mobile phone radiation for several hours per day over several weeks, and reported mildly elevated mRNA levels for calcium ATP-ase, endothelin, neural cell adhesion molecule, and neural growth factor (Yan et al. 2009). Thus, the authors suggested that the potential injuries in brains might be due to mobile phone radiation exposure. However, the exposure assessment of this study had some deficiencies. Ammari et al. reported an increase in glial fibrillary acidic protein (GFAP) expression in rat brains after 900 MHz GSM signal exposure at 1.5 or 6 W/kg for several weeks, suggesting potential gliosis (Ammari et al. 2010). Currently, the number of studies *in vivo* on this topic is limited. The results have been contradictory and thus insufficient.

Studies *in vitro* have reported both effects and no effects on signal transduction pathways or certain structural proteins. For instance, the following studies *in vitro* have reported effects. Leszczynski et al. detected an increase in

p38MAPK expression after mobile phone radiation exposure along with changes in HSP27 expression and the phosphorylation status (Leszczynski et al. 2002). The inhibition of p38MAPK also blocked HSP27 phosphorylation, and it was thus speculated whether the p38MAPK stress response pathway could be a target for mobile phone radiation. Czyz et al. reported an increase in the *p21* mRNA level in p53-deficient embryonic stem cells after mobile phone radiation exposure, while no alteration was found in the *Erg-1* mRNA level (Czyz et al. 2004). Caraglia et al. noted changes in the expression and activity of several signaling proteins after exposing a human oropharyngeal epidermoid carcinoma cancer cell line to 1950 MHz RF-EMF (potential modulation of the signal was not specified) (Caraglia et al. 2005). The authors suggested the induction of apoptosis via inactivation of RAS–ERK survival signaling. Nikolova et al. exposed mouse embryonic stem cells to a 1710 MHz GSM signal and found elevated mRNA levels of *Bax* and *Gadd45* and a decrease in the neural-specific *Nurr1* mRNA level (Nikolova et al. 2005). However, the responses were not associated with any detectable changes in cell physiology. Friedman et al. reported activation of the ERK1/2 signal transduction pathway in rat and human cells after short-term mobile phone radiation exposure, while the p38MAPK and JNK1/2 pathways were not activated (Friedman et al. 2007). As a drawback, this study did not provide any SAR estimates. Buttiglione et al. examined the downstream MAPK cascades by exposing human neuroblastoma cells to a 900 MHz GSM signal for different durations at an average SAR of 1 W/kg (Buttiglione et al. 2007). Short-term exposures seemed to induce a transient increase in the *ERG-1* mRNA level with a simultaneous activation of ERK1/2, SAPK/JNK, and ELK-1. Yu et al. reported the activation of ERK1/2 and JNK1/2 after mobile phone radiation exposure, but the exposure assessment was not reported (Yu et al. 2008). Cervellatti et al. exposed human trophoblast cells to a GSM signal of 1817 MHz at an SAR of 2 W/kg for 1 hour and reported a sharp decrease in the intercellular gap junction-like structures and changes in connexin expression, localization and cellular structure (Cervellati et al. 2009). This implied effects on gap junctions following mobile phone radiation exposure. Del Vecchio et al. exposed rat primary neurons for several days to a 900 MHz GSM signal and reported an increase in beta-thymosin expression and a corresponding reduction in the number of generated neurites (Del Vecchio et al. 2009).

However, several studies reporting no effects on these proteins have also been published. For instance, Lee JS et al. reported no differences in the expression or phosphorylation of MAPKs, ERK1/2, JNK1/2, or p38 in two cell types under different exposure conditions (Lee et al. 2006). Hirose et al. exposed two different cells to a WCDMA signal of 2142.5 MHz at different SARs and durations (Hirose et al. 2006). Neither p53 protein expression nor

phosphorylation, nor p53 downstream targets were affected. Recently, Hirose et al. investigated microglial activation *in vitro* by examining the expression of an immune reaction-related molecule and cytokine production after exposure to a 1950 MHz WCDMA signal for 2 hours at different SARs (Hirose et al. 2010). No marked differences were found in the production of tumor necrosis factor-alpha (TNF-alpha), interleukin-1 beta (IL-1 beta), or interleukin-6 (IL-6). Huang et al. exposed mouse auditory hair cells to a 1763 MHz CDMA signal at an SAR of 20 W/kg for various durations (Huang et al. 2008b). The authors reported no effects on ERK, JNK, or p38 protein expression or phosphorylation. Lee KY et al. carried out a single exposure of human breast cancer cells to an 837/1950 MHz CDMA/WCDMA signal or a combination of both signals at an SAR of 4 W/kg for 1 hour (Lee et al. 2011). The levels of cell cycle regulatory proteins, p53, p21, cyclins, as well as cyclin-dependent kinases were unaffected after the exposure. Additionally, no effects on cell cycle distribution were observed.

2.1.4.4. Transcriptome

Several studies have adopted large-scale screening techniques to examine gene or protein expression after mobile phone radiation exposure. Naturally, some of the specific proteins discussed in earlier sections have also been assessed in the large-scale screening. Most of the studies in this field using high-throughput screening have so far focused on the gene expression level. Protein expression studies on this topic are presented in section 2.2.3.

Usually, transcriptomics techniques applied in mobile phone radiation research have been based on cDNA microarrays. This technology provides the possibility to screen up to several thousands of genes simultaneously, thus offering a large amount of information. Unfortunately, an insufficient number of biological and technical replicates is rather often used, and it is not therefore possible to perform appropriate statistical analysis. In some publications the results have even been based on a single hybridization without the further validation of target genes using other methods. Such results cannot be considered reliable. Three replicates would be a minimum requirement for statistical analysis. The experiments using transcriptomics are summarized in the text and Table 6 in Appendix 1.

To date only two *in vivo* microarray studies have been conducted in this research field. Belyaev et al. exposed rats to a GSM signal of 915 MHz for 2 hours at a whole-body SAR of 0.4 W/kg (Belyaev et al. 2006). The gene expression profiles in the cerebellum were obtained in triplicate, and a total of 12 genes having diverse functions were reported to be affected, with fold ratios being 1.34–2.74. However, the gene expression changes were not confirmed with any other methods. Paparini et al. exposed mice to a 1800 MHz GSM signal for 1 hour

at a whole body SAR of 1.1 W/kg and examined the gene expression in the whole brain (SAR 0.2 W/kg) (Paparini et al. 2008). Three replicates showed no changes in gene expression when using more stringent data analysis. When less stringent conditions were applied, a total of 75 genes were found to be affected (1.5–2.8 up or 0.67–0.29 down). The expression validation of 30 of these potentially affected genes with RT-PCR did not show any alterations. Thus, the authors concluded that there is no consistent indication of gene expression modulation in the whole mouse brain associated with a GSM exposure of 1800 MHz.

In addition to the studies carried out *in vivo*, transcriptomics has been applied several times in studies *in vitro*. Some of these have reported changes in gene expression, although they have often been based on an insufficient number of replicates. For instance, Pacini et al. exposed human skin fibroblasts using a commercial mobile phone (inadequate exposure assessment) for 1 hour and found 14 differently expressed genes in a single experiment (Pacini et al. 2002). Furthermore, a significant increase in DNA synthesis and intracellular mitogenic second messenger formation was reported with a matching high expression of genes in the MAP kinase family. Lee S et al. exposed a human promyelocytic leukemia cell line to a 2450 MHz PW field for 2 or 6 hours at an SAR of 10 W/kg and examined gene expression using a single replicate with the Serial Analysis of Gene Expression (SAGE) technique (Lee et al. 2005). Several hundreds of genes were reported to be affected without further validation. Remondini et al. published a pooled analysis of gene expression of several different cell types after mobile phone radiation exposure (900 or 1800 MHz) (Remondini et al. 2006). Six different cell types were exposed with various exposure protocols (SARs 1–2.5 W/kg, durations 1–44 hours). The cellular RNA was pooled from several experiments, but only a single hybridization was performed for each cell type. The cell responses varied based on the exposures and cell types, e.g., the EA.hy926 cells responded to the 900 MHz GSM exposure but not to the 1800 MHz GSM exposure, while some other cells did not respond at all to the mobile phone radiation exposure. In general, the authors suggested that some human cell types might alter their gene expression in response to mobile phone radiation, but no consistent signature (e.g., stress response) could be detected. Zhao TY et al. exposed mouse primary neurons and astrocytes to a commercial mobile phone (inadequate exposure assessment) for 2 hours (Zhao, Zou & Knapp 2007). The authors found Caspase-2,-6 and *Asc* to be affected in both cells as well as *Bax* in the astrocytes in the duplicate array analysis. The results were validated with RT-PCR. The authors suggested that a relatively short-term mobile phone radiation exposure can up-regulate the elements of apoptotic pathways in brain-originated cells. They also suggested that the neurons appeared to be more sensitive than the astrocytes. Zhao R et al. exposed rat

neurons to an intermittent (5 min on/10 min off) GSM signal of 1800 MHz for 24 hours at an SAR of 2 W/kg (Zhao et al. 2007). A single experiment displayed in total 34 altering genes with rather low fold ratios of 1.15–1.62. The affected genes were associated with multiple cellular functions, such as the cytoskeleton, signal transduction pathways, and metabolism. Most of the changes were further validated with RT-PCR.

However, several transcriptomics studies *in vitro* have not reported effects on gene expression. These findings have often been based on a higher number of replicates or further validation of potentially affected genes. For instance, Whitehead et al. exposed a non-osteogenic mouse pluripotent cell line to two different signals for 24 hours at an SAR of 5 W/kg (Whitehead et al. 2006a, 2006b). Three replicates displayed differences (fold ratio >1.3) in several genes, but based on the sham-sham and false positive rate calculations, the authors concluded that the number of affected genes after the exposure did not exceed the false-positive rate, and no differences were therefore actually observed. In the positive control the number of affected genes was higher than the false positive rate. However, none of the potential target genes for the mobile phone radiation exposure were confirmed with any other methods, as they were solely rejected based on the false positive rate calculations. Qutob et al. and Chauhan et al. exposed a human glioblastoma-derived cell line and human monocyte-derived cell line to a 1900 MHz PW signal for various SAR values and durations (Qutob et al. 2006, Chauhan et al. 2007b). In the data analysis a gene appearance in all five performed replicates was required. No evidence of effects due to mobile phone radiation was found, while several affected genes were found in the positive control samples (heat shock). In addition, several *HSP* genes were confirmed to be unaffected by RT-PCR, and thus confirmed to be actual false negatives, as in the microarray results. Hirose et al. exposed human glioblastoma cells and human fibroblasts to various signals, durations, and SAR levels (Hirose et al. 2006, 2007). Two experiments showed no consistent effects after mobile phone radiation exposure, and the expression of p53-related genes was further confirmed not to be affected by RT-PCR. Zeng et al. exposed a human breast cancer cell line to a GSM signal of 1800 MHz at average SARs of 2 and 3.5 W/kg for 24 hours using an intermittent exposure (5 min on/10 min off) (Zeng et al. 2006). Five potentially responding genes were found after the exposure of 3.5 W/kg in the duplicate analysis. However, RT-PCR did not confirm the differences in these genes, and the authors therefore suggested that no effects were actually observed. Gurisik et al. exposed a human neuroblastoma cell line to a 900 MHz GSM signal for two hours at an SAR of 0.2 W/kg, allowing a recovery time of two hours afterwards (Gurisik et al. 2006). Six genes were found to be slightly down-expressed in a single experiment. The expression of two of these

genes was further validated with RT-PCR, but no confirmation for the array results was obtained. Thus, the authors suggested that no effects were observed due to the exposure. Huang et al. exposed mouse auditory hair cells and human T lymphoma cells to a CDMA signal of 1763 MHz with various SAR levels and durations (Huang et al. 2008a, 2008b). A few dozens of genes were found to be affected in the analysis using either three or five replicates. However, the fold ratios were small and no consistent groups of functional categories were found in the analysis. Without any further validation, the authors suggested that the results might also be false positives and that the exposure had no effect on the global gene expression of the cells examined. Sekijima et al. investigated gene expression in three cell types after exposure to various signals and SAR levels for 96 hours (Sekijima et al. 2010). The duplicate hybridizations suggested that mobile phone radiation exposure had only a minor effect ($p < 0.05$, max fold ratio 1.14) on two cell types with the highest SAR value used, while the heat shock treatment caused changes in several genes. Therefore, the authors concluded that exposure to mobile phone radiation is unlikely to cause a general stress response in the tested cells under these conditions.

2.1.5. Conclusions based on the review of the literature

Mobile phones generate a modulated radio frequency field, which is a form of non-ionizing radiation. Mobile phone radiation does not have enough energy to cause ionizations and to induce direct chemical changes (e.g., DNA strand breaks). Currently, it is not known whether mobile phone radiation might cause other than thermal effects, and potential mechanism for such effects is unknown.

During the past two decades, a considerable amount of research has been conducted related to the biological and health effects of mobile phone radiation exposure. Several biological and medical endpoints have been addressed in these studies. In general, the results in this field have been contradictory. Most of the studies published to date have not reported any effects after mobile phone radiation exposure, but certain studies have reported such effects. Furthermore, attempts to repeat some of the earlier experiments suggesting effects have not reported similar findings.

For instance, it seems that mobile phone radiation has no immediate carcinogenic risk at low SAR levels, whereas knowledge of the effects of long-term use (over ten years) is still limited (International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009). Based on the current scientific knowledge on tumor formation in humans or animals, at the end of May 2011, the International Agency for Research on Cancer (IARC) classified radio

frequency electromagnetic fields as possibly carcinogenic to humans (Group 2B) (Baan et al. 2011). This classification was based on the increased risk of glioma associated with mobile phone use found in some studies, and coffee, for instance, belongs to the same class. Human volunteer studies have shown consistent effects on thermoregulatory systems after RF-EMF exposure (for a review, see e.g., International Commission for Non-Ionizing Radiation Protection (ICNIRP) 2009), which have been due to the RF-EMF-induced heating. Other human volunteer studies have led to no response or inconsistent responses following mobile phone radiation exposure (behavioral studies, neurophysiological studies). Various endpoints have also been examined *in vivo* and *in vitro*. A large majority of studies with different cellular endpoints or genotoxicity have reported no effects after exposure, although some of the publications have suggested changes after mobile phone radiation exposure. However, different biological systems, exposure set-ups and conditions have been applied in these studies, and comparison of the results is therefore not always straightforward.

Based on the gaps in current knowledge, the World Health Organization has identified specific research needs in this field (World Health Organization (WHO) 2010). These high priority research needs include epidemiological cohort studies on children and adolescents as well as RF-EMF exposure provocation studies on human volunteers, including children of different ages. Emphasis on juveniles has also been addressed via animal studies focusing on early-life and prenatal RF-EMF exposures.

2.2. Proteomics

2.2.1. Overview

Proteomics refers to the large-scale study of proteins, their structures, functions, and modifications, as well as their interactions with each other. The technology allows simultaneous screening of several hundreds, even thousands, of proteins and thus enables, for instance, biomarker discovery in different clinical or pathological conditions and various types of systems biology approaches. A basic proteomic analysis is focused on analytical protein chemistry by characterizing proteins and their post-translational modifications (e.g., phosphorylation, methylation, glycosylation, ubiquitination). In expression proteomics, i.e., differential display proteomics studies, the protein expression profiles are examined using a case-control experimental set-up. Proteomics is also used to examine protein–protein interactions and identify protein complexes. (Simpson 2002)

The field of proteomics has rapidly expanded over the past two decades. The words “proteome” and “proteomics” were introduced in the mid-1990s, although the principal technologies used existed before that. According to the original definition, a proteome meant the total protein complement of a genome (Wasinger et al. 1995). Since then, the term proteome has also been widely used to describe the set of proteins that are expressed during a certain time under given environmental conditions, and the total protein complement has been termed a complete proteome.

The core technique for proteomics studies was originally two-dimensional gel electrophoresis (2DE), which was developed in the 1950s (Smithies, Poulik 1956) with further significant developments in the 1970s (O’Farrell 1975, Klose 1975, Garrels 1979). In the 1990s, mass spectrometry (MS)-based techniques for proteomics studies were also developed. In MS studies, unlike in 2DE studies, the protein identity is obtained immediately and the protein expression levels can be compared between samples if labeling is used. Nowadays, several different techniques based on heavy and light stable isotopic labeling (e.g., ICAT, iTRAQ, SILAC) can be used for MS/MS-driven proteomics studies. However, MS studies also have limitations, mostly related to instrument requirements and data analysis. Proteomics can additionally be used to examine protein interactions, e.g., with (yeast) two-hybrid systems by engineering a host organism to express a protein of interest.

In general, these screening techniques, which are used in parallel, create a massive amount of data and thus require sophisticated analysis methods and programs, which have remained a challenge. Additionally, proteome-wide measurements with standard shotgun techniques (2DE, MS) only provide information about those proteins that are expressed in the cell at a specific time, while information about protein dynamics (e.g., half-lives) is not accessible (Wilkins 2009). Some of the typical problems in high-throughput studies were addressed by a group of experts as they developed guidelines for proteomics publications (Wilkins et al. 2006). In addition to these high-throughput techniques, specific and comprehensive serial applications for certain model organisms also exist, such as organism engineering for protein tagging and comprehensive antibody arrays. Often, these serial applications provide higher-quality data concerning abundance, half-life, and localization of proteins, but unfortunately they are only useful for specific model organisms that can be genetically manipulated. However, high-throughput techniques are currently the most capable techniques to provide different insights in case-control set-ups, especially for genetically non-engineered organisms. (For a review, see e.g., Wilkins 2009.)

2.2.2. Two-dimensional gel electrophoresis

2.2.2.1. Principles

Two-dimensional gel electrophoresis (2DE) was invented in the 1950s and several marked improvements have been introduced since then (for a review, see e.g., Görg et al. 2009, Klose 2009). Since the 1990s, 2DE has been widely applied in proteomics studies. In 2DE, proteins are separated from each other based on their charge, i.e., the isoelectric point (pI) of the protein and the molecular weight (MW) (Simpson 2002, Westermeier 2005).

Sample preparation is a crucial step in 2DE. Typically, a sample requires preparative steps before it can be applied in 2DE. Proteins can be extracted from simple cellular samples with different lysing techniques, such as sonication, freeze and thaw, or detergent lysing in the presence of protease inhibitors. More complex samples might also require cleaning or precipitation. In certain applications, sample fractionation based on, for instance, cellular components or molecular weight is useful.

In the first-dimension separation the protein charge is neutralized in a gradient pH gel using high voltage isoelectric focusing (IEF). Earlier, carrier ampholytes IEF was performed, but nowadays immobilized pH gradient gels (i.e., the IPG strips) are typically applied. IPG strips allow high reproducibility and enable protein separation with various wide and narrow pH ranges. For proteins having very basic isoelectric points, non-equilibrium pH gradient electrophoresis (NEPHGE) can also be applied.

The second-dimension separation is based on the molecular weight, often using standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), although other buffer systems have also been developed. The used gels are either single percentage gels (e.g., 10% or 12%) or gradient gels (e.g., 4–20%), depending on the appropriate molecular weight range. Either lab-made or commercial gels can be used.

Protein detection is based on either protein labeling before electrophoresis separation or post-staining of gels. Radioactive labeling with ^{35}S or ^{32}P is a very sensitive technique and stable isotopes $^{14}N/^{15}N$ or $^{12}C/^{13}C$ are useful for quantitative analysis. However, these techniques require a living sample and are thus not applicable for biopsy samples. Additionally, radioactive labeling requires facilities to handle radioactive material. Proteins can also be labeled with fluorescent dyes (Cy dyes), which has become a state-of-the-art technique in 2DE. Post-staining of gels can be performed with several techniques, e.g., with coomassie blue, silver, or fluorescent dyes, each of which have different advantages and limitations. Coomassie blue staining is the easiest to use but not very sensitive. Silver staining is sensitive but requires several steps and is

not linear. Fluorescent dyes are linear but often expensive. Additionally, stains detecting special modifications, such as phosphorylation, are commercially available. (See further discussion in section 2.2.2.2.)

The gels with visualized proteins are imaged, usually with high resolution (laser) scanners or specific cameras. For differential display proteomics studies, 2DE gel maps are analyzed with specific computer software. The software is designed to quantitatively analyze protein expression profiles in gels and also perform statistics. Proteins of interest can be identified using MS technologies. The simplest MS identification is based on peptide mass fingerprinting (PMF), in which proteins are digested with a specific enzyme to generate peptides. Peptide masses are compared to databases to identify the proteins. Amino acid sequencing can also be performed to obtain an exact sequence using MS/MS instruments. After MS identification, special applications and further studies can be applied to proteins of interest.

2.2.2.2. Challenges and recent developments

Currently, 2DE is the most widely applied technique for proteomics studies. It is highly parallel, allowing several gels to be run simultaneously, and it enables the separation of several hundreds of proteins in a single gel. Furthermore, protein abundances are immediately available, as well as post-translational modifications based on the change in the pI of the protein. However, the 2DE technique also has limitations and challenges in the analysis of certain types of proteins (Görg et al. 2009). Proteins having an extreme pI (very acidic or alkaline) have usually been considered a challenge for two-dimensional separations, but nowadays several solutions have been introduced to overcome these problems, e.g., the narrow interval IPG strips (down to pH 2.5 for acidic proteins and up to pH 12 for alkaline proteins), specific reagents to stabilize the cysteine sulfhydryl groups in alkaline proteins, specific sample application techniques, and the application of high voltages (up to 12 000 V) (for a review, see e.g., Görg et al. 2009). Membrane proteins constitute a significant proportion of a cell's protein content, but are still underrepresented in 2DE gels. Because of their hydrophobic nature, the solubility of the membrane proteins is poor and they tend to aggregate and precipitate in aqueous media. Some potential modifications to the solutions used have been described (for a review, see e.g., Görg et al. 2009). It seems that no single proteomics technology currently exists to separate the complete membrane proteome, and membrane proteins therefore need to be investigated using a combination of several techniques. Additionally, the low abundance proteins are a major problem for all proteomics analysis technologies. The problem can be approached by targeting specific sub-proteomes (e.g., the isolation of certain cellular components using cellular

fractionation) or by applying sample prefractionation methods (e.g., preparative IEF, free-flow electrophoresis) simultaneously with various narrow pH gradient IPG strips, and thus allowing higher protein loads. Several of these protein classes, whose analysis in a simple total cell lysate 2DE is limited, are targets of growing interest in specific applications. This emphasizes the importance of an appropriate experimental design in studies in which the study hypothesis is based on the investigation of these proteins.

Protein visualization is one of the crucial steps for accurate data analysis and for protein abundance determination. Coomassie blue staining has been used for a long time for protein detection in 2DE. However, the limited sensitivity of this staining restricts the detection of even moderately abundant proteins. Silver staining with several different modifications has also been widely applied in 2DE analysis. Silver staining is one of the most sensitive staining methods for certain proteins, but problems arise from its limited linear dynamic range. Nowadays, it is generally accepted that less than two-fold differences cannot be detected using silver staining because of linearity problems. Thus, fluorescent dyes, such as Sypro® Ruby, with a high linearity have been introduced. In the late 1990s, Unlü et al. described a technique called difference gel electrophoresis (DIGE) utilizing fluorescent Cy dyes for protein labeling (Unlü, Morgan & Minden 1997). Three dyes with similar molecular weights but distinct fluorescent characteristics were developed. The dyes react with either lysine amino acids (minimal labeling) or cysteines (saturation labeling) and preserve the charge of the target amino acid. Proteins are labeled with dyes before electrophoretic separation and a pool of proteins labeled with three different dyes can be separated in a single gel. The technique was further developed by Alban et al. as a common internal standard was applied to all electrophoretic separations (Alban et al. 2003). This internal standard was used for data normalization and it was shown to greatly improve data analysis. Protein abundances were better controlled and technical variation diminished, as the same standard sample was run in all gel separations, enabling better gel-to-gel analysis. So far, DIGE has become a state-of-the-art technique in differential display 2DE studies, as the three fluorescent dyes and the application of an internal standard provide better accuracy than other technologies. However, the technique is rather expensive, and is not therefore included among the essential methods in all laboratories.

As in all fields of science, good experimental design is crucial in proteomics for a successful study. The use of proteomics is still restricted because of several limitations, such as its technical complexity and the high cost of data production (reagents, instruments, time), all of which have contributed to a poor experimental design in several published proteomics studies (Wilkins et al. 2006). In recent years, particular attention has been paid to experimental

design and data analysis methods in differential display proteomics studies (e.g., Wilkins et al. 2006, Karp, Lilley 2007, Minden et al. 2009). Earlier, most of the 2DE-based proteomics studies relied on the fold ratios in data analysis. However, fold ratio analyses ignore both biological and technical variations in samples, as they are based on the average fold ratios over the experimental groups, and thus increase the risk of selecting variable proteins due to the sample selection and not due to the experimental conditions. Nowadays, statistical testing, most often the Student's t-test, has been applied in 2DE data analysis to assess the nature of the observed differences, as the variance is then better controlled. However, it must be kept in mind that all statistical tests are based on certain assumptions that need to be recognized. For instance, the commonly used Student's t-test assumes a normal distribution of the data. This is not usually the case for 2DE data, as they are frequently skewed. However, this issue can be approached with appropriate data transformation methods (for reviews, see e.g., Karp, Lilley 2007, Minden et al. 2009). Another possibility is to use non-parametric tests (e.g., the Mann-Whitney U-test), which are more robust but less powerful in detecting changes. Another problem arising from statistical analysis is multiple testing (for reviews, see e.g., Karp, Lilley 2007, Karp et al. 2007). Multiple testing leads to the finding of false positives (e.g., in a typical analysis of 1000 spots at the 95% confidence level, 50 false positives may be expected due to multiple testing). This problem can be approached in several ways. Probably the most commonly used method in the proteomics field is the application of false discovery rate (FDR) correction. The focus in this method is on achieving an acceptable ratio of true and false positives. For example, a 5% FDR means that on average 5% of the changes identified as significant can be expected to be actual false positives. Recently, 2DE studies have applied both statistical testing and fold ratio analysis, which seems to be an appropriate methodology for the data analysis.

To achieve successful results from the data analysis, it is essential to consider the experimental design and the number of replicates. To date, many experiments that have used 2DE have had a low number of biological and/or technical replicates, while in certain MS/MS-driven studies the results have sometimes even been based on a single experiment, limiting the information that can be gained. Technical replicates are useful when the system includes a high level of noise, but most often the biological replicates provide more information about true effects. As the DIGE technique significantly reduces technical variation, it is usually enough to consider the number of biological replicates in 2DE-DIGE studies. The number of replicates needed has been estimated in a few studies (Karp, Kreil & Lilley 2004, Karp, Lilley 2007, Karp et al. 2007, Stühler et al. 2006). If technical variation is the only source of variation, three to five replicates are needed to determine a fold change of 1.5–2. However, if the

biological variation exceeds the systematic variation, the number of biological replicates needs to be increased respectively. For instance, the biological variation in animal studies is higher than in cell culture studies (Karp et al. 2007, Meyer, Stühler 2007). Among studies carried out *in vitro*, cell line experiments have shown less variation than primary cell cultures (Molloy et al. 2003).

Some limitations have also been identified in the DIGE system itself. For instance, preferential labeling has been observed (Tonge et al. 2001, Karp, Griffin & Lilley 2005), as well as a biased background for different dyes (Karp, Kreil & Lilley 2004). However, these problems can be overcome by using the dye-swap protocol, i.e., in the typical DIGE experiment an internal standard is labeled with the Cy2 dye and the cases and controls with the Cy3 and Cy5 dyes, swapping the dyes between the sample groups. Additionally, it has been suggested that the three-dye approach is not completely independent of the means of statistical testing, as the same internal standard is used for two samples, thus causing bias in the analysis (Karp et al. 2007). This was suggested to be resolved using only a two-dye approach.

In spite of its limitations, 2DE is the most commonly applied protein separation technique in proteome research (Görg et al. 2009). All available proteomics analysis technologies show specific technical advantages, but also have limitations. In comparison with MS/MS studies, 2DE studies require less hardware and are thus easily available for research groups. The protein abundances and post-translational modifications observed as the pI shift (e.g., protein phosphorylation and glycosylation) are initially available. Furthermore, the method is highly parallel, as several gels can be run simultaneously. With an appropriate experimental design, 2DE is a very powerful tool to reveal the protein content of the organism in certain conditions. However, none of the current proteomics technologies alone is able to address all research needs. Thus, the combination of several techniques is the most effective approach to solve the experimental question addressed.

2.2.3. Proteomics in mobile phone radiation studies

Despite the fact that proteomics has been applied extensively in several fields of research, its use in mobile phone radiation research has remained minor. To date, ten articles have been published reporting proteome responses after mobile phone radiation exposure. Four of these articles are presented in this thesis, and six of the ten proteomics studies have been performed at STUK. The proteomics studies related to mobile phone radiation exposure have been summarized in the following table (Table 1) and in the text.

Table 1. Proteomics studies after mobile phone radiation exposure.

Reference	Study material	Frequency, modulation SAR Time points (exposure/ sampling)*	Proteomics technique	Results
Leszczynski et al. 2002	Human endothelial cell line EA.hy926	900 MHz GSM 2.4 W/kg 1 hr	2DE, silver staining and ^{32}P-labeling, Western blotting, 4 replicates for 2DE	• Increase in the total protein phosphorylation level • Increase in the HSP27/P-HSP27/ p38MAPK protein expression
Nylund & Leszczynski 2004 (II)	Human endothelial cell line EA.hy926	900 MHz GSM 2.4 W/kg 1 hr	2DE, silver staining, Western blotting, immunocytochemistry 10 replicates	• 38 proteins statistically significantly affected, 4 protein spots identified (2* vimentin, IDH3A, HNRNPH1) • Changes in vimentin expression confirmed with Western blots and immunocytochemistry
Zeng et al. 2006	Human breast cancer cell line MCF-7	1800 MHz GSM, continuous and intermittent (5 min on/10 min off) 3.5 W/kg 1, 3, 6, 12, 24 hrs	2DE, silver staining, 3 technical replicates from a single protein lysate	• No changes observed (a few changes in all conditions, no overlap between exposure conditions, authors concluded effects as random)
Nylund & Leszczynski 2006 (III)	Human endothelial cell line EA.hy926 and EA.hy926v1	900 MHz GSM 2.4 W/kg 1 hr	2DE, silver staining, 10 replicates	• 38 proteins statistically significantly affected in EA.hy926, 45 proteins statistically significantly affected in EA.hy926v1, no correlation between cell line variants • No identifications or further confirmation
Li et al. 2007	Human lens epithelial cells HLEC	1800 MHz GSM 1, 2, 3.5 W/kg 2 hrs	2DE, silver staining, 3 replicates	• 4 proteins changed (>3-fold in 3.5 W/kg, >2-fold in 2.0 W/kg, no effect on 1 W/kg), identified as hnRNP K and HSP70 • No further confirmation
Karinen et al. 2008	Human skin, exposure *in vivo*	900 MHz GSM 1.3 W/kg 1 hr	2DE, silver staining, 10 volunteers	• 8 proteins statistically significantly affected • No identifications or further confirmation

*Sampling immediately after exposure unless otherwise stated

Table 1. Continued.

Reference	Study material	Frequency, modulation SAR Time points (exposure/ sampling)*	Proteomics technique	Results
Nylund et al. 2009 (IV)	Human endothelial cell line EA.hy926	1800 MHz GSM 2.0 W/kg 1 hr	2DE, silver staining, western blotting 10 replicates	• 8 proteins statistically significantly affected, 3 proteins identified (SRM, GRP78, PSA1) • WB validation failed for the GRP78 protein expression • No effects on HSP27 or vimentin protein expression (2DE/WB)
Gerner et al. 2010	Human T-lymphocyte cell line Jurkat T, Human primary diploid fibroblasts ES1 cells, Human peripheral blood mononuclear cells WBC	1800 MHz GSM intermittent (5 min on/10 min off) 2 W/kg 8 hrs	2DE, ^{35}S-labeling, fluorescent dye, cytoplasmic fraction 3 replicates	• Jurkat T cells: No differences in total protein expression, significant increase in *de novo* synthesis (^{35}S-labeling), 14 proteins identified (including, e.g., HSPs, T-complex proteins) • Fibroblasts: increase in protein synthesis, 17 proteins identified (including, e.g., HSPs, T-complex proteins, annexins, BIP) • WBC: minor increase in activated cells, increase in HSP60 synthesis, no effects on quiescent cells • No further confirmation for any proteins
Kim et al. 2010	Human breast cancer cell line MCF-7	849 MHz CDMA 2, 10 W/kg 1 hr/day, 3 days sampling 24 hrs afterwards	2DE, silver staining, Western blotting, RT-PCR, 3 replicates	• No reproducible changes observed • Changes in GRP78, PIN1, and glucosidase II observed in single gels, but no confirmation was observed with WB/RT-PCR
Nylund et al. 2010 (V)	Human primary umbilical vein endothelial cells HUVEC, Human primary brain microvascular endothelial cells HBMEC	1800 MHz GSM 2.0 W/kg 1 hr	2DE-DIGE, 13 replicates for HUVEC, 11 replicates for HBMEC	• No effects on either of the cell types after exposure (numerous differences between the cell types)

*Sampling immediately after exposure unless otherwise stated

Pioneering work in proteomics and mobile phone radiation research was published by Leszczynski et al. (Leszczynski et al. 2002). In this study the human endothelial cell line EA.hy926 was exposed to a GSM signal of 900 MHz at an average SAR of 2.4 W/kg for one hour. An increase in the general protein phosphorylation level was reported immediately after exposure using the ^{32}P-labeling of samples and the 2DE separation of proteins. Furthermore, a transient increase in HSP27, P-HSP27 and p38MAPK expression levels was also reported. The study was performed using four replicates and also included Western blots and immunocytochemical staining of selected proteins. Publications (II–V) by the same research laboratory are discussed in detail in this thesis.

Zeng et al. exposed the human breast cancer cell line MCF-7 to an 1800 MHz GSM signal at an average SAR of 3.5 W/kg for 1, 3, 6, 12, and 24 hours by using both continuous and intermittent exposure (5 min on/10 min off) (Zeng et al. 2006). The protein expression was studied immediately after the end of the exposures. Three silver-stained 2DE gels were prepared from a single cell lysate. A few changes, based on the fold ratio between the sample groups (2-fold up or down) or *de novo* synthesis, were observed in the analysis, but there was no correlation between the exposure conditions. The authors also performed a transcriptomics analysis for the cells exposed for 24 hours at average SARs of 2 and 3.5 W/kg and found five potentially affected genes. However, RT-PCR did not confirm differences in these genes after mobile phone radiation exposure. Thus, the authors suggested that the observed effects in the proteomics and transcriptomics analysis might have occurred by chance and were not caused by the exposure.

Li et al. exposed the human lens epithelial cells (HLEC) to a GSM signal of 1800 MHz at average SARs of 1, 2, and 3.5 W/kg for 2 hours and examined the protein expression immediately after the exposure (Li et al. 2007). The proteins were separated in triplicate silver-stained 2DE gels. In total, 4 proteins appeared with altering expression levels (>3-fold at 3.5 W/kg, >2-fold at 2.0 W/kg, no effect at 1 W/kg, no statistical testing or analysis of variances). Two of these proteins were identified as hnRNP K and one as HSP70, and the identification of one protein was not successful. The expression of these proteins was not validated with any other methods.

Gerner et al. exposed several human cells (the human T lymphocyte cell line Jurkat T, human primary diploid fibroblasts ES1 cells, human peripheral blood mononuclear cells WBC) to a GSM signal of 1800 MHz at an average SAR of 2 W/kg for 8 hours (additionally, for 2 and 4 hours) using an intermittent exposure (5 min on/10 min off) (Gerner et al. 2010). The protein expression from the cytosolic fractions was examined in triplicate immediately after exposure using 2DE with ^{35}S-labeling and fluorescent dye. Several changes were reported

in protein synthesis (^{35}S-labeled proteins) based on one-way analysis of variance (ANOVA, $p < 0.05$), while the fluorescently labeled total proteins displayed no alterations. Fourteen proteins were identified to have an altered protein synthesis level from the Jurkat T cells and a few more proteins from the fibroblasts. These identified proteins included for instance HSP-family proteins, T-complex proteins, annexins and BIP. In white blood cells (WBC), a minor increase in *de novo* synthesis was observed in the activated cells but the fold ratio was over 2 only for HSP60. No effects were observed in the quiescent white blood cells. The authors suggested that a sufficiently long time is needed to observe the effects, as there were no effects after 2- and 4-hour exposures. Additionally, it was suggested that proliferating cells with high protein synthesis rates are more sensitive to the mobile phone radiation exposures than the non-active cells.

Kim et al. exposed the human breast cancer cell line MCF-7 to a CDMA signal of 849 MHz at average SARs of 2 and 10 W/kg (Kim et al. 2010). The cells were exposed for 1 hour per day on 3 consecutive days and the cells were collected 24 hours afterwards. The protein expression was examined from three replicates using silver-stained 2DE gels. No reproducible changes were observed between these three replicates (the analysis method was not clarified), but a few proteins were found to have an altered expression level in single gels. These proteins were identified as GRP78, PIN1, and glucosidase II. Western blotting and RT-PCR were performed for these proteins, but no changes were observed, and no effects were therefore recorded in this system.

So far, a single human volunteer study *in vivo* has been published that examined the proteome effects after mobile phone radiation exposure. Karinen et al. exposed an area of the forearm skin of 10 human female volunteers to a GSM signal of 900 MHz at an average SAR of 1.3 W/kg for 1 hour (Karinen et al. 2008). Immediately after the exposure, punch biopsies were collected from the exposed area, while the non-exposed forearm served as a control. Extracted proteins were separated using silver-stained 2DE gels as a single gel per lysate. In total, 8 proteins were found to be altered in statistical tests (ANOVA and Wilcoxon tests without correction for multiple comparisons). Two of the proteins were present in all ten volunteers, while the others were expressed in 4–8 cases. However, none of the proteins was identified and their expression was not further validated. The authors suggested that mobile phone radiation exposure might alter protein expression in the human skin.

As noted, the availability of proteomics studies related to the effects of mobile phone radiation exposure is currently very limited. The 2DE technique has been applied in all ten published articles, while not a single research article has applied MS-based proteomics techniques in mobile phone radiation-related research. Additionally, most of the studies have had limitations in the

experimental design, especially in the number of replicates in relation to the staining technique used.

3. Aims of the Present Study

The general aim of the study was to examine changes in the proteome of human endothelial cells after short-term exposure to mobile phone radiation. The specific aims were to:

- Apply new genome-wide screening techniques, i.e., proteomics for the screening of several protein targets simultaneously responding to mobile phone radiation exposure. The finding of potential target proteins would allow the formulation of hypotheses of potential mechanisms by which mobile phone radiation could affect biological systems. (I)
- Investigate potential target proteins for short-term exposure to mobile phone radiation and the potential effects of mobile phone radiation on the cellular proteome. (II–V)
- Examine whether the proteome response to mobile phone radiation varies depending on the different backgrounds of the model system. (III, V)
- Examine whether the response to mobile phone radiation depends on different GSM systems/frequencies. (II, IV)
- Apply methodological development to the examined model system and experimental design to improve the data quality. (V)

4. Materials and Methods

The experimental methods used in this study are listed in Table 2 and brief descriptions of the methods are presented below. Detailed descriptions of the techniques are provided in the original publications (I–V).

Table 2. Methods used in this study.

Method	Publication
Cell culture EA.hy926/EA.hy926v1	I–IV/III
Primary cell cultures HUVEC & HBMEC	V
900 MHz GSM mobile phone radiation exposure	I–III
1800 MHz GSM mobile phone radiation exposure	IV, V
Two-dimensional gel electrophoresis, 2DE	I–V
MS identification	II, IV
Western blotting	II, IV
Immunocytochemistry	I, II

4.1. Cell cultures

In this thesis, human endothelial cells were used as a model system. Both a human endothelial cell line and primary human endothelial cells were used. Human endothelial cells were selected as a model system, because the endothelium is present in several body parts that are exposed to mobile phone radiation in everyday life. Specifically, endothelium is present in the blood-brain barrier, the function of which has been speculated to be affected, and is therefore often examined after mobile phone radiation exposure (see section 2.1.3.4).

4.1.1. Human endothelial cell line

The human endothelial cell line EA.hy926 was established by fusing primary human umbilical vein cells (HUVEC) with a thioguanine-resistant clone of the lung carcinoma cell line A549 by the exposure to polyethylene glycol (PEG) (Edgell, McDonald & Graham 1983). Hybrid clones were selected in HAT medium and screened for factor VIII-related antigen (Edgell, McDonald & Graham 1983), and the cell line was shown to exhibit typical characteristics of endothelial cells (van Oost et al. 1986, Edgell et al. 1990, Ahn et al. 1995). The EA.hy926 cell line was donated by the laboratory of Dr Cora-Jean S. Edgell of the University of North Carolina at Chapel Hill, NC, USA in 1987, and since

then it has been maintained at the University of Helsinki and at STUK. Another batch of a cell line that had been generated by subcloning of the EA.hy926 cell line was obtained from Dr Edgell's laboratory in 2000. This new batch of the cell line is referred to as EA.hy926v1.

Both of the cell lines were grown in Dulbecco's MEM (DMEM), supplemented with antibiotics, 10% fetal bovine serum, L-glutamine and HAT supplement. For the mobile phone radiation experiments, the cells were removed from the culture flasks by brief trypsinization, washed in cell culture medium and seeded to specific Petri dishes designed to be used in the exposure set-ups. Cell densities (cells/cm^2) were approximately the same on both dishes, i.e., 1.2 x 10^6 cells/55-mm-diameter glass Petri dish (DURAN, Germany) for the 900 MHz GSM experiments and 0.4 x 10^6 cells/35-mm-diameter Petri dish (NUNC, Denmark) for the 1800 MHz GSM experiments. Furthermore, cell densities were adjusted to obtain similar semi-confluent monolayers after overnight culturing in both dish types. These semi-confluent monolayers of EA.hy926/EA.hy926v1 were exposed to mobile phone radiation. After exposure the cells were harvested either by scraping (I–III) or enzymatically (IV). For the 2DE studies, 10 independent replicates were generated.

The cell cycle analysis was performed for the EA.hy926/EA.hy926v1 cell lines by determining the DNA content of the cells with the standard propidium iodide staining method as described by Leszczynski (Leszczynski 1995). (III)

4.1.2. Primary human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza, Switzerland, and cultivated according to the manufacturer's instructions. The HUVECs used were a pool of cells from several donors to gain a more heterogeneous sample and thus exclude potential individual variability. For the mobile phone radiation experiments, the cells were removed from the culture flasks by brief trypsinization, washed in cell culture medium and seeded into 35-mm-diameter "CellBind" Petri dishes (Corning, USA). After overnight culturing the medium was replaced with fresh medium and the semi-confluent monolayers of the HUVECs were exposed to mobile phone radiation using an 1800 MHz set-up. After exposure the cells were quickly washed with warm (37 °C) phosphate buffered saline (PBS) and harvested with warm versene (a chelating agent containing EDTA). For the 2DE analysis, 13 independent replicates were generated from the HUVECs.

4.1.3. Primary human brain microvascular endothelial cells

Primary human brain microvascular endothelial cells (HBMEC) were purchased from ScienCell Research Laboratories, USA, and cultivated as per the manufacturer's information. The used HBMECs were from a single donor (only available) and all cells used for the experiments were from the same batch. A vial of cells was cultivated to confluency, after which the cells were de-attached with trypsin and cultivated into 35-mm-diameter "CellBind" Petri dishes (Corning, USA). All the used plates were beforehand coated with 1.5% fibronectin (Sigma, USA). After cultivation for 72 hrs the medium was replaced with new medium and the semi-confluent monolayers of the HBMECs were exposed to 1800 MHz GSM mobile phone radiation. After exposure the cells were quickly washed with warm (37 °C) PBS and harvested with trypsin. For the 2DE analysis, 11 independent replicates were generated from the HBMECs.

4.2. Mobile phone radiation exposures

In all experiments, cell samples were exposed for one hour to mobile phone radiation. The cells were harvested immediately after exposure (without a post-incubation period) to examine the acute response at the protein level.

4.2.1. GSM 900 MHz exposure set-up

Cells were irradiated with simulated mobile phone radiation (900 MHz GSM signal) in a specially constructed exposure system. The system is described in detail by Leszczynski et al. (Leszczynski et al. 2002).

The specially constructed irradiation chamber was placed vertically inside a cell-culture incubator. Two 55-mm-diameter glass Petri dishes were placed inside the chamber in specific locations in such a manner that the E-field vector was parallel to the plane of the culture medium. Temperature-controlled water was circulated through the 9-mm glass-fiber-molded waterbed under the Petri dishes. The RF-EMF signal was generated with the ED Laboratory SG-1240 signal generator and modulated with a pulse duration of 0.577 ms and a repetition rate of 4.615 ms to match the GSM signal modulation scheme. The signal was amplified with an RF Power Labs R720F amplifier and fed to the exposure waveguide via a monopole type feed post. The SAR distribution in the cell culture and the E-field above the cell culture were determined using computer simulations (finite-difference time-domain (FDTD) method). The standard deviation for the SAR distribution inside the Petri dish was 45% based on the computer simulations (Toivo 2011). The simulation results were further validated with measurements.

For all experiments the cells were exposed to GSM mobile phone radiation of 900 MHz for 1 hour at 37 ± 0.3 °C at an average SAR of 2.4 W/kg. In the exposure, for 15% of the cells the SAR was higher than 3 W/kg, and for 0.7% of the cells the SAR was over 5 W/kg based on the non-uniformity of the SAR distribution (Toivo 2011). Sham exposures were generated similarly to the mobile phone radiation exposures but with the signal generator and amplifier turned off.

4.2.2. GSM 1800 MHz exposure set-up

The sXc-1800 exposure system, developed and provided by the IT'IS Foundation (Zurich, Switzerland), was used to expose cells to a GSM signal of 1800 MHz (IV Figure 1, V Figure 1C). A detailed description of the system and dosimetry is presented by Schuderer et al. (Schuderer et al. 2004).

The system consists of two identical exposure chambers mounted inside the same cell culture incubator (NuAire US Autoflow CO_2 Water-Jacketed Incubator, NuAire, USA). One of the chambers acted as a sham control (no radiation) and the other as an experimental chamber (with radiation). The sham exposure chamber and the mobile phone radiation exposure chamber were randomly assigned by the computer program that controlled the exposures. This computer program generated encrypted files with information about the irradiation chamber selection and the environmental monitoring during the experiment. These encrypted files were decoded afterwards by the chamber manufacturer, IT'IS, Zurich, Switzerland, and blinded execution of the experiments was thereby permitted.

The exposure system is fully automated and enables the controlled exposures of cells (H-polarization or at the H-field maximum of the standing wave (Schönborn et al. 2001)) at freely programmable amplitude modulations. Identical environmental conditions existed in both chambers (sham and experimental), since they were both located inside the same cell culture incubator and the inlets of the airflow through them are at the same location. The SAR distribution within the cell culture dish was characterized with a full three-dimensional (3D) electrothermal finite-difference time-domain (FDTD) analysis using the simulation platform SEMCAD (SPEAG, Switzerland). Additionally, the SAR intensity and distribution were verified with measurements using a 1-mm-diameter field probe inserted into the culture medium of the cell culture dish. The non-uniformity of the SAR distribution in the set-up was 23–30% depending on the volume of medium used for the monolayer exposure. In these studies it was estimated to be ca. 28% based on the 3 ml of medium used in cell dishes. (Schuderer et al. 2004)

The simulated mobile phone signal used in the studies was a GSM Talk signal of 1800 MHz. It is characterized by a random change between the discontinuous transmission mode (DTX) and non-DTX or GSM Basic phases. The distribution in time was exponential, with a mean duration of 10.8 seconds for the non-DTX ("talking") and 5.6 seconds for the DTX ("listening"). The dominant modulation components of this signal were 2, 8, 217, 1733 Hz, and higher harmonics (Tillmann et al. 2007).

The monolayers of human endothelial cells were placed to two 6-dish holders and placed inside the exposure chambers of the set-up in specific locations. In one chamber, randomly selected by the computer program, the cells were exposed to an average SAR of 2.0 W/kg at 37 ± 0.3 °C for 1 hour, while in the other chamber the cells were sham-exposed in similar conditions but without mobile phone radiation. The experiments were performed in a blinded manner and the code was broken at IT'IS afterwards.

4.3. Two-dimensional gel electrophoresis

4.3.1. Sample preparation

The sample preparation methods varied among the different experimental set-ups. The harvested cells were lysed with urea-containing lysing buffer (various contents, for details see publications II–V) for 1 hour at room temperature with occasional vortexing, after which the samples were centrifuged twice for 15 min at 20 000 x*g*. The protein concentrations were measured using the Bradford method. The amount of total protein used varied from 75 µg (V) up to 250 µg (IV).

4.3.2. Sample labeling

Primary endothelial cell samples (V) were examined using the DIGE technique (Unlü, Morgan & Minden 1997, Alban et al. 2003), whereas the EA.hy926 cell samples were visualized by silver staining (I–IV). The internal standard used for the DIGE experiments was prepared by pooling of the same amount of each sample into one sample. The same amount of total protein from each sample was labeled with Cy fluorescent dyes (GE Healthcare, USA). Each sample was labeled with either Cy3 or Cy5 dye using the "dye swap" principle based on the blinded exposure coding, while the internal standard was always labeled with Cy2 dye. The labeling was performed according to the manufacturer's instructions. Briefly, 600 pmol of dye per 75 µg of total protein was added to the

sample and labeling was performed for 30 min on ice in darkness. Afterwards, the labeling was quenched with 10 mM free lysine for 10 minutes on ice. A batch of Cy3- and Cy5-labeled samples was pooled with the Cy2-labeled internal standard, and this pool of proteins was separated in a single 2DE.

4.3.3. Isoelectric focusing

Isoelectric focusing was performed using IPGphor apparatus (GE Healthcare) and ready IEF strips (18 cm/pH 3–10 NL or 24 cm/pH 4–7, GE Healthcare). The samples were loaded using an in-gel rehydration in a buffer containing 9 M urea, 2% CHAPS, 0.5% IPG buffer, and 65 mM DTT. The IEF was performed at 20 °C until the desired volt-hours were achieved (65 kVhrs/18 cm, 95 kVhrs/24 cm). For details, see publications II–V.

4.3.4. SDS-PAGE

Before the second-dimension SDS-PAGE, the IEF strips were equilibrated for 15 min with 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, and 10 mg/mL dithioreitol (DTT) for 15 min and then for another 15 min in the same buffer, in which 25 mg/mL iodoacetamide (IAA) replaced DTT. In publications I–III, SDS-PAGE was performed using 8% gels similar to Leszczynski et al. (2002). In publications IV & V, 10% gels were used to obtain a better MW separation range. After electrophoresis the gels were processed for protein visualization. For details, see publications II–V.

For molecular weight range determination, MW markers (Bio-Rad, USA) were applied with a paper plug along with SDS-PAGE separation. Separate protein lysates were used (not analytical protein lysates).

4.3.5. Gel staining and image acquisition

Silver-stained gels (I–IV) were first fixed (30% ethanol, 0.5% acetic acid), washed with 20% ethanol and ddH_2O, sensitized with sodium thiosulfate (0.2 g/L), incubated in silver nitrate solution (2 g/L) and developed (potassium anhydride 30 g/L, 37% formaldehyde 0.7 mL/L, sodium thiosulfate 0.01 g/L). The development was stopped with Tris 50 g/L + 0.5% acetic acid, after which the gels were washed twice with ddH_2O and scanned using a GS-710 densitometer (Bio-Rad).

Cy dye-labeled proteins (V) were scanned with a Typhoon Trio scanner (GE Healthcare) with the appropriate excitation and emission wavelengths for Cy2, Cy3, and Cy5 dyes. The PMT voltages were optimized in such a manner

that the maximum signal intensity was approximately on the same level for all of the dyes.

4.3.6. Data analysis

4.3.6.1. PDQuest software

Silver-stained gels (I–IV) were analyzed using PDQuest software (Bio-Rad). In all experiments, 10 replicates from both the mobile phone radiation-exposed and the sham-exposed samples were used. A single gel was selected as a master gel and all the other gels were matched against this master gel. The gels were normalized by the software based the total volume of a gel. The spot volumes of the mobile phone radiation-exposed and sham-exposed sample groups were then compared using statistical testing (t-test, 95% confidence level). The protein spots that visually appeared as technical artifacts (e.g., background areas of silver staining, irregularly shaped dust particles, air bubbles), but were erroneously detected by the software, were manually removed from the analysis.

4.3.6.2. DeCyder software

The datasets containing images from the Cy2, Cy3, and Cy5-labeled samples were acquired with a Typhoon Trio scanner (GE Healthcare) and cropped with ImageQuant software (GE Healthcare) to contain the same pattern of proteins. The datasets were then imported into the DeCyder 6.5 software (GE Healthcare), in which the batch processor was used to detect the spots and match them against a selected master gel. The number of spots was estimated to 10 000 and the volume of 30 000 was used as a cut-off filter. After a brief manual check of the matched spots, the workspace was imported to the DeCyder extended data analysis (EDA) module for statistical analysis. The protein spots found in at least 70% of spot maps were included in the EDA analysis. The Student's t-test (with and without FDR correction) was used to identify differing protein spots. Statistics were performed on log-transformed data, while fold ratios were calculated from standardized abundances. Principal component analysis (PCA) was also performed for the spot maps. The lists containing the statistically significantly differing spots were imported back to the DeCyder biological variation analysis (BVA) module, where the results were evaluated based on the average ratio between the sample groups as well as visually to identify the possible artifacts (e.g., dust or other background artifacts). (V)

4.4. Mass spectrometry

4.4.1. In-gel digestions

Proteins of interest were extracted from several gels and in-gel digested. Before digestion the proteins were reduced with 20 mM DTT in 0.1 M ammonium bicarbonate (NH_4HCO_3) and alkylated with 55 mM IAA in NH_4HCO_3. The proteins were digested overnight at +37 °C with modified trypsin (sequencing grade modified trypsin, porcine, Promega, USA) in 50 mM NH_4HCO_3. After overnight digestion, the peptides were extracted with 25 mM NH_4HCO_3 and twice with 5% formic acid. The peptides were concentrated and de-salted using C-18 ZipTips (Millipore, USA) according to the manufacturer's instructions, with the exception of the elution solution being 60% acetonitrile (ACN). (IV)

Additionally, MS identification services were purchased from the Protein Chemistry Laboratory of the Institute of Biotechnology at the University of Helsinki, Finland. (II)

4.4.2. MS data analysis

The tryptic digestions were mixed 1:1 with α-cyano-4-hydroxycinnamic acid matrix and analyzed with the MALDI-TOF-LR-MS (Waters, USA) operating in positive ion reflectron mode. The mass spectra were externally calibrated with an ACTH clip 18-39 (MW 2465.199 Da, Sigma, USA) and internally calibrated with trypsin autolysis peaks (MW 1045.564/2211.108 Da). The peptide mass fingerprints (PMF) for protein identification were searched automatically at the accuracy of 50 ppm from the UniProt database with the ProteinLynx-software (Waters) operating along the instrument. The identifications were also confirmed by a manual search using the Matrix Science Mascot Peptide Mass Fingerprint tool (www.matrixscience.com). (IV)

4.5. Western blotting

The cell samples from the mobile phone radiation exposures were lysed with 2% SDS, 1% protease inhibitor cocktail (Sigma, USA), and the protein concentrations were measured using the Lowry method. The proteins were separated on 1D SDS-PAGE and blotted on a polyvinylidene fluoride (PVDF) membrane, blocked with non-fat dry milk, and exposed to primary antibodies. The respective secondary antibodies containing a horseradish peroxidase (HRP) conjugate (Dako, Denmark) were used. The signal was detected using enhanced

chemiluminescence (ECL). The autoradiography films were scanned with a densitometer and analyzed with Phoretix software (Molecular Probes, USA). For details, see publications II & IV.

4.6. Immunocytochemistry

After the exposure the cell samples were washed with PBS and fixed on glass plates overnight at +4 °C (3.7% paraformaldehyde in fixing buffer: 0.1 M Pipes, 1 mM ethylene glycol tetraacetic acid, 4% polyethyl glycol 8000, 0.1 M NaOH, pH 6.9). After fixing, the cells were permeabilized (0.5% Triton X-100 in fixing buffer for 10 min and 0.1% sodium borohydride in PBS for 10 min) and blocked with 5% bovine serum albumin (BSA). After blocking, the primary antibodies were applied and afterwards the respective fluorescently labeled secondary antibodies. The images were acquired using a Zeiss Axioplan 2 imaging microscope and evaluated by eye. (II)

5. RESULTS

The main results of this study are presented and discussed in publications I–V. A brief summary of the results is presented below.

5.1. Effects on the proteome after 900 MHz GSM exposures (I–III)

EA.hy926 and EA.hy926v1 cells were exposed to a GSM signal of 900 MHz. The exposures were performed during 2001–2004 and the data and the results are presented in publications I–III.

5.1.1. Human endothelial cell line EA.hy926

EA.hy926 cell samples were exposed to GSM mobile phone radiation of 900 MHz for one hour at an average SAR of 2.4 W/kg, and effects on the cell proteome were examined immediately after exposure. Ten independent replicates from the mobile phone radiation and the sham-exposed samples were generated using 2DE with silver staining. In total, 38 protein spots had statistically significantly altered expression levels (t-test, $p \leq 0.05$) between the sample groups (II, Figure 1). The fold ratios between the mobile phone radiation and the sham-exposed samples varied from 0.08 up to 8.9, in addition to a few cases of *de novo* synthesis (fold ratios unpublished). Clear technical artifacts (e.g., from background staining) were removed from the data, but the data still contained some weakly expressed proteins, which were hardly detectable from the background level (III, Figure 2B). (I–III)

A few of the proteins with differing expression levels between the sample groups were identified using mass spectrometry. These included the following (II, Figure 2 and Table 1):

- Vimentin (protein components of class III-intermediate filaments) was found to be expressed in at least two different isoforms differing in molecular weight and isoelectric point. The expression of both isoforms was increased in the samples exposed to mobile phone radiation (2.5-fold, $p = 0.006$, experimental MW/pI ca. 47 kDa/4.4 and 2.2-fold, $p = 0.02$, experimental MW/pI ca. 48 kDa/4.8).
- Isocitrate dehydrogenase 3 (NAD1) was slightly down-regulated in the samples exposed to mobile phone radiation (0.72-fold, $p = 0.03$).
- Heterogeneous ribonucleoprotein H1 was moderately down-regulated in the samples exposed to mobile phone radiation (0.61-fold, $p = 0.03$).

Vimentin expression was further validated using Western blotting and immunocytochemistry (II, Figure 3). Western blotting showed that vimentin was expressed in two forms, and the higher MW form was unaffected after mobile phone radiation exposure. However, the lower MW form was only present in the samples exposed to mobile phone radiation and not in the sham-exposed samples. In addition, a rearrangement of vimentin inside the cells after mobile phone radiation exposure was observed using immunocytochemical staining. Moreover, differences in F-actin and HSP27 expression and cellular localization were observed in immunocytochemical staining (II, Figure 4).

5.1.2. Human endothelial cell line EA.hy926v1

EA.hy926v1, a subclone of the EA.hy926 cell line, was used to examine effects on the proteome level after mobile phone radiation exposure. The cell lines had the same origin, but over the years they have spontaneously begun to exhibit different characteristics. This can be observed, for instance, from their different growth pattern (III, Figure 1).

Similar techniques were used to expose and examine the proteome-level effects in EA.hy926v1 cells to those used in EA.hy926 cells. In total, 45 protein spots had statistically significantly altered expression levels (t-test, $p \leq 0.05$) when comparing the mobile phone radiation and the sham-exposed samples (III, Figure 2C/D). The fold ratios between the sample groups varied from 0.04 up to 6.7 (fold ratios unpublished). In addition, some protein spots appeared with *de novo* synthesis. However, none of these 45 protein spots was the same as those protein spots affected in the EA.hy926 cell samples after exposure. Moreover, the protein expression pattern in the 2DE gels differed between the cell types, and only about a half of the proteins could be matched confidently between the different variants. The time between the two protein separation sets (EA.hy926 and EA.hy926v1) was approximately six months, which might have an influence on the comparability of 2DE protein maps. (III)

5.2. Effects on the proteome after 1800 MHz GSM exposures (IV, V)

EA.hy926 cells and the primary human endothelial cells HUVEC and HBMEC were exposed to a GSM signal of 1800 MHz. The data and the results from these experiments are presented in publications IV & V.

5.2.1. Human endothelial cell line EA.hy926

Cell samples of the EA.hy926 cell line were exposed to GSM mobile phone radiation of 1800 MHz for one hour at an average SAR of 2.0 W/kg, and effects on the proteome of the cells were examined immediately after exposure. Ten independent replicates were generated using 2DE with silver staining. Eight protein spots were found to have statistically significantly altered expression levels (t-test, $p < 0.05$) between the mobile phone radiation and the sham-exposed samples (IV, Figure 2). After exposure to the GSM signal of 900 MHz, 28 proteins spots were found to be altered in the pH range of 4–7 that was used in this study. Out of the eight proteins found here, four of the proteins were down-regulated with fold ratios of 0.33–0.47 and four of the proteins were up-regulated with fold ratios ranging from 1.47 to 2.46. For most of the spots, protein quantities were rather low. (IV)

Out of eight proteins that were affected after mobile phone radiation exposure, three proteins were identified by MS. These proteins were the following (IV, Figure 2 and Table 2):

- Spermidine synthase (SRM) was down-regulated in the samples exposed to mobile phone radiation (0.35-fold, $p = 0.036$).
- A 78 kDa glucose-regulated protein (GRP78) was identified as a fragment of protein. A ca. 55 kDa fragment was up-regulated in the samples exposed to mobile phone radiation (2.46-fold, $p = 0.029$).
- Proteasome subunit alpha type 1 (PSA1) was down-regulated in the samples exposed to mobile phone radiation (0.47-fold, $p = 0.045$).

Identification of the remaining five protein spots with the Maldi-ToF peptide fingerprint (PMF) technique was not successful, and no commercial antibodies were available for SRM or PSA1 proteins. (IV)

The expression of GRP78 protein was further validated using Western blotting. Based on MS identification, it was not possible to identify an exact location for the protein fragment, but a monoclonal antibody (corresponding residues surrounding Gly584, Cell Signaling Technology, USA) identified only a total protein (unpublished data). Using a polyclonal antibody, two forms of the protein representing the whole protein and a fragment were detected in the blot (IV, Figure 4A). However, the expression level of neither of them was altered based on Western blotting. The GRP78 protein amount in 2DE was also rather low and the standard deviation of protein quantity was high. This indicates that the 2DE result is possibly actually a false positive. (IV)

Furthermore, several other proteins were identified on the EA.hy926 2DE gel map to examine the expression of some other interesting proteins. These are listed in publication IV (IV, Figure 3 and Table 1). The 2DE showed no statistically significant differences in vimentin or HSP27 expressions, which

were earlier observed to be altered after exposure to a 900 MHz GSM signal (II, Leszczynski et al. 2002). The Western blots of these proteins also showed no changes in the expression levels of these proteins between the mobile phone radiation and the sham-exposed samples (IV, Figure 4B/C). In addition, based on the MS identifications, the expression levels of HSP60, P-HSP27, and p38 proteins were examined in the 2DE gels, but no statistically significant changes were observed (unpublished data). However, HSP27 phosphorylation/activity was not assessed with a specific phosphorylation assay in this thesis research.

5.2.2. Primary human endothelial cells

The cell samples from the primary human umbilical vein endothelial cells (HUVEC) and the primary human brain microvascular endothelial cells (HBMEC) were exposed to a GSM signal of 1800 MHz for one hour. The proteome of the cell samples was examined using 2DE-DIGE, and 13 independent replicates were produced from the HUVEC and 11 from the HBMEC. In the HUVEC proteome, 35 statistically significantly affected (t-test, $p \leq 0.05$) protein spots were found (V, Figure 3). The maximum average fold ratio between the sample groups was 1.33 for these significantly affected protein spots. In the HBMEC proteome, two statistically significantly affected (t-test, $p \leq 0.05$) protein spots with average fold ratios of -1.16 and +1.1 were observed when comparing the sample groups (V, Figure 4). However, when the false discovery rate (FDR) correction ($p \leq 0.05$) was applied to the statistical tests, all statistically significantly affected spots were recognized as false positives. All the spots found to be differentially expressed before the FDR correction were also manually checked. The average fold ratios of protein spots between the sample groups were close to 1.0 and the spots with the highest average fold ratios were visually recognized as technical artifacts (e.g., dust particles based on the extremely sharp peak geometry). In addition, principal component analysis (PCA) of the spot maps demonstrated differences between the cell types, but not between the exposure conditions (V, Figure 5). (V)

The differences between the cell types were also examined in the same analysis. In total, 368 protein spots were found to differ between the cell types (t-test, $p \leq 0.0001$, with FDR correction). Out of these 368 protein spots, 145 spots were differentially expressed between the cell types by more than 2-fold up or down (V, Figure 2). A few of these proteins were also identified with MS and, for example, tropomyosin showed a 5.8-fold decrease in HUVEC in comparison to HBMEC. The different expression levels were clearly observed in all samples, irrespective of the exposure conditions (unpublished data).

6. DISCUSSION

6.1. Effects on the proteome after mobile phone radiation exposure

In this thesis research, the effects on the proteome of four different types of human endothelial cells were examined after short-term exposure to mobile phone radiation. In general, proteomics was found to be an effective tool to screen the expression of several hundreds of proteins simultaneously, and applicable in mobile phone radiation research. It was observed that the cell type as well as exposure conditions have an impact on the responses at the proteome level following mobile phone radiation exposure. Changes in the proteome of the human endothelial cell line EA.hy926 were detected after exposure to a 900 MHz GSM signal, and changes in vimentin expression were further confirmed with other methods. A few other proteins were also identified, but their expression levels were not further validated. The proteome of the EA.hy926v1 cell line was also affected after 900 MHz GSM exposure, but differently from the EA.hy926 cells, although both cell lines have the same origin. Furthermore, a few changes were observed in the proteome of EA.hy926 cells after 1800 MHz GSM exposure. However, the number of affected proteins was lower in comparison to the 900 MHz GSM studies, and none of the affected proteins was the same in the two studies. Alteration of the GRP78 expression level was observed in 2DE, but it could not be confirmed with other techniques. This underlines the importance of data validation. The proteome of the primary human endothelial cells did not show any changes after exposure to an 1800 MHz GSM signal.

The presented results show that more changes were observed on the proteome level after exposure to 900 MHz GSM than to 1800 MHz GSM. There are a few possible explanations for this:

i) The different exposure frequencies (900 vs. 1800 MHz). However, there is currently no known mechanism by which a particular frequency could cause these observed differences, while another frequency does not cause similar effects. However, this issue of different frequencies should be further investigated. If possible, the same exposure set-up could be applied with different frequencies to address this issue.

ii) The differences in SAR distribution in the cell culture dishes of the exposure set-ups. Certain differences in SAR distributions were evident; however, these differences were minor and, for instance, only 0.7% of cells gained a higher SAR than 5 W/kg in the 900 MHz GSM set-up (Toivo 2011). Thus, it is unlikely that these differences would be observed using proteomics techniques. Furthermore, the EA.hy926 cells were exposed to a

1800 MHz GSM signal at an SAR of 5 W/kg for one hour, and the Western blot did not reveal any changes in the expression level of HSP27, vimentin or p38MAPK (unpublished data), similarly to the earlier 900 MHz GSM exposures (II, Leszczynski et al. 2002). Therefore, it is unlikely that the different SAR distributions in the used set-ups would alone explain the observed differences in the responses after the exposures.

iii) The more reliable 2DE technology in the later studies (silver staining vs. DIGE, data analysis). 2DE technology has improved over time. The primary cells were examined using 2DE-DIGE, which is a more reliable technique than silver staining used in the earlier studies. The improved reliability was observed as a decrease in variation and in the number of observed false positives. Additionally, in the EA.hy926 1800 MHz GSM study, the fold ratios were examined more carefully before assigning the affected proteins. In the earlier studies only statistics were used, and as some of the fold ratios were close to 1.0, the proteins could not be considered actually affected. Therefore, methodological variation is likely to partly explain the observed differences (see further discussion in chapter 6.2).

iv) Potential differences in the cells used. It is also possible that some spontaneous modifications have occurred in the EA.hy926 cell line used, and it might have become less responsive over the time. These spontaneous modifications are known to occur in cell lines if they are over-subcultured (for review, see e.g., Hughes et al. 2007). This may be likely, as the EA.hy926 cells originally had an abnormal chromosomal number around 80 (Edgell, McDonald & Graham 1983) and were based on HUVEC cells, which often exhibit an aneuploidic or polyploidic nature (e.g., Nichols et al. 1987, Wagner et al. 2001, Kimura et al. 2004). The possibility of such modifications in the EA.hy926 cell line is supported by the presence of the EA.hy926v1 cell line, which is a variant of the same cell line but shows different growth characteristics and thus indicates the potential genetic instability of this cell line. The potential genetic modifications are also supported by the recent Western blots, in which no alterations were observed in the expression of vimentin or p38MAPK in the EA.hy926 cells after exposure to a 900 MHz GSM signal (unpublished data), similarly to the earlier studies (II, Leszczynski et al. 2002).

To address the variation in all these results, the EA.hy926 cells should be examined simultaneously using modern proteomics techniques, both exposure set-ups, and different passages of the cell line, as it seems that the EA.hy926 cell line is a potential responder to mobile phone radiation exposure, unlike the primary cells.

The most convincing evidence of cellular-level effects is shown by the vimentin expression level, which was affected after 900 MHz GSM mobile phone radiation exposure, as especially observed from the Western blots. However, this effect was not observed after the 1800 MHz GSM exposure. Other research groups have not examined vimentin expression after mobile phone radiation exposure, but some changes have been observed in other proteins relating to cellular structures, e.g., connexins and gap junction like-structures (Cervellati et al. 2009). Using immunocytochemistry, the expression of vimentin and cellular localization of HSP27 were found to be affected after the 900 MHz GSM exposure. However, the results of the immunocytochemical staining were only evaluated by eye and are not therefore completely reliable. Leszczynski et al. and Yu et al. also reported changes in HSP27 expression after mobile phone radiation exposure (Leszczynski et al. 2002, Yu et al. 2008). However, the exposure assessment by Yu et al. was not reported, and their results cannot therefore be considered conclusive. Meanwhile, several other studies have reported no changes in the HSP27 expression level (e.g., Lee et al. 2006, Vanderwaal et al. 2006, Hirose et al. 2007). In this thesis research, no changes were observed in HSP27 expression after 1800 MHz GSM exposure. There have also been other studies suggesting that the cellular responses might depend on the cell type and/or exposure. For example, Sanchez et al. observed a decrease in HSC70 expression in human dermal fibroblasts after 900 MHz GSM exposure (Sanchez et al. 2006), whereas after 1800 MHz GSM exposure no effects were observed in these cells (Sanchez et al. 2007). The different responses on the mRNA level depending on the cell type and exposure conditions also support the observation that the responses after the mobile phone radiation exposure depend on cell type and exposure conditions (III, Remondini et al. 2006).

To date, only a few proteomics studies *in vitro* related to mobile phone radiation research have been published. Zeng et al. concluded that no changes were observed after mobile phone radiation exposure (Zeng et al. 2006). Their observation is similar to the ones made in this thesis research using human primary endothelial cells, although more modern and reliable techniques were applied here. Kim et al. used the same cells as Zeng et al. and found certain proteins, GRP78, PIN1, and glucosidase II, to be affected in single gels, but the results were not reproducible with any other techniques (Kim et al. 2010). Li et al. reported four proteins to be affected, two hnRNP K, HSP70, and one unidentified protein, but the results were not confirmed with other techniques. Further validation would give more impact to this study, as 2DE was only performed in triplicate using silver staining (Li et al. 2007). So far, the highest number of affected and identified proteins after mobile phone radiation exposure

has been detected by Gerner et al. (Gerner et al. 2010). They reported 14 affected proteins spots, e.g., HSP-family proteins, T-complex proteins, annexins, and BIP, i.e., GRP78. Gerner et al. used an approach that differed from all the other studies, as they used ^{35}S-labeling and actually measured the protein synthesis. Differences were observed in protein synthesis, while the fluorescently labeled gels showed no differences in total protein expression. This would suggest that mobile phone radiation has a greater effect on protein synthesis than on the actual total expression of proteins, indicating an imbalance in the protein level due to the exposure (Gerner et al. 2010). However, the reliability of this study was also limited, because only triplicate gels were used and the observations were not confirmed with any other techniques.

Similarly to the research presented in this thesis, Kim et al. and Gerner et al. also observed changes in GRP78 protein expression. However, validation of the expression of this protein in this study and by Kim et al. revealed no changes. Thus, the finding of effects on GRP78 might more likely represent a limitation of the 2DE technique, since it has been shown that several proteins are repeatedly reported to be affected in different kinds of proteomics studies (Petrak et al. 2008). These proteins include, e.g., HSP27, enolase 1, peroxiredoxins, vimentin, annexins, HSC71, keratins, GRP78, and RHOGDI. For instance, HSP27 was identified in 31%, vimentin in 19% and GRP78 in 13% of the studies published during a three-year period in the journal *Proteomic*s (Petrak et al. 2008). Extreme caution was suggested in the interpretation of differential expression of the most frequently identified proteins, as these might represent more the lack of depth in 2DE analysis (experimental design) than real differences due to biological condition examined. The expression of these proteins should at least be confirmed with other methods before assigning them as affected, as was done in this study and in that by Kim et al.

In summary, in the studies presented in this thesis, proteome responses after mobile phone radiation exposure seem to vary depending on the cell type and exposure. Although some minor effects exist, they are not necessarily global effects. This is in line with the observations of mobile phone radiation research in general, as most of the observed effects have so far been contradictory. Therefore, at this point it is not possible to identify any unique cellular-level response caused by mobile phone radiation. Neither is it possible to identify a likely mechanism or potential physiological or pathological effects on the cellular level due to mobile phone radiation exposure. Furthermore, it is not possible to predict the presence or absence of any potential health effect due to mobile phone radiation exposure.

6.2. General remarks on proteomics results in the light of technological development

Proteomics data need to be evaluated in light of technological development. Proteomics technologies have been widely available for about two decades, and several methodological improvements have been introduced during this time. The most important of these are probably the development of staining methods and improvements in data analysis.

In the earlier studies presented in this thesis, silver staining was used to visualize proteins, as it was still regularly used at that time. However, only fluorescent dyes are nowadays used for quantitative proteomics. Among the silver-stained protein analyses, there were protein spots that were very faint and spots for which changes in the expression level were less than 2-fold, which were assigned as affected only by statistical analysis. Moreover, the normality of data was not investigated when the statistical analysis was performed. These analyses were directly based on raw data, and not on log-transformed data, which might have caused bias in the results. Furthermore, false discovery rate correction was not applied in the data analysis, and in fact, the false positive rate was not exceeded in any of the studies. Therefore, some of the observations of affected proteins might actually have been false positives. Thus, based on current knowledge, effects on the cellular proteome in EA.hy926 and EA.hy926v1 cells after mobile phone radiation exposure might not be as significant as originally presented. However, despite the limitations of the methodology, the effects on vimentin protein expression were also observed with other methods. Conversely, no effects were proven for GRP78 protein expression in Western blot analysis after 1800 MHz GSM exposure. This indicates that GRP78 is possibly a false positive detection in 2DE, which is supported by the low protein quantity and high standard deviation in 2DE quantification (IV). This underlines the importance of data validation using other methods, especially when 2DE is not performed using state-of-the-art technology. The last study, using 2DE-DIGE technology and the latest requirements in data analysis methodology (e.g., log transformation of data and false discovery rate correction), showed no effects on the proteome of two human primary cell lines. Due to the technology used in this study, the results are very significant in showing no immediate effects on the proteome of primary human endothelial cells immediately after exposure to an 1800 MHz GSM signal. Meanwhile, the strength of the 2DE-DIGE system was demonstrated by the observed differences between the cell types.

The main strength of the studies presented in this thesis is the number of replicates collected for the proteomics analyses. All the proteome studies were based on at least 10 biological replicates, which is unprecedented in the field of mobile phone radiation research. All remaining published proteome studies *in*

vitro in this research field, although only a few exist, are based on triplicate gels and other staining techniques than DIGE. In the DIGE system, three replicates are enough to show 2-fold differences if the biological variation is small (e.g., in cell lines) and the technical variation due to the system is reduced (Karp et al. 2007). In systems other than DIGE, the gel-to-gel variation is much higher and more replicates are needed to observe such differences.

6.3. Future aspects

The results presented here demonstrate that the proteome response after mobile phone radiation exposure depends on the cells used as well as on the exposure conditions. The proteome of EA.hy926 cells was affected after exposure to 900 MHz GSM mobile phone radiation, whereas the changes in the proteome after 1800 MHz GSM exposure were much weaker. The observed differences in EA.hy926 responses should be addressed with modern techniques to determine their causes. A study comparing the effects between the exposure set-ups using state-of-the-art proteomics techniques is currently in progress. The preliminary results of this study indicate that the responses after mobile phone radiation exposures are not similar to those observed earlier (unpublished data). However, this study has not taken into account the potential spontaneous genetic changes in the cell line, which should also be examined.

Furthermore, these experiments only focused on a single exposure condition, i.e., exposure for one hour at an SAR average close to 2 W/kg, and the proteome responses were only examined immediately after exposure. The time and dose selection was based on a previous study published by Leszczynski et al., as several changes were reported in that study (Leszczynski et al. 2002). However, other researchers (e.g., Gerner et al. 2010) have suggested that longer exposure times are needed to observe responses on the proteome level. Additionally, it might be worthwhile to allow a post-incubation time after the exposures to enable potential changes to appear on the translational level. Recent Western blot data on heat shock exposures (43 °C, 1 hour) of EA.hy926 cells have demonstrated that HSP70 protein expression levels are increased a few hours after the treatment, but not immediately after (unpublished data). However, some studies have suggested that even with a post-incubation time, responses following mobile phone radiation exposure might not be observed (e.g., Chauhan et al. 2006a, 2006b, Kim et al. 2010). Furthermore, chronic exposures with occasional sampling could reveal a response to long-lasting exposures without the need for time point selection. Additionally, cell selection for further studies is important, as it seems that responses depend on the cell type and even on the stage of the cells. For example, Gerner et al. found that metabolically

inactive mononuclear cells were less affected than active mononuclear cells after mobile phone radiation exposure (Gerner et al. 2010). The use of several cell types would also reveal potentially responding cells.

Regarding the proteomics studies, cellular and technical fractionation would be useful to gain further depth in 2DE analysis. With these improvements, the decreased number of typical responders (Petrak et al. 2008) in 2DE studies would be observed. Furthermore, other techniques should also be used in protein expression studies, as the basic shot-gun proteomics techniques do not provide information on, for instance, the protein half-lives or localization (if total cell lysates are used). Additionally, it might be worthwhile to examine the potential effects after mobile phone radiation exposure at the individual cell level, as the more crude proteomics techniques require rather large and comprehensive effects in a total cell population before they are observed. For instance, Newman et al. have introduced in yeast the use of single-cell proteomics, which allows quantitative single-cell measurements of proteins (Newman et al. 2006). However, this technique is currently available only for certain model organisms, although it might be applicable in the future.

7. CONCLUSIONS

In this thesis, the results of proteomics analysis of human endothelial cells after short-term mobile phone radiation exposure are presented. Proteomics was found to be an effective and applicable tool to examine responses at the proteome level after mobile phone radiation exposure, although so far it has not been extensively applied in this research field. Proteomics techniques allow the large-scale screening of several hundreds, even thousands, of proteins simultaneously, and are thus more efficient than single endpoint techniques, especially if an appropriate experimental design is applied. However, proteomics requires rather large and comprehensive effects in total cell populations before they are observed, i.e., effects at the individual cell level cannot be detected with the proteomics techniques used.

In this study, several changes were observed in the proteome of the human endothelial cell line EA.hy926 after the exposure to 900 MHz GSM mobile phone radiation. In addition, the proteome of a variant of the same cell line, EA.hy926v1, was affected after the same exposure, but different proteins were altered compared to EA.hy926 cells. However, changes in the proteome of EA.hy926 cells were weaker after exposure to an 1800 MHz GSM signal than after the 900 MHz GSM exposure. The proteome of primary cells was not affected after 1800 MHz GSM exposure when examined using 2DE-DIGE technology. The earlier studies using EA.hy926 cells were partly limited due to certain technological aspects of 2DE (staining, data analysis), but the extensive replication as well the validation of some of the protein endpoints with other methods are strengths of this study. Due to the technology used here, the last study using primary cells and 2DE-DIGE technology is very significant in showing no immediate effects on the proteome of primary human endothelial cells after 1800 MHz GSM exposure.

The results presented in this thesis regarding the proteome-level effects after mobile phone radiation exposure are contradictory. The results for EA.hy926 cells suggest that minor effects occur, whereas no effects were observed using the more advanced 2DE-DIGE technology and primary cells. The responses with EA.hy926 cells varied according to the cell type and exposure conditions, and the consistent responses at the cellular level could not therefore be identified. Further research is recommended to understand the variation in responses and whether consistent cellular-level responses exist.

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APPENDIX 1

Table 3. Studies on heat shock proteins after mobile phone radiation exposure.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vivo*				
Fritze et al. 1997	Rats (Wistar), brains	890–915 MHz GSM, 900 MHz CW 0.3 / 0.17 W/kg (GSM), 1.5 / 0.84 W/kg (GSM), 7.5 / 4.2 W/kg (CW) 4 hrs sampling immediately and 24 hrs after exposure	*Hsp70*, gene expression (mRNA *in situ* hybridization), protein expression (immunohistochemistry)	Increase in *Hsp70* mRNA after 7.5 W/kg CW exposure, transient in 24 hrs No effects on protein expression
De Pomerai et al. 2000	C. *Elegans* (PC72, PC161)	750 MHz CW 0.001 W/kg 18 hrs sampling after exposure	*Hsp16*, reporter gene activity (β-galactosidase activity, GFP fluorescence)	Activation of *Hsp16* gene Publication retracted later
Weisbrot et al. 2003	*Drosophila melanogaster* (Oregon R)	900/1900 MHz GSM commercial mobile phone ~ 1.4 W/kg (not measured) 1 hrs/day, 10 days sampling after exposure	HSP70, protein expression (Western blotting)	Increase in HSP70 protein expression level Inadequate dosimetry
Lee JS. et al. 2005	Mice (*Hsp70.1*-deficient), major tissues	849 MHz, 1763 MHz CDMA 0.4 W/kg (whole body) 2x45min/day, 5 days/week, 10 weeks sampling 4, 8, and 10 weeks	HSP25, HSP70, HSP90, protein expression (Western blotting)	No effects
Dawe et al. 2006	C. *Elegans* (PC72, PC161)	1000 MHz CW 4–40 mW/kg 2.5, 20 hrs sampling at 2- or 4-hr intervals during exposure and after exposure	*Hsp16*, reporter gene activity (MUG assay, GFP fluorescence)	No non-thermal effects

Table 3. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vivo*				
Sanchez et al. 2008	Rats (10-week-old hairless female), skin	900 MHz, 1800 MHz GSM 2.5, 5 W/kg 2 hrs (5 W/kg) sampling immediately afterwards 2 hrs/day, 5 days/week, 12 weeks (2.5, 5 W/kg) sampling 72 hrs after the last exposure	HSP25, HSP70, HSC70, protein expression (immunohistochemistry)	No effects (Effects on positive control)
Dawe et al. 2008	C. *Elegans* (PC72)	1800 MHz GSM, CW 1.8 W/kg 2.5 hrs sampling after exposure	*Hsp16*, reporter gene activity (MUG assay)	No effects Minor effect observed if background levels otherwise elevated
Finnie et al. 2009	Female pregnant mice (BALB/c), descendant brains	900 MHz PW (GSM type) 4 W/kg (whole body) 60 min/day, from day 1 to day 19 of gestation sampling immediately prior to parturition (day 19)	HSP25, HSP32, HSP70, protein expression (immunohistochemistry)	No effects on HSP expression in mice brains (No induction of HSP32 or HSP70 at all, the HSP25 expression similar in all mice brains)
Watilliaux et al. 2010	Developing rats (Wistar) (post-natal days 5, 15, or 35), brains	1800 MHz GSM 1.7–2.5 W/kg 2 hrs sampling 24 hrs after exposure	HSP60, HSC70, HSP70, HSP90, protein expression (Western blotting)	No effects (No effects either on several glial markers, e.g., GFAP)

Table 3. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Leszczynski et al. 2002	Human endothelial cell line EA.hy926	900 MHz GSM 2.4 W/kg 1 hr sampling 1, 2, 6, and 8 hrs after the start of exposure (HSP27) sampling 1, 2, and 5 hrs after the start of exposure (P-HSP27)	HSP27, protein expression (Western blotting), P-HSP27, protein phosphorylation (^{32}P-labeling)	Increase in HSP27 phosphorylation and expression, transient in a few hours after the end of the exposure
Capri et al. 2004	Human mononuclear cells PBMC	1800 MHz GSM intermittent (10 min on/ 20 min off) (Basic, DTX, Talk modulations) 2.0 W/kg (Basic, Talk), 1.4 W/kg (DTX) 44 hrs sampling after exposure	HSP70, protein expression (flow cytometry)	No effects
Czyz et al. 2004	Mouse embryonic stem (ES) cells: Pluripotent R1 ES cells, wild type (wt) D3, and *p53*-deficient ES cells	1710 MHz GSM (217 Hz, Talk modulation) 1.5, 2.0 W/kg 6, 48 hrs sampling immediately after 6-hr exposure sampling after 48-hr exposure during cultivation (0, 2, 5 days) and differentiation (2, 5, 7, 10, 15 days)	*Hsp70*, gene expression (RT-PCR)	Increase in *Hsp70* mRNA level throughout differentiation period in the p53-deficient ES cells after the GSM 217 Hz modulated signal, no effects on other cells or after the GSM Talk signal exposure
Caraglia et al. 2005	Human oropharyngeal epidermoid carcinoma cancer cell line KB	1950 MHz (modulation not specified) 3.6 W/kg 1, 2, 3 hrs sampling after exposure	HSP27, HSP70, HSP90, protein expression (Western blotting)	Increase in HSP27 expression (maximum 3.5-fold at 1 hr time point) Increase in HSP70 expression (maximum at 2 hrs time point) Decrease in HSP90 expression (5-fold at 3 hrs)

Table 3. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Lim et al. 2005	Human peripheral blood (monocytes, lymphocytes)	900 MHz GSM, CW 0.4, 2.0, 3.6 W/kg 20 min, 1, 4 hrs sampling after exposure	HSP27, HSP70, protein expression (flow cytometry)	No effects (Effects on positive control)
Lixia et al. 2006	Human lens epithelial cells hLEC	1800 MHz GSM 1, 2, 3 W/kg 2 hrs sampling after exposure	HSP70, gene expression (RT-PCR), protein expression (Western blotting)	Increase in HSP70 protein expression after 2 and 3 W/kg exposures, no effects on mRNA levels
Sanchez et al. 2006	Human normal primary epidermal keratinocytes NHEK, Human normal primary dermal fibroblasts NHDF, Human reconstructed epidermis	900 MHz GSM 2 W/kg 48 hrs sampling after exposure	HSP27, HSP70, HSC70, protein expression (immunohistochemistry)	Decrease in HSC70 expression in fibroblasts Increase in HSP70 expression in the reconstructed epidermis, if cultivated 3 or 5 weeks (Effects on positive control)
Vanderwaal et al. 2006	Human epithelial carcinoma cell line HeLa S3, Human endothelial cell line EA.hy926	847 MHz TDMA, 1900 MHz GSM 5 W/kg (TDMA), 3.7 W/kg (GSM) 1, 2, 24 hrs (TDMA), 1, 2, 5 hrs (GSM) sampling after exposure	HSP27, protein expression and phosphorylation (Western blotting)	No effects (Effects on positive control)
Lee JS. et al. 2006	Human T-lymphocyte Jurkat cells, Rat primary astrocytes	1763 MHz CDMA 2, 20 W/kg 30 min, 1 hr sampling 6, 12, 24 hrs after exposure	HSP27, HSP70, HSP90, protein expression (Western blotting)	No effects (Effects on positive control)

Table 3. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Simko et al. 2006	Human monocyte cell line Mono Mac 6	1800 MHz CW, GSM (217 Hz, non-DTX modulation) 2 W/kg 1 hr sampling 2 hrs after exposure	HSP70, protein expression (Western blotting, flow cytometry)	No effects (Effects on positive control)
Lantow et al. 2006a	Human umbilical cord blood-derived primary monocytes	1800 MHz GSM (DTX modulation) 2 W/kg 1 hr sampling 0, 1, 2 hrs after exposure	HSP70, protein expression (flow cytometry)	No effects (Effects on positive control)
Lantow et al. 2006b	Human monocyte cell line Mono Mac 6, Human myelogenous leukemia cell line K562	1800 MHz GSM (DTX modulation) 2.0 W/kg 1 hr sampling 0, 1, 2 hrs after exposure	HSP70, protein expression (flow cytometry)	No effects (Effects on positive control)
Chauhan et al. 2006a	Human lymphoblastoma cells TK6	1900 MHz PW intermittent (5 min on, 10 min off) 1, 10 W/kg 6 hrs sampling immediately and 18 hrs post-exposure	*HSP27, HSP70B*, gene expression (RT-PCR)	No effects (Effects on positive control)
Chauhan et al. 2006b	Human promyelocytic leukemia cell line HL-60, Human monocyte cell line Mono Mac 6	1900 MHz PW intermittent (5 min on, 10 min off) 1, 10 W/kg 6 hrs sampling immediately and 18 hrs post-exposure	*HSP27, HSP70B*, gene expression (RT-PCR)	No effects (Effects on positive control)

Table 3. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Chauhan et al. 2007b	Human glioblastoma cell line U87MG	1900 MHz PW 0.1, 1, 10 W/kg 24 hrs sampling after exposure	*HSP27, HSP40, HSP70B, HSP71, HSP90AA1, HSP105,* gene expression (RT-PCR)	No effects (Effects on positive control)
Sanchez et al. 2007	Human normal primary epidermal keratinocytes NHEK, Human primary dermal fibroblasts NHDF	1800 MHz GSM (217 Hz modulation) 2 W/kg 48 hrs sampling after exposure	HSP27, HSC70, HSP70, protein expression (immunohistochemistry)	No effects (Effects on positive control)
Hirose et al. 2007	Human glioblastoma cell line A172, Human fibroblast cell line IMR-90	2142.5 MHz WCDMA, CW 80, 250, 800 mW/kg (A172, WCDMA) 80 mW/kg (A172, CW) 2, 24, 48 hrs (A172) 80, 800 mW/kg (IMR-90, WCDMA) 80 mW/kg (IMR-90, CW) 2, 28 hrs (IMR-90) sampling after exposure	HSP27, P-HSP27, protein expression and phosphorylation (bead-based multiplex assay) HSP27, HSP70, protein expression and cellular localization (immunocytochemistry) *HSP27, HSP40, HSP70, HSP105/110,* gene expression DNA chip analysis	No effects on HSP27 (Effects on positive control) No effects on the cellular location of HSP27, HSP70 No effects on gene expression (Effects on positive control)
Huang et al. 2008b	Mouse auditory hair cells HEI-OC1	1763 MHz CDMA 20 W/kg 6, 12, 24 hrs sampling after exposure	HSP27, HSP70, HSP90, protein expression (Western blotting)	No effects

Table 3. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Valbonesi et al. 2008	Human trophoblast cell line HTR-8/SVneo	1800 MHz GSM (217 Hz modulation) 2 W/kg 1 hr sampling 0, 1, 3 hrs after exposure	HSC70, HSP70, protein expression (Western blotting) *HSC70, HSP70A, HSP70B, HSP70C*, gene expression (RT-PCR)	No effects (Effects on positive control)
Franzellitti et al. 2008	Human trophoblast cell line HTR-8/SVneo	1800 MHz CW, GSM (217 Hz, Talk modulation) 2 W/kg 4, 16, 24 hrs sampling after exposure	HSP70, HSC70, protein expression (Western blotting) *HSC70, HSP70A, HSP70B, HSP70C*, gene expression (RT-PCR)	Altered *HSP70C* mRNA transcript levels after certain exposure types (up after 24 hrs exposure to the GSM 217 Hz signal, down after 4 and 16 hours exposure to the GSM Talk signal) No effects on protein expression or gene expression of other HSP70 family members (Effects on positive control)
Yu et al. 2008	Human lens epithelial cells hLEC	1800 MHz RF-EMF 1, 2, 3, 4 W/kg 2 hrs sampling after exposure	HSP27, HSP70, HSP90, protein expression (Western blotting)	Increase in HSP27 and HSP70 expression in all conditions, no effects on HSP90 Exposure assessment reported insufficiently

Table 4. Studies on proto-oncogenes after mobile phone radiation exposure.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vivo*				
Fritze et al. 1997	Rats (Wistar), brains	890–915 MHz GSM, 900 MHz CW 0.3 / 0.17 W/kg (GSM), 1.5 / 0.84 W/kg (GSM), 7.5 / 4.2 W/kg (CW) 4 hrs sampling immediately and 24 hrs after exposure	*C-Fos, C-Jun*, gene expression (mRNA *in situ* hydridization) C-FOS, FOS B, C-JUN, JUN B, JUN D, protein expression (immunohistochemistry)	No effects No effects either on the proliferation or on the expression of specific astroglial and microglial marker proteins (Effects due to immobilization)
Finnie et al. 2005	Mice (C57BL/6NTac), brains	900 MHz PW (GSM type) 4 W/kg (whole body) 1 hr sampling after exposure	C-FOS, protein expression (immunohistochemistry)	No effects after exposure (Effects due to immobilization)
Finnie et al. 2006b	Female pregnant mice (BALB/c), descendant brains	900 MHz PW (GSM type) 4 W/kg (whole body) 60 min/day, from day 1 to day 19 of gestation sampling immediately prior to parturition (day 19)	C-FOS, protein expression (immunohistochemistry)	No effects
Lopez-Martin et al. 2006	Rats (male Sprague–Dawley), picrotoxin treatment, brains	900 MHz GSM untreated: 0.42 W/kg (brain), 0.24 W/kg (mean) treated: 0.27 W/kg (brain), 0.15 W/kg (mean) 2 hrs sampling 1 hr after exposure	C-FOS, protein expression (immunohistochemistry)	Increase in C-FOS expression in the presence of picrotoxin, no effects without pre-treatment

Table 4. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vivo*				
Finnie et al. 2007	Mice (C57BL/6NTac), brains	900 MHz PW (GSM type) 4 W/kg (whole body) 60 min/day, 5 days/week, 104 weeks sampling after exposure	C-FOS, protein expression (immunohistochemistry)	No effects
Yilmaz et al. 2008	Rats (Sprague–Dawley), brains and testes	900 MHz GSM, commercial mobile phone 0.52 W/kg (whole body) 20 min/day, 1 month sampling after exposure	BCL-2, protein expression (immunohistochemistry)	No effects on rat brain or testes
Lopez-Martin et al. 2009	Rats (male Sprague–Dawley), picrotoxin treatment, brains	900 MHz GSM untreated: 0.05 W/kg (brain), 0.05 W/kg (mean) treated: 0.03 W/kg (brain), 0.03 W/kg (mean) 2 hrs sampling 1 hr after exposure	C-FOS, protein expression (immunohistochemistry)	Increase in C-FOS expression in the presence of picrotoxin
Studies *in vitro*				
Ivaschuk et al. 1997	Rat pheochromocytoma cells PC12	836.55 MHz TDMA average densities of 0.09, 0.9, and 9 mW/cm^2, for 0.9 mW/ cm^2 SAR 2.6 mW/kg 20, 40, 60 min intermittent (20 min on/ 20 min off) total incubation times 20, 60, 100 min sampling after exposure	*C-Fos, C-Jun*, gene expression (Northern blotting)	Decrease in *C-Jun* transcript levels after 20-min exposure to 9 mW/cm^2, no other effects

Table 4. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Goswami et al. 1999	Mouse embryo fibroblasts C3H 10T 1/2 (exponential growth phase and serum-deprived cells)	835.62 MHz CW, 847.74 MHz CDMA 0.6 W/kg 4 days continuous exposure sampling 24 hrs, 4 days	*Jun, Fos, Myc*, gene expression (RT-PCR)	Increase in *Fos* mRNA levels in exponential growth phase cells in transit to the plateau phase and in plateau-phase cells, effects larger in the CW-exposed samples than in the CDMA-exposed samples
Czyz et al. 2004	Mouse embryonic stem (ES) cells: Pluripotent R1 ES cells, wild type (wt) D3, and *p53*-deficient ES cells	1710 MHz GSM (217 Hz, Talk modulation) 1.5, 2.0 W/kg 6, 48 hrs sampling immediately after 6-hr exposure sampling after 48-hr exposure during cultivation (0, 2, 5 days) and differentiation (2, 5, 7, 10, 15 days)	*Bcl-2, C-Jun, C-Myc*, gene expression (RT-PCR)	Transient up-regulation of *C-Jun* and *C-Myc* mRNA levels in early stages (day 2, 5, and 5+2 of EB differentiation) with the GSM 217 Hz signal in the *p53*-deficient cells, no effects on other cells or after the GSM Talk signal exposure
Whitehead et al. 2005	Mouse embryo fibroblasts C3H 10T 1/2 (exponential growth phase, transition to plateau phase)	847.74 MHz CDMA, 835.62 MHz FDMA, 836.55 MHz TDMA 5.2, 10 W /kg 4 days continuous exposure sampling 24 hrs, 4 days	*C-Fos*, gene expression (RT-PCR)	No effects (Effects on positive control)
Merola et al. 2006	Human neuroblastoma cell line LAN-5	900 MHz GSM 1.0 W/kg 48 and 72 hrs sampling after exposure	B-MYB, N-MYC, protein expression (Western blotting)	No effects

Table 4. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Chauhan et al. 2006a	Human lymphoblastoma cells TK6	1900 MHz PW intermittent (5 min on, 10 min off) 1, 10 W/kg 6 hrs sampling immediately and 18 hrs post-exposure	*JUN, FOS, MYC*, gene expression (RT-PCR)	No effects (Effects on positive control)
Chauhan et al. 2006b	Human promyelocytic leukemia cell line HL-60, Human monocyte cell line Mono Mac 6	1900 MHz PW intermittent (5 min on, 10 min off) 1, 10 W/kg 6 hrs sampling immediately and 18 hrs post-exposure	*C-MYC, C-FOS, C-JUN*, gene expression (RT-PCR)	No effects (Effects on positive control)
Buttiglione et al. 2007	Human neuroblastoma cells SH-SY5Y	900 MHz GSM 1 W/kg 5, 15, 30 min, or 6, 24 hrs sampling after exposure	*BCL-2*, survivin, *BAK, BAX,* gene expression (RT-PCR)	Decrease in *BCL-2* and survivin after 24 hrs exposure (*BCL-2* also 6 hrs after), in parallel with an impaired cell cycle progression No effects on *BAK, BAX*
Del Vecchio et al. 2009	Rat primary cortical neurons	900 MHz GSM 1 W/kg 5 days continuous exposure sampling 24 hrs	*C-Jun, C-Fos*, gene expression (RT-PCR)	No effects

Table 5. Studies on signal transduction pathway and certain structural proteins after mobile phone radiation exposure.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vivo*				
Weisbrot et al. 2003	*Drosophila melanogaster* (Oregon R)	900/1900 MHz GSM commercial mobile phone ~ 1.4 W/kg (not measured) 1 hrs/day for 10 days sampling after exposure	ELK-1, protein phosphorylation (Western blotting)	Increase in ELK-1 phosphorylation
Lee JS. et al. 2005	Mice (*Hsp70.1*-deficient), major tissues	849 MHz, 1763 MHz CDMA 0.4 W/kg (whole body) 2x45min /day, 5 days/week, 10 weeks sampling 4, 8, and 10 weeks	ERK1/2, P-ERK, JNK1/2, P-JNK, p38MAPK, P-p38MAPK, protein expression and phosphorylation (Western blotting)	No effects on the expression or phosphorylation of ERK, JNK, p38MAPK
Dasdag et al. 2009	Rats (male Wistar albino), brains	900 MHz GSM 0.17–0.58 W/kg 2 hours/day, 7 days/week, 10 months sampling after exposure	p53, protein expression (immunohistochemistry, semi-quantitative scoring system)	No effects on p53 (Decrease in apoptosis)
Yan et al. 2009	Rats (Sprague-Dawley), brains	800 MHz AMPS, 1900 MHz Personal communication services mode (PCS), commercial mobile phone 1.8 W/kg AMPS, 1.18 W/kg PCS 6 hours/day, 7 days/week, 18 weeks sampling after exposure	Calcium ATP-ase, Endothelin, Neural Cell Adhesion Molecule, Neural Growth Factor, gene expression (RT-PCR)	Mild increase in the mRNA levels of all examined genes (Some insufficiency in the exposure assessment)
Ammari et al. 2010	Rats (Sprague-Dawley), brains	900 MHz GSM 1.5 W/kg (brain average), 45 min/day 6 W/kg (brain average), 15 min/day 5 days/week, 8 weeks sampling 3, 10 days after exposure	GFAP, protein expression (immunohistochemistry)	Increase in GFAP expression

Table 5. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vivo*				
Watilliaux et al. 2010	Developing rats (Wistar) (post-natal days 5, 15, or 35), brains	1800 MHz GSM 1.7 – 2.5 W/kg 2 hrs sampling 24 hrs after exposure	GFAP, GLAST, GLT1, serine racemase, protein expression (Western blotting)	No effects
Studies *in vitro*				
Leszczynski et al. 2002	Human endothelial cell line EA.hy926	900 MHz GSM 2.4 W/kg 1 hr sampling 1, 2, 6, and 8 hrs after the start of exposure	p38MAPK, protein expression (Western blotting)	Increase in p38MAPK expression, transient in a few hours after the end of the exposure
Czyz et al. 2004	Mouse embryonic stem (ES) cells: Pluripotent R1 ES cells, wild type (wt) D3, and *p53*-deficient ES cells	1710 MHz GSM (217 Hz, Talk modulation) 1.5, 2.0 W/kg 6, 48 hrs sampling immediately after 6-hr exposure sampling after 48-hr exposure during cultivation (0, 2, 5 days) and differentiation (2, 5, 7, 10, 15 days)	*p21, Erg-1*, gene expression (RT-PCR)	Transient up-regulation of the *p21* mRNA level in the early stages (day 2, 5, and 5+2 of EB differentiation) with the GSM 217 Hz signal in the *p53*-deficient cells, no effects on other cells or after GSM Talk signal exposure
Caraglia et al. 2005	Human oropharyngeal epidermoid carcinoma cancer cell line KB	1950 MHz (modulation not specified) 3.6 W/kg 1, 2, 3 hrs sampling after exposure	RAS, RAS activity, RAF1, ERK1/2, AKT, PGSK3, PI3K, p38K, P-p38K, JNK1, P-JNK1, protein expression and activity (Western blotting, gel kinase assay)	Decrease in the expression and the activity of RAS Decrease in RAF1 expression Decrease in ERK1/2 activity (no changes in expression) Decrease in AKT expression (no changes in activity) Increase in PI3K expression Decrease in p38 activity (no changes in expression) Increase in JNK1 activity (no changes in expression)

Table 5. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Nikolova et al. 2005	Mouse embryonic stem (ES) cells	1710 MHz GSM (217 Hz modulation) intermittent (5 min on/30 min off) 1.5 W/kg 6, 48 hrs sampling 7, 11, 17, and 23 days after plating	*Bcl-2, Bax, Gadd45, p53, Nurr1, TH,* Nestin, gene expression (RT-PCR)	Increase in *Bax* mRNA levels at day 17, increase in *Gadd45* mRNA levels at day 23, decrease in *Nurr1* mRNA levels at day 7, no changes in cell physiology
Lee JS. et al. 2006	Human T-lymphocyte Jurkat cells, Rat primary astrocytes	1763 MHz CDMA 2, 20 W/kg 30 min, 1 hr sampling 6, 12, 24 hrs after exposure	ERK, P-ERK, JNK, P-JNK, p38, P-p38, protein expression and phosphorylation (Western blotting)	No effects (Effects on positive control)
Hirose et al. 2006	Human glioblastoma cell line A172, Human fibroblast cell line IMR-90	2142.5 MHz WCDMA, CW 80, 250, 800 mW/kg (A172, WCDMA) 80 mW/kg (A172, CW) 24, 48 hrs (A172) 80 mW/kg (IMR-90, WCDMA, CW) 28 hrs (IMR-90) sampling after exposure	p53, protein expression and phosphorylation (bead-based multiplex assay) *TP53, TP53BP2, APAF1, CASP9,* gene expression (RT-PCR)	No effects (Effects on positive control)
Friedman et al. 2007	Human epithelial carcinoma cell line HeLa, Fischer rat fibroblast 3T3-like cell line Rat1	800, 875, 950 MHz RF-EMF 0.07 mW/cm^2 / 5, 10, 20, 30 min (ERK) 0.1, 0.2, 0.31 mW/cm^2 / 10 min (P-ERK) 0.23 mW/cm^2 / 5, 10, 20, 30 min (JNK, p38) 0.005, 0.03, 011 mW/cm^2 / 5, 10, 20, 30 min (ERK) 0.17 mW/cm^2 / 2, 12 min + incubation 5, 10 min (ERK) sampling after exposure unless otherwise stated	ERK, P-ERK, JNK, P-JNK, p38, P-p38, protein expression and phosphorylation (Western blotting)	Activation of the ERK1/2 pathway (rapid response in various exposure conditions) No effects on JNK, p38 No SAR estimates

Table 5. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Buttiglione et al. 2007	Human neuroblastoma cells SH-SY5Y	900 MHz GSM 1 W/kg 5, 15, 30 min, or 6, 24 hrs sampling after exposure	*ERG-1, p53,* gene expression (RT-PCR) P-ERK1/2, P-JNK, P-ELK-1, protein phosphorylation (Western blotting)	Increase in *ERG-1* mRNA levels after 5 min exposure, transient in 6 hrs No effects on p53 Phosphorylation of ERK1/2, JNK, ELK-1, transient in 6 hrs
Huang et al. 2008b	Mouse auditory hair cells HEI-OC1	1763 MHz CDMA 20 W/kg 15, 30, 60, 120 min sampling after exposure	ERK, P-ERK, JNK, P-JNK, p38, P-p38, protein expression and phosphorylation (Western blotting)	No effects (Effects on positive control)
Yu et al. 2008	Human lens epithelial cells hLEC	1800 MHz RF-EMF 1, 2, 3, 4 W/kg 5, 15, 30, 60, 120 min sampling after exposure	ERK1, P-ERK1, ERK2, P-ERK2, JNK1, P-JNK1, JNK2, P-JNK2, p38, P-p38, protein expression and phosphorylation (Western blotting)	ERK1/2 activated after 5 min exposure, peaked at 30 min, lasted 2 hrs JNK1/2 phosphorylated after 2 hrs exposure (highest after 2 W/kg exposure) No effects on p38 Exposure assessment not reported
Cervelatti et al. 2009	Human trophoblast cell line HTR-8/SVneo	1817 MHz GSM (217 Hz modulation) 2 W/kg 1 hr sampling after exposure	*Cx32, Cx37, Cx40, Cx43, Cx45,* gene expression (RT-PCR) Cx40, Cx43, protein expression and localization (Western blotting, immunocytochemistry)	Increase in *Cx40* and the *Cx43* mRNA levels, no changes in the protein expression level or the protein delocalization Decrease in intercellular gap junction-like structures

Table 5. Continued.

Reference	Study material	Frequency, modulation SAR (local/whole body, if applicable) Exposure time Sampling time	Endpoints	Results (& comments)
Studies *in vitro*				
Del Vecchio et al. 2009	Rat primary cortical neurons	900 MHz GSM 1 W/kg 5 days continuous exposure sampling 1, 3, 5 days	Beta-thymosin, gene expression (RT-PCR)	Increase in beta-thymosin mRNA levels at 4th cellular division, a corresponding decrease in the number of neurites
Hirose et al. 2010	Rat primary microglial cells	1950 MHz WCDMA 0.2, 0.8, 2.0 W/kg 2 hrs sampling 24, 72 hrs after the exposure	TNF-α, IL-1β, IL-6, protein expression (antibody bead kit)	No effects
Lee KY et al. 2011	Human breast cancer MCF7 cells	837 MHz CDMA, 1950 MHz WCDMA (single exposure or combination of signals) 4 W/kg (single), 2*2 W/kg (combination) 1 hr sampling 0, 2, 10, 24, 48 hrs after exposure	p53, p21, cyclin A, cyclin B1, cyclin D1, CDK2, CDK4, protein expression (Western blotting)	No effects (Effects on positive control)

Table 6. Transcriptomics studies after mobile phone radiation exposure.

Reference	Study material	Frequency, modulation Time points (exposure/sampling)*	Experimental platform, # of replicates	Results (& comments)
Studies *in vivo*				
Belyaev et al. 2006	Rats (Fischer 344), cerebellum	915 GSM 0.4 W/kg whole body 2 hrs	Affymetrix U34 GeneChips containing 8800 rat genes n=3	• 12 genes affected, fold ratios 1.34–2.74, diverse functions • No further validation
Paparini et al. 2008	Mice (Balb/cJ), brains	1800 MHz GSM 1.1 W/kg whole body, 0.2 W/kg whole brain 1 hr	Affymetrix MOE 430A arrays containing over 22000 probes, n=3, pooled RNA	• More stringent data analysis showed no changes • Less stringent conditions showed 75 genes affected (1.5–2.8 up or 0.67–0.29 down), the expression of 30 potentially affected genes validated using RT-PCR, none of them showed alterations • The authors concluded that there is no consistent indication of a gene expression modulation
Studies *in vitro*				
Pacini et al. 2002	Normal human skin fibroblasts Detroit 550	902.4 MHz, GSM commercial mobile phone 0.6 W/kg 1 hr	Atlas Human Array Trial Kit, 82 genes, n=1	• 14 genes differentially expressed (mitogenic signal transduction genes, cell growth inhibitors, apoptosis) • No further validation, n=1, commercial mobile phone
Lee S. et al. 2005	Human promyelocytic leukemia cell line HL-60	2450 MHz PW 10 W/kg 2, 6 hrs	Serial Analysis of Gene Expression (SAGE), n=1	• 2 hrs: 221 genes affected • 6 hrs: 759 genes affected • No further validation, n=1

*Sampling immediately after exposure unless otherwise stated

Table 6. Continued.

Reference	Study material	Frequency, modulation Time points (exposure/sampling)*	Experimental platform, # of replicates	Results (& comments)
Studies *in vitro*				
Whitehead et al. 2006a, 2006b	Non-osteogenic mouse pluripotent cell line C3H 10T1/2	835.6 MHz FDMA 847.7 MHz CDMA 5 W/kg 24 hrs	Affymetrix U74Av2 GeneChips containing ca. 9200 unique genes, n=3	• The number of probes with an expression change > 1.3-fold was less than or equal to the expected number of false positives • Positive control • No further validation on the potential target genes as all were rejected based on false positive rate calculations
Qutob et al. 2006	Human glioblastoma-derived cell line U87MG	1900 MHz PW 0.1, 1.0, 10.0 W/kg 4 hrs	Agilent human 22K microarray slides (Human 1A), over 18000 human genes, n=5	• No effects after exposure • Positive control • 6 *HSP* genes confirmed with RT-PCR, no effects • In the data analysis a gene appearance in all the five replicates was required
Hirose et al. 2006	Human glioblastoma cells A172 Human fibroblasts IMR-90	2142.5 MHz WCDMA, CW A172: 80, 250, 800 mW/kg (WCDMA), 80 mW/kg (CW) A172: 24, 48 hrs IMR-90: 80 mW/kg (WCDMA&CW) IMR-90: 28 hrs	Affymetrix Human genome HG-U133 Plus2.0 microarrays, ca. 33000 human genes, n=2	• No consistent effects in two experiments • Positive control • The expression of four p53-related genes confirmed with RT-PCR

*Sampling immediately after exposure unless otherwise stated

Table 6. Continued.

Reference	Study material	Frequency, modulation Time points (exposure/sampling)*	Experimental platform, # of replicates	Results (& comments)
Studies *in vitro*				
Remondini et al. 2006	Human neuroblastoma cells NB69, Human endothelial cell line EA.hy926, Human quiescent T-lymphocytes, Human monocytes U937, Human microglial cells CHME5, Human hematopoietic leukemia cells HL-60	NB69: 1800 MHz GSM intermittent (5 min on/10 min off), 2 W/kg, 24 hrs EA.hy926: 900 MHz GSM, 2.4 W/kg, 1hr 1800 MHz GSM, 2 W/kg, 1 hr T-lymphocytes: 1800 MHz GSM intermittent (10 min on/20 min off), 1.4 W/kg, 44 hrs U937: 900 MHz GSM, 2 W/kg, 1 hr CHME5: 900 MHz GSM, 2 W/kg, 1 hr HL-60: 1800 MHz GSM intermittent (5 min on/5 min off), 1.0 W/kg, 24 hrs 1800 MHz GSM, 1.3 W/kg, 24 hrs	Human Unigene RZPD-2 cDNA array containing about 75000 cDNA clones, RNA pooled from several exposures, n=1 for hybridizations	• EA.hy926: effects after the 900 MHz exposure (32 clones), no effects after the 1800 MHz exposure • U937: effects (34 clones) • HL-60: effects after the intermittent exposure (12 clones), no effects after the continuous exposure although SAR higher • No effects on other cells • No consistent signature after exposure • No validation of results, n=1 for hybridizations
Zeng et al. 2006	Human breast cancer cell line MCF-7	1800 MHz GSM intermittent (5 min on/10 min off) 2, 3.5 W/kg 24 hrs	Affymetrix GeneChip Test3 containing 14500 genes, n=2	• Five responding genes (PPP1R12A, TBL1X , EFNB2, TOP1, MTDH) • RT-PCR did not confirm these, thus, the authors concluded no effects were actually observed • n=2

*Sampling immediately after exposure unless otherwise stated

Table 6. Continued.

Reference	Study material	Frequency, modulation Time points (exposure/sampling)*	Experimental platform, # of replicates	Results (& comments)
Studies *in vitro*				
Gurisik et al. 2006	Human neuroblastoma cell line SK-N-SH	900 MHz PW (GSM 217 Hz modulation) 0.2 W/kg 2 hrs (sampling 2 hrs post-exposure)	Affymetrix Human Focus Gene arrays including ca. 8400 genes, n=1	• Six genes slightly down-expressed (LIM, Nap1L1, CCPG1, ACADM, BMAL1, Rbbp4) • RT-PCR did not validate two of these genes (CCPG1 and BMAL1) and, thus, the authors concluded that no effects were observed • n=1
Hirose et al. 2007	Human glioblastoma cells A172, Human fibroblasts IMR-90	2142.5 MHz WCDMA, CW 80 and 800 mW/kg (WCDMA) 80 mW/kg (CW) A172: 2, 24, 48 hrs IMR-90: 2, 28 hrs	Affymetrix Human genome HG-U133 Plus2.0 microarrays, ca. 33000 human genes, n=2	• No effects • Positive control • n=2
Chauhan et al. 2007b	Human glioblastoma-cell line U87MG, Human monocyte cell line Mono Mac 6 (MM6)	1900 MHz PW intermittent (5 min on/10 min off) for MM6 0.1, 1.0, 10.0 W/kg (0.1 W/kg U87MG only) 6 hrs (MM6), 24 hrs (U87MG) sampling also 18 hrs post-exposure for MM6	Agilent human 22K microarray slides (Human 1Av2), over 18000 human genes, n=5	• No effects after exposure • Positive control • 6 *HSP* genes confirmed with RT-PCR for the U87MG cells, no effects • In the data analysis a gene appearance in all the five replicates was required
Zhao TY et al. 2007	Primary mouse neurons and astrocytes	1900 MHz GSM, commercial mobile phone, on and stand by -modes SAR unknown 2 hrs	GEArray Q series mouse apoptosis array, containing 96 genes, n=2	• Up-regulation of caspase-2, caspase-6 and *Asc* in both cell types, also *Bax* up-regulated in astrocytes • RT-PCR confirmed results • Exposure assessment inadequate, n=2

*Sampling immediately after exposure unless otherwise stated

Table 6. Continued.

Reference	Study material	Frequency, modulation Time points (exposure/sampling)*	Experimental platform, # of replicates	Results (& comments)
Studies *in vitro*				
Zhao R et al. 2007	Primary rat neurons	1800 MHz GSM intermittent (5 min on/ 10 min off) 2 W/kg 24 hrs	Affymetrix Rat Neurobiology U34 array, containing over 1200 transcripts n=1	• 34 affected genes (fold ratios 1.15–1.62) • Most of the changes further validated with RT-PCR • n=1 for array, n=3 RT-PCR
Huang et al. 2008a	Human T lymphoma cell line Jurkat T	1763 MHz CDMA 10 W/kg 24 hrs	Applied Biosystems 1700 full genome expression array, 30000 genes n=5	• 10 genes affected 1.3–1.8 –fold, among them two cytokine receptors • No further validation for the gene expression, the authors concluded no effects on global gene expression were observed
Huang et al. 2008b	Mouse auditory hair cells HEI-OC1	1763 MHz CDMA 20 W/kg 24 hrs sampling 5 hrs after the exposure	Applied Biosystems 1700 full genome expression array, 32000 genes n=3	• 29 genes affected (18 annotated genes and 11 ESTs) more than 1.5-fold • No consistent groups of functional categories • No further validation, the authors concluded that as the number of affected genes is low those genes might be actually false positives • No effects either on cell cycle, DNA damage, or stress response
Sekijima et al. 2010	Human glioblastoma cell line A172, Human neuroglioma H4, Human fibroblasts IMR-90	2142.5 MHz WCDMA and CW 80, 250, 800 mW/kg 96 hrs	Affymetrix Human Genome Array, ca. 16000 to 19000 genes, n=2	• Minor effect ($p<0.05$, max fold ratio 1.14) on A172 and H4 cells, no effect on IMR-90 cells • Positive control • No further validation • The authors concluded no effects were observed • No effects on either cell growth or viability

*Sampling immediately after exposure unless otherwise stated

I

LESZCZYNSKI D., NYLUND R., JOENVÄÄRÄ S., AND REIVINEN J.:
Applicability of discovery science approach to determine biological effects of mobile phone radiation
Proteomics 2004, 4: 426–431

Short Communication

Dariusz Leszczynski
Reetta Nylund
Sakari Joenväärä**
Jukka Reivinen***

Bio-NIR Research Group
at the Radiobiology Laboratory,
STUK – Radiation and
Nuclear Safety Authority,
Helsinki, Finland

Applicability of discovery science approach to determine biological effects of mobile phone radiation

We argue that the use of high-throughput screening techniques, although expensive and laborious, is justified and necessary in studies that examine biological effects of mobile phone radiation. The "case of hsp27 protein" presented here suggests that even proteins with only modestly altered (by exposure to mobile phone radiation) expression and activity might have an impact on cell physiology. However, this short communication does not attempt to present the full scientific evidence that is far too large to be presented in a single article and that is being prepared for publication in three separate research articles. Examples of the experimental evidence presented here were designed to show the flow of experimental process demonstrating that the use of high-throughput screening techniques might help in rapid identification of the responding proteins. This, in turn, can help in speeding up of the process of determining whether these changes might affect human health.*

Keywords: Gene expression / hsp 27 / Mobile phone radiation / Protein expression PRO 0646

Elucidation of the biological and health effects of mobile phone radiation (radio-frequency modulated electromagnetic fields; RF-EMF) has been done for decades but the reliable answers concerning potential health hazard are still missing [1]. Extensive epidemiological studies are commonly expected to provide the answer whether RF-EMF might be hazardous to people. However, finding and scientific validation of any potential health hazard, whether it would be cancer or non-cancer effect, might be not possible using epidemiological approach alone. This is because the "low sensitivity" of epidemiological methodology might be insufficient to reliably detect health impact of the weak biological effects caused by RF-EMF [1]. Therefore, although epidemiological studies will be needed to ultimately validate the extent of any potential health hazard of RF-EMF to human population, they also need to be supplemented and supported by data from animal and *in vitro* studies.

It has been suggested that high-throughput screening techniques (HTSTs) of transcriptomics and proteomics could be used to rapidly identify a broad variety of potential molecular targets of RF-EMF and generate a variety of biological end-points for further analyses [2]. Combination of transcriptomics and proteomics in search for biological effects is called the "discovery science". This term has been coined-in by Aebersold *et al.* [3] to define the new approach that will help in revealing biological mechanisms, some of which might be unpredictable using the presently available knowledge. This approach seems to be particularly suited for elucidation RF-EMF health hazard issue because it might reveal effects that are not possible to predict, based on the present very limited knowledge about the biological effects of RF-EMF [1]. However, before committing large funds that are needed for HTST studies it is necessary to determine whether indeed this approach will be successful in unraveling physiologically significant biological events induced by RF-EMF. HTSTs are able to pick-up very small changes in protein or gene expression the changes of which might be of insufficient magnitude to alter cell physiology. Thus, although using HTSTs it might be possible to find biologi-

Correspondence: Professor Dariusz Leszczynski, PhD, STUK – Radiation and Nuclear Safety Authority, Laippatie 4, FIN-00880, Helsinki, Finland
E-mail: dariusz.leszczynski@stuk.fi
Fax: +358-9-75988464

Abbreviations: HTST, high-throughput screening technique; **RF-EMF**, radio-frequency-modulated electromagnetic fields

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** Presently employed as Scientist by the MediCel Ltd, Helsinki, Finland

*** Presently working as Scientific Adviser in the Research Council for Health of the Academy of Finland, Helsinki, Finland

cal effects induced by RF-EMF but these effects might be of limited or no significance at all for the cell physiology. Therefore, to determine the usefulness of HTST approach to the issue of bioeffects induced by RF-EMF, we have performed a 5-step feasibility study and have shown that HTSTs might indeed help to identify experimental targets for physiological studies of RF-EMF-induced biological responses (Fig. 1).

Firstly, in step 1, we have determined whether cells respond at all to RF-EMF and, if so, what is the extent of cell response [4]. This has been done by analyzing global changes in the pattern of protein phosphorylation because these changes occur rapidly in cells stimulated by either internal or external factors. Therefore, observation of any change in protein phosphorylation indicates that cells are recognizing a given factor and are responding to it. As an experimental model we have used cultures of human endothelial cell line EA.hy926 [5]. Cells were exposed for 1 h to 900 MHz GSM mobile phone simulating signal at an average specific absorption rate (SAR) of 2.4 W/kg [4] that is slightly above the European safety limit (SAR = 2.0 W/kg). Proteins extracted from cells were separated using two-dimensional electrophoresis and with PDQuest software (Bio-Rad, Hercules, CA, USA). Some 1266 different protein spots were identified (for details see [4]). Using ^{32}P-labelling it was possible to determine that among the 1266 proteins spots, in non-irradiated control exposed cells, ~110 phosphoproteins were detected (for details see [4]) whereas in exposed cells some 372 phosphoproteins were detected (for details see [4]). The observed broad change in the pattern of global protein phosphorylation has suggested that cells respond to RF-EMF and that possibly any of the hundreds of phosphoproteins that have altered their phosphorylation status could, at least potentially, affect cell physiology. By using Western blot or mass spectrometry to identify the phosphoproteins present in the 2-DE spots, it might be possible to find a variety of protein targets that could be used in examining effects of mobile phone radiation on cell physiology. With this approach, the selection of molecular targets for further studies would not be

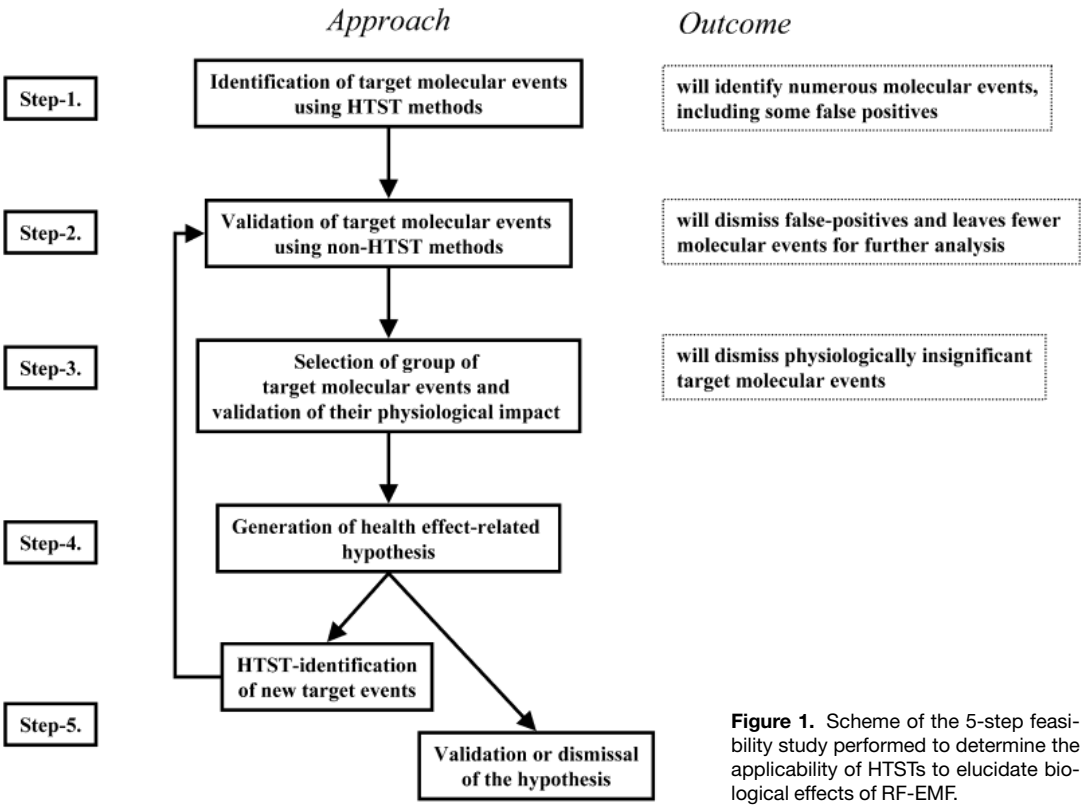


Figure 1. Scheme of the 5-step feasibility study performed to determine the applicability of HTSTs to elucidate biological effects of RF-EMF.

based only on deduction of potentially affected events but also on the knowledge of the identities of proteins that indeed respond to RF-EMF. Thus, in the continuation of step 1, using simple Western blot screening with antibodies directed against various stress response proteins, we have identified heat shock protein 27 (hsp27) as one of the phosphoproteins responding to RF-EMF. In the feasibility study presented here we have focused on hsp27 stress protein because it is known to be involved in regulation of endothelial cell permeability. Because some studies performed in the last 20 years have suggested that microwaves might affect the blood-brain barrier permeability [1], therefore, studying the hsp27-related cellular response was scientifically justified. Hsp27 is continuously expressed in endothelial cells [6].

The change in phosphorylation and expression status of hsp27 was confirmed in step 2 in order to assure the validity of this observation (for details see [4]) by examining: (i) Western blotting; (ii) immunoprecipitation of ^{32}P-labelled phosphorylated hsp27; (iii) immunoprecipitation of ^{32}P-labelled phosphorylated p38MAPK, an up-stream kinase indirectly involved in phosphorylation of hsp27; and (iv) inhibition of hsp27 phosphorylation by introduction to cell cultures of inhibitor of hsp27-up-stream kinase p38MAPK (SB203580) and determining hsp27 phosphorylation status by immunoprecipitation. Thus, in step 2 we have confirmed that hsp27 is the valid molecular target event of the RF-EMF and that it is scientifically justified to further examine whether these changes have any impact on cell physiology [4].

The phosphorylation and increase in expression of hsp27 [4] observed by us is a well-established mechanism of cell response to a broad variety of stress stimuli [7]. Therefore, the observed doubling of hsp27 expression and the 2- to 7-fold increase in the amount of phosphorylated hsp27 in cells (for details see [4]) have suggested that EA.hy926 cells have recognized RF-EMF as an external stress factor and that they have launched an hsp27-dependent counter response. Therefore, in step 3 we have examined whether these changes will have any effect on cell physiology. Phosphorylation of hsp27 has been shown to regulate polymerization of F-actin and stability of generated protein stress fibers [8]. Thus, we have examined the status of the stress fibers in exposed cells by staining F-actin with AlexaFluor-labeled phalloidin. As shown in Fig. 2A, RF-EMF exposure has caused an increase in cellular staining with phalloidin what indicates increase in stability of F-actin stress fibers. The stability of stress fibers, as determined by the pattern of staining with phalloidin-AlexaFluor, increased after 1 h irradiation and did not decline during the 1 h of post-irradiation incubation. Induction of the stability of stress fibers caused cells to shrink; visible cell shrinking was observed among the cells brightly stained with AlexaFluor-phalloidin (Fig. 2A; middle and right panels). The increase in the stability of stress fibers was prevented in the presence of p38MAPK inhibitor SB203580 (Fig. 2B). Also it was possible to observe that in cells expressing high levels of hsp27 (Fig. 2C), the cell edges were brightly stained with phalloidin-AlexaFluor, what indicates relocalization of F-actin stress fibers to cell ruffles whereas in cells expressing low levels of hsp27, network of stress fibers was seen throughout the whole cytoplasm but not in the ruffles. Such behavior of hsp27 and stress fibers in cells exposed to RF-EMF is in agreement with the general pattern of cellular response to stimuli that activate hsp27-dependent stress response [8].

The above results [4] have formed the basis and support for our working hypothesis that was formulated as step 4 (for details see [4]). Stabilization of stress fibers and cell shrinking caused by it, when occurring in endothelial cells lining brain's capillary blood vessels, might be of importance for the functioning of blood-brain barrier [8, 9]. Also, the activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming a complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9, and cytochrome *c*), or some of its components, and preventing proteolytic activation of the pro-caspase-9 into active form of caspase-9 [10, 11]. This, in turn, prevents activation of pro-caspase-3 which is activated by caspase-9. Thus, induction of the increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells. Therefore, based on the known cellular role of overexpressed/phosphorylated hsp27 we have proposed a hypothesis [4] that: the activation (phosphorylation) of hsp27 by mobile phone radiation might be the molecular mechanism (i) regulating increase in blood-brain barrier permeability, which would explain, observed in some animal experiments, the increase in blood-brain barrier permeability, and (ii) regulating apoptosis through interference with the cytochrome *c*/caspase-9/caspase-3 pathway (for details see [4]).

Further experiments using HTST (step 5 of the feasibility study) have revealed additional information pertinent to the cytoskeleton- and apoptosis-related properties of RF-EMF exposed endothelial cells. The suggested changes in the cytoskeletal proteins were detected using 2-DE separated proteins. Approximately 1300 protein spots were detected by 2-DE. Comparison of the control

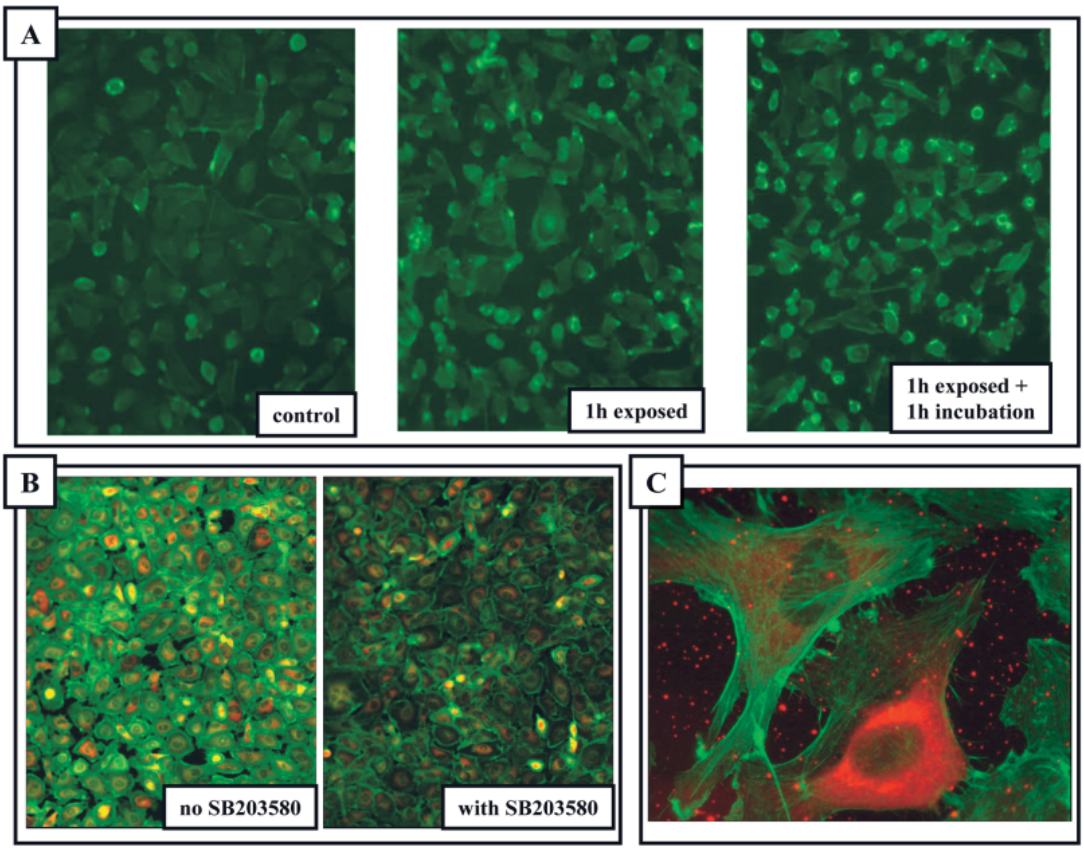


Figure 2. Cellular response to RF-EMF – validation of physiological event – step 3. Exposure of cells to RF-EMF has caused increase in cell staining with AlexaFluor-labeled phalloidin (A). This suggests the increase in the expression/stability of F-actin, a protein forming cellular stress fibers. Rounding up is visible among the cells expressing highest F-actin content (the brightest staining with AlexaFluor-phalloidin). This effect persisted during the 1 h post-exposure incubation of cells in control conditions. The presence of p38MAPK inhibitor, SB203580, in cell culture medium has prevented increase in AlexaFluor-phalloidin staining (B). The large magnification of cells shown in (C) demonstrates the difference in distribution of AlexaFluor-phalloidin stained stress fibers (green color) in cells with high (cell on the right) and low (cell on the left) content of hsp27 protein (indirect immunohistochemical staining; red fluorescence).

and exposed samples revealed some 49 protein spots which were statistically significant (Student's *t*-test, $p < 0.05$, $n = 10$) affected by the exposure (increased or declined expression). Among the statistically significantly altered proteins, cytoskeletal proteins-vimentins (Fig. 3) were identified using MALDI-TOF. The suggested RF-EMF interference with cell apoptosis was further examined using cDNA Expression Arrays (Clontech) and screening expression of 3600 different genes. Among the genes that were downregulated in cells exposed to RF-EMF, were numerous genes encoding proteins of the Fas/TNFα-apoptotic pathway (Fig. 4). This pathway was suggested as target for the RF-EMF-induced phosphorylated hsp27. Therefore, the concomitantly observed increase in hsp27 phosphorylation, that is an anti-apoptotic event, and downregulation of proteins of Fas/TNFα apoptotic pathway suggest that further studies aiming at elucidation of RF-EMF effect on cell apoptosis are justified.

In conclusion, we have demonstrated that by using HTST it is possible to identify RF-EMF-induced molecular events that might alter cell physiology. Even though the increase in expression/phosphorylation of the examined hsp27

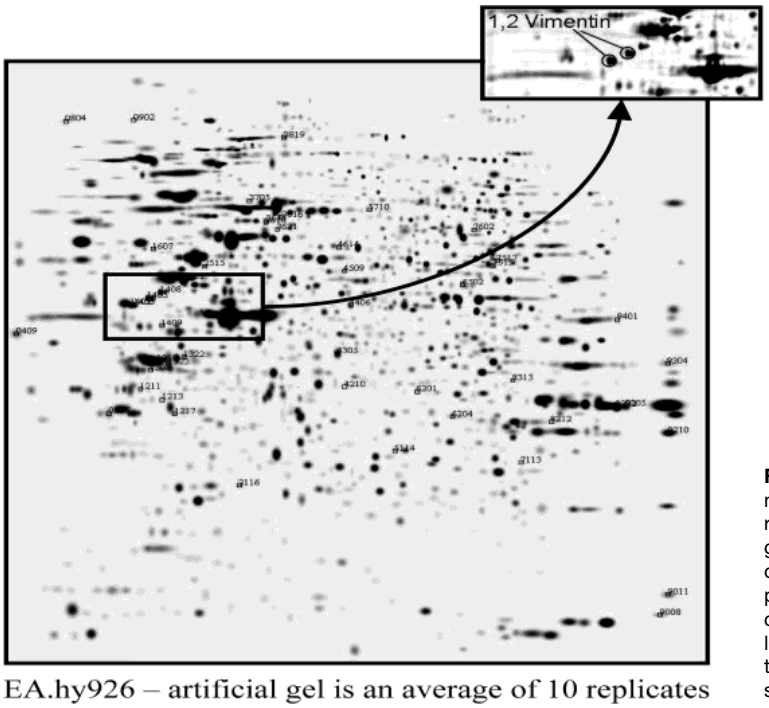
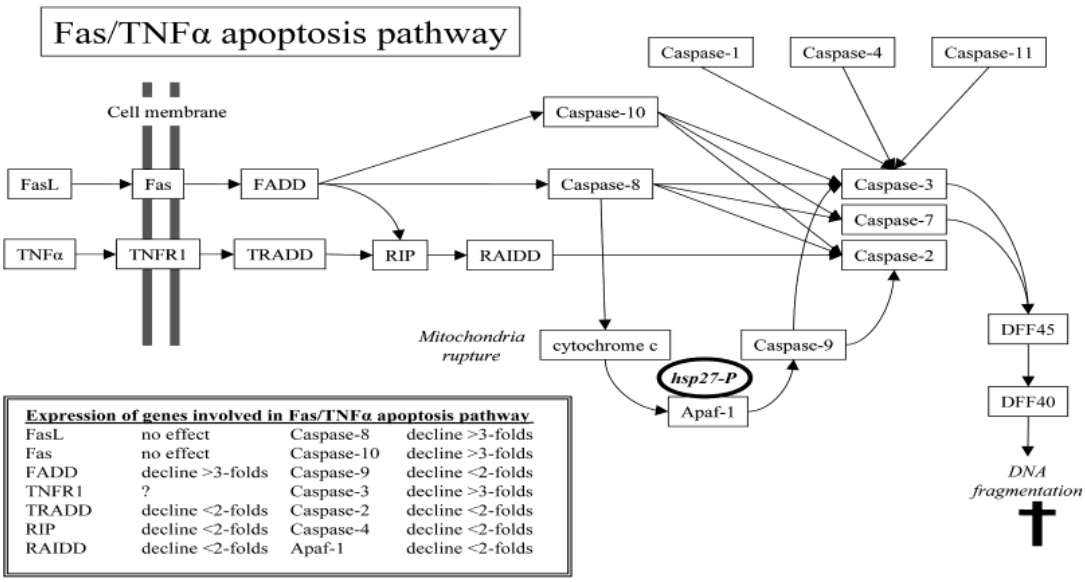


Figure 3. HTST identification of new target events – cytoskeleton-related – step 5. Using larger 2-DE gels (20 × 20 cm) and 10 replicates of each run we have identified ~ 49 proteins that have altered statistically significant their expression following RF-EMF exposure. Among the MS-identified spots were cytoskeletal proteins vimentins (inset).



Expression of genes involved in Fas/TNFα apoptosis pathway

FasL	no effect	Caspase-8	decline >3-folds
Fas	no effect	Caspase-10	decline >3-folds
FADD	decline >3-folds	Caspase-9	decline <2-folds
TNFR1	?	Caspase-3	decline >3-folds
TRADD	decline <2-folds	Caspase-2	decline <2-folds
RIP	decline <2-folds	Caspase-4	decline <2-folds
RAIDD	decline <2-folds	Apaf-1	decline <2-folds

Figure 4. HTST identification of new target events – apoptosis-related – step 5. Analysis of RF-EMF-induced expression changes, using cDNA Expression Array for 3600 tumor-related genes (three separate experiments), has revealed that the majority of genes that encode proteins forming the Fas/TNFα apoptotic pathway, that is regulated by phosphorylated hsp27, are downregulated (table insert).

protein was very modest (*ca.* 2–3-fold increase), it was possible to determine the impact of this event on cell physiology. Whether any impact on organs (*e.g.*, brain) or whole body will be exerted by this change remains to be determined by *in vivo* studies. Although the use of discovery science approach employing HTSTs will not provide direct evidence of health hazard or its absence, it will be essential in unraveling of possibly all biological effects exerted by RF-EMF exposure. Further elucidation of the physiological significance of these biological effects for the health and well-being, in short- and long-term exposure conditions, will allow the determination whether any health hazard might be associated with the use of mobile phones at the presently allowed radiation safety levels.

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II

NYLUND R. AND LESZCZYNSKI D.:
Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation
Proteomics 2004, 4: 1359–1365

Short Communication

Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation

Reetta Nylund and Dariusz Leszczynski

BioNIR Research Group, STUK – Radiation and Nuclear Safety Authority, Helsinki, Finland

The human endothelial cell line EA.hy926 was exposed to mobile phone radiation and the effect on protein expression was examined using two-dimensional electrophoresis (2-DE). Up to 38 various proteins have statistically significantly altered their expression levels following the irradiation. Four proteins were identified with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS). Two of the affected proteins were determined to be isoforms of cytoskeletal vimentin. This finding supports our earlier presented working hypothesis which indicated that the mobile phone radiation might affect the cytoskeleton and might have an effect on the physiological functions that are regulated by the cytoskeleton.

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Keywords: Endothelium / Human endothelial cell line / Microwaves / Mobile phone radiation

During the recent years the worldwide use of mobile phones has dramatically increased. At the same time there is an increasing concern of the potential health hazard of the low-energy microwave radiation emitted by mobile phones. The majority of published studies suggest that there are no biological effects induced by the mobile phone radiation because the amount of energy delivered to the biological system is too low to trigger any chemical reactions [1]. However, there are also investigations showing that this radiation might induce biological effects by affecting cellular stress response [2, 3]. Whether these stress response-related effects might/will have any impact on cell's or whole organism's physiology remains to be clarified by further studies. So far, end-points for the studies of biological effects of the mobile phone radiation have been selected based on the hypotheses deriving from the data available from the published literature. This approach requires lots of guess work and often the preselected end-points were proven to be irrelevant. We have suggested that the selection of the end-points could be done using high-throughput screening methods like those used in proteomics and transcriptomics [4]. Such an approach would allow prescreening of thousands of proteins and genes and then selection for further studies only of these proteins/genes that responded to the radiation, *e.g.*, by changing either their expression level or activity (phosphorylation status). In the present pilot study we have used 2-DE and MALDI-MS proteomics to find new protein targets of the mobile phone radiation using as a model the human endothelial cell line EA.hy926 [5].

Cells were grown in glass Petri dishes (diameter 5 cm, plating cell density 1.2×10^6/dish) overnight to semi-confluency before exposure to the mobile phone radiation (Fig. 1A). Cells were exposed for 1 h to 900 MHz GSM mobile phone radiation-like signal at an average specific absorption rate (SAR) of 2.4 W/kg (slightly above the European mobile phone radiation exposure limit of 2.0 W/kg) and at temperature stabilized at 37°C $\pm$ 0.3°C, as described previously [3]. Control cells were sham-exposed in the same exposure chamber but with switched-off mobile phone radiation. Immediately after the end of the exposure, cells were placed on ice, washed with ice-cold PBS, and lysed with a buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer pH 3–10 NL, 1% dithiohreitol (DTT), 1 mM sodium orthovanadate, and 1 mM PMSF. The protein concentration in lysates was measured using the Bradford method; 175 μg of total protein was used for 2-DE. IEF was performed using an IPGphor apparatus and nonlinear pH 3–10, 18 cm long IEF strips (Amersham Biosciences, Uppsala, Sweden). The samples were loaded using in-

Correspondence: Dr. Dariusz Leszczynski, STUK – Radiation and Nuclear Safety Authority, Laippatie 4 14, FIN-00880 Helsinki, Finland
E-mail: dariusz.leszczynski@stuk.fi
Fax: +358-9-75988464

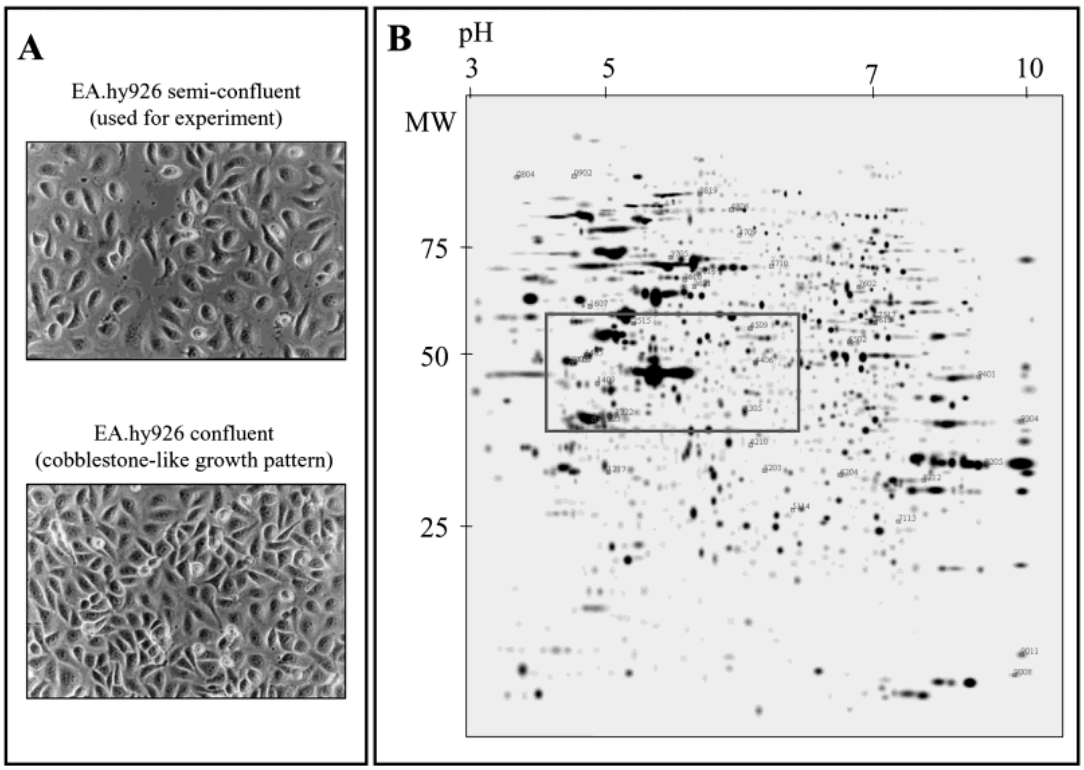

Figure 1. (A) Growth pattern of the semiconfluent monolayer of EA.hy926 cells on the day of the experiment (upper panel). Cell cultures allowed to grow for additional 24 h have reached confluence and formed characteristics for endothelial cells "cobblestone" growth pattern (lower panel). (B) PDQuest-generated 2-DE artificial gel of proteins extracted from the human endothelial cell line EA.hy926. First dimension, IEF pH gradient 3–10 NL; second dimension, 8% SDS-PAGE. Statistically significantly (*t*-test $p < 0.05$) differing spots are numbered using PDQuest SSP numbers. In EA.hy926 cell line were 38 statistically significantly differing spots (gray numbered) detected. Four spots (within the gray rectangle): vimentin (#1402 and #1405), isocitrate dehydrogenase 3 (NAD+) α (#4305), and heterogeneous nuclear ribonucleoprotein H1 (#4406), were identified using MALDI-MS.

gel rehydration in a buffer containing 9 M urea, 2% CHAPS, 0.2% DTT, 0.5% IPG buffer pH 3–10 NL for 12 h. IEF was run at 20°C using step-and-hold and gradient methods as follows: 30 V − 2 h, 100 V − 0.5 h, 300 V − 0.5 h, 600 V − 0.5 h, 1500 V − 0.5 h, 8000 V gradient 4 h, 8000 V − until 65 000 Vh were achieved. For SDS-PAGE, the IEF strips were equilibrated for 15 min in 6 M urea, 30% glycerol, 50 mM Tris-HCl pH 8.8, 2% SDS, and 10 mg/mL DTT for 15 min, and then for another 15 min in the same buffer with 25 mg/mL iodoacetamide replacing DTT. SDS-PAGE was run in 8% gel using Protean IIxi Multicell apparatus (Bio-Rad, Hercules, CA, USA) and a constant current of 40 mA/gel at 10°C. After electrophoresis, gels were overnight fixed with a mixture of 30% ethanol and 0.5% acetic acid, then washed with 20% ethanol in doubly distilled H_2O (ddH_2O), sensitized with sodium thiosulfate (0.2 g/L), incubated in silver nitrate solution (2 g/L) and developed in a solution of potassium anhydride (30 g/L), 37% formaldehyde (0.7 mL/L), and sodium thiosulfate (0.01 g/L). The development was stopped with Tris (50 g/L), and acetic acid (0.5%) solution. Silver-stained gels were stored in ddH_2O at 4°C. The gels were scanned using a GS-710 densitometer (Bio-Rad) and analyzed using PDQuest 6.2 software (Bio-Rad). 2-DE artificial gels were generated from ten independent protein samples from ten independent replicates of controls and irradiated cell cultures (Fig. 1B). The protein expression pattern in ten replicate control samples was then com-

pared with the protein pattern in ten replicate irradiated samples. The normalized spot volumes of the proteins from control and exposed sample gels were statistically analyzed using the Student's *t*-test at a confidence level of 95%.

As in previous studies [3, 4] we have used the human endothelial cell line EA.hy926. On the day of experiment cells were forming a subconfluent monolayer (Fig. 1A, upper panel). Extending the cell culture period by 24 h led to formation of a confluent monolayer with the characteristics for endothelial cells "cobblestone" growth pattern (Fig. 1A, lower panel). The comparison of the 2-DE-separated proteins from the exposed and sham (control) cells shows that 38 protein spots change their expression level in response to mobile phone radiation exposure (statistically significant change; *t*-test $p < 0.05$) (Fig. 1B). The identity of all of the protein spots that have responded to the mobile phone radiation is currently being determined by MALDI-MS and will be reported in due time.

Four of the protein spots, whose expressions were statistically significantly altered by irradiation, were selected for the present study and identified using MALDI-MS (Fig. 1B, selected area, and Fig. 2A). In order to increase the probability that only a single protein is present in the single 2-DE spot, the selected spots had to fulfil the following requirements: (i) spots had to be well separated from other spots in both 2-DE dimensions, and (ii) spots had to be sufficiently large and contain a sufficient amount of protein for analysis. The MALDI-MS analysis service was purchased from the Protein Chemistry Laboratory of the

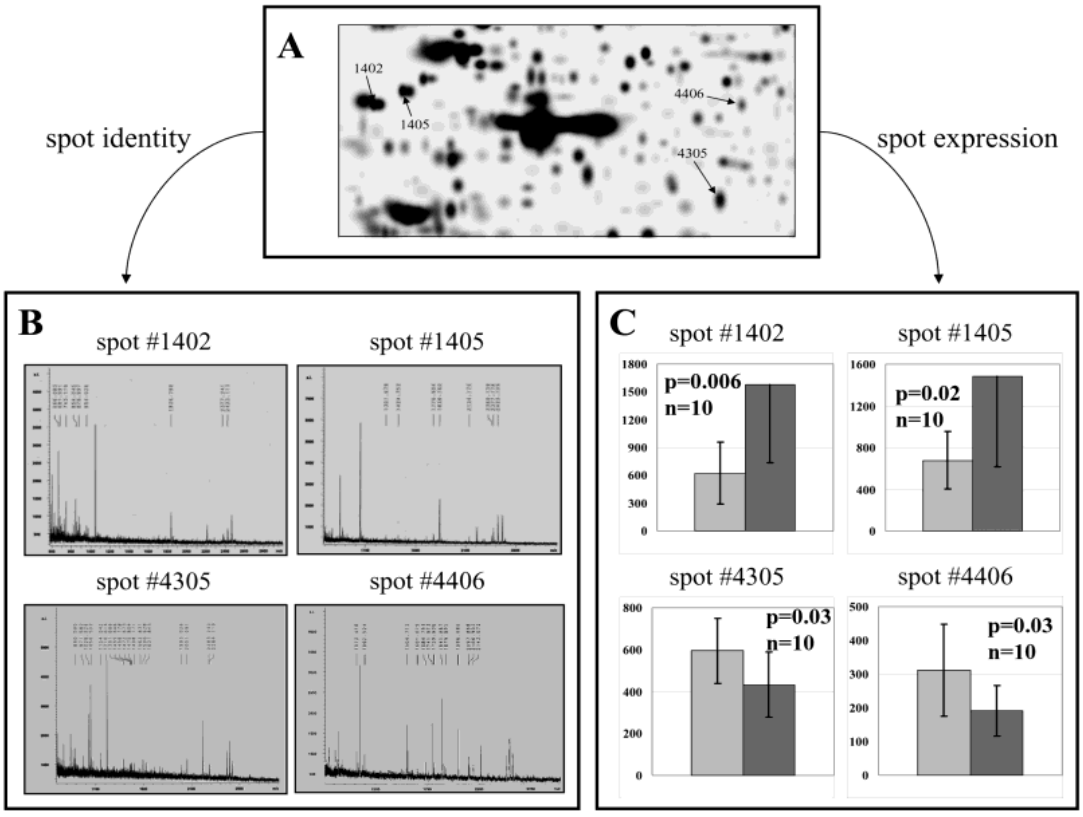

Figure 2. (A) Enlarged fragment of the 2-DE gel with marked spots selected for MALDI-MS. (B) MALDI-MS spectra showing peptide finger prints of the four identified protein spots. (C) PDQuest analysis of changes in expression of the selected protein spots. Light-grey bars, protein from sham-exposed cells; dark-grey bars, protein from mobile phone radiation-exposed cells. Bars represent average ± SD of ten separate experiments. *P*-values are statistical significance calculated with Student's *t*-test.

Institute of Biotechnology at the Helsinki University, Finland. The proteins from the selected spots were reduced with DTT and alkylated with iodoacetamide before overnight digestion with a sequence-grade modified trypsin (Promega, Madison, WI, USA). The peptide mixture was concentrated and desalted using Millipore ZipTip™ μ-C18 pipette tips. The peptide mass fingerprints were measured with a Bruker Biflex™ MALDI-TOF mass spectrometer in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The database searches were performed using ProFound (prowl.rockerfeller.edu/cgi-bin/ProFound) and Mascot (www.matrixscience.com) searches.

The proteins identified with MALDI-MS were as follows (Fig. 2 and Table 1):
(i) Increased expression in response to mobile phone radiation: protein spots #1402 and #1405 (vimentins [6], protein components of class III-intermediate filaments). In EA.hy926 cells vimentins were found to be expressed in at least two different isoforms differing in molecular weight and isoelectric point. Expression of both isoforms was increased. Isoform in spot #1402 (experimental MW/p*I ca.* 47 kDa/4.4) increase in expression was 2.5-fold with *p*-value of 0.006 and isoform in spot #1405 (experimental MW/p*I ca.* 48 kDa/4.8) 2.2-fold with a *p*-value of 0.02.
(ii) Declined expression in response to the mobile phone radiation: protein spot 4305 (isocitrate dehydrogenase 3 (NAD+) α [7]) is a subunit of the mitochondrial enzyme, which catalyzes the conversion of isocitrate to 2-oxoglutarate in the citric acid cycle. The expression level of this protein was moderately downregulated in the exposed samples having a ratio exposed *vs.* control 0.72 with a *p*-value of 0.03. The downregulation of this protein might affect cellular energy production.
(iii) Protein spot 4406 (heterogeneous ribonucleoprotein H1 [8]) is a component of the heterogeneous nuclear ribonucleoprotein (HNRNP) complexes which provide a substrate for the processing events which pre-mRNAs go through before becoming functional mRNAs in the cytoplasm. The expression level of this protein is slightly downregulated in the exposed samples with a ratio exposed *vs.* control 0.61 with a *p*-value of 0.03. The downregulation of this protein might affect protein translation processes.

Alterations in the vimentin expression suggest that some form of cytoskeleton-related response might take place in cells exposed to the mobile phone radiation. This notion agrees with our earlier observation of the effect of mobile phone radiation on the stability of F-actin stress fibers [3, 4]. Changes in the vimentin expression observed in 2-DE were further confirmed by SDS-PAGE and Western blotting and by cell staining using indirect immunofluorescence. For SDS-PAGE/Western blotting a standard protocol was used. Briefly, the cell lysates were separated using 7.5% SDS-PAGE, blotted to a PVDF-membrane, blocked with 5% non-fat dry milk, and exposed to the primary vimentin antibody (Zymed, San Francisco, CA, USA) and the secondary antibody containing a horseradish peroxidase (HRP)-conjugate (Dako, Glostrup, Denmark). The signal was detected using enhanced chemiluminescence (Pierce, Rockford, IL, USA). For immunocytochemistry cells were fixed in 3% paraformaldehyde, membranes were permeabilized in 0.5% Triton X-100, and as a primary antibody was used vimentin antibody (Zymed) and the secondary antibody was TRICT-conjugated (Dako). F-actin was visualized for both immunohistochemistry and for flow cytometry by using phalloidin labeled with AlexaFluor (Molecular Probes, Eugene, OR, USA). The images were captured using a Leitz fluorescence microscope and computerized image acquisition system (Metafer, Germany). Flow cytometry analysis was done using FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA).

SDS-PAGE and Western blot have confirmed that EA.hy926 cells express two isoforms of vimentin. The higher-molecular-mass form (experimental MW *ca.* 57 kDa) was present both in control and in irradiated cells

Table 1. List of four proteins that were extracted from the 2-DE gels and identified using MALDI-MS

SSP	Protein name	SWISS-PROT ID code	Sequence coverage	SWISS-PROT annotations
1402	Vimentin	P08670	19%	Class III intermediate filament
1405	Vimentin	P08670	14%	Class II intermediate filament
4305	Isocitrate dehydrogenase 3(NAD^+) α	P50213	21%	Component of enzyme complex catalyzing conversion of isocitrate to 2-oxoglutarate
4406	Heterogeneous nuclear ribonucleoprotein H1	P31943	34%	Component of HNRNP complex providing a substrate for pre-mRNA processing

and its expression was not affected by irradiation (Fig. 3A). The lower-molecular-mass vimentin (experimental MW *ca.* 48 kDa) was not detectable in the nonirradiated cells but was expressed in the irradiated cells (Fig. 3A). Indirect immunohistochemistry staining of vimentin has shown the change in the distribution pattern of the vimentin filaments after the exposure to mobile phone radiation (Fig. 3B). In conclusion, the observed changes in the vimentin expression suggest that the mobile phone radiation might potentially alter cell physiology by affecting cellular cytoskeleton.

In addition to changes in the expression pattern of vimentin, also the expression of stress fibers forming F-actin was altered after exposure (Fig. 3B). Flow cytometry analysis of cells stained with phalloidin labeled with Alexa-Fluor has shown that following the exposure there is an increase in the expression of the phalloidin-binding form of actin – the F-actin (Figs. 4A and B). Simultaneous detection (using the method described previously [3, 4]) of F-actin and stress protein Hsp27 has revealed that the mobile phone radiation exposure causes increase in expression of Hsp27 and of F-actin (Fig. 4C). This result is in agreement with our previously published data [3, 4].

Our study has shown that proteomics might be an efficient tool when searching for the proteins responding to a weak stimulus, like the mobile phone radiation. In this pilot study, we have found several tens of protein targets of the mobile phone radiation. Functions of the few of the MALDI-MS-identified protein spots suggest possible effects of mobile phone radiation on such physiological functions as (i) cellular energy production, (ii) protein translation, and (iii) cytoskeleton-dependent processes (*e.g.*, cell size, shape, and cell-cell interactions). Further studies will be needed to determine whether there is any impact of these changes on cell physiology.

The results suggesting an effect of mobile phone radiation on the cytoskeleton are in agreement with our earlier observations. We have shown that the mobile phone radiation activates the p38 MAP kinase stress signalling pathway, leading to phosphorylation of small stress protein Hsp27 [3]. This in turn was shown to increase the stability of F-actin stress fibers leading to changes in cell size and shape (shrinking and rounding-up) [4]. The observed effects on the expression of vimentins and on the distribution pattern of vimentin filaments give further support to the notion that the cytoskeleton might be one of the

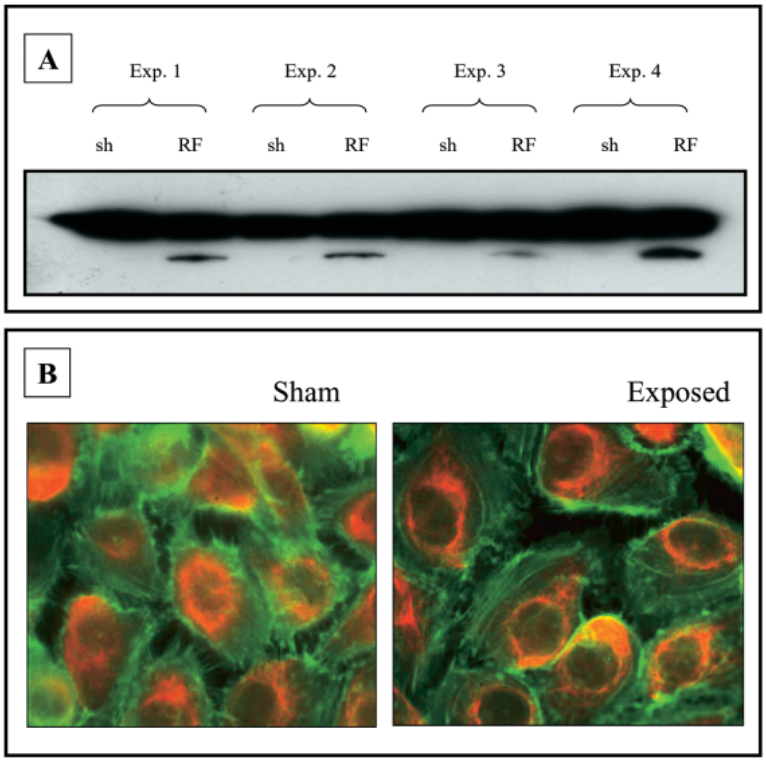

Figure 3. (A) Western blot-detected expression of vimentin in sham-exposed EA.hy926 cells (sh-lanes) and in exposed cells (RF-lanes). Upper band, MW *ca.* 57 kDa; lower band, MW *ca.* 48 kDa. (B) Immunostaining of vimentin (red) and F-actin (green) in sham- and mobile phone radiation-exposed EA.hy926 cells. Vimentin was detected by indirect immunofluorescence using a specific antibody and polyclonal TRITC-labeled second antibody. F-actin was detected with phalloidin labeled with Alexa-Fluor. Note change in staining patterns from "fuzzy-like" to "fibrillar-like" staining for both vimentin and F-actin, suggesting cytoskeleton rearrangement.

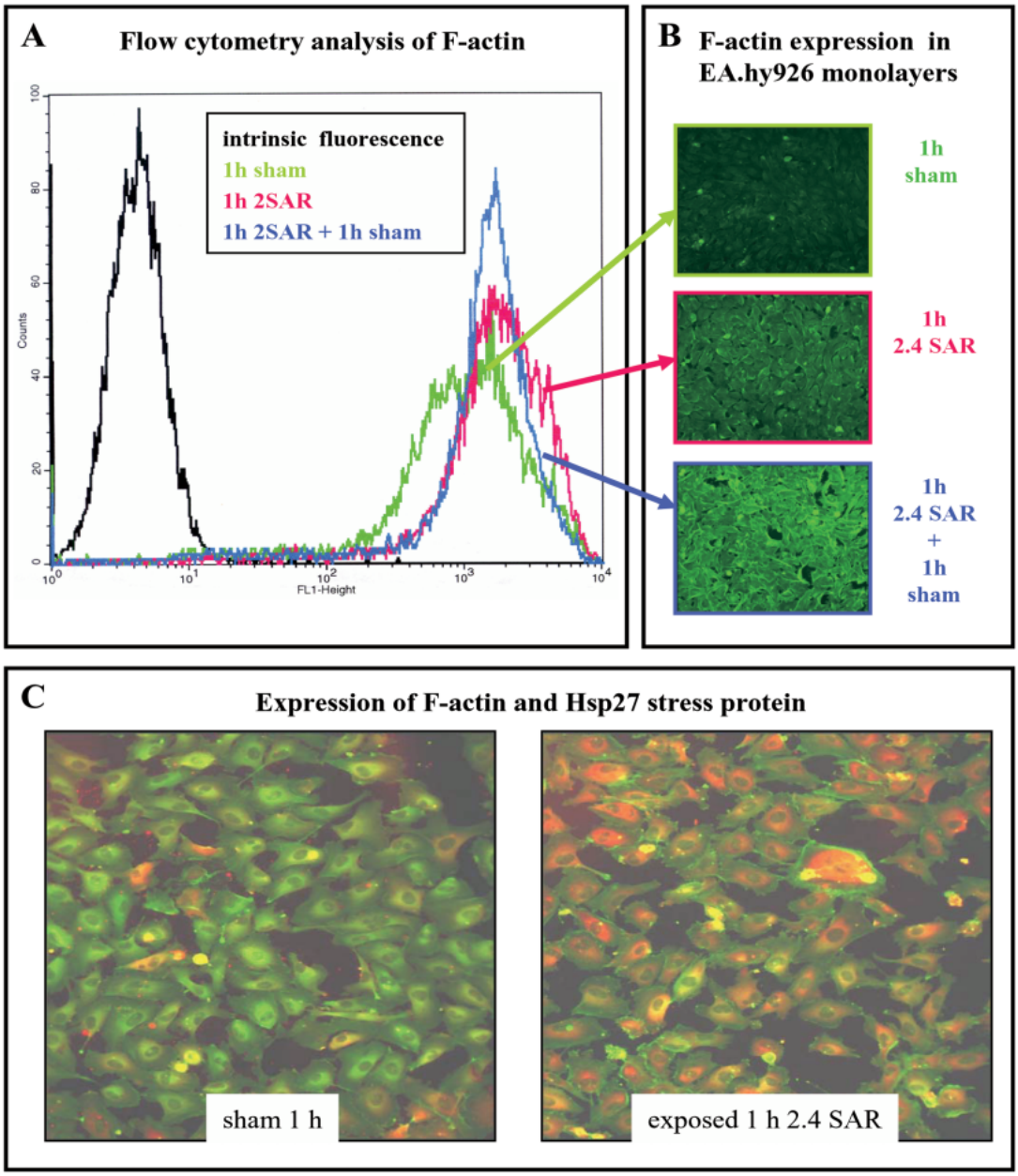

Figure 4. (A) Representative flow cytometry analysis of F-actin expression in sham-exposed cells (green), mobile phone radiation-exposed cells immediately after exposure (red) and exposed cells analyzed 1 h after end of exposure (blue). Fluorescence shift to the right for the exposed cells indicates increase in the expression of F-actin. (B) Expression of F-actin in cell cultures like these used for flow cytometry. Note increased staining for F-actin (green) in exposed cells, in agreement with the flow cytometry analysis. (C) Expression of F-actin (green) and Hsp27 (red) in sham and exposed cells. Note increase in staining for Hsp27 and rearrangement in the distribution of stress fibers from fuzzy perinuclear localization in sham cells to fibrillar localization in cell ruffles.

mobile phone radiation-responding cytoplasmic structures. If such cytoskeleton effects would take place *in vivo* in brain endothelial cells, they could have impact on the function of the blood-brain barrier (BBB). The proper functioning of the tight junctions of endothelial cells lining brains capillary blood vessels is crucial for the proper functioning of the BBB. The cytoskeleton of endothelial cells plays an important role in the regulation of endothelial cell contacts through the tight junctions [9]. Some animal studies and *in vitro* experiments have suggested that the mobile phone radiation might affect the permeability of BBB (for review see [10]). Effects on the cytoskeleton and on the stress protein Hsp27 might be a part of the underlying regulatory mechanism as previously hypothesized [3,4]. Further *in vitro* and *in vivo* studies are underway to clarify mobile phone radiation-induced cytoskeletal effects and function of the BBB.

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III

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Mobile phone radiation causes changes in gene and protein expression in human endothelial cell lines and the response seems to be genome- and proteome-dependent
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RESEARCH ARTICLE

Mobile phone radiation causes changes in gene and protein expression in human endothelial cell lines and the response seems to be genome- and proteome-dependent

Reetta Nylund and Dariusz Leszczynski

Functional Proteomics Group, Radiation Biology Laboratory, STUK, Radiation and Nuclear Safety Authority, Helsinki, Finland

We have examined *in vitro* cell response to mobile phone radiation (900 MHz GSM signal) using two variants of human endothelial cell line: EA.hy926 and EA.hy926v1. Gene expression changes were examined in three experiments using cDNA Expression Arrays and protein expression changes were examined in ten experiments using 2-DE and PDQuest software. Obtained results show that gene and protein expression were altered, in both examined cell lines, in response to one hour mobile phone radiation exposure at an average specific absorption rate of 2.8 W/kg. However, the same genes and proteins were differently affected by the exposure in each of the cell lines. This suggests that the cell response to mobile phone radiation might be genome- and proteome-dependent. Therefore, it is likely that different types of cells and from different species might respond differently to mobile phone radiation or might have different sensitivity to this weak stimulus. Our findings might also explain, at least in part, the origin of discrepancies in replication studies between different laboratories.

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1 Introduction

Induction of biological and health effects by mobile phone radiation remains a controversial issue. In spite of years of research, there is still ongoing discussion whether low-energy mobile phone radiation could induce any detrimental health effects, especially in long-time avid users and in children (Stewart Report, National Radiation Protection Board, London, UK, 2000 (http://www.iegmp.org.uk)).

We have proposed earlier [1, 2] and subsequently experimentally demonstrated [3] that the research approach using genome-wide and proteome-wide screening techniques of transcriptomics and proteomics will speed up the identification of genes and proteins that are responding to mobile phone radiation. Using this approach we have shown that mobile phone radiation activates cellular stress response by increasing the expression and phosphorylation (activity) of small stress protein Hsp27 [4]. We also have shown that mobile phone radiation-induced phosphorylation of Hsp27 has a physiological impact on cells. It induces phospho-Hsp27-dependent stabilization of stress fibers [3]. We have also shown that mobile phone radiation affects expression of the cytoskeletal protein vimentin, an intermediate filament protein [5].

In the present study, we have examined the effects of 900 MHz GSM mobile phone radiation on the gene and protein expression in two closely related human endothelial cell lines, EA.hy926 and EA.hy926v1. Our results show that gene and protein expression are altered in both cell lines in response to mobile phone radiation exposure. Furthermore, different genes and different proteins were affected by the same exposure in both cell lines. It suggests that, at least in

Correspondence: Dr. Dariusz Leszczynski, STUK-Radiation and Nuclear Safety Authority, Laippatie 4, FIN-00880 Helsinki, Finland
E-mail: dariusz.leszczynski@stuk.fi
Fax: +358-9-75-988-464

Abbreviation: RF-EMF, radio-frequency electromagnetic field

the laboratory, the cell responses to mobile phone radiation seem to depend on the repertoire of genes and proteins that are expressed, and are also most likely active, in the cells at the time of the exposure.

2 Material and methods

2.1 Cells and culture conditions

Human endothelial cell line, EA.hy926 [6], was obtained from the laboratory of Dr. Cora-Jean S. Edgell (North Carolina University at Chapel Hill, NC, USA) in 1987 and has since been maintained at the University of Helsinki and at STUK. In 2000, the second batch of human endothelial cell line was obtained by STUK from Dr. Edgell's laboratory. This new batch of endothelial cells was generated in Dr. Edgell's labaratory by sub-cloning of the EA.hy926 cell line. To distinguish between these two different batches of cells, the cell line that has been obtained and maintained in Helsinki since 1987 is called EA.hy926. The batch of cells that was obtained in the year 2000, is called EA.hy926v1.

Both cell lines were grown in Dulbecco's MEM, supplemented with antibiotics, 10% fetal bovine serum, L-glutamine and HAT-supplement (Sigma, USA). For mobile phone radiation experiments, cells were removed from culture flasks by brief trypsinization, washed in cell culture medium and seeded at a density of 1.2×10^6 cells per 55 mm-diameter glass Petri dish (DURAN, Germany). After overnight culturing, the semi-confluent monolayers of EA.hy926 and EA.hy926v1 cells were exposed either to sham or to RF-EMF radiation. Cell cultures for sham and irradiation were prepared in similar glass dishes, derived from the same passage of cells, were seeded at the same cell density, and were grown for the same period of time before the exposure experiment. The only difference between sham and RF-EMF irradiated samples was that the irradiated cells have resided for 1 h in the irradiation chamber with RF-EMF radiation turned on whereas the sham cells resided in the irradiation chamber for the same period of time but with radiation turned off.

2.2 Cell cycle analysis

Cell cycle analysis was performed by determining the DNA content of cells with the standard propidium iodide staining method as described previously [7]. Briefly, cells were harvested with trypsin, washed with PBS and fixed in 90% alcohol v/v for 10 min. on ice. After fixation the cells were washed in PBS, suspended in RNase solution in PBS (100 units/mL), and incubated for 30 min. at 37°C. Afterwards, the propidium iodide solution (10 mg/mL) was added to the cells and the cells were incubated for 18 h at 4°C. After propidium iodide staining, cells were washed with PBS and analyzed with FACScan using the CellFit Cell-Cycle analysis software (Becton Dickinson, USA). The growth pattern and cell cycle distribution of both cell lines is shown in Fig. 1.

2.3 RF-EMF exposure system and exposure protocol

Cells were irradiated with a simulated mobile phone microwave radiation as previously described [4]. In all experiments reported here, cells were exposed for 1 h to a 900 MHz GSM signal at an average specific absorption rate of 2.8 W/kg. The specific absorption rate level for cell exposure was originally set at 2.0 W/kg because it is the safety limit for mobile phone emissions set by the International Commission on Non-Ionizing Radiation Protection. However, the improved measurements and simulations since our first experimental publication in 2002 [4], have shown that the specific absorption rate level to which cells were exposed was 2.8 W/kg. Importantly, the temperature of the cells and the culture medium had remained relatively constant throughout the one hour exposure period at 37 $\pm$ 0.2°C and therefore the effects observed by us are not caused by bulk heating of the cells.

2.4 Gene expression analysis by cDNA Expression Arrays

The total RNA was isolated from sham and RF-EMF exposed cells using the NucleoSpin RNA II kit according to the manufacturer's instructions (Clontech, USA). Briefly, semi-confluent cell cultures were lysed directly in the glass Petri dishes. RNA from cleared cell lysates was immobilized in spincolumns provided by the NucleoSpin RNA II kit. After DNAse treatment, the total RNA was eluted from the columns and analyzed for possible remaining DNA contamination by PCR using β-actin primers against genomic DNA [8]. The PCR products were run by agarose gel electrophoresis and only DNA free samples were used in Atlas™ Microarray procedure.

For the synthesis of cDNA probes and differential analysis of gene expression we used Atlas Pure Total RNA Labelling System (Clontech) and Atlas cDNA Expression Arrays (Clontech), respectively. The poly A^+ RNA enrichment of 50 μg total RNA and ^{32}P-labelled cDNA probe synthesis made by reverse transcription were performed according to Atlas™ Pure Total RNA Labeling System (Clontech). The precisely same amount of ^{32}P-labelled cDNA from sham and RF-EMF exposed cells were used as a probe in Atlas™ Human 1.2 cDNA Expression Array, containing probes for 1167 genes immobilized on a nylon membrane. Hybridization and washing procedures were performed according to the recommendations of the membrane manufacturer. Briefly, the array membranes were prehybridized for 30 min at 68°C in ExpressHyb hybridization solution containing 0.1 mg/mL salmon testis DNA. The ^{32}P-labelled cDNA was added to the hybridization solution, the array membrane was hybridized overnight at 68°C and thenext day array membranes were washed four times with SSC/1% SDS solutions. The filters were exposed at −70°C for 7–21 days in a manner such that similar signal intensity was produced from filters containing hybridized cDNA from both sham and RF-EMF exposed cells.

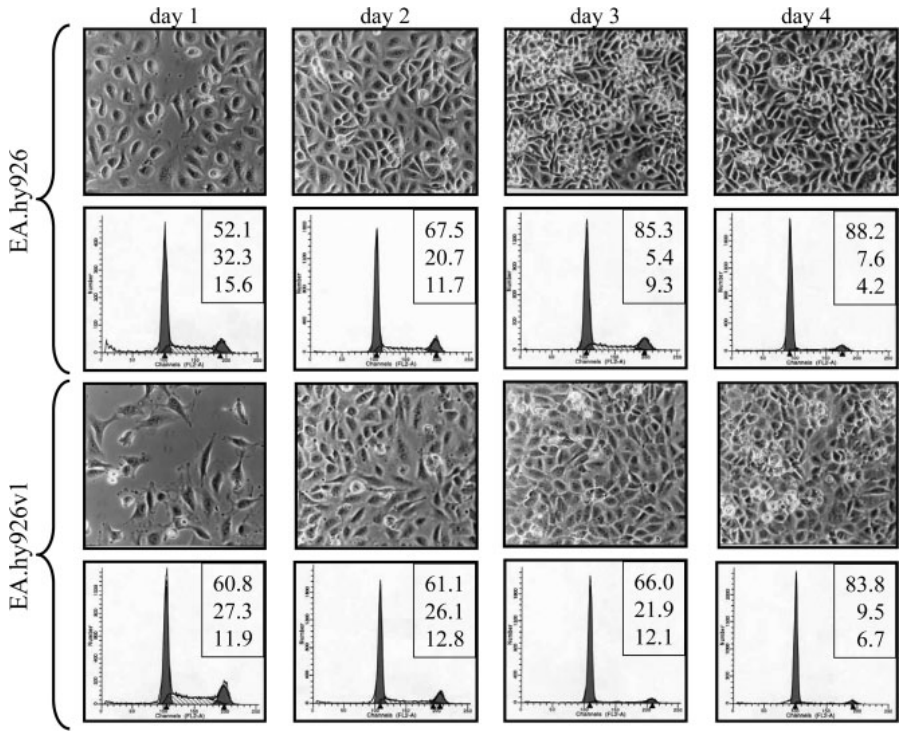


Figure 1. Growth pattern (cobble stone-like growth pattern of endothelial cells) and cell cycle distribution of EA.hy926 and EA.hy926v1 cell lines grown for 1–4 days. Cells were used for experiments on day one of the culture.

Hybridization signals on the autoradiograms were scanned by GS-710 Calibrating Imaging Densitometer (Bio-Rad, USA) and the intensity of gene spots was analyzed by Atlas-Image 2.0 Software (Clontech). After orientation and alignment of the two array membranes, array background was subtracted, and the intensity of the detected gene spots were adjusted by the normalization coefficient, which was calculated by software based on the intensity of total genes (the global normalization, sum method).

Each cDNA expression array experiment was repeated three times ($n = 3$) for each cell line using three different cultures of cells. As per cDNA Expression Array manufacturer's instructions, only genes whose adjusted intensity was higher than 3000 were selected for further analysis. After excluding genes that had adjusted intensity lower than 3000, the expression levels of the remaining genes were normalized to the average expression level of the nine housekeeping genes. Thereafter, the average ± SD for three replicates of sham and exposed genes was calculated for each cell line. Then the ratio between average exposed and average sham sample was calculated. For each cell line, as specified in the cDNA Expression Array manufacturer's instructions, only the genes which had a ratio with at least 2-fold increase or 2-fold decline in expression level were included in the final table of the irradiation affected genes.

2.5 Protein expression analysis by 2-DE

Protein extraction was performed immediately after the end of the exposure. Petri dishes with cell cultures were placed on ice, washed with ice-cold PBS and lysed with buffer consisting of: 7 M urea, 2 M thiourea, 4% chaps, 2% IPG buffer pH 3–10 NL, 1% DTT, 1 mM sodium orthovanadate, and 1 mM PMSF. Protein concentration in lysates was measured using the Bradford method and 175 μg of total protein was used for 2-DE.

Proteins were separated by 2-DE. The IEF was performed using an IPGphor apparatus and non-linear pH 3–10 18 cm long IEF strips (Amersham Biosciences, Sweden). The samples were loaded using in-gel rehydration in a buffer containing 9 M urea, 2% chaps, 0.2% DTT, 0.5% IPG buffer pH 3–10 NL for 12 h. IEF was run at 20°C using step-and-hold with gradient methods as follows: 30 V, 2 h; 100 V, 0.5 h; 300 V, 0.5 h; 600 V, 0.5 h; 1500 V, 0.5 h; 8000 V gradient 4 h; 8000 V, until the 65000 V·h were achieved. For SDS-PAGE the IEF strips were

equilibrated for 15 min with 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, and 10 mg/mL DTT for 15 min and then for another 15 min in the same buffer, but where 25 mg/mL iodoacetamide replaced DTT. SDS-PAGE was run in 8% gel using Protean IIxi Multicell apparatus (Bio-Rad) and a constant current of 40 mA/gel at 10°C. After electrophoresis, gels were fixed overnight with a mixture of 30% ethanol and 0.5% acetic acid, then washed with 20% ethanol in ddH_2O, sensitized with sodium thiosulfate (0.2 g/L), incubated in silver nitrate solution (2 g/L) and developed in a solution of potassium anhydride (30 g/L), 37% formaldehyde (0.7 mL/L) and sodium thiosulfate (0.01 g/L). The development was stopped with Tris (50 g/L) and acetic acid (0.5%) solution. Silver stained gels were stored in ddH_2O at 4°C. The gels were scanned using GS-710 densitometer (Bio-Rad) and analyzed using PDQuest 6.2 software (Bio-Rad). 2-DE artificial gels were generated from ten independent protein samples from ten independent replicates of sham and irradiated cell cultures. The protein expression pattern in ten replicate sham samples was then compared with the protein pattern in ten replicate irradiated samples. The normalized spot volumes of the proteins from sham and exposed sample gels were statistically analyzed using student *t*-test at the confidence level of 95%.

3 Results

3.1 Effect of RF-EMF on gene expression

The expression of 1167 genes was analyzed in EA.hy926 and EA.hy926v1 cells. After disregarding all genes that had expression levels below 3000, we compared effects of RF-EMF exposure on the expression of 136 genes in both cell lines.

Mobile phone radiation exposure had caused changes in the expression level of numerous genes in both cell lines. The comparison between the genes affected in each of the used cell lines revealed that both cell lines responded differently to the same RF-EMF exposure. It appeared that RF-EMF exposure had caused a predominant decline in gene expression in EA.hy926 cells whereas in EA.hy926v1 cell line it caused a predominant increase in gene expression. Most strikingly, the majority of genes that were found to be down-regulated in EA.hy926 cell line were either up-regulated or not affected in the EA.hy926v1 cell line (Table 1).

In EA.hy926 cell line, four genes were found that had increased expression levels by more than 2-fold (ratio $\geq$2.0; red color bold numbers in Table 1). Of these, one gene (cell surface glycoprotein MUC18; code A08g) had also increased expression level in EA.hy926v1 but the increase was much more dramatic ($\geq$10-fold). The other three genes had too low expression levels in the EA.hy926v1 cell line to be considered.

In the EA.hy926v1 cell line, 61genes were found that had increased expression levels by more than 2-fold (ratio $\geq$2.0; red color bold numbers in Table 1). Of these, only one gene displayed up-regulated expression levels in EA.hy926 cells–*i.e.* the cell surface glycoprotein MUC18. The 19 genes, that were up-regulated 2-fold or more in EA.hy926v1 cells, were down-regulated 2-fold or more in EA.hy926 cells. For 41 genes that were up-regulated in EA.hy926v1, the expression level of the same genes in EA.hy926 cells was either not accounted for *i.e.* too low expression level to be considered; $<$3000, or their up- or down-regulation was less than 2-fold.

In the EA.hy926 cell line, 89 genes were found where expression levels had been down-regulated by the exposure by 2-fold or more (ratio $\leq$0.5; blue color bold numbers in Table 1). Of these, the only expression level of one gene that encodes homeobox B7 protein had declined in both cell lines (Table 1; code E04a). The expression levels of 19 genes, which had declined in EA.hy926 cells, were found to increase in EA.hy926v1 cells. For 69 genes, that were down-regulated in EA.hy926 cell line, the expression levels of the same genes in EA.hy926v1 cells were either not accounted for *i.e.* too low expression level to be considered; $<$3000, or their up- or down-regulation was less than 2-fold.

Statistical significance of the changes in gene expression levels in three experiments was analyzed using student's *t*-test (Table 1; purple color italic numbers). Only one gene expression change (serine/threonine protein phosphatase; Table 1; code C12a) was statistically significant in the EA.hy926 cell line. In the EA.hy926v1 cell line there were 13 genes with statistically significant change in expression (Table 1; codes: A07d, A09b, A12l, A13l, B08m, B10m, C05k, D08i, D14j, E12n, F07b, F07e, and F10e). For clarity, the summary of the statistically significantly affected genes in both cell lines is shown in Table 2.

3.2 Effect of RF-EMF on protein expression

Protein expression in EA.hy926 and EA.hy926v1 cells was analyzed using 2-DE. The effect of mobile phone radiation exposure on the EA.hy926 cell line was shown by us previously [5]. Here we have made the comparison between this cell line and the EA.hy926v1 cell line. The comparison has revealed that the protein expression profiles in both cell lines are different, in spite of the closely related origin of the cell lines (Fig. 2). Only approximately half of all of the protein spots could be matched confidently between the cell lines. This indicates that, in each cell line different proteins were active at the time of irradiation. Since mobile phone radiation induced changes in gene expression, it was expected that also protein expression would be affected. Indeed, the comparison of the sham and mobile phone radiation-exposed samples (ten experiments for each cell line for each treatment) had shown that several tens of protein spots had altered their expression level ($p < 0.05$). In the EA.hy926 cell line there were 38 protein spots with altered expression level due to radiation exposure (Fig. 2A–B) whereas in the EA.hy926v1 cell line there were 45 differentially expressed protein spots (Fig. 2C–D). Similarly, as was the case with gene expression changes, also different proteins were affected by the exposure in both cell lines.

Table 1. List of genes that in response to mobile phone radiation, increased (red) or decreased (blue) expression in EA.hy 926 cells. The effect of the exposure on the same genes in EA.hy926v1 cells is shown in parallel. Empty fields indicate that for one of the cell lines, the data were not valid for the analysis (the difference between gene expression in sham and exposed cells was less than 3000 density units).

Code[a]	GENE NAME	EA.hy 926 cells							EA.hy 926v1 cells						
		Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	Ratio[c] exposed/sham ↓	*t*-test[d]	Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	Ratio[c] exposed/sham ↓	*t*-test[d]
A01g	Transforming protein rhoA H12	**4.16**	**4.66**	**1.32**	**0.05**		**0.32**	*0.402*							
A01f	Vascular endothelial growth factor receptor 1								**0.03**	**0.03**	**0.11**	**0.13**	**3.22**		*0.404*
A02h	G2/mitotic-specific cyclin B1 (CCNB1)	1.09	0.57	0.83	0.24		0.76	*0.521*	**0.04**	**0.04**	**0.71**	**0.62**	**18.72**		*0.201*
A02i	Cell division control protein 2 homolog (CDC2)								**0.02**	**0.02**	**0.49**	**0.47**	**22.93**		*0.225*
A02j	Serine/threonine-protein kinase PLK1 (STPK13)	**0.52**	**0.22**	**0.22**	**0.38**		**0.42**	*0.317*	**0.06**	**0.04**	**0.51**	**0.41**	**8.17**		*0.196*
A03b	EB1 protein								**0.14**	**0.13**	**0.43**	**0.21**	**3.13**		*0.130*
A03l	Prothymosin alpha (ProT-alpha; PTMA)	**6.78**	**9.04**	**1.38**	**0.06**		**0.20**	*0.409*	1.67	0.36	1.54	0.07		0.92	*0.593*
A04b	Ezrin; cytovillin 2; villin 2 (VIL2)	0.52	0.30	0.43	0.08		0.82	*0.651*	**0.10**	**0.08**	**0.34**	**0.17**	**3.30**		*0.126*
A04i	Cell division protein kinase 4	0.18	0.14	0.14	0.24		0.76	*0.797*	**0.18**	**0.19**	**0.08**	**0.14**		**0.47**	*0.536*
A05e	Tyrosine-protein kinase receptor UFO precursor								**0.34**	**0.50**	**0.72**	**0.36**	**2.11**		*0.354*
A06e	fms proto-oncogene (c-fms)								**0.03**	**0.03**	**0.13**	**0.15**	**4.49**		*0.359*
A07d	fos-related antigen (FRA1)	**3.25**	**3.06**	**1.33**	**0.04**		**0.41**	*0.389*	0.99	0.21	1.48	0.07	1.49		**0.043**
A07k	Cyclin-dependent kinase inhibitor 1 (CDKN1A)	**0.39**	**0.35**	**0.00**	**0.00**		**0.00**	*0.191*	**0.23**	**0.24**	**0.62**	**0.21**	**2.68**		*0.103*
A07l	40S ribosomal protein S19 (RPS19)	**4.92**	**5.86**	**1.34**	**0.07**		**0.27**	*0.401*	0.76	0.38	1.47	0.12	1.93		*0.074*
A08d	v-erbA related protein (EAR2)								**0.03**	**0.04**	**0.11**	**0.11**	**3.98**		*0.334*
A08g	Cell surface glycoprotein MUC18	**0.24**	**0.22**	**0.49**	**0.02**	**2.02**		*0.191*	**0.04**	**0.03**	**0.41**	**0.39**	**10.09**		*0.236*
A08k	Cyclin-dependent kinase inhibitor 3	**1.05**	**0.44**	**0.23**	**0.40**		**0.22**	*0.078*	**0.06**	**0.02**	**0.39**	**0.33**	**6.63**		*0.226*
A08n	Chloride conductance regulatory protein ICLN	**0.36**	**0.13**	**0.74**	**0.18**	**2.04**		*0.049*							
A09b	c-myc purine-binding transcription factor puf	**6.81**	**9.10**	**1.37**	**0.06**		**0.20**	*0.409*	0.97	0.25	1.54	0.07	1.59		**0.052**
A09h	fte-1	**4.46**	**5.06**	**1.34**	**0.04**		**0.30**	*0.398*	**0.30**	**0.39**	**1.28**	**0.21**	**4.22**		*0.031*
A09l	Proliferating cell nucleolar antigen P120	0.52	0.38	0.88	0.14	1.69		*0.235*	**0.06**	**0.02**	**0.28**	**0.30**	**5.10**		*0.316*
A10b	Nucleoside diphosphate kinase A	**2.96**	**3.13**	**1.18**	**0.11**		**0.40**	*0.429*							
A10g	T-lymphoma invasion and metastasis inducing								**0.05**	**0.04**	**0.41**	**0.42**	**8.91**		*0.273*
A12e	ERBB-3 receptor protein-tyrosine kinase precursor	**0.65**	**0.61**	**0.24**	**0.10**		**0.36**	*0.360*							
A12l	Transducer of erbB2 (TOB)	0.00	0.00	0.15	0.13		DIV/0	*0.189*	**0.07**	**0.09**	**0.62**	**0.19**	**8.52**		**0.022**
A13g	Cyclin K								**0.05**	**0.08**	**0.13**	**0.09**	**2.81**		*0.312*
A13j	Cyclin-dependent kinase regulatory subunit 1	**1.83**	**1.45**	**0.70**	**0.41**		**0.38**	*0.309*	**0.14**	**0.21**	**0.75**	**0.21**	**5.46**		*0.021*
A13l	p55CDC	**3.80**	**4.35**	**1.08**	**0.18**		**0.28**	*0.392*	**0.15**	**0.21**	**1.00**	**0.27**	**6.57**		**0.014**
A14j	Cyclin-dependent kinase regulatory subunit 2								**0.08**	**0.10**	**0.63**	**0.43**	**8.26**		*0.151*
B02n	ras-related protein RAP-1B	0.68	0.37	0.47	0.21		0.68	*0.440*	**0.07**	**0.11**	**0.24**	**0.13**	**3.61**		*0.163*
B03n	ras-related protein RAB2	0.97	0.39	0.88	0.26		0.90	*0.741*	**0.06**	**0.11**	**0.41**	**0.23**	**6.39**		*0.102*
B05j	c-jun N-terminal kinase 2 (JNK2)	**1.93**	**2.15**	**0.35**	**0.39**		**0.18**	*0.329*							
B06d	Thrombin receptor	**1.80**	**1.93**	**0.66**	**0.10**		**0.36**	*0.413*							
B06h	MAPKAP kinase	**0.09**	**0.15**	**0.01**	**0.01**		**0.07**	*0.450*							
B06k	Serine kinase	**0.13**	**0.13**	**0.01**	**0.01**		**0.04**	*0.241*							
B07h	Mitogen-activated protein kinase p38 (p38 MAP kinase)	**2.01**	**2.57**	**0.47**	**0.23**		**0.24**	*0.409*							

Table 1. Continued

Code[a]	GENE NAME	EA.hy 926 cells							EA.hy 926v1 cells						
		Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	↓	*t*-test[d]	Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	↓	*t*-test[d]
B07m	Ral A; GTP-binding protein	**0.33**	**0.30**	**0.64**	**0.26**	1.95		*0.243*							
B08m	Transforming protein rhoB								**0.07**	**0.13**	**0.45**	**0.17**	**6.19**		**0.040**
B10m	ras-related protein RAB-7	**2.43**	**1.88**	**0.73**	**0.53**		**0.30**	*0.253*	**0.16**	**0.26**	**1.25**	**0.26**	**7.63**		**0.007**
B13m	ras-related C3 botulinum toxin substrate 1	**1.07**	**1.18**	**0.23**	**0.31**		**0.21**	*0.343*							
B14d	Ephrin type-A receptor 1 precursor	**3.38**	**4.30**	**0.68**	**0.34**		**0.20**	*0.391*							
C01e	PKC inhibitor protein-1 (KCIP-1)	1.03	1.05	0.67	0.33		0.65	*0.624*	**0.16**	**0.19**	**0.43**	**0.17**	**2.74**		*0.137*
C02e	PKC substrate 80-kDa protein heavy chain	**4.61**	**6.42**	**1.05**	**0.49**		**0.23**	*0.437*	**0.44**	**0.57**	**1.41**	**0.02**	**3.18**		*0.100*
C02j	CAD; DNA fragmentation factor 40-kDa subunit	**0.20**	**0.25**	**0.00**	**0.00**		**0.00**	*0.295*							
C02k	Inducible nitric oxide synthase (INOS)	**0.28**	**0.34**	**0.00**	**0.00**		**0.01**	*0.300*							
C03f	SH3P18 SH3 domain-containing protein								**0.02**	**0.02**	**0.09**	**0.13**	**4.55**		*0.436*
C03k	Defender against cell death 1 (DAD1)								**0.43**	**0.50**	**1.44**	**0.15**	**3.37**		*0.063*
C04e	Hint protein; PKC inhibitor 1 (PKCI1)	**2.73**	**3.77**	**0.78**	**0.30**		**0.29**	*0.466*							
C05a	Leukocyte surface CD47 antigen precursor	**0.52**	**0.42**	**0.06**	**0.05**		**0.11**	*0.193*							
C05e	macMARCKS; MARCKS-related protein	**0.47**	**0.54**	**0.07**	**0.12**		**0.15**	*0.326*							
C05h	Caspase-3 (CASP3)	**0.74**	**0.99**	**0.14**	**0.13**		**0.19**	*0.404*							
C05k	Cytoplasmic dynein light chain 1 (HDLC1)								**0.32**	**0.24**	**1.23**	**0.25**	**3.88**		**0.010**
C06h	Caspase-4 precursor (CASP4)	**5.52**	**7.25**	**1.09**	**0.38**		**0.20**	*0.401*	**0.26**	**0.36**	**1.05**	**0.39**	**4.06**		*0.060*
C06k	Cytochrome P450 reductase	**0.79**	**1.17**	**0.18**	**0.30**		**0.22**	*0.465*							
C06l	Proliferating cyclic nuclear antigen (PCNA); cyclin	**6.02**	**8.90**	**1.26**	**0.16**		**0.21**	*0.452*	1.08	0.14	1.49	0.08	1.38		*0.021*
C06n	mutL protein homolog; DNA mismatch repair protein	**6.66**	**9.26**	**1.37**	**0.06**		**0.21**	*0.427*	1.25	0.19	1.54	0.07	1.23		*0.110*
C08e	Tuberin	**1.18**	**1.13**	**0.48**	**0.43**		**0.41**	*0.402*	**0.05**	**0.07**	**0.35**	**0.33**	**6.50**		*0.255*
C09d	rho GDP dissociation inihibitor 1	**3.02**	**3.91**	**0.64**	**0.58**		**0.21**	*0.404*	**0.36**	**0.43**	**0.76**	**0.28**	**2.14**		*0.249*
C09g	Adenosine A1 receptor (ADORA1)	**0.06**	**0.10**	**0.27**	**0.29**	4.66		*0.334*							
C09h	Caspase-8 precursor (CASP8)	**0.20**	**0.26**	**0.04**	**0.06**		**0.19**	*0.397*							
C11h	Caspase-10 precursor (CASP10)								**0.02**	**0.02**	**0.53**	**0.44**	**24.16**		*0.183*
C11k	ALG-2 calcium-binding protein	1.15	1.34	0.81	0.24		0.70	*0.706*	**0.20**	**0.19**	**0.51**	**0.24**	**2.55**		*0.162*
C12a	Serine/threonine protein phosphatase PP1-alpha 1 catalytic subunit	**1.16**	**0.18**	**0.46**	**0.19**		**0.40**	**0.010**							
C12d	Cortactin; amplaxin; ems-1 oncogene	**3.20**	**3.92**	**0.89**	**0.23**		**0.28**	*0.416*							
C13a	Dual-specificity protein phosphatase 2	**0.05**	**0.09**	**0.01**	**0.01**		**0.12**	*0.472*							
C14h	Calpain 2 large (catalytic) subunit	**1.72**	**2.16**	**0.49**	**0.43**		**0.28**	*0.428*							
D02g	Glial growth factor 2 precursor	0.95	0.49	0.85	0.33		0.89	*0.786*	**0.07**	**0.02**	**0.35**	**0.26**	**5.16**		*0.198*
D02l	Hepatic leukemia factor (HLF)	**0.46**	**0.69**	**0.01**	**0.01**		**0.01**	*0.375*							
D02n	60S ribosomal protein L6 (RPL6)	**6.82**	**9.12**	**1.36**	**0.07**		**0.20**	*0.408*	1.54	0.31	1.54	0.07	1.00		*0.993*
D03i	Myelin-oligodendrocyte glycoprotein precursor (MOG)	**0.66**	**0.86**	**0.08**	**0.14**		**0.12**	*0.361*							
D03k	YL-1 protein	**0.37**	**0.48**	**0.12**	**0.21**		**0.33**	*0.482*							
D04a	HHR23A; UV excision repair prot. RAD23A	0.91	0.08	0.96	0.10	1.05		*0.538*	**0.20**	**0.11**	**0.66**	**0.43**	**3.35**		*0.198*
D05a	Ubiquitin-conjugating enzyme E2 17-kDa (UBE2A)	**2.85**	**2.53**	**1.26**	**0.05**		**0.44**	*0.389*							
D05l	ets-related gene transforming protein (ERG1)	**0.31**	**0.37**	**0.07**	**0.12**		**0.22**	*0.371*							

Table 1. Continued

Code[a]	GENE NAME	EA.hy 926 cells							EA.hy 926v1 cells						
		Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	↓	*t*-test[d]	Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	↓	*t*-test[d]
D07n	cAMP-responsive element-binding protein (CREB1)	**0.34**	**0.22**	**0.13**	**0.22**		**0.38**	*0.317*							
D08i	Major prion protein precursor (PRP)	**3.05**	**2.86**	**1.32**	**0.04**		**0.43**	*0.403*	1.10	0.11	1.44	0.07	1.30		**0.016**
D09f	acyl-CoA-binding protein	**4.67**	**5.47**	**1.36**	**0.05**		**0.29**	*0.405*	0.86	0.30	1.50	0.05	1.75		*0.061*
D09i	Alzheimer's disease amyloid A4 protein precursor	**3.24**	**3.39**	**1.20**	**0.12**		**0.37**	*0.408*							
D09n	Transcription factor E4TF1-60	**0.26**	**0.43**	**0.09**	**0.16**		**0.35**	*0.583*							
D14a	Deoxyribonuclease II (DNase II)	0.46	0.17	0.56	0.16	1.23		*0.481*	**0.14**	**0.16**	**0.28**	**0.18**	**2.05**		*0.357*
D14j	Activated RNA polymerase II transcriptional coactivator p15	**6.01**	**7.79**	**1.35**	**0.08**		**0.22**	*0.409*	**0.30**	**0.34**	**1.28**	**0.45**	**4.25**		**0.044**
E01l	Interleukin-2 receptor alpha subunit precursor	**2.24**	**2.64**	**0.81**	**0.11**		**0.36**	*0.446*	0.11	0.09	0.07	0.11		0.69	*0.710*
E02e	cAMP-dependent transcription factor ATF-4	**5.27**	**6.48**	**1.36**	**0.05**		**0.26**	*0.406*	**0.40**	**0.47**	**1.32**	**0.18**	**3.29**		*0.061*
E03d	Transcription initiation factor TFIID	1.04	0.62	0.70	0.17		0.68	*0.452*	**0.13**	**0.19**	**0.48**	**0.33**	**3.65**		*0.207*
E03f	DNA-binding protein A	**3.97**	**4.44**	**1.09**	**0.17**		**0.27**	*0.377*							
E03g	Cadherin 5 (CDH5); vascular endothelial cadherin (VE-cadherin)	**2.78**	**2.74**	**1.23**	**0.12**		**0.44**	*0.432*	0.60	0.37	0.99	0.38	1.65		*0.278*
E03k	Neuromedin B receptor (NMBR)	**2.10**	**2.01**	**0.70**	**0.31**		**0.33**	*0.351*							
E04a	Homeobox B7 protein HOXB7	**1.56**	**1.93**	**0.77**	**0.67**		**0.50**	*0.561*	**0.25**	**0.25**	**0.00**	**0.00**		**0.00**	*0.229*
E04b	Nuclease-sensitive element DNA-binding protein (NSEP)	**6.61**	**8.76**	**1.37**	**0.04**		**0.21**	*0.409*	1.58	0.30	1.54	0.07		0.97	*0.844*
E05h	NADH-ubiquinone oxidoreductase B18								**0.07**	**0.03**	**0.23**	**0.25**	**3.22**		*0.380*
E06f	Polyadenylate binding protein-interacting protein 1 (PAIP1)	**0.32**	**0.31**	**0.06**	**0.11**		**0.20**	*0.289*							
E07b	fli-1 oncogene; ergB transcription factor	1.75	1.01	1.10	0.09		0.63	*0.380*	**0.17**	**0.26**	**0.84**	**0.47**	**5.04**		*0.114*
E08b	Paired box protein PAX-5; B-cell specific transcription factor	**0.31**	**0.30**	**0.05**	**0.09**		**0.17**	*0.272*							
E08e	Guanine nucleotide-binding protein Gα-subunit	**6.08**	**7.91**	**1.36**	**0.05**		**0.22**	*0.410*	1.25	0.23	1.54	0.07	1.24		*0.149*
E08f	High mobility group protein (HMG-I)	**3.68**	**4.06**	**1.31**	**0.04**		**0.36**	*0.418*							
E09b	Special AT-rich sequence binding protein 1								**0.13**	**0.21**	**0.36**	**0.37**	**2.74**		*0.421*
E09i	Fibronectin receptor beta subunit (FNRB)	**2.01**	**2.07**	**0.63**	**0.36**		**0.32**	*0.371*							
E10a	Early growth response protein 1 (hEGR1)	**0.13**	**0.16**	**0.62**	**0.56**	**4.66**		*0.264*							
E10i	Integrin alpha 6 precursor	**0.89**	**0.87**	**0.43**	**0.11**		**0.48**	*0.457*							
E11a	Transcription factor ETR101								**0.13**	**0.21**	**0.47**	**0.04**	**3.60**		*0.097*
E11d	Purine-rich single-stranded DNA-binding-α								**0.02**	**0.02**	**0.16**	**0.17**	**8.20**		*0.279*
E11i	Integrin beta 4 (ITGB4); CD104 antigen								**0.06**	**0.02**	**0.27**	**0.22**	**4.55**		*0.243*
E11n	Microsomal glutathione S-transferase 12	**6.40**	**8.44**	**1.31**	**0.08**		**0.21**	*0.406*							
E12e	Nucleobindin precursor (NUC)	**1.18**	**1.56**	**0.31**	**0.09**		**0.27**	*0.435*	**0.22**	**0.30**	**0.70**	**0.21**	**3.19**		*0.090*
E12n	Glutathione S-transferase pi (GSTP1; GST3)	**6.53**	**8.63**	**1.37**	**0.05**		**0.21**	*0.410*	1.23	0.15	1.55	0.07	1.25		**0.050**
E13g	alpha1 catenin (CTNNA1);								**0.11**	**0.08**	**0.27**	**0.10**	**2.52**		*0.093*
E14a	Transcriptional repressor prot. yin & yang 1								**0.05**	**0.05**	**0.28**	**0.22**	**5.91**		*0.204*
E14b	FUSE binding protein								**0.01**	**0.01**	**0.18**	**0.21**	**32.61**		*0.280*
E14i	Leukocyte adhesion glycoprotein LFA-11α	0.74	0.76	0.56	0.21		**0.75**	*0.717*	**0.21**	**0.30**	**1.43**	**0.20**	**6.95**		*0.006*
F01c	MPV17 protein	**0.37**	**0.38**	**0.04**	**0.08**		**0.12**	*0.270*							
F01f	Insulin-like growth factor binding protein 1	**0.91**	**1.19**	**0.22**	**0.02**		**0.24**	*0.420*							
F01l	Proteasome component C2	**1.72**	**1.68**	**0.86**	**0.16**		**0.50**	*0.469*							

Table 1. Continued

Code[a]	GENE NAME	EA.hy 926 cells						EA.hy 926v1 cells						
		Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	↓	*t*-test[d]	Sham[b] average ± SD		Exposed[b] average ± SD		Ratio[c] exposed/sham ↑	↓	*t*-test[d]
F02a	Heat shock cognate 71-kDa protein								**0.06**	**0.10**	**0.94**	**0.55**	**16.84**		*0.103*
F02b	Heat-shock protein 40 (HSP40)								**0.13**	**0.20**	**0.49**	**0.44**	**3.82**		*0..291*
F02f	Vascular endothelial growth factor precursor	**0.45**	**0.59**	**0.00**	**0.01**		**0.01**	*0.322*							
F02l	Proteasome component C3;	**3.25**	**3.67**	**1.14**	**0.17**		**0.35**	*0.425*							
F03b	Heat shock protein 60 (HSP-60)								**0.03**	**0.04**	**0.52**	**0.51**	**15.44**		*0.241*
F03e	Macrophage-specific colony-stimulating factor (CSF-1; MCSF)	**4.29**	**5.30**	**0.83**	**0.25**		**0.19**	*0.375*							
F03l	Proteasome component C5	**3.17**	**3.22**	**1.05**	**0.25**		**0.33**	*0.372*							
F04b	Heat shock 90-kDa protein A (HSP90A)	**6.06**	**7.98**	**1.33**	**0.08**		**0.22**	*0.412*	**0.39**	**0.68**	**1.53**	**0.07**	**3.91**		*0.099*
F04k	Heregulin-beta3; glial growth factor; neuregulin	**1.38**	**1.28**	**0.36**	**0.63**		**0.26**	*0.306*							
F04l	Proteasome component C8	**4.99**	**6.25**	**1.28**	**0.11**		**0.26**	*0.412*	**0.12**	**0.17**	**1.15**	**0.58**	**9.52**		*0.081*
F05b	27-kDa heat-shock protein (HSP27)	**6.82**	**9.11**	**1.38**	**0.05**		**0.20**	*0.410*	1.68	0.37	1.55	0.07		0.92	*0.594*
F05e	Hepatoma-derived growth factor (HDGF)	**1.35**	**1.42**	**0.61**	**0.26**		**0.45**	*0.465*							
F05k	alpha-1-antitrypsin precursor								**0.02**	**0.02**	**0.11**	**0.17**	**5.95**		*0.432*
F06c	Eosinophil granule major basic protein precursor	**0.32**	**0.47**	**0.00**	**0.01**		**0.01**	*0.365*							
F07b	Cytosolic superoxide dismutase 1 (SOD1)	**5.38**	**7.02**	**0.90**	**0.40**		**0.17**	*0.385*	**0.16**	**0.20**	**1.15**	**0.18**	**7.23**		**0.003**
F07e	Neuroleukin (NLK)	**3.00**	**3.40**	**1.09**	**0.17**		**0.36**	*0.431*	**0.15**	**0.20**	**0.92**	**0.29**	**5.96**		**0.024**
F08a	Thioredoxin peroxidase 2 (TDPX2)	**4.94**	**6.77**	**0.81**	**0.24**		**0.16**	*0.401*							
F10e	T-cell-specific rantes protein precursor	**0.36**	**0.37**	**0.12**	**0.08**		**0.33**	*0.376*	**0.15**	**0.15**	**1.24**	**0.49**	**8.46**		**0.050**
F10f	Embryonic growth/differentiation factor 1								**0.04**	**0.02**	**0.11**	**0.13**	**2.79**		*0.445*
F10i	Interleukin-1 beta precursor (IL-1 ; IL1B); catabolin	**2.06**	**2.21**	**0.88**	**0.09**		**0.43**	*0.453*	0.10	0.01	0.19	0.22	1.87		*0.564*
F13b	Glutathione S-transferase mu1 (GSTM1; GST1)	**0.53**	**0.70**	**0.09**	**0.08**		**0.16**	*0.386*							
F13f	Thymosin beta-10	**6.83**	**9.12**	**1.38**	**0.06**		**0.20**	*0.410*	1.69	0.39	1.54	0.07		0.91	*0.572*
F13j	Thymosin beta 4	**6.82**	**9.10**	**1.38**	**0.06**		**0.20**	*0.410*							
F14b	Glutathione S-transferase A1	**0.69**	**0.85**	**0.12**	**0.15**		**0.18**	*0.369*							

a) Gene code according to the location on cDNA Array
b) Genes in bold were considered as down- or up-regulated as their ratio of exposed/sham was either ≥2 or ≤0.5
c) Ratios in bold were either ≥2 (red; increase in expression) or ≤0.5 (blue; decline in expression)
d) Purple figures in bold were statistically significant changes ≤0.05

Table 2. List of genes that in response to mobile phone radiation, have statistically significant altered expression.

Cell Line	Code[a)]	GENE NAME	Sham average ± SD		Exposed average ± SD		Ratio exposed/sham ↑	↓	*t*-test	Potential targets of RF-EMF
EA.hy926	C12a	Serine/threonine protein phosphatase	1.16	0.18	0.46	0.19		0.40	0.010	yes
EA.hy926v1	A07d	fos-related antigen (FRA1)	0.99	0.21	1.48	0.07	1.49		0.043	?
	A09b	c-myc purine-binding transcription factor puf	0.97	0.25	1.54	0.07	1.59		0.052	?
	A12l	Transducer of erbB2 (TOB)	0.07	0.09	0.62	0.19	8.52		0.022	yes
	A13l	p55CDC	0.15	0.21	1.00	0.27	6.57		0.014	yes
	B08m	Transforming protein rhoB	0.07	0.13	0.45	0.17	6.19		0.040	yes
	B10m	ras-related protein RAB-7	0.16	0.26	1.25	0.26	7.63		0.007	yes
	C05k	Cytoplasmic dynein light chain 1 (HDLC1)	0.32	0.24	1.23	0.25	3.88		0.010	yes
	D08i	Major prion protein precursor (PRP)	1.10	0.11	1.44	0.07	1.30		0.016	?
	D14j	RNA polymerase II transcriptional coactivator p15	0.30	0.34	1.28	0.45	4.25		0.044	yes
	E12n	Glutathione S-transferase pi (GSTP1; GST3)	1.23	0.15	1.55	0.07	1.25		0.050	?
	F07b	Cytosolic superoxide dismutase 1 (SOD1)	0.16	0.20	1.15	0.18	7.23		0.003	yes
	F07e	Neuroleukin (NLK)	0.15	0.20	0.92	0.29	5.96		0.024	yes
	F10e	T-cell-specific rantes protein precursor	0.15	0.15	1.24	0.49	8.46		0.050	yes

a) Gene code according to the location on cDNA array

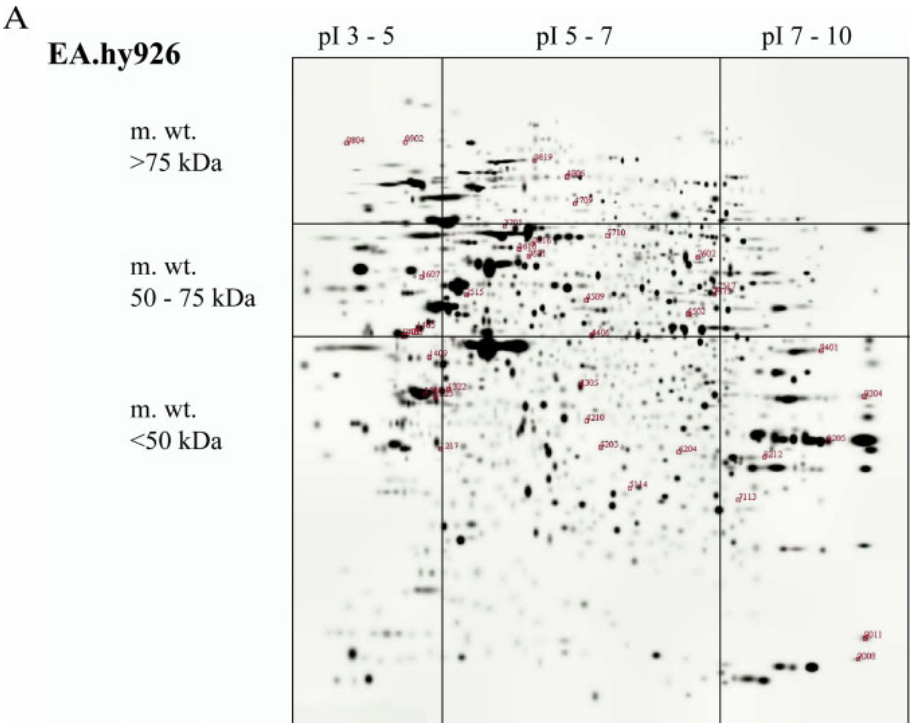
4 Discussion

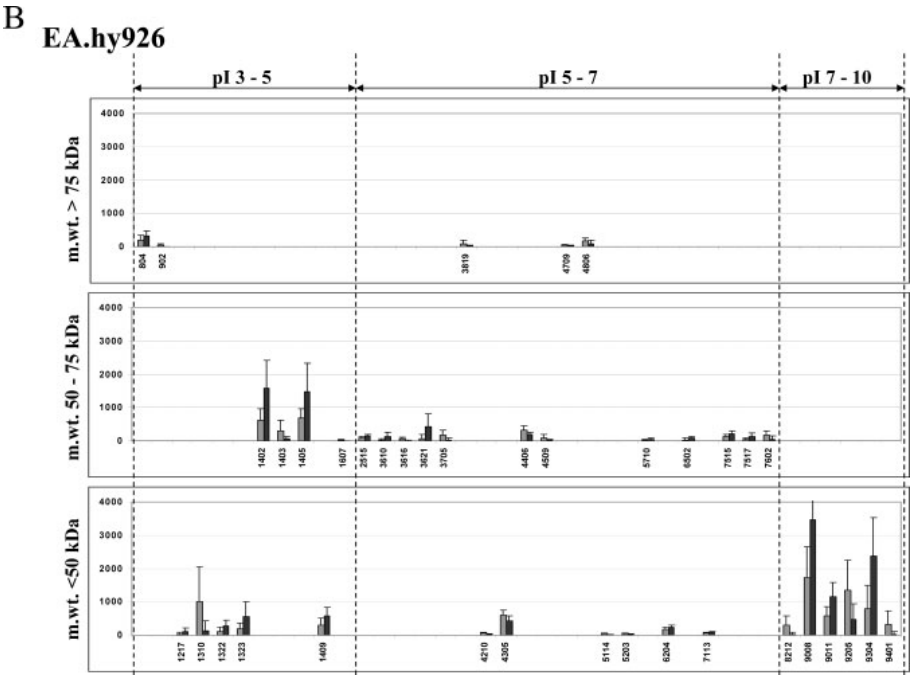
In spite of years of research, there is still an uncertainty about whether low-energy EMF can induce biological effects that would be able to alter cell physiology. Importantly, even when biological effects have been reported, the biophysical mechanisms behind their occurrence are still unknown. We have postulated that the combined use of the genome-wide and proteome-wide screening techniques of proteomics and transcriptomics (the so-called "Discovery Science" approach; [9]) will be a useful approach to determine a broad variety of biological targets of RF-EMF on the sub-cellular level, both *in vitro* and *in vivo*. These targets could then be used to formulate new health-related hypotheses for testing in animal and human-volunteer studies.

Search through publication databases shows that only very few studies have used methods of transcriptomics and proteomics to determine gene or protein responses to EMF exposure. Only four studies from two research groups have used this approach to examine effects of mobile phone radiation exposure [3–5, 12, 13]. Few of the studies that examined effects of electromagnetic fields have presented data obtained using the transcriptomics examined changes in the protein approach [10–15] and few have used the proteomics approach [3–5, 14, 16, 17]. Interestingly, only in two proteomics studies [4, 16] activity by determining changes in phospho-proteome. Altogether, the number of published studies is still very low and the data is insufficient to make any generalizing conclusions as to the biophysical and cell-wide biological impact of EMF radiation (including mobile phones radiation) on living organisms. It is also necessary to remember that some of the published studies, most likely due to limited funding, either suffer from technical problems or are based on a single experiment, which significantly diminishes their scientific value.

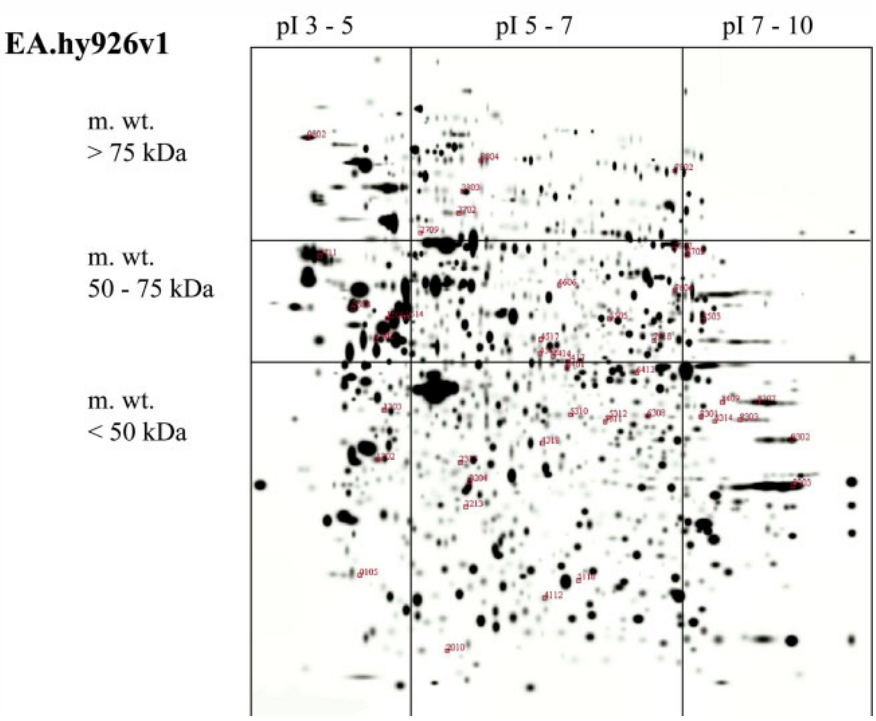
We have previously shown that exposure of EA.hy926 human endothelial cell line to 900 MHz mobile phone radiation induces activation of the p38 MAP kinase stress response pathway and leads to an increase in expression and phosphorylation of the small stress response protein Hsp27 [4]. We have also shown that cells' response to the mobile phone radiation affects components of the cytoskeleton, the F-actin stress fibers [3–5], that are stabilized in the presence of phosphorylated Hsp27. In other studies, it has been shown that the phosphorylated form of Hsp27 has the ability to translocate to the nucleus and to induce changes in gene expression [18, 19]. Thus, we examined whether the mobile phone radiation exposure, that induces Hsp27 phosphorylation, will cause changes in gene expression and, subsequently, changes in protein expression.

Figure 2. Effect of mobile phone radiation on protein expression in EA.hy926 and EA,hy926v1 cell lines. Each result is a compilation of ten different experiments. (A): PDQuest-generated 2-DE artificial gel of proteins extracted from human endothelial cell line EA.hy926. The 1-D IEF pH gradient 3–10 NL, 2-D 8% SDS-PAGE. Statistically significantly (*t*-test $p < 0.05$; $n = 10$) differentially expressed spots are numbered using PDQuest SSP numbers. In EA.hy926 cell line 38 statistically significantly differing spots were detected (numbered). Corresponding bar graphs of the statistically significantly changed protein spots in EA.hy926 cells are shown in (B). (C): PDQuest-generated 2-DE artificial gel of proteins extracted from human endothelial cell line EA.hy926v1. The 1-D IEF pH gradient 3–10 NL, 2-D 8% SDS-PAGE. Statistically significantly (*t*-test $p < 0.05$; $n = 10$) differentially expressed spots are numbered using PDQuest SSP numbers. In EA.hy926 cell line were detected 45 statistically significantly differing spots (numbered). Corresponding bar graphs of the statistically significantly changed protein spots in EA.hy926 cells are shown in (D).

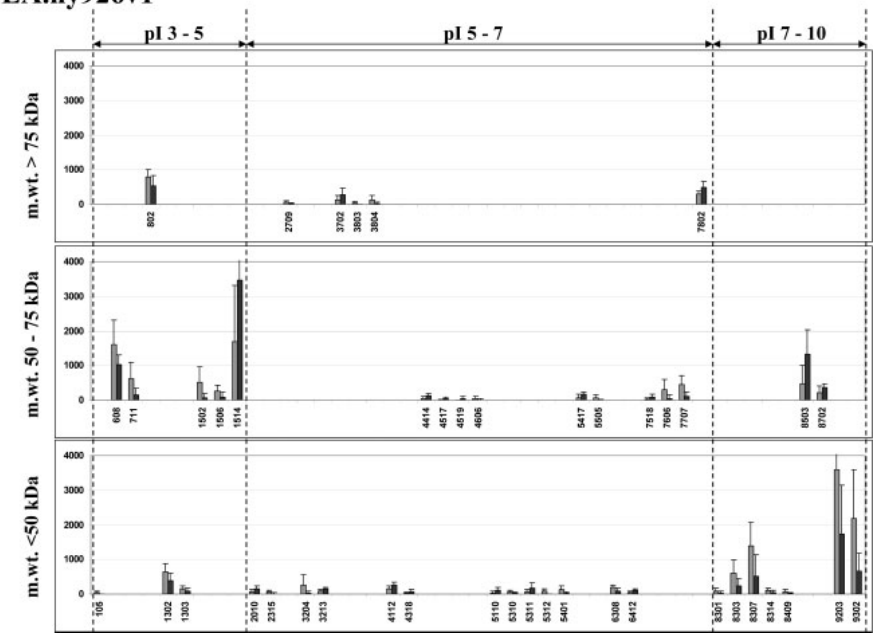




C



D



The results of our study suggest that mobile phone radiation alters expression levels of numerous genes and proteins in both of the examined human endothelial cell lines. The broadness of the response suggests that a variety of cellular functions and cellular signaling pathways might be affected by the mobile phone radiation exposure. This observation agrees with our previously published study showing the changes in phosphorylation levels of hundreds of proteins, which were induced by mobile phone radiation exposure [4]. Also, our results are in agreement with the earlier published study suggesting that mobile phone radiation exposure affects gene expression in human fibroblasts [12].

Another observation of this study is that both human endothelial cell lines have responded differently to the same exposure. This opens the possibility to hypothesize that cells, depending on their origin, functions and metabolic status at the time of exposure, might respond differently to the same mobile phone radiation exposure. As shown here, different genes and different proteins responded to the mobile phone radiation in both endothelial cell lines used in our study. At the present, we do not know which of the genes and proteins might be responsible for the observed diversity of the response. Interestingly, expression of p38 MAP kinase gene is 10-fold higher in EA.hy926 cell line than in the EA.hy926v1 cell line (data not shown). We have shown previously that p38 MAP kinase protein is activated by the RF-EMF exposure [3, 4]. Whether the 10-fold difference in p38 MAP kinase gene expression between both cell lines has any influence on the observed difference in the gene and protein response between the examined human endothelial cell lines remains to be determined.

In conclusion, our results suggest that the mobile phone radiation causes broad changes in gene and protein expression *in vitro*. The difference in response observed between closely related cells suggests the possibility that the genome and proteome expressed by the cells at the time of exposure to mobile phone radiation might play a role in cellular responses. This observation also suggests that the genome- and proteome-dependent differences in cell response to mobile phone radiation might be, at least in part, responsible for the failures of replication experiments in different laboratories. It might be that the same cell lines, when grown in different laboratories for extended periods of time, spontaneously acquire new properties that are responsible for the different behavior of cells under exposure to mobile phone radiation. Finally, the hypothesis of the possible link between cell genome and proteome, and the cellular response to mobile phone radiation supports the possibility that some cell types or cells expressing certain pattern of genes and/or proteins might be in some cases more sensitive and in some cases less sensitive to the mobile phone radiation. Such possibility might not be surprising, as it is well known that genotypically different cells might respond differently to the same stimulus,*e.g.* ionizing radiation, chemicals, carcinogens, drugs *etc.*, for reviews see [20–23].

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IV

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Proteomic Analysis of the Response of Human Endothelial Cell Line EA.hy926 to 1800 GSM Mobile Phone Radiation

Reetta Nylund[1], Hanna Tammio[1], Niels Kuster[2], Dariusz Leszczynski[1,*]

[1]STUK - Radiation and Nuclear Safety Authority, Helsinki, Finland
[2]IT'IS Foundation, Swiss Federal Institute of Technology, Zurich, Switzerland

Abstract

Background: We have earlier shown that exposure of human endothelial cell line EA.hy926 to 900 MHz GSM mobile phone radiation causes changes in the expression of numerous proteins. Here, we have examined the effects of 1800 MHz GSM mobile phone signal on the proteome of the same cell line.

Results: EA.hy926 cells were exposed for one hour to 1800 MHz GSM signal, simulating mobile phone talking conditions, at an average specific absorption rate (SAR) of 2.0 W/kg at 37±0.3°C. Sham samples were produced simultaneously in the same conditions but without the radiation exposure. Cells were harvested immediately after 1-hour exposure to the radiation, and proteins were extracted and separated using 2-dimensional electrophoresis (2DE). In total, 10 experimental replicates were generated from both exposed and sham samples. About 900 protein spots were detected in the 2DE-gels using PDQuest software and eight of them were found to be differentially expressed in exposed cells ($p<0.05$, t-test). Three out of these eight proteins were identified using Maldi-ToF mass spectrometry (MS). These proteins are: spermidine synthase (SRM), 78 kDa glucose-regulated protein (55 kDa fragment) (GRP78) and proteasome subunit alpha type 1 (PSA1). Due to the lack of the availability of commercial antibodies we were able to further examine expression of only GRP78. Using SDS-PAGE and western blot method we were not able to confirm the result obtained for GRP78 using 2DE. Additionally, we have not seen any effect of 1800GSM exposure on the expression of vimentin and Hsp27 - proteins that were affected by the 900 MHz GSM exposure in our earlier studies.

Conclusions: Our results suggest that the 900GSM and 1800GSM exposures might affect the expression of some proteins in the EA.hy926 cell line. The observed here discrepancy between the expression changes of GRP78 detected with 1DE and 2DE confirms the importance of validation of the results obtained with 2DE using other methods, e.g. western blot.

Abbreviations: 2DE: Two-dimensional electrophoresis; CHAPS: 3-[(3- Cholamidopropyl)dimethylammonio]-1-propanesulfonate; Da: Dalton; ddH_2O: Double distilled water; DMEM: Dulbecco's Modified Eagle's Medium; DTT: Dithioreitol; EA.hy926: Human endothelial cell line; ECL Enhanced chemiluminescence; GSM: Global System for Mobile Communications; HAT: (mixture of) sodium hypoxanthine, aminopterin, and thymidine; HRP: Horseradish peroxidase; IAA: Iodoacetamide; IEF: Isoelectric focusing; IPG: Immobilized pH gradient; LR: Linear-reflectron; MALDI-TOF: Matrix-assisted laser desorption/ionization time of flight; MS: Mass spectrometry/ mass spectrometer; NH_4HCO_3: Ammoniumbicarbonate; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate buffered saline; pI: Isoelectric point; PMF: Peptide mass fingerprint; PMSF: Phenylmethylsulphonyl fluoride; PVDF: Polyvinylidene Fluoride; RF-EMF: Radiofrequency modulated electromagnetic field; SAR: Specific absorption rate; SDS: Sodium dodecyl sulphate; Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride; Versene: Chelating agent containing EDTA

Background

The use of mobile phones has widely increased over the past decade. However, the issue of potential health effects induced by mobile phone radiation remains controversial and further research is needed to fill-up the existing gaps in the knowledge about the biological and physiological effects of this low-level energy radiation.

We have proposed that the use of high-throughput screening techniques of transcriptomics and proteomics, as tools to find genes and proteins responding to mobile phone radiation, might help the process of finding out whether mobile phone radiation might cause any health risk (Leszczynski and Joenväärä, 2001; Leszczynski, 2006; Leszczynski and Meltz, 2006). Proteomics approach has been so far used only in a few *in vitro* studies (Leszczynski et al., 2002; Leszczynski et al., 2004; Nylund and Leszczynski, 2004; Nylund and Leszczynski, 2006; Zeng et al., 2006; Li et al., 2007) and in a single *in vivo* human volunteer study (Karinen et al., 2008). Such a small number of published studies does not allow for making any generalized conclusions about of the possible effects of mobile phone exposures on the cell proteome and on the cell physiology. Only by performing more of this kind of studies, the proteomic database can be

***Corresponding author:** Dariusz Leszczynski, PhD, STUK-Radiation and Nuclear Safety Authority, Laippatie 4, 00880 Helsinki, Finland, E-mail: dariusz.leszczynski@stuk.fi

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expanded and, with the help of that, the impact of mobile phone radiation on cell proteome will be possible to assess.

We have previously determined that the 900 MHz GSM mobile phone radiation signal alters expression of several tens of proteins in the human endothelial cell line EA.hy926 (Leszczynski, et al., 2002; Nylund and Leszczynski, 2004; Nylund and Leszczynski, 2006). In the present study we have examined whether the 1800 MHz GSM mobile phone radiation signal exposure will also affect protein expression in EA.hy926 cells. Protein expression was determined using 2DE proteomics and results were compared with the earlier study that used 900 MHz GSM mobile phone radiation.

Materials and Methods

In Vitro Cell Model and Cell Culture Conditions

Brain capillary endothelial cells are one of the potential targets of the mobile phone radiation. In some animal studies it has been shown that mobile phone radiation might affect function of the blood-brain barrier. That is why we have selected to examine *in vitro* effects of mobile phone radiation on endothelial cells. Human endothelial cell line EA.hy926 was selected because of the uniformity of cell cultures from batch to batch and because of easy and fast means to generate large quantities of cells for experiments. Neither of the above is possible to achieve with primary endothelial cells, known for slow growth and for the variability between batches isolated from different human donors.

Human endothelial cell line EA.hy926 (a gift from Dr. Cora-Jean S. Edgell North Carolina University at Chapel Hill, NC, USA) was grown in Dulbecco's MEM (DMEM), supplemented with antibiotics, 10% foetal bovine serum, L-glutamine and HAT-supplement (Sigma, USA). For the mobile phone radiation experiments, cells were removed from culture flasks by brief trypsinization, washed in cell culture medium and seeded at a density of 0.4×10^6cells/dish in 35 mm-diameter Petri dishes (NUNC, Denmark). After an overnight culturing the semi-confluent monolayers of EA.hy926 were exposed to mobile phone radiation or sham exposed.

Exposure to Mobile Phone Radiation Signal

The sXc-1800 exposure system, developed and provided by the IT'IS Foundation and installed at STUK (Helsinki), was employed (Figure 1). This consists of two identical exposure chambers mounted in the same cell culture incubator. It is fully automated and enables exposures of cells in monolayers (H-polarization or at H-field maximum of the standing wave) at freely programmable amplitude modulations. The exposure chambers are based on resonant R18 waveguides, allowing for SAR values of several hundred W/kg at the cell monolayer level with a few watts input power. The identical environmental conditions (temperature, humidity, CO_2) are achieved in both exposure chambers because the inlet of the airflow to both chambers is at the same location. The system monitors, every 10 seconds, the incident field strengths, the proper functioning of the ventilators, the outlet air temperatures and the functional state of the whole exposure set-up. The Pt100 temperature sensors (accuracy ±0.1 °C) have been calibrated prior to the installation and the recorded differences in temperature are well

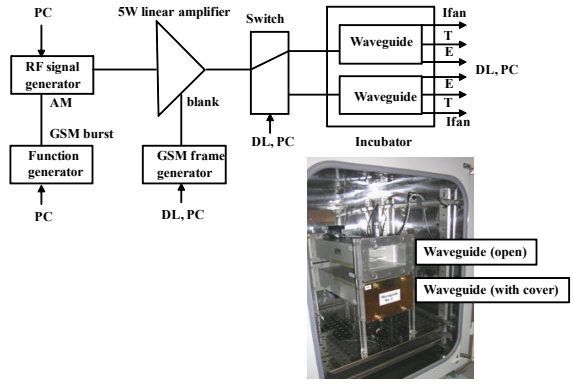

Figure 1: A diagram of sXc1800 mobile phone radiation exposure system (E: E-field sensors, T: temperarure sensors, Ifan: fan current sensors, DL: data logger i/o, PC: personal computer via GPIB) and photo of the waveguides inside a cell culture incubator.

within the specified long-term stability of the calibration. The induced temperature load due to mobile phone radiation absorption has been characterized as a function of SAR (t) for different signals and volumes of medium. This enables a reliable estimate of the maximum temperature rise as a function of the exposure. The ambient electromagnetic field of the cell culture incubator was measured in several positions within the incubator using an EFA-3 field measurement system (Wandel & Goltermann, Germany). Further details of the exposure system are described elsewhere (Schuderer et al., 2004). The signal applied in this study was GSM Talk. GSM Talk signal is characterized by a random change between the discontinuous transmission mode (DTX) and non-DTX or GSM Basic phases. The distribution in time was exponential with a mean duration of 10.8 seconds for non-DTX and 5.6 seconds for DTX. The dominant modulation components of this signal are 2, 8, 217, 1733 Hz, and higher harmonics. The more detailed description of the signal can be found elsewhere (Tillmann et al., 2006).

After overnight cultivation, the semi-confluent monolayers of EA.hy926 cells were placed in two 6-dish holders and inserted into the exposure chambers. In one of the exposure chambers, randomly selected by the system's computer, the cells were exposed to an average SAR of 2.0 W/kg at 37±0.3°C (to assure examination of non-thermal effects), while in the other chamber they were sham-exposed, in the similar conditions but without mobile phone radiation signal exposure. Precise control of the temperature of the cell cultures during the exposure to mobile phone radiation is of paramount importance to assure that the temperature increases are not responsible for the observed effects. Therefore, because in our experiments the temperature of cell cultures did not increase by more than 0.3°C we can state that the observed effects are of non-thermal nature (are not caused by any significant temperature increase). The experiments were performed in the blinded manner and the code was broken after the files from the exposure system were sent to IT'IS, Zurich, Switzerland.

Protein Extraction

Immediately after the end of the 1-hour exposure cells were

quickly washed with PBS and harvested with versene. Proteins were extracted with a buffer consisting of 8 M Urea, 1 M Thiourea, 4% Chaps, 10 mM DTT, 2% IPG buffer pH 4-7, 1 mM sodium orthovanadate and 1 mM PMSF. Protein concentrations were measured using Bradford method. The 250µg of total protein was used for two-dimensional gel electrophoresis (2DE).

2DE

The isoelectric focusing was performed using an IPGphor apparatus (GE Healthcare, USA) and 24 cm long ready IEF strips pH 4-7 (GE Healthcare). The samples were loaded using in-gel rehydration in a buffer containing 9 M Urea, 2% Chaps, 0.2% DTT, 0.5% IPG buffer pH 4-7 for 4 hours. IEF was run at 20°C using step-and-hold methods as follows: 50 V 8 h; 100 V 1 h; 500 V 1 h; 1000 V 1 h; 2000 V 1 h; 8000 V until 95000 Vhrs were achieved. Before SDS-PAGE the IEF strips were equilibrated for 15 min with 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, and 10 mg/mL DTT and then for another 15 min in the same buffer, in which DTT was replaced by 25 mg/mL iodoacetamide (IAA). SDS-PAGE was run in 10% gel using Ettan DALTsix Electrophoresis system (GE Healthcare) at the constant power setting of 3.5W/gel for the first 0.5 hours and then 13W/gel. After electrophoresis the gels were silver stained. Gels were fixed (30% ethanol, 0.5% acetic acid), washed with 20% ethanol and ddH2O, sensitized with sodium thiosulfate (0.2 g/L), incubated in the silver nitrate solution (2 g/L) and developed (potassium anhydride 30 g/L, 37% formaldehyde 0.7 mL/L, sodium thiosulfate 0.01 g/L). The development was stopped with Tris 50 g/L + 0.5% acetic acid, and then the gels were washed twice with ddH2O and scanned.

Data Analysis

The silver stained gels were scanned using GS-710 densitometer (Bio-Rad, USA) and analyzed using PDQuest 7.2 software (Bio-Rad). In total, ten gels from both sham and exposed samples were analysed. The normalized spot volumes of the proteins from sham and exposed sample gels were statistically analyzed using student *t*-test at the confidence level of 95%. Protein spots, that visually appeared as technical artefacts (e.g. background areas of silver staining, irregular-shaped dust particles, air bubbles) but were erroneously detected by the software, were manually removed from the analysis.

In-gel Digestions for Mass Spectrometry Protein Identification

Proteins of interest were extracted from several gels and in-gel digested. Before digestion the proteins were reduced with 20 mM DTT in 0.1M ammonium-bi-carbonate (NH_4HCO_3) and alkylated with 55 mM IAA in NH_4HCO_3. Proteins were digested overnight at +37°C with modified trypsin (sequencing grade modified trypsin, porcine, Promega, USA) in 50 mM NH_4HCO_3. After overnight digestion, resulting peptides were extracted from gels with 25 mM NH_4HCO_3 and twice with 5% formic acid. Peptides were concentrated and de-salted using C-18 ZipTips (Millipore, USA) according to the manufacturer's instructions with the exception of elution solution (60% acetonitrile).

Mass Spectrometry Identification of Proteins

Tryptic digestions were mixed 1:1 with α-cyano-4-hydroxycinnamic acid matrix and analyzed with MALDI-TOF-LR-MS (Waters, USA) operating in a positive ion reflectron mode. The mass spectra were externally calibrated with ACTH clip 18-39 (MW 2465.199 Da, Sigma, USA) and internally calibrated with trypsin autolysis peaks (1045.564/2211.108 Da). The peptide mass fingerprints for protein identification were searched automatically at the accuracy of 20-50ppm from UniProt database with ProteinLynx-software (Waters) operating along the instrument. Statistically significantly affected proteins were also searched manually using Matrix Science Mascot Peptide Mass Fingerprint search tool (www.matrixscience.com).

Western Blotting

Immediately after the end of the RF-EMF exposure the cells were washed with PBS and harvested with versene. Proteins were extracted with 2% SDS, 1% protease inhibitor cocktail (Sigma, USA). Protein concentrations were measured using Lowry method (Bio-Rad). In total, five replicates were produced. Proteins were separated on 7.5% (GRP78) or 10% (Hsp27, Vimentin) 1D SDS-PAGE and blotted on a PVDF-membrane, blocked with 2% non-fat dry milk, and exposed to primary antibody. The polyclonal Bip (GRP78, Cell Signalling Technology, USA), monoclonal Hsp27 (StressGene, Canada), and vimentin (Zymed, USA) antibodies were used. The respective secondary antibody containing a horseradish peroxidase (HRP)-conjugate (Dako, Denmark) was used. The signal was detected using enhanced chemiluminescence (ECL) (Millipore, USA). Autoradiography films were scanned with GS-710 densitometer (Bio-Rad) and analysed with Phoretix software (Molecular Probes, USA).

Results and Discussion

In this study we have examined protein expression levels in EA.hy926 cells after the exposure to 1800 MHz GSM mobile phone radiation. Protein expression pattern of EA.hy926 cells was analysed using 2DE with the pH range of 4 - 7 and the gel percentage of 10%, allowing a good separation at the molecular weight (MW) range of approximately 15-150 kDa. In total, 10 replicates were generated from both exposed and sham samples. Such high number of replicates is necessary in order to diminish technical and biological variability, when using silver staining technique to visualize proteins in 2DE gels.

Using PDQuest 7.2 software, about 900 protein spots were detected in the gels. Protein spots, that visually appeared as technical artefacts but were detected by the software, were manually removed from the analysis. Statistical significance of the observed differences in proteins expression levels was determined using student t-test, at the confidence level of 95%, with the assumption of the independent samples. The analysis has revealed eight protein spots which were found to be differentially expressed ($p<0.05$) (Figure 2). Expression of the four of the proteins was found to be down-regulated and four up-regulated by the mobile phone radiation exposure. Down-regulation ratios varied between 0.33-0.47 and up-regulation ratios varied from 1.47 to 2.46.

Comparison of the changes in protein expression pattern observed here and in the earlier study (Nylund and Leszczynski, 2004), shows that exposure to 900 MHz GSM signal has caused expression changes in a larger number of proteins spots and the

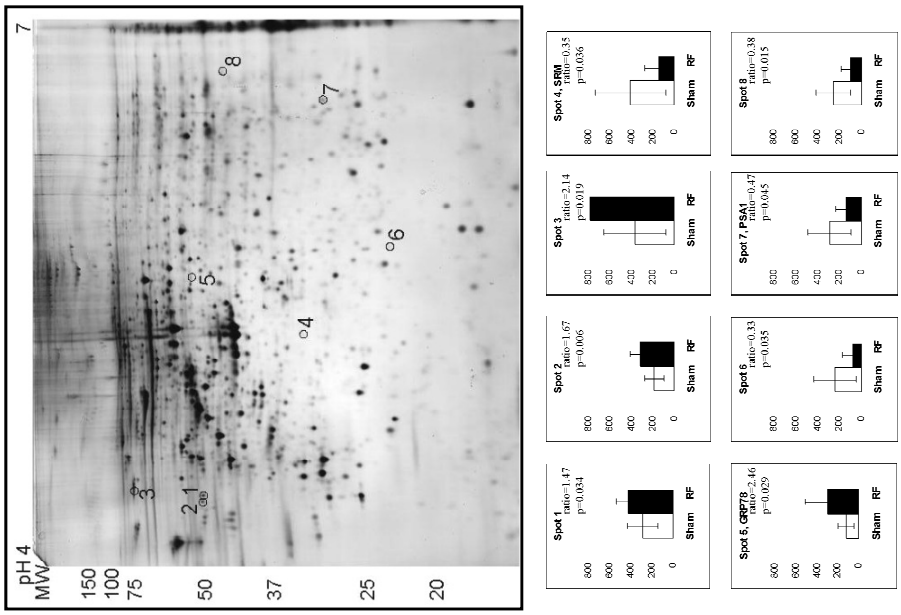

Figure 2: A representative gel image showing protein spots with altering expression levels and histograms showing average expression levels and standard deviations of the sham and exposed samples as well as ratio between RF and sham exposed sample (ratio >1 describes up-regulation and ratio <1 down-regulation of the protein). Also t-test p-values are shown.

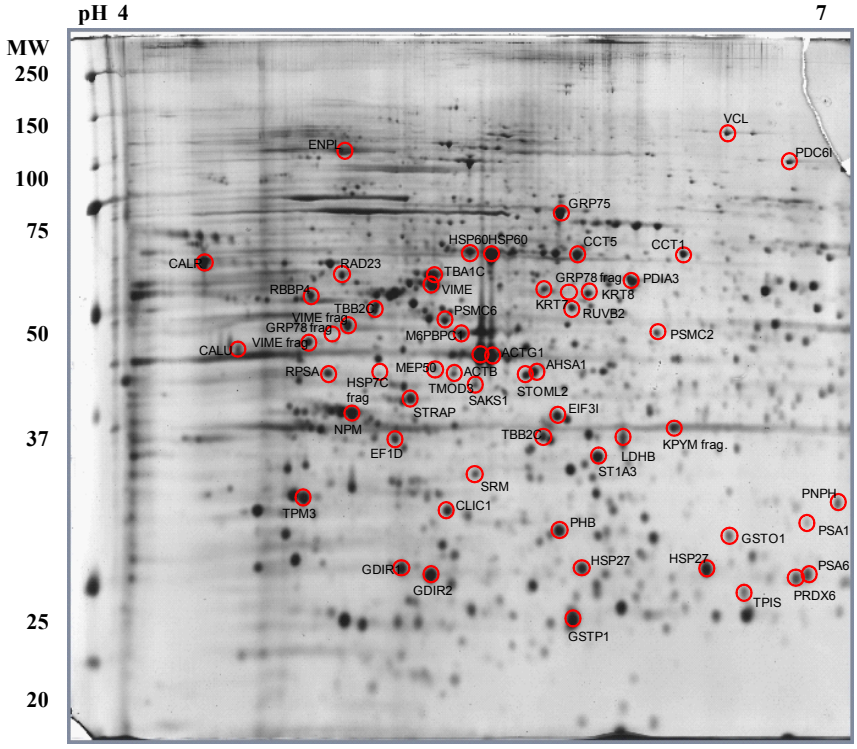

Figure 3: A gel image showing the identified protein spots in the EA.hy926 2DE map.

Gene name	Protein name	Access code	Sequence coverage (%)	MW(kDa)/pI theoretical	MW(kDa)/pI measured
ACTB	Actin, cytoplasmic 1	P60709	31.5	41.7/5.4	43/5.4
ACTG1	Actin, cytoplasmic 2	P63261	40	41.8/5.4	43/5.5
AHSA1	Activator of 90 kDa heat shock protein ATPase homolog 1, p38	O95433	72.8	38.3/5.5	40/5.8
CALR	Calreticulin precursor	P27797	49	48.1/4.3	58/4.3
CALU	Calumenin	O43852	45	37.1/4.5	44/4.5
CLIC1	Chloride intracellular channel protein 1	O00299	46.5	26.9/5.1	29/5.3
CCT1/ TCPA	T-complex protein 1 subunit alpha	P17987	60.3	60.3/6.0	60/6.3
CCT5/ TCPE	T-complex protein 1 subunit epsilon	P48643	48.2	59.6/5.6	60/5.9
EF1D	Elongation factor 1-delta	P29692	44.8	31.1/4.9	36/5.2
EIF3I	Eukaryotic translation initiation factor 3 subunit I	Q13347	23	36.5/5.4	37/5.8
ENPL	Endoplasmin precursor	P14625	27.5	92.4/4.8	120/4.9
GDIR1	Rho GDP-dissociation inhibitor 1	P52565	52.5	23.2/5.0	26/5.2
GDIR2/ ARHGDIB	Rho GDP-dissociation inhibitor 2	P52566	35.3	23.0/5.1	25/5.3
GRP75	Stress-70 protein, mitochondrial (Precursor)	P38646	56	73.6/6.1	74/5.8
GRP78 (frag.)	78kDa glucose-regulated protein (Precursor) (frag)	P11021	26	72.4/5.1	54/5.9
GRP78 (frag.)	78kDa glucose-regulated protein (Precursor) (frag)	P11021	33.6	72.4/5.1	48/4.8
GSTO1	Glutathione transferase omega-1	P78417	45.6	27.5/6.6	27/6.5
GSTP1	Glutathione S-transferase P	P09211	53.8	23.3/5.5	23/5.9
HSP27	Heat shock protein beta-1	P04792	48.3	22.8/6.3	26/5.9
HSP27	Heat shock protein beta-1	P04792	37.1	22.8/6.3	26/6.4
HSP60	60 kDa heat shock protein	P10809	51	61.0/5.8	61/5.6
HSP60	60 kDa heat shock protein	P10809	52.7	61.0/5.8	61/5.4
HSP7C frag.	Heat shock cognate 71 kDa protein (frag)	P11142	25.7	71.2/5.4	40/5.1
KPYM frag.	Pyruvate kinase isozymes M1/M2 (frag)	P14618	46.5	58.0/8.2	36/6.3
KRT7	Keratin, type II cytoskeletal 7	P08729	64.8	51.4/5.6	54/5.8
KRT8	Keratin, type II cytoskeletal 8	P05787	57.1	53.7/5.6	54/5.9
LDHB	L-lactate dehydrogenase B chain	P07195	46.7	36.5/6.0	36/6.1
M6PBP1C	mannose-6-phosphate receptor binding protein 1C	O60664	64.3	47.0/5.4	48/5.4
MEP50	Methylosome protein 50	Q9BQA1	31.6	36.7/5.1	41/5.3
NPM	Nucleophosmin	P06748	44.6	32.5/4.7	37/4.9
PDCD6IP	Programmed cell death 6-interacting protein	Q8WUM4	58.8	96.0/6.4	105/6.8
PDIA3	Protein disulfide-isomerase A3 (Precursor)	P30101	49.5	56.7/6.3	56/6.2
PHB	Prohibitin	P35232	42.6	29.8/5.7	28/5.8
PNPH	Purine nucleoside phosphorylase	P00491	59.5	32.1/6.9	30/6.9
PRDX6	Peroxiredoxin-6	P30041	48.7	25.0/6.3	25/6.8
PSA1	Proteasome subunit α type 1	P25786	27	29.5/6.6	28/6.8
PSA6	Proteasome subunit α type 6	P60900	52.8	27.4/6.7	26/6.8
PSMC3/ PRS6A	26S protease regulatory subunit 6A	P17980	85	49.2/5.2	49/5.3
PSMC2/ PRS7	26S protease regulatory subunit 7	P35998	53.6	48.6/5.9	48/6.2
RPSA	40S ribosomal prot SA	P08865	31.2	32.9/4.8	40/4.8
RAD23	UV excision repair protein RAD23 homolog B	P54727	27.1	43.1/4.8	57/4.9

RBBP4	Histone-binding protein BBP4	Q09028	31.1	47.7/4.8	53/4.8
RUVB2	RuvB-like 2	Q9Y230	69.1	51.1/5.6	52/5.9
SAKS1	SAPK substrate protein 1	Q04323	50.5	33.3/5.3	39/5.4
SRM	Spermidine synthase	P19623	19.9	33.8/5.4	33/5.4
ST1A3	Sulfotransferase 1A3/1A4	P50224	52.6	36.4/5.8	35/6.0
STOML2	Stomatin-like protein 2	Q9UJZ1	46.6	38.5/6.9	40/5.7
STRAP	Serine-threonine kinase receptor-associated protein	Q9Y3F4	56.3	38.4/5.0	38/5.2
TBA1C	Tubulin alpha-1C chain	Q9BQE3	44.5	49.9/5.0	58/5.3
TBB2C	Tubulin beta-2C chain	P68371	50.1	49.8/4.8	51/5.1
TBB2C (frag)	Tubulin beta-2C chain (frag)	P68371	35.3	49.8/4.8	36/5.8
TMOD3	Tropomodulin3	Q9NYL9	36.9	39.6/5.1	40/5.4
TPIS	Triosephosphate isomerase	P60174	80	26.7/6.9	24/6.6
TPM3	Tropomyosin 3	Q5VU58	69	29.2/4.8	30/4.8
VIME	Vimentin	P08670	78	53.6/5.1	54/5.3
VIME	Vimentin (fragment)	P08670	51.1	53.6/5.1	49/4.9
VIME	Vimentin (fragment)	P08670	66.3	53.6/5.1	47/4.8
VCL	Vinculin	P18206	32.8	123.7/5.6	130/6.5

Table 1: All proteins that were identified by MS in EA.hy926 2DE gels.

spot #	Expression (exposed/sham)	Protein name	Access code	Sequence coverage (%)	Mascot score
4	down	SRM	P19623	19.9	74
5	up	GRP78 fragment	P11021	26	101
7	down	PSA1	P25786	27	111

Table 2: Identified proteins that altered their expression after exposure to 1800 MHz GSM radiation.

changes induced by both exposures were detected in different proteins spots. Previously, using 900 MHz GSM signal, total of 38 protein spots were found to be affected after the mobile phone exposure (Nylund and Leszczynski, 2004), out of which 28 was in the pH range of 4 - 7, as compared with 8 proteins spots that were found here to be statistically significantly affected by 1800 MHz GSM exposure in the same pH range. The number of statistically significantly affected proteins is small (below the number of expected false positives). However, it is possible that some of these proteins might indeed be responding to mobile phone radiation. As shown in our earlier study (Nylund and Leszczynski, 2004), the number of statistically significantly affected proteins might be lower than the expected number of false positives but further analysis using western blot might show that some of the affected proteins (in that particular study - vimentin), might indeed respond to the mobile phone radiation.

Using peptide mass fingerprint (PMF) technique and Maldi-ToF MS, total of 50 protein spots were identified in 2DE gels of EA.hy926 exposed to 1800 MHz GSM mobile phone radiation (Figure 3; Table 1). Among the identified proteins were proteins that we have shown earlier to be affected by 900 MHz GSM radiation: vimentin and Hsp27 (Leszczynski et al., 2002; Nylund and Leszczynski, 2004). Expression of neither of them was altered in a statistically significant manner in 2DE by 1800 MHz GSM radiation (not shown).

Among the 50 identified protein spots were 8 proteins that expression was statistically significantly affected by 1800 MHz GSM radiation. Three of these eight protein spots were successfully identified (Table 2):

- spot #4 - spermidine synthase (P19623 SRM) (Wahlfors et al., 1990), regulates amine and bioamine biosynthesis,
- spot #5 - 78 kDa glucose regulated protein (fragment) (P11021 GRP78) (Ting and Lee, 1988), member of the heat shock protein 70 family, facilitates the assembly of multimeric protein complexes inside the endoplasmic reticulum. The molecular weight of this protein 72.4 kDa, while the affected protein spot observed here was only a fragment of ca. 55 kDa.
- spot #7 - proteasome subunit alpha type 1 (P25786 PSA1) (Silva-Pereira et al., 1992), is a part of large proteasome complex.

Identification of the other five proteins spots with Maldi-ToF was not successful due to low amount of protein in the spots.

Using western blot technique we have attempted to confirm the 2DE results for some of the proteins. Expression changes of GRP78 were examined using polyclonal antibody (Bip/GRP78, Cell Signalling Technology). Two protein bands were detected with MW of 75 kDa (represents the whole protein) and 55 kDa (represents GRP78 fragment identified from our 2DE gels). However, neither of the protein bands appeared to be affected by radiation exposure (Figure 4A). Thus, the western blot technique did not confirm the results obtained with 2DE. Two other identified proteins, SRM and PSA1, were not analyzed using western blot because the corresponding antibodies were not commercially available. Also the western blot experiments for vimentin and Hsp27 have shown a lack of effect of 1800 MHz GSM radiation. For vimentin, using the same antibody as previously (Nylund and Leszczynski, 2004), only a single band was observed in western blot, while in the earlier study the 900 MHz GSM radiation has caused appearance of an additional low-molecular weight vimentin band (Nylund and Leszczynski, 2004). For the single vimentin band observed here there was no

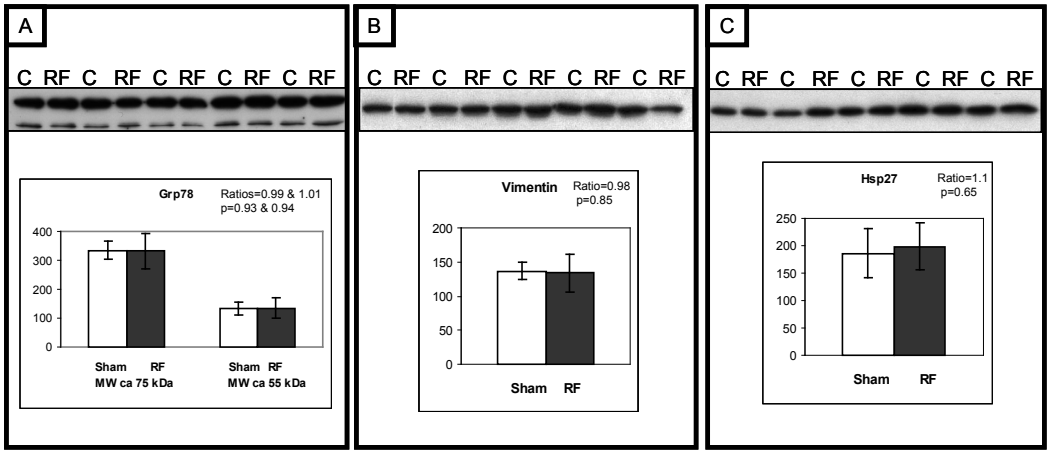

Figure 4: Western blots and densitogram bar-graph analyses (mean ± SD) for GRP78 protein (A), vimentin (B) and Hsp27 protein (C). For all western blots EA.hy926 cells were exposed for one hour to 2.0 W/kg 1800 MHz GSM signal using talk-conditions. The experiments were repeated five times. S = sham sample; RF = exposed sample.

change in the expression following the radiation exposure (Figure 4B). For Hsp27, the 2DE gel analyses have shown a statistically non-significant slight increase in the expression but western blot did not show any difference between Hsp27 expression in sham and exposed cells (Figure 4C).

Future Perspectives

In our previous and in the present study we have used two common mobile phone frequencies, 900 MHz and 1800 MHz, to determine if these radiation frequencies could have any impact on cell proteome. The observed here discrepancy between the responses of EA.hy926 cells to 1800 MHz GSM radiation and the previously published responses of EA.hy926 cells to 900 MHz GSM might be caused either by the different exposure frequencies or by technical differences between the exposure set-ups or by both of the above. The major difference, besides the frequency, between the 900 GSM and 1800 MHz GSM exposure chambers, appears to be the distribution of radiation field within the cell culture dish. In 900 MHz GSM set-up there was non-uniform SAR distribution (Leszczynski et al., 2002). It means that the cells growing in the certain areas of the culture dish were exposed to much higher SAR (over 5.0 W/kg) as compared to the average SAR for the whole cell culture dish (2.4 W/kg) (Leszczynski et al., 2002). In the contrast, the 1800 MHz GSM set-up had very uniform SAR distribution and the cells throughout the cell culture dish were exposed to the same level (2.0 W/kg) of radiation. The possibility of the field-distribution-related effect is supported by our new results showing that stress kinases are activated by the 1800 MHz radiation at 5.0 W/kg but not at 2.0 W/kg (manuscript in preparation). Therefore, there is a need to compare side-by-side the effects of 900 MHz and 1800 MHz frequencies on protein expression and on stress response in EA.hy926 cells using different SAR values.

Summary Conclusions

Our results suggest that the 900 MHz GSM and 1800 MHz GSM exposures might affect the expression of some proteins in the EA.hy926 cell line. The observed here discrepancy between the expression changes of GRP78 detected with 1DE and 2DE confirms the importance of validation of the results obtained with 2DE using non-high-throughput methods, as e.g. western blot. However, one serious limitation of this approach is the availability of specific antibodies or possession of an animal facility permitting to produce specific antibodies.

Authors' Contributions

RN developed the proteomics system used here, performed all the analyses presented here, and wrote the draft manuscript. HT performed the 2DE experiments. NK provided the exposure set-up used here. DL obtained the funding of the study and coordinated execution of this project and wrote the final version of the manuscript. All authors have read and approved the final version of the manuscript.

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Analysis of proteome response to the mobile phone radiation in two types of human primary endothelial cells
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Analysis of proteome response to the mobile phone radiation in two types of human primary endothelial cells

Reetta Nylund[1], Niels Kuster[2], Dariusz Leszczynski[1*]

Abstract

Background: Use of mobile phones has widely increased over the past decade. However, in spite of the extensive research, the question of potential health effects of the mobile phone radiation remains unanswered. We have earlier proposed, and applied, proteomics as a tool to study biological effects of the mobile phone radiation, using as a model human endothelial cell line EA.hy926. Exposure of EA.hy926 cells to 900 MHz GSM radiation has caused statistically significant changes in expression of numerous proteins. However, exposure of EA.hy926 cells to 1800 MHz GSM signal had only very small effect on cell proteome, as compared with 900 MHz GSM exposure. In the present study, using as model human primary endothelial cells, we have examined whether exposure to 1800 MHz GSM mobile phone radiation can affect cell proteome.

Results: Primary human umbilical vein endothelial cells and primary human brain microvascular endothelial cells were exposed for 1 hour to 1800 MHz GSM mobile phone radiation at an average specific absorption rate of 2.0 W/kg. The cells were harvested immediately after the exposure and the protein expression patterns of the sham-exposed and radiation-exposed cells were examined using two dimensional difference gel electrophoresis-based proteomics (2DE-DIGE). There were observed numerous differences between the proteomes of human umbilical vein endothelial cells and human brain microvascular endothelial cells (both sham-exposed). These differences are most likely representing physiological differences between endothelia in different vascular beds. However, the exposure of both types of primary endothelial cells to mobile phone radiation did not cause any statistically significant changes in protein expression.

Conclusions: Exposure of primary human endothelial cells to the mobile phone radiation, 1800 MHz GSM signal for 1 hour at an average specific absorption rate of 2.0 W/kg, does not affect protein expression, when the proteomes were examined immediately after the end of the exposure and when the false discovery rate correction was applied to analysis. This observation agrees with our earlier study showing that the 1800 MHz GSM radiation exposure had only very limited effect on the proteome of human endothelial cell line EA.hy926, as compared with the effect of 900 MHz GSM radiation.

Background

The use of mobile phones has widely increased over the past decade. In spite of the extensive research, the question of the possible health effects of the mobile phone radiation remains open. In 2001 we have proposed [1] and subsequently demonstrated [2] that proteomics could be used as a tool to find the protein targets that are affected by the mobile phone radiation. Based on the knowledge which proteins respond to the mobile phone radiation, new hypotheses about the possible biological effects might be put forward for testing. So far, the proteomics approach has been used only in a very few studies examining effects of the mobile phone radiation [2-10]. Therefore, based on this very limited material, it is not yet possible to draw any general conclusions about the effects of this radiation on cell proteome or on the physiological processes regulated by the affected proteins.

In our earlier studies we have determined that the 900 MHz GSM mobile phone radiation induces proteome

* Correspondence: dariusz.leszczynski@stuk.fi
[1]STUK - Radiation and Nuclear Safety Authority, Helsinki, Finland
Full list of author information is available at the end of the article

changes in human endothelial cell line EA.hy926 [2-5]. Furthermore, it appears that cell response to this radiation might depend on the transcriptome and proteome expressed by the cells at the time of exposure [5,11]. Using two variants of the EA.hy926 cell line, we have observed that the variants responded differently, on transcriptome and proteome level, to the same 900 MHz GSM signal [5]. On the other hand, we have observed that exposure of EA.hy926 cells to 1800 MHz GSM radiation had very low, if at all, statistically significant effect on cell proteome [8]. Therefore, it is unclear whether 900 MHz and 1800 MHz GSM radiation differ in their ability to induce biological effects.

In the present study, using primary human endothelial cells derived from two different vascular beds, we have examined cell responses to 1800 MHz GSM signal of mobile phone radiation. The examined cells were primary human umbilical vein endothelial cells (HUVEC) and primary human brain microvasculature endothelial cells (HBMEC). Both of the primary endothelial cell types were exposed for 1 hour to the 1800 MHz GSM mobile phone radiation at an average specific absorption rate (SAR) of 2.0 W/kg and harvested, as in our earlier studies [2-5,8], immediately after the end of exposure. The protein expression patterns in both cell types were examined using two dimensional difference gel electrophoresis (2D DIGE) -based proteomics [12].

Materials and methods

Cell culture and conditions

Primary human umbilical vein endothelial cells (HUVEC) were purchased form Lonza, Switzerland and cultivated according to manufacturer's instructions. The purchased HUVEC were a pool of cells from several donors. For mobile phone radiation experiments, cells were removed from culture flasks by brief trypsinization, washed in cell culture medium and seeded in the 35 mm-diameter "CellBIND" Petri dishes (Corning, USA). After overnight incubation the medium in the dishes was replaced with a fresh one and the monolayers of HUVEC (Figure 1A) were exposed to the mobile phone radiation in a special exposure chamber. The sham samples (unexposed control) were produced simultaneously in an identical sham exposure chamber (see below description of the system). Immediately after the end of exposure the cells were quickly washed with warm (37°C) PBS and harvested with warm versene solution. In total, 13 independent sham and exposed samples were generated from HUVEC in 13 different exposure experiments.

Primary human brain microvascular endothelial cells (HBMEC) were purchased from ScienCell Research Laboratories, USA and cultivated according to manufacturer's instructions. The purchased HBMEC were from a single donor and all cells used for the experiments were from the same batch. Before experiments cells were grown to confluency, detached with trypsin and seeded in the 35 mm-diameter "CellBIND" Petri dishes (Corning, USA) that were additionally coated with fibronectin (1.5%, overnight at 37°C) (Sigma, USA). Seventy-two hours after seeding the medium was replaced with a fresh one and the monolayers of HBMEC (Figure 1B) were exposed to the mobile phone radiation using the same exposure chamber as for HUVEC. Immediately after the end of exposure the cells were rinsed with warm PBS and harvested with trypsin. In total, 11 independent sham and exposed samples were generated from HBMEC in 11 different exposure experiments.

Mobile phone radiation exposure

The sXc-1800 exposure system, developed and provided by the IT'IS Foundation (Zurich, Switzerland) was used for exposing cells to 1800 MHz GSM signal (Figure 1C). The detailed description of the system and the dosimetry of it have been presented elsewhere [13]. Briefly: The system consists of two identical exposure chambers mounted inside the same cell culture incubator (NuAire US Autoflow CO_2 Water-Jacketed Incubator, NuAire, USA). One of the chambers acted as a sham control (no radiation) and the other as an experimental (with radiation). Sham exposure chamber and RF exposure chamber were randomly assigned by the computer program that controlled exposures. This computer program has generated during the experiment encrypted files with information in which of the two chambers was radiation and which acted as sham-control. These encrypted files were decoded after the experiment by chamber manufacturer, IT'IS, Zurich, Switzerland. This set-up permitted blinded execution of experiments. The exposure system is fully automated and enables controlled exposures of cells (H-polarization or at H-field maximum of the standing wave [14]) at freely programmable amplitude modulations. Identical environmental conditions existed in both chambers (sham and experimental) since they were both located in the same cell culture incubator and the inlets of the airflow through them are at the same location. At 10 seconds intervals the system has monitored the incident field strengths, the proper functioning of the ventilators, the outlet air temperatures and the state of all equipment. The Pt100 temperature sensors (accuracy ± 0.1 °C) have been calibrated prior to the installation and the recorded differences in temperature are well within the specified long-term stability of the calibration. The induced temperature load, due to radiation absorption, has been characterized as a function of SAR (t) for different signals and volumes of medium. This enables a reliable estimate of the maximum temperature rise as a function of the exposure [13]. SAR distribution within the cell culture dish was

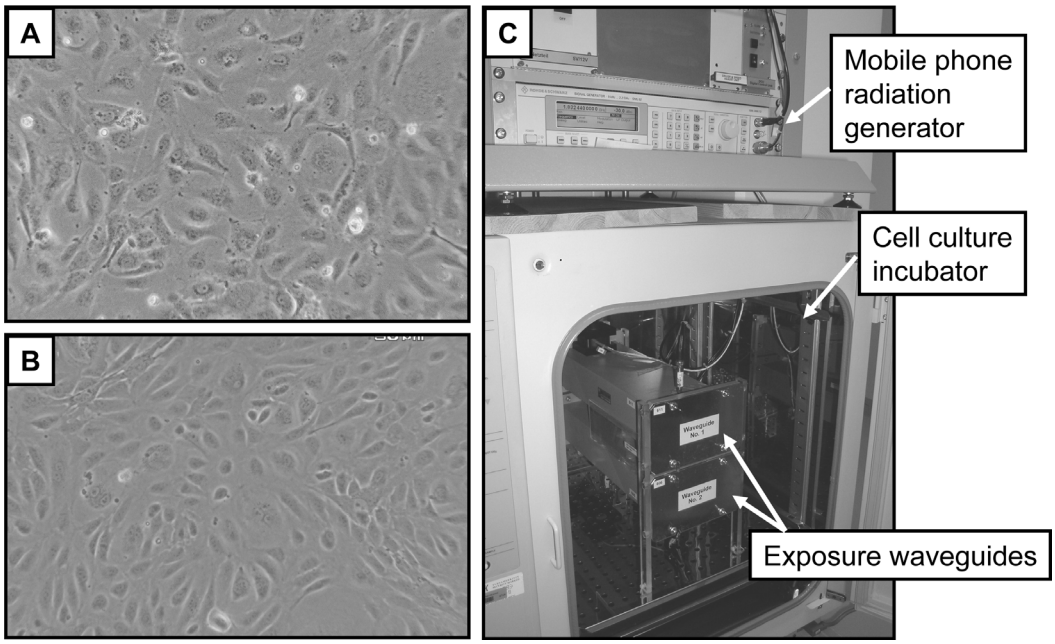

Figure 1 Monolayers of HUVEC (A) and HBMEC (B). Exposure set-up for 1800 MHz GSM mobile phone radiation (C).

characterized with a full three-dimensional 83-D) electrothermal finite-difference time-domain (FDTD) analysis using the simulation platform SEMCAD (by SPEAG). Additionally, SAR intensity and distribution was verified with measurements using a 1-mm-diameter-field probe inserted into the culture medium of the cell culture dish [13]. The simulated mobile phone signal used in this study was 1800 MHz GSM Talk-signal. It is characterized by a random change between the discontinuous transmission mode (DTX) and non-DTX or GSM Basic phases. The distribution in time was exponential with a mean duration of 10.8 seconds for non-DTX ("talking") and 5.6 seconds for DTX ("listening"). The dominant modulation components of this signal are 2, 8, 217, 1733 Hz and higher harmonics [15].

The monolayers of primary human endothelial cells were placed to two 6-dish holders and placed inside the exposure chambers of the exposure set-up. In one chamber, randomly selected by the computer program, cells were exposed to an average SAR of 2.0 W/kg at 37 ± 0.3°C for 1 hour, while in the other chamber the cells were sham-exposed in the similar conditions but without mobile phone radiation exposure. The experiments were performed in the blinded manner and the code was broken at IT'IS after the analyses of the experiments were completed.

Sample preparation & labeling for 2-dimensional electrophoresis

Cell pellets were lysed in 50 μl of a lysis buffer (8 M Urea, 1 M Thiourea, 4% Chaps, 30 mM Tris, 1 mM sodium orthovanadate, and 1 mM PMSF, pH 8.5) for 1 hour at the room temperature, followed by centrifugation (twice) for 15 min at 20000 g each. Protein concentrations were measured using Bradford method. The 75 μg of total protein from each sample was used for two-dimensional gel electrophoresis. The internal standard was prepared by pooling of the same amount of each sample into a one common internal standard sample.

The 75 μg of total protein from each sample was used for the analysis. Samples were labeled with DIGE Cy-fluorescent dyes (GE Healthcare, USA) and internal standard sample was labeled with Cy2 dye in all cases. Each experimental sample was labeled with either Cy3 or Cy5 dye. The coding of the sample labeling was according to the exposure chamber: cells placed in chamber #1 of the set-up were always labeled with Cy3 dye and cells placed in chamber #2 were always labeled with Cy5 dye. The labeling procedure was done according to the manufacturer's instructions. Briefly: 600 pmol of dye was added to the sample and labeling was performed for 30 min on ice. Afterwards the labeling was quenched with 10 mM lysine for 10 minutes on ice.

Samples deriving from the same exposure (Cy3 and Cy5 labeled) were pooled together with Cy2 labeled internal standard and separated all together in a single 2DE gel.

2-dimensional electrophoresis

The isoelectric focusing was performed using an IPGphor3 apparatus (GE Healthcare) and 24 cm long IEF strips pH 4-7 (GE Healthcare). The samples were loaded using in-gel rehydration loading in a buffer containing 9 M Urea, 2% Chaps, 0.5% IPG buffer pH 4-7, and 65 mM DTT for 5 h. IEF was run with 50 μA/strip at 20°C using step-and-hold method as follows: 50 V 8 h; 100 V 1 h; 500 V 1 h; 1000 V 1 h; 2000 V 1 h; 5000 V 1 h, 10000 V until 95000 Vhrs were achieved. After the end of IEF run the strips were equilibrated for 15 min with 6 M urea, 30% glycerol, 50 mM Tris-HCl, 2% SDS, and 10 mg/mL DTT for 15 min and then for another 15 min in the same buffer, in which 25 mg/mL iodoacetamide (IAA) has replaced DTT. SDS-PAGE was run in 10% gel using Ettan DALTsix Electrophoresis system (GE Healthcare) using 1 mm low fluorescent glass plates with the constant settings of 10 mA/1W/gel for the first hour and then 12 mA/1.5W/gel overnight at 20°C. After the electrophoresis the gels were scanned between the glass plates with Typhoon Trio scanner (GE Healthcare) with the appropriate excitation and emission wavelengths for Cy2, Cy3, and Cy5 dyes. The PMT voltages were optimized in such manner that the maximum signal intensity was approximately on the same level for all dyes.

Data acquiring and analysis

The images were acquired with Typhoon Trio scanner (GE Healthcare). The datasets containing images from Cy3, and Cy5 labeled samples and Cy2 labeled internal standard were cropped with ImageQuant tool-software (GE Healthcare). The datasets were cropped to contain the same pattern of proteins in all cases. The datasets were then imported to DeCyder 6.5 software (GE Healthcare), in which the batch processor was used to detect and to match the spots. The 10000 spots were assumed to be found in the spot detection, and the volume of 30000 was used as a cut-off filter. After a brief manual visual check of the matched spots the workspace was imported to DeCyder Extended Data Analysis module (EDA) for statistical analysis. For EDA analysis protein spots, which were found at least in 70% of gels, were included. The student t-test was used to find differentially expressed protein spots. False discovery rate correction (FDR) was applied when t-test was performed in EDA module. Also principal component analysis (PCA) was performed in EDA for the spot maps. The lists of the statistically significantly affected spots were imported back to DeCyder Biological Variation Analysis module (BVA) in which the results were filtered on the basis of the average ratio between the samples.

Results

Two different types of primary human endothelial cells were used in this study, the HUVEC and the HBMEC. The protein expression patterns of these cells were examined using 2DE-based proteomics with DIGE-technique. In total, 13 separate replicates of sham and exposed proteomes were generated from HUVEC and 11 separate replicates of sham and exposed proteomes from HBMEC. The same internal standard was used for all samples allowing better technical quality and less variance between the gels. All gel images were analyzed together in DeCyder 6.5. In total, 2863 protein spots were detected in the master gel. Protein spots which were detected in 70% of spot maps were included in the EDA analysis (total of 1746 spots).

The proteome analysis has shown differences in 2D protein expression pattern between HUVEC and HBMEC. In total, 368 spots were found to differ between both cell types using an independent t-test with $p \leq 0.0001$ and with false discovery rate correction. Out of these 368 protein spots, the 145 spots were found to be differentially expressed between the cell types by more than 2-folds up or down (Figure 2).

Based on our previous study [4], it was expected that the different physiological properties of HUVEC and HBMEC, may lead to the induction of different protein expression profiles following the exposure to mobile phone radiation. In both cell types the differences in the protein expression in the response to the mobile phone radiation were analyzed for the 1746 spots included in EDA analysis, using independent t-test. In HUVEC proteome there were found 35 statistically significantly affected protein spots ($p \leq 0.05$; t-test) (Figure 3). The maximum average ratio, between sham and exposed samples, was for these protein spots = 1.33. In HBMEC proteome there were found 2 statistically significantly affected protein spots ($p \leq 0.05$; t-test) and the average ratios of -1.16 and +1.1 were observed between sham and exposed samples (Figure 4).

However, when the false discovery rate correction (FDR) was performed, all the statistically significantly affected spots were recognized as false positives. This outcome of FDR analysis might be explained by the low average ratio between exposed and sham samples (difference considered as a noise) or because some of the protein spots have appeared in manual visual examination as technical artefacts (e.g. dust particles). Indeed, all spots, found to be differentially expressed before FDR analysis, were also manually checked and the average ratios between exposed and sham samples were shown to be very close to 1.0 and the highest average ratio peaks were recognized as dust particles due to extremely sharp peak geometry.

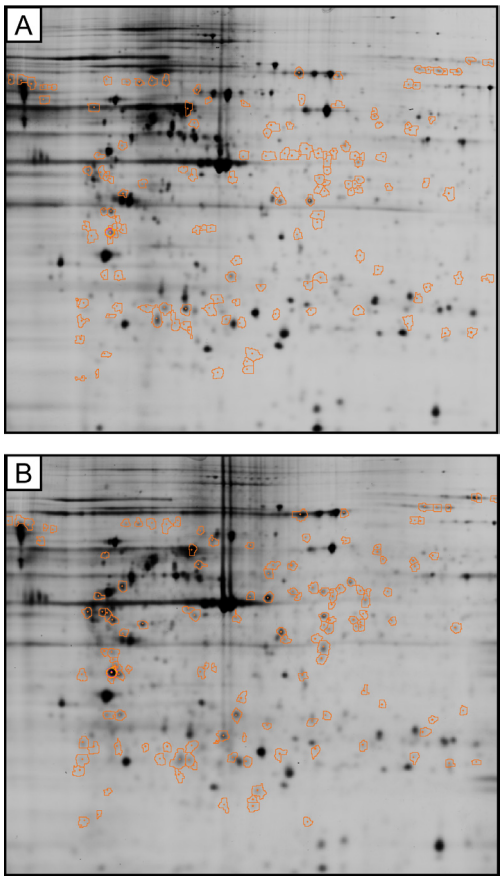

Figure 2 2D-gels of HUVEC (A) and HBMEC (B) with marked 145 spots (orange colored rings) that are differing between the cell types.

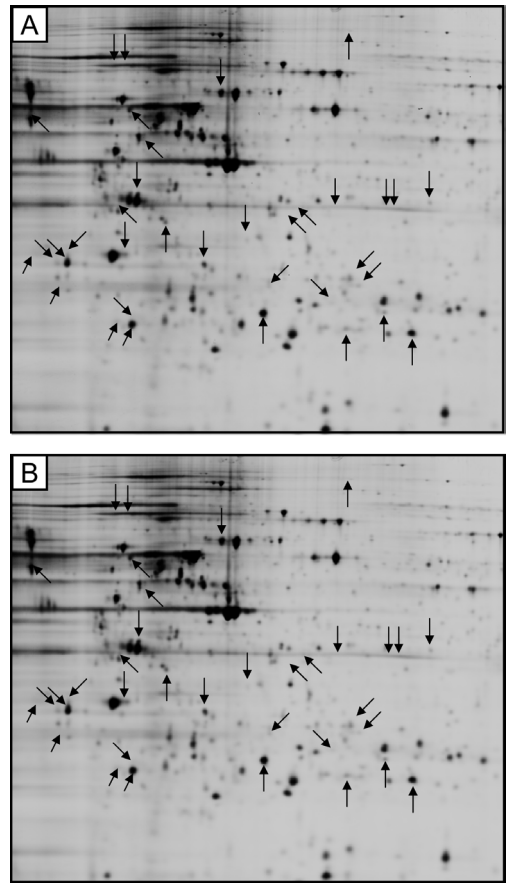

Figure 3 2D-gels of HUVEC cells that were either sham (A) or RF-EMF exposed (B). Arrows point to 35 affected spots; before FDR correction.

Also a principal component analysis (PCA) of the protein spot maps was performed in EDA. The comparison of the protein spot maps showed that the first principal component in the analysis was clearly set as cell type, and not the exposure condition (Figure 5). Additionally, analysis has shown that in HBMEC there is a great dispersion in protein maps between individual exposures (replicates). Thus, PCA also demonstrates that the differences were found only between the cell types (analysis of which was not the aim of the study) but not between the exposure conditions (aim of the study).

Discussion and Conclusions

Based on our earlier study [5] it was hypothesized that the endothelial cells, derived from the different vascular beds and having differing proteomes, would respond differently to mobile phone radiation exposure. At the same time, the study was to determine whether the observed earlier [8] very limited effect of 1800 MHz GSM radiation on the proteome of EA.hy926 human endothelial cell line will be reproduced using primary human endothelial cells.

As the proteome analysis has demonstrated, there are numerous differences in protein expression between proteomes of HUVEC and HBMEC. These differences are most likely reflecting the differences in physiological functions performed by endothelial cells in different vascular beds. The differences between proteomes of primary cells remained in cultures for several *in vitro* passages, indicating that they might be of significance for the specific cell functions and not just transient alterations of the dynamic proteome.

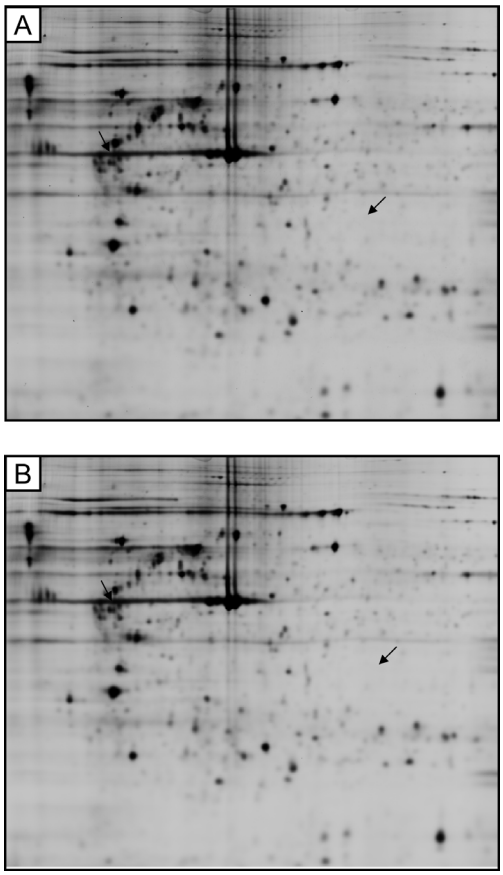

Figure 4 2D-gels of HBMEC cells that were either sham (A) or RF-EMF exposed (B). Arrows point to two affected spots; before FDR correction.

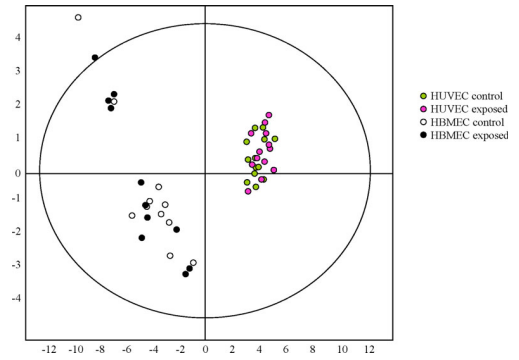

Figure 5 Principal component analysis (PCA) of the protein spot maps shows the majority of differences to be found between the cell types, but not between the exposure conditions.

The exposure of HUVEC and HBMEC to 1800 MHz GSM mobile phone radiation did not cause any statistically significant changes in proteomes of either of cell types. This result differs from our earlier published studies where human endothelial cell lines (EA.hy926 and EA.hy926v1) were exposed to 900 MHz GSM mobile phone signal and statistically significant changes in proteome were detected [2-5]. However, this result agrees with our recent study [8] showing that the 1800 MHz GSM radiation has very small effect, if at all, on the proteome of EA.hy926 cell line, as compared with the 900 MHz GSM radiation.

The discrepancy between the responses of cells, to 1800 MHz GSM signal and the 900 MHz GSM signal, observed in our previous and in the current study, might be likely caused by: (i) different exposure frequencies (900 MHz vs. 1800 MHz), (ii) differences in SAR distribution in cell culture dishes in the used exposure set-ups, (iii) differences in used cell types (primary cells vs. cell line), and (iv) differences in the 2DE proteomics methodology (silver stain vs. DIGE).

In the 900 MHz GSM set-up there is a more non-uniform SAR distribution [3] than in the 1800 MHz set-up and therefore, cells in the certain areas of the culture dish are exposed to higher SAR (over 5.0 W/kg) when the average SAR for the whole cell culture dish is 2.4 W/kg [3]. The 1800 MHz GSM set-up has more uniform SAR distribution and the vast majority of cells, throughout the cell culture dish, were exposed to the same level of radiation SAR = 2.0 W/kg. Thus it might be possible to speculate that the SAR of 2.0 W/kg might be not sufficient to induce statistically significant changes in the cell proteome whereas the SAR of ≥5.0 W/kg might be sufficient to do so. Additionally, in the previous studies with 900 MHz GSM radiation, proteins spots were detected using silver staining whereas in the present study the DIGE-technique was applied. DIGE-technique is commonly considered to be more reliable and to produce less technical variability. In comparison with silver staining techniques, the use of DIGE-technique reduces the number of the observed false positive results.

Results of the present study are in agreement with Gerner et al. [9] who did not observe statistically significant changes in protein expression levels in proteomes of cells exposed to 1800 MHz GSM signal. Interestingly, they have detected changes in the rate of protein synthesis following long-term (8 hours) but not short term (1 hour) exposures. Our present study was not designed to determine effect on *de novo* protein synthesis observed by Gerner et al. [9].

In conclusion, our results suggest that, as expected, the proteomes of the same cell type (endothelium) but derived from different vascular beds (umbilical vein and brain microvasculature) express very different proteomes. However, the 1800 MHz GSM mobile phone radiation appeared to have no statistically significant effect on the proteome of HUVEC and HBMEC, when cells were exposed for 1 h at an average SAR of 2.0 W/kg and examined immediately after that.

List of Abbreviations

2DE: two-dimensional electrophoresis; BVA: Biological Variation Analysis module in DeCyder; CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; ddH_2O: Double distilled water; DIGE: Difference Gel Electrophoresis; DTT: Dithioreitol; EA.hy926: Human endothelial cell line; EDA: Extended Data Analysis module in DeCyder; FDR: False discovery rate; GSM: Global System for Mobile Communications; HBMEC: Human Brain Microvascular Endothelial Cell; HUVEC: Human Umbilical Vein Endothelial Cell; IAA: Iodoacetamide; IEF: Isoelectric focusing; IPG: Immobilized pH gradient; PAGE: Polyacrylamide gel electrophoresis; PBS: Phosphate buffered saline; pI: Isoelectric point; PMSF: phenylmethylsulphonyl fluoride; SAR: Specific absorption rate; SDS: Sodium dodecyl sulfate; Tris-HCl: Tris (hydroxymethyl)aminomethane hydrochloride, Versene Chelating agent containing EDTA

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Author details

[1]STUK - Radiation and Nuclear Safety Authority, Helsinki, Finland. [2]IT'IS Foundation, Swiss Federal Institute of Technology, Zurich, Switzerland.

Authors' contributions

DL and RN designed the study; RN carried out the experiments; NK has provided radiation exposure equipment and radiation dosimetry, DL and RN have written and re-written the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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