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The RF-EMF case II: Genomics approach - a users view -

International Workshop:
„Omics for Assessing Unclear Risks"

Berlin, 26.-28. May 2008

introduction – where are we?

- somewhere inside the hay-stack
- searching for a (very) subtle effect
- no biological end-point available
- although theories are available for RF-EMF/biology interaction on a physical basis -
- unclear mechanism
  (what does happen at which place?)

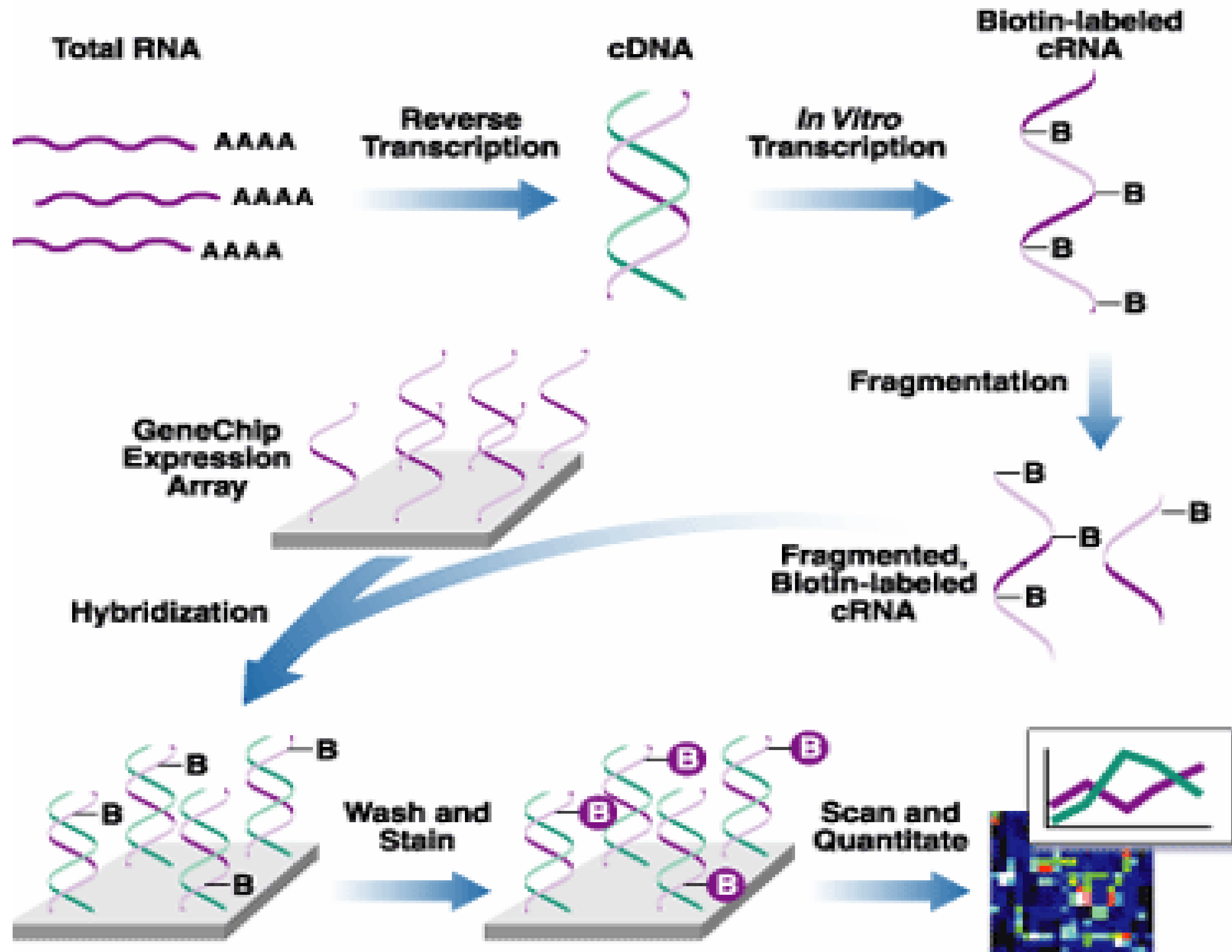
<u>transcriptomics</u>:

- offers an hypothesis free approach

~~genomics~~ transcriptomics methods

- whole genome chip arrays
- targeted chip arrays
- SAGE (serial analysis of gene expression)
  - amplification of 10-14bp DNA fragments
  - characteristic for each gene
- RT-PCR arrays „TaqMan® Low Density Arrays“

RNA-analysis with chip-microarrays



publications concerning RF-EMF exposure and transcriptomics

- from 1999-2008
- limited no. of studies (20-25)
- typically performed in cell cultures
- and by gene chip arrays using glass carriers

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Harvey et al. (Cell Biol. Int. 1999)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
HMC-1 human mast cells	Human Atlas cDNA Array Chip w. 588 Genes	no	864 MHz (CW)	7 W/kg	7d @ 3x20min/d	

- 3 regulated genes observed
  - c-kit: activation of mast cells

- cells used in different states of cell cycle
- variation in passage number
- unclear number of replicates
- exposure outside of incubator
- inhomogeneous field in exposure unit

Pacini et al. (Oncol. Res. 2002)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
skin fibroblasts, human	91 genes	no	902.4 MHz	0.6 W/kg	1h	

- morophological changes
- enhanced expression of 14 genes:
  - mitogenic genes
  - signal transduction
  - inhibitors of cell growth
  - apoptosis genes

Lee et al. (FEBS Lett. 2005)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
HL-60 cells, human BM-leukemia	SAGE (w/ whole genome screening)	no	2.45 GHz (PW)	10 W/kg	2h / 6h	

- 221 genes regulated after 2h exposure
- 759 genes regulated after 6h exposure
- regulation of cell cycle and apoptosis genes
- no HSP affected

- no validations experiments

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Zeng et al. (Proteomics 2006)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
MCF-7 cells, human breast cancer	Affy. HG-U133A Genechip + Proteome	RT-PCR 2D-GE	1800 MHz (PW) GSM	3.5 W/kg 2 W/kg	1- 24h 5'on/10'off	

- 5 genes upregulated at 3.5 W/kg
- fold-changes < 2x
- not confirmed by RT-PCR

- 2 biological and 2 technical replicates (4 Chips) for validation
- regulation of few proteins but different from reg. genes might prove accidential effects
- results not convincing acc. to authors

Whitehead et al. (Proteomics 2006, Rad. Res. 2006)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
C3H 10T ½ cells, mouse fibrobl.	Affy. U74Av2 Genechip 9198 Genes		836 MHz FDMA 848 MHz CDMA	5 W/kg	24h	0.68 Gy

- number of reg. genes not higher than expected false positives
- only quantity of regulated genes is reported

Remondini et al. (Proteomics 2006)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
EA.hy926, U937 lymphobl., HL-60, CHME5, T-lymphoc., NB69	Human Unigene RZPD-2 cDNA array	no	900 MHz (PW) 1800 MHz (PW)	1-2.5 W/kg	1-24h cont & int	

- differential gene expression found in EA.hy296, U937, HL-60
- not in CHME5, T-lymphocytes, NB69
- severely criticized by Chauhan et al.:
  - RNA pooled from independent expts. prior to cDNA generation
    - insufficient biological replicates
  - data analysis based on one single independent hybridazation per group
  - insufficient technical replicates
  - lack of validation experiments (RT-PCR)

Qutob et al. (Rad. Res. 2006)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
U87MG glioblast. cells, human	Agilent Human 1A Oligonuc.22K Microarray	yes	1.9 GHz (PW)	0.1- 1- 10 W/kg	4h kont.	43°C

- no evidence for non-thermal influence of RF-EMF on gene expression of U87MG cells
- evaluation by SAM an MA-Anova
- upregulation of typical stress reactive genes in positive controls
  - 66 upregulated, 33 downregulated

Chauhan et al. (Proteomics 2007)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
U87MG + MM6 monocytes, human	Agilent Human 1A Oligonuc.22K Microarray	yes	1.9 GHz (PW)	0.1-10W/kg U87MG 1 – 10 W/kg MM6	24h cont 6h MM6	43°C

- results of Qutob et al. were confirmed for both, U87MG glioblastomas and MM6 monocytes
- more genes regulated at heat shock conditions for 24h

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Hirose et al. (Bioelectromagnetics 2006, 2007)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
A172 glioblastoma, IMR90 fibrobl, hum.	Affy. HG-U133 plus 2.0 whole genome for selected genes only	not for verification	2.1425 GHz	80, 250, 800 mW/kg	2, 24, 48h	42°C 43°C

- genechip analysis is by-product in this study
- chip arrays are used after manual selection of genes

Zhao et al. (Toxicology 2007)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
Cerebral cortical and hippocampal neuronal cell cultures	Affym. Rat Neurobiol U34 arrays; ~1,200 genes	yes	1800 MHz (PW) GSM	2 W/kg	24h 5‘on/10‘off	

- 24 genes upregulated
- 10 genes downregulated
- confirmed by RT-PCR
- gene expression of rat neurons can be altered by RF EMF

Nylund & Leszczynski (Proteomics 2006)

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
EA.hy926 hum. umb. vein EA.hy926v1 cell cultures	Altas Human 1.2 cDNA Expr. Array	no 2D-GE	900 MHz GSM	2.8 W/kg	1h	

- changes in gene (and protein) expression in both cell lines
- different results for each cell line

own experiments

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
primary rat brain endothelial cells (BBB)	Affym. Rat Genome 230 2.0 Array ~28,000 genes	yes	UMTS GSM 1800	0.4-8 W/kg	72h cont.	38°C 40°C

- 68/61 genes regulated > 2x (GSM1800/UMTS)
- 47 genes each selected for validation experiments
- 7/13 genes confirmed by RT-PCR
- vasoactive receptors, tight-junction protein, solute carriers
- different results for each field

....more details at BEMS 2008 meeting

Belyaev et al. (Bioelectromagnetics 2006) in vivo

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
Rat cerebellum	Affym. U34 Genechips ~8,800 genes	no	915 MHz (PW) GSM	0.4 mW/kg	2h	

- no effect on conformation of chromatin and DSB
- 11 genes upregulated (~1.3 – 2.7x)
- 1 gene downregulated (~2x)
- including genes for neurotransmitter regulation, BBB, melatonin production
- no validation experiment

Paparini et al. (Bioelectromagnetics 2008) in vivo

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
whole mouse brain from Balb/cJ mice	Affymetrix MOE 430A ~22,000 genes	yes	1800 MHz (PW)	0.2 W/kg	1h	

- 75 regulated genes, not confirmed by RT-PCR

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Nittby et al. (Environmentalist 2008) in vivo

Target	System	RT-PCR	Field	SAR	Dur.	Pos. Contr.
Fischer 344 rats hippocampal and cortical tissue	Affym. Rat Genome 230 2.0 Array ~28,000 genes	no	GSM 1800	30 mW/kg	6h	

- total of 8 rats for exposure and sham
- no significance due to limited replicates
- investigation on functional categories (gene ontology)

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summary of transcriptomics methods concerning RF-EMF exposure

- different targets
- different omics-systems
- different/missing validation experiments
- different field parameters
- different SAR
- different exposure duration (dose)
- different/missing positive controls

- difficult to compare and to formulate a common result

summary of results

- effects of moderate intensity
- different results for various fields and cells
- at least a confirmation that effects to be observed are subtle

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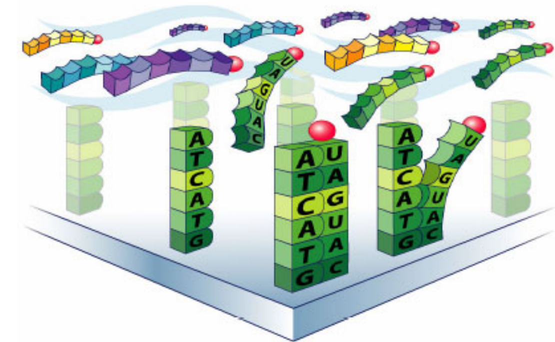
can the analysis of differential gene expression (transcriptomics) help us out?

what do we have?

- **two complex issues to handle so far:**
  - adequate maintenance of the biological system
  - need for valid and precise dosimetry

- **transcriptomics adds:**
  - generating a large amount of data
  - need for sophisticated processing, application of advanced evaluation methods/tools
  - not giving a black/white answer
    (be aware of complexity)
  - variations in interpretation possible



what do we need?

- special needs of transcriptomics analyses
  - sufficient biological replicates
  - standardization of methods (experimental and data handling)
  - quality control for RNA samples
  - positive controls in biology
  - validation experiments for chiparrays: typically RT-PCR
  - handling of bioinformatic tools
  - preferably: a biological system as simple as possible

- clear need for expertise in transcriptomics
- cooperation with omics facilities

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benefits of an *in vitro* approach

- reduction of in vivo complexity
- high compexity of tissues might hide effects in cells of interest
- primary cultures: no dediffentiation
- precise determination of field parameters
- no stress factors (restrainment)
- reproducible exposure conditions
- facilitated field and temperature monitoring
- no non-responders

what do we get?

- not a tool to find the holy grail
- no direct contribution to risk assessment
- identify answers of cells by means of gene activity
- chance to ask new questions, to perform better experiments
- hypothesis free approach:
  - very strong method to identify new biological endpoints
  - possiblity to detect unexpected targets
  - delivery of additional information by analysis of functional pathways
  - may be a first step to understand mechanistic interaction

- Genechip analysis was approved as a tools for breast-cancer prognosis by FDA

io summary of technical issues

- **concerning the target**
  - transcriptomics are especially suitable for cell culture analysis
  - primary cultures preferable, cell lines may be genetically instable
  - tissue: problematic diversity
- **concerning the experiment**
  - quality control of RNA samples
  - biological replicates are important
  - positive controls important
  - need for standardization of omics methods / protocols
  - professional handling of arrays and data
  - complex datamining: mandatory cooperations with bioinformatics experts (cannot be standardized)
  - validation experiments are mandatory, eg. RT-PCR
- **high price: problem of replication**

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will omics methods contibute to our knowledge for assessment of unclear risks of RF-EMF?

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yes,

by getting new insights into the answer of cellular systems on the EMF-stimulus

on the levels of

gene expression

protein expression

pathway analysis

thank you for your attention!