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Analysis of gene expression in human umbilical vein endothelial cells exposed to a 50-Hz magnetic field

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Abstract. Human umbilical vein endothelial cells (HUVECs) were exposed to 100-μT, 50-Hz sinusoidal intermittent (5 min off, 10 min on) magnetic fields for 24 h. Total RNA was extracted from exposed and sham-exposed cells and was then analyzed via a DNA microarray technique. Interestingly, the expression of a subunit of the L-type Ca channel was 2.3-fold higher in exposed cells; this finding may be relevant to similar previously published results (an increase of α1C and α1D subunits of L-type Ca channel in neuronal stem cells as preveiously demonstrated by Piacentini and colleagues in 2008. However, analysis of the false discovery rate precluded us from drawing firm conclusions about the effects of magnetic fields on gene expression in HUVECs. Nevertheless, non-coding RNAs were more common than coding RNAs among the genes that were differentially expressed with a > 2-fold or < 0.5-fold difference between exposed and sham-exposed cells. A similar result was obtained when the expression data from exposed cells or those from sham-exposed cells were analyzed separately. Thus, the higher frequency of non-coding RNAs than that of coding RNAs among the differentially expressed genes was attributed to the higher degree of *fluctuation* in the expression of non-coding RNA. Interestingly, this *fluctuation* seems to be a characteristic intrinsic to non-coding RNAs themselves.

Keywords: magnetic fields, HUVEC, DNA microarray, lincRNA

1. Introduction

The effects of magnetic fields on living organisms have been studied for many years. There is now little doubt that many living organisms, from bacteria to mammals, can perceive and respond to geomagnetic fields, and the biochemical, molecular genetic, physiological, and cellular processes mediating perception of magnetic fields by organisms are being revealed (Castelvecchi, 2012; Lohmann, 2010; Johnson and Lohmann, 2008). On the other hand, the report that 50-Hz magnetic fields (low frequency magnetic fields; LFMF) from power lines increase the risk of childhood leukemia [(Wertheimer and Leeper, 1979) was followed by many epidemiological and cell physiological studies. For example, Lai and Singh (Lai and Singh, 2004) used a comet assay to analyze the damage to DNA that was from brain cells of rats exposed to 100 μT LFMF. They found that upon exposure to LFMF, single- and double-strand breaks of ~~in~~ DNA increased. They further demonstrated that strand breakage was diminished by anti-oxidant reagents such as vitamin E. Hence, they proposed that magnetic fields promote the generation of hydroxyl radicals from H_2O_2 in the presence of Fe^{2+} ions (Fenton reaction) and that these radicals eventually lead to DNA damage. Another study demonstrated that LFMF causes increased expression of heat-shock protein 70 (HSP70) in HL-60 cells (Goodman and Blank, 1998). In this case, it was hypothesized that the magnetic fields acted as a stressor ; quite interestingly, the upregulated promoter resides upstream of the HSP70 promoter (Blank and Goodman, 2009).

It is highly important to determine whether LFMF alters gene expression, because such information may provide a key to understanding the molecular mechanisms of the biological effects of LFMF. In fact, increasing number of studies on gene expression in cells exposed to LFMF are being conducted. Recently developed DNA microarray techniques allow researchers to analyze the expression pattern of a whole genome derived from tissues or cells exposed to LFMF. Savage et al. (Savage et al., 2005) have used two-dimensional gel electrophoresis to show that the levels of several proteins are altered in human glioma cells exposed to a 60-Hz, 1.2-μT magnetic field for 3 h. Furthermore, they used microarray analysis to demonstrate that 30 genes were either upregulated or downregulated when the cells were exposed to LFMF. They concluded that the relationships between changes in protein expression and changes in gene expression are unclear. Henderson et al. (Henderson et al., 2006) exposed human endothelial cells to 10 – 700 μT LFMF for 24 h. They also used DNA microarray techniques to analyze gene expression and found that the expression of some genes was altered; however, no gene consistently exhibited the same change in expression in several replicate experiments. Therefore, they concluded that no gene was actually affected by the LFMF. Bouwens et al. (Bouwens et al., 2012) exposed cultured THP-1 monocytes and endothelial cells to 5 μT LFMF for up to 6 h. Using a quantitative PCR technique, they found no change in expression of several genes that encoded inflammatory or anti-inflammatory cytokines. Fedrowitz and Loscher (Fedrowitz and Loscher, 2012) applied 100 μT LFMF to rat mammary gland tissue for 2 weeks and then used DNA microarrays to analyze the cells isolated from the exposed tissue; they reported that the expression of a dozen genes changed. Notably, expression of the genes encoding α-amylase and the parotid secretory protein decreased by several hundred fold.

In spite of these efforts, no consensus has been reached on the relationship between LFMF exposure and gene expression. Nevertheless, it is important to establish if and how magnetic fields affect gene expression because the regulation of gene expression is central in the activity of life. One would expect to gain a deeper understanding of the molecular mechanism of the effects of LFMF from genome-wide analyses of changes in gene expression in response to LFMF.

Previously, we investigated the effect of LFMF on the production of nitric oxide (NO) in human umbilical vein endothelial cells (HUVEC) (Miyata et al., 2012; 2011). The effect of LFMF on NO production has also been studied by other groups (Patruno et al., 2009; Real et al., 2006; Sakamoto et al., 2002). NO is produced when cells are exposed to various stresses (Moncada et al., 1991). Hence, the results of these studies are consistent with the notion that the LFMF acts as a stressor of cells (Blank and Goodman, 2009).

Here, human DNA microarrays were used to assess the differences in gene expression in HUVEC exposed to LFMF and those not exposed to LFMF.

2. Materials and methods

2.1. Experimental set-up for magnetic exposure

Two Merritt coils (custom-made; Hozen Industries Co., Ltd., Kyoto, Japan) were placed side-by-side in a CO_2 incubator (model IT600, Yamato Scientific Co., Ltd., Tokyo Japan; figure 1A). Each Merritt coil comprised four sub-coils. Two electrical wires were wound in parallel around each sub-coil; as electrical current flows through each of two such wires, either in the same direction or in opposite directions, the magnetic fields generated by each wire either added up or canceled each other, respectively. Hereafter, we refer to the configuration of the coils for which all electric currents flow in the same direction as "Exp"; the configuration in which the currents cancelled each other are referred to as "Active-sham". With these configurations, each Merritt coil generated the same amount of Joule heat, and the temperature difference between the Exp and Active-sham coils was minimized. In each experiment, Merritt coils were operated by an electric current that was generated by a function generator (Model WF1943A, NF Co., Yokohama, Japan) and amplified with an electric amplifier (Model PMA390AE, DENON, Kawasaki, Japan). The 50-Hz sinusoidal current was generated intermittently (5 min on, 10 min off; figure 1B), and cells were exposed to magnetic fields for 24 h. The strength of the magnetic field was measured with a Tesla meter (FW4190; Pacific Scientific-OECO, Milwaukie, OR, USA or Model 421 with a Hall-effect probe (MMA-2502-VH); Lake Shore Cryotronics, Westerville, OH, USA); it was 100 μT in the Exp coil and 0.4 μT in the Active-sham coil. A sheet of magnetic shielding (Finemet MS-F; Hitachi Metals Admet, Ltd., Tokyo, Japan) was placed between the Exp and the Active-sham coils to reduce stray fields. In each coil, four 10-cm cell culture dishes were placed (figure 1 C) on a rack made of acrylic plastic. For later handling, dishes were numbered as shown in figure 1D. The temperatures in the Exp and Active-sham setups were measured with thermistor probes (TR-72Ui, T&D Co., Matsumoto, Japan) that were placed immediately above the top dishes. During LFMF exposure, the time-averaged temperature in the Exp coil was 36.9°C, and that in the Active-sham coil was 36.7°C.

HUVECs (doubling time = 13.8 h; Lonza Japan, Tokyo, Japan) were plated in 8 cell culture dishes (1.8×10^5 cells per dish; 4 dishes for Exp and 4 dishes for Active sham) and were cultivated in cell culture medium (EBM-2 + additives (Bullet kit), Lonza) for 3 days before being exposed to LFMF.

2.2. DNA microarray experiment

Immediately after the exposure, TRIZOL® was used according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA) to extract total RNA from the cells in each culture dish; the resulting 8 RNA samples were then processed and analyzed separately.

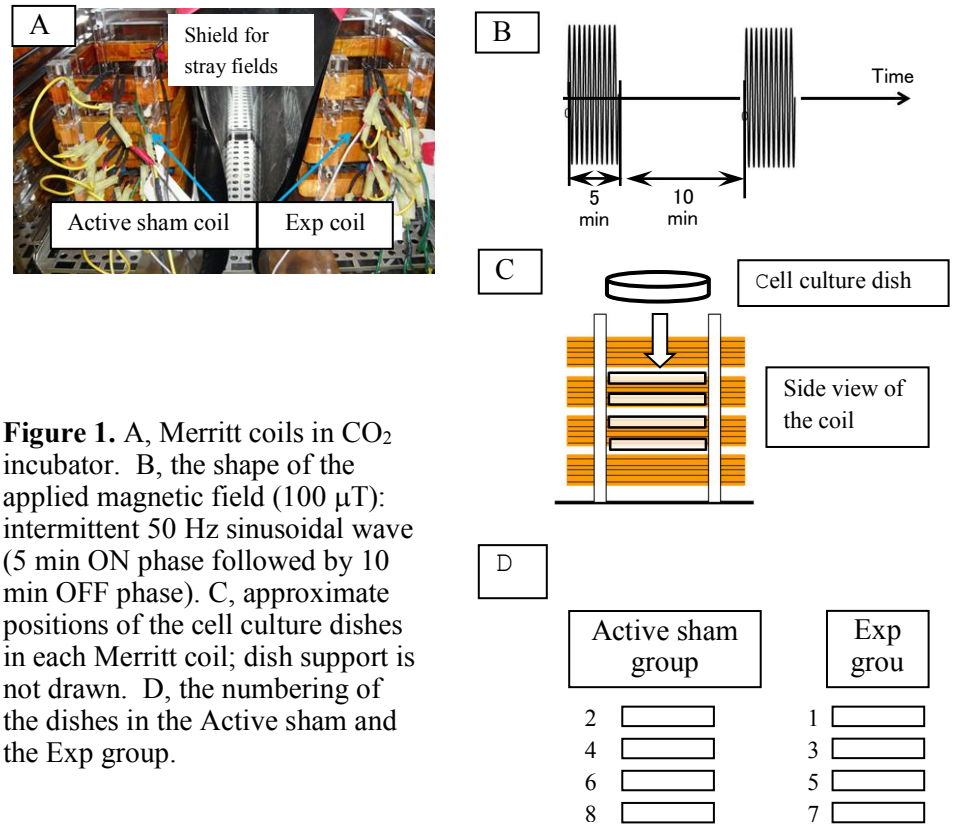

Figure 1. A, Merritt coils in CO_2 incubator. B, the shape of the applied magnetic field (100 μT): intermittent 50 Hz sinusoidal wave (5 min ON phase followed by 10 min OFF phase). C, approximate positions of the cell culture dishes in each Merritt coil; dish support is not drawn. D, the numbering of the dishes in the Active sham and the Exp group.

The purity of total RNA was confirmed by electrophoresis with an Experion system (Biorad, Hercules, CA, USA). First-strand cDNA was synthesized via a reverse transcriptase reaction, which was followed by double-strand cDNA synthesis. Finally, Cy3-labeled cRNA was synthesized via a T7 RNA polymerase reaction. The cRNA was hybridized to Gene chip (Sure Print G3, Human ver. 2.0, Agilent Technologies, Santa Clara, CA, USA), and the fluorescence intensity of each spot was measured. The fluorescence intensities were subjected to quantile normalization with the open-source programming language R (Bioconductor).

The four fluorescence intensities measured for each transcript in the Exp group (samples from dishes placed in the Exp coil) and those for the Active-sham group (those from the dishes in the Active-sham coil) were averaged within each group. For about 45% of all transcripts, the fluorescence intensity was insufficient for further analysis; this could have been due to low expression levels or to artifacts arising for technical reasons (e.g., uneven fluorescence distribution within the spot), and the signals from these transcripts were not subjected to further analysis. The remaining transcripts were designated as "Analyzed transcripts" for convenience, and were subjected to further analysis. For each Analyzed transcript, the fold-change (FC) value (the ratio of the averaged fluorescence intensity of the Exp to that of Active sham) was calculated. Also, a protocol based on LIMMA (Linear Models for Microarray) (Smyth et al., 2005) was used to calculate a p-value for each transcript. DAVID (The Database for Annotation, Visualization and Integrated Discovery, v6.7; National Institute of Allergy and Infectious Diseases, NIH) was used to annotate the function of each transcript, and Gene Spring (ver. 12,1; Agilent Technologies) was used to perform the principal component analysis (PCA). The expression data were sorted according to FC values (table 1; the complete data set will be deposited in Gene Expression Omnibus). Not every transcript was assigned with both a gene symbol and a

(functional) description in the final output table from DAVID. Hence, we tentatively classified each transcript into one of the three following categories: (1) coding RNA; these transcripts had either "mRNA", "uncharacterized protein" or "cDNA" in the description; some did not have gene symbols; (2) non-coding RNA; these transcripts had "non-coding RNA", "non-protein coding RNA", "misc(ellaneous) RNA", "uncharacterized RNA", "long intervening non-coding (linc) RNA", "ribosomal RNA", "small-nuclear RNA", "guide RNA", "small-nucleolar RNA" or "small-cytoplasmic RNA" in the description; or (3) other; these transcripts were either not assigned with a description or they did not fall into either of the other categories. Examples of coding RNAs are listed in table 2.

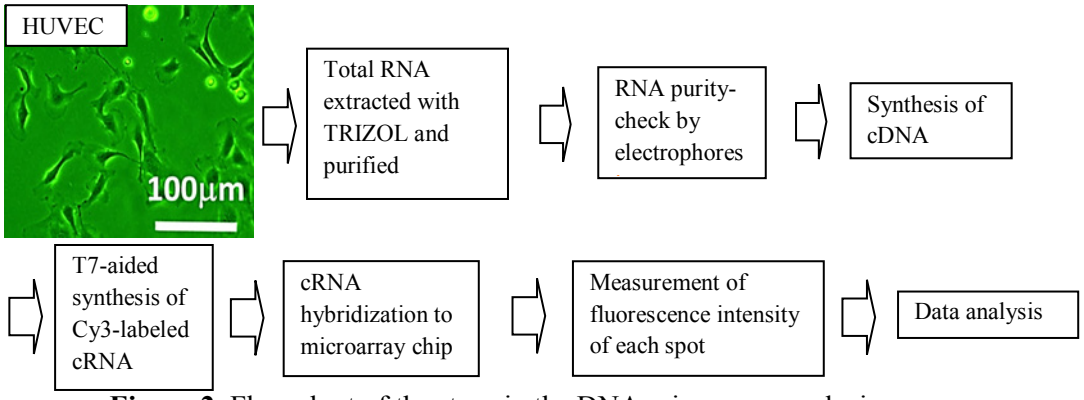

Figure 2. Flow chart of the steps in the DNA microarray analysis

3. Results

3.1. DNA microarray analysis

The total number of transcripts was 50599, and the number of Analyzed transcripts was 28069 (table 1). Among all Analyzed transcripts, the number of transcripts with an FC > 2 was 375 (0.74% of the total transcripts) and that with an FC < 0.5 was 385 (0.76%), and the average FC was 1.03 ± 0.26 (± SD). There were 120 transcripts (0.24% of all transcripts) with an FC > 2 or < 0.5 with statistical significance ($p < 0.05$). Hereafter, we use "extreme FC values" to represent FC > 2 or FC < 0.05.

Table 1. Summary of all transcripts, Analyzed transcripts, and transcripts with extreme FC values with statistical significance ($p < 0.05$)

All transcripts	Analyzed transcripts				Transcripts with extreme FC values with statistical significance ($p < 0.05$)	
	Total	FC > 2 (all p)	FC < 0.5 (all p)	Averaged FC value	FC > 2 ($p < 0.05$)	FC < 0.5 ($p < 0.05$)
50599	28069	375	385	1.03 ± 0.26	58	62

Table 2. List of 43 genes (transcripts with extreme FC values (p < 0.05), symbols and descriptions)

Gene Symbol	Description	p value	FC*
WRAP53	WD repeat containing, antisense to TP53 [Source:HGNC Symbol;Acc:25522] [ENST00000316024]	0.039434	5.612265
GABRA1	Homo sapiens gamma-aminobutyric acid (GABA) A receptor, alpha 1 (GABRA1), transcript variant 3, mRNA [NM_001127644]	0.00299	4.345748
LAIR2	Homo sapiens leukocyte-associated immunoglobulin-like receptor 2 (LAIR2), transcript variant 1, mRNA [NM_002288]	0.001139	4.014793
BTBD8	Homo sapiens BTB (POZ) domain containing 8 (BTBD8), mRNA [NM_183242]	0.04365	3.253272
ADAM19	Homo sapiens ADAM metallopeptidase domain 19 (ADAM19), mRNA [NM_033274]	0.003376	3.094322
GBP5	Homo sapiens guanylate binding protein 5 (GBP5), transcript variant 1, mRNA [NM_052942]	0.017624	2.696712
FAM55D	Homo sapiens family with sequence similarity 55, member D (FAM55D), transcript variant 1, mRNA [NM_001077639]	0.029315	2.662311
TMC2	Homo sapiens transmembrane channel-like 2 (TMC2), mRNA [NM_080751]	0.019751	2.570234
SPRR2G	Homo sapiens small proline-rich protein 2G (SPRR2G), mRNA [NM_001014291]	0.002643	2.568802
TMEM86A	Homo sapiens transmembrane protein 86A (TMEM86A), mRNA [NM_153347]	0.002527	2.545616
USP51	Homo sapiens ubiquitin specific peptidase 51 (USP51), mRNA [NM_201286]	0.038522	2.443366
KIF1A	Homo sapiens kinesin family member 1A (KIF1A), transcript variant 1, mRNA [NM_001244008]	0.030806	2.373186
CACNA1S**	Homo sapiens calcium channel, voltage-dependent, L type, alpha 1S subunit (CACNA1S), mRNA [NM_000069]	0.012357	2.358972
SLC25A41	Homo sapiens solute carrier family 25, member 41 (SLC25A41), mRNA [NM_173637]	0.019805	2.312485
ZNF695	Homo sapiens zinc finger protein 695 (ZNF695), transcript variant 1, mRNA [NM_020394]	0.015386	2.306848
PRR21	Homo sapiens proline rich 21 (PRR21), mRNA [NM_001080835]	0.016449	2.296751
OTUD6A	Homo sapiens OTU domain containing 6A (OTUD6A), mRNA [NM_207320]	0.03519	2.263953
CXCL12	Homo sapiens chemokine (C-X-C motif) ligand 12 (CXCL12), transcript variant 3, mRNA [NM_001033886]	0.046159	2.236144
CLDN23	Homo sapiens claudin 23 (CLDN23), mRNA [NM_194284]	0.040492	2.111673
LOXL4	Homo sapiens lysyl oxidase-like 4 (LOXL4), mRNA [NM_032211]	0.028883	2.000424
PIK3CD	Homo sapiens phosphoinositide-3-kinase, catalytic, delta polypeptide (PIK3CD), mRNA [NM_005026]	0.040687	0.4847
OR5B21	Homo sapiens olfactory receptor, family 5, subfamily B, member 21 (OR5B21), mRNA [NM_001005218]	0.032437	0.466581
CCDC34	Homo sapiens coiled-coil domain containing 34 (CCDC34), transcript variant 2, mRNA [NM_080654]	0.036646	0.461922

IL16	Homo sapiens interleukin 16 (IL16), transcript variant 1, mRNA [NM_004513]	0.0177	0.458963
CALHM1	Homo sapiens calcium homeostasis modulator 1 (CALHM1), mRNA [NM_001001412]	0.043882	0.45218
PRG2	Homo sapiens proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein) (PRG2), transcript variant 1, mRNA [NM_002728]	0.023906	0.414114
WDR52	Homo sapiens WD repeat domain 52 (WDR52), transcript variant 1, mRNA [NM_001164496]	0.005142	0.411141
MLN	Homo sapiens motilin (MLN), transcript variant 2, mRNA [NM_001040109]	0.011032	0.409454
INPP4B	Homo sapiens inositol polyphosphate-4-phosphatase, type II, 105kDa (INPP4B), transcript variant 1, mRNA [NM_003866]	0.002558	0.396759
PLEKHM3	Homo sapiens pleckstrin homology domain containing, family M, member 3 (PLEKHM3), mRNA [NM_001080475]	0.046749	0.392448
KRTAP19-8	Homo sapiens keratin associated protein 19-8 (KRTAP19-8), mRNA [NM_001099219]	0.008039	0.383195
ADAMTSL1	Homo sapiens ADAMTS-like 1 (ADAMTSL1), transcript variant 4, mRNA [NM_001040272]	0.012441	0.381861
OR5A1	Homo sapiens olfactory receptor, family 5, subfamily A, member 1 (OR5A1), mRNA [NM_001004728]	0.002518	0.375593
CDNF	Homo sapiens cerebral dopamine neurotrophic factor (CDNF), mRNA [NM_001029954]	0.016079	0.365736
TSPYL6	Homo sapiens TSPY-like 6 (TSPYL6), mRNA [NM_001003937]	0.027078	0.347506
ZNF665	Homo sapiens zinc finger protein 665 (ZNF665), mRNA [NM_024733]	0.024112	0.341962
SSPO	Homo sapiens SCO-spondin homolog (Bos taurus) (SSPO), mRNA [NM_198455]	0.046293	0.335951
MGAT4C	Homo sapiens mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme C (putative) (MGAT4C), mRNA [NM_013244]	0.04461	0.333515
FOXO4	Homo sapiens forkhead box O4 (FOXO4), transcript variant 1, mRNA [NM_005938]	0.047038	0.312889
CDH26	cadherin 26 [Source:HGNC Symbol;Acc:15902] [ENST00000244047]	0.008763	0.240127
ANKRD30A	Homo sapiens ankyrin repeat domain 30A (ANKRD30A), mRNA [NM_052997]	0.012533	0.222024
ATP6V1B1	Homo sapiens ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1 (ATP6V1B1), mRNA [NM_001692]	0.040238	0.202444
WNT7B	Homo sapiens wingless-type MMTV integration site family, member 7B (WNT7B), mRNA [NM_058238]	0.022919	0.165418

Shaded area indicates FC > 2.
*Table is sorted according to FC values; the p-values and the FC values are not rounded off
**The increase of the expression of a subunit of L-type Ca channel in neural stem cells exposed to 1 mT, 60 Hz magnetic fields is also reported by Piacentini et al. (Piacentini et al., 2008).

Table 2 shows 43 genes (transcripts with extreme FC values ($p < 0.05$), gene symbols and descriptions); the gene with the highest FC was WRAP53 (FC = 5.6, $p = 0.039$), and that with lowest FC was WNT7B (FC = 0.17, $p = 0.022$). Supplementary table 1 lists all transcripts with extreme FC values regardless of p-value. Notably, the expression of CACNA1S, which encodes a light chain of a

subunit of L-type Ca channel, is elevated (table 2). Expression of related subunits of the same L-type Ca channel is also elevated in neural stem cells exposed to 60-Hz, 1-mT magnetic fields (Piacentini et al., 2008).

We used the Gene Functional Classification and Functional Annotation utilities of DAVID to assess whether any group of functionally related genes existed among the 120 transcripts with extreme FC values ($p < 0.05$), but no clear biological relation among the genes became apparent. Therefore, we did not further investigate the biological significance of the changes in the expression of individual genes. We also calculated the false-discovery rate (Storey and Tibshirani, 2003) of individual transcripts with extreme FC values ($p < 0.05$) using Gene Spring (ver. 12.1). No transcript with an extreme FC value ($p < 0.05$) had a false discovery rate < 1, leaving open the possibility that the altered expression of these transcripts was a false-positive result.

3.2. Principal component analysis

The expression data arising from 28069 Analyzed transcripts (regardless of FC value and p-value) in the Exp or in Active-sham group were subjected to PCA. The plots of Exp-group data (#1, 3, 5, 7) were separated from the plots of Active-sham group data (#2, 4, 6, 8; figure 3) along the second component (Y-axis): the contribution of the second component was 13.3%, and that of the first (X-axis) and the third (Z-axis) component were 40.1 and 10.6%, respectively.

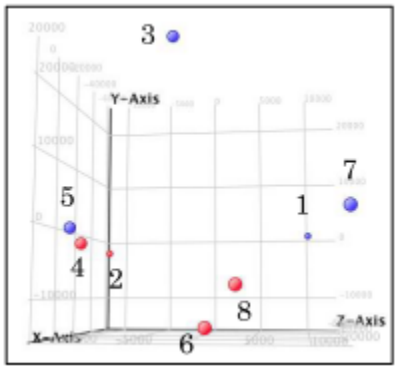

Figure 3. The plot of PCA.
The plots of Exp group (1, 3, 5, 7) are separated from those of the Active-sham group (2, 4, 6, 8). Numbers correspond to those shown in figure 1(D). The plots for the exposure group and those for the Active-sham group are separated.

3.3. Non-coding RNAs

Shown in the second and third columns of table 3 are the numbers and percentages of coding RNA and ncRNA in all transcripts and the Analyzed transcripts of all FC values. Among all transcripts, ~58% were coding RNAs and 31% were ncRNAs, and among the Analyzed transcripts of all FC values, ~71% were coding RNAs and 19% were ncRNAs. The proportion of ncRNA was lower in the Analyzed transcripts of all FC values than in all transcripts.

Remarkably, ncRNAs made up 43% of the Analyzed transcripts with extreme FC values (table 3, fourth column); this representation was higher than that among all transcripts (31%), as well as among the Analyzed transcripts of all FC values (19%). Among the Analyzed transcripts, lincRNAs showed the most remarkable difference between the Analyzed transcripts of all FC values and those with extreme FC values, making up 8.5% of the Analyzed transcripts of all FC values and 30% of the Analyzed transcripts with extreme FC values; they also made up 28% of the transcripts with extreme FC values and statistical significance ($p < 0.05$). Hence, the increase in representation of lincRNAs and ncRNAs seemed to occur regardless of the p-value.

Table 3. Distribution of ncRNAs, lincRNAs, and coding RNAs in each set of transcripts

	All transcripts	Analyzed transcripts, (all p values)		Transcripts with extreme FC values with statistical significance ($p < 0.05$)
		With all FC values	With extreme FC values	
Total number	50599*(100%)	28069**(100%)	760 (100%)	120#(100%)
Coding RNA	29565 (58%)	19982 (71%)	333 (44%)	67 (56%)
ncRNA	15757 (31%)	5398 (19%)	326 (43%)	45 (38%)
lincRNA (in ncRNAs)	9172 (18%)	2380$ (8.5%)	230$ (30%)	34$ (28%)
Others¶	5277 (10%)	2689 (9.6%)	98 (13%)	8 (6.7%)

*: due to the overlap among transcripts and inclusion of transcripts for non-coding RNA, the number of total transcripts (50599) exceeds that of human coding genes (~25000).
**: the number of spots with adequate fluorescence intensity.
#: total number of transcripts with extreme FC values ($p < 0.05$)**.**
¶: transcripts with no description and the transcripts that did not fall into the categories of coding RNA or ncRNA.

3.4. Increase of the population of lincRNAs occurs either in the Exp or the Active-sham group
We investigated whether the remarkable enrichment of lincRNAs in the transcripts with extreme FC values as well as in the transcripts with extreme FC values with statistical significance ($p < 0.05$) was an intrinsic property of lincRNAs. We compared the population of lincRNAs in the transcripts with extreme FC values *in* the Exp group and *in* the Active-sham group. The second column in table 4 shows the combination of samples for each comparison (numbers are as shown in figure 1D). The results (third and fourth columns) again demonstrated an increase in the population of lincRNA even among samples selected solely from the Exp group or the Active-sham group. Thus, at least for HUVECs, the increased population of lincRNA among the transcripts with extreme FC values was an intrinsic property of lincRNA, and was not due to the effect of LFMF or a related side effect (e.g., temperature difference).

Table 4. Combinations of the samples for within-group comparisons

Comparison #	Combination of samples	Population of lincRNA (transcripts with all FC values)	Population of lincRNA (transcripts with extreme FC values
1 (between Exp)	(1, 3) vs. (5, 7)	6.1%	25%
2 (between Active sham)	(2, 4) vs. (6, 8)	5.7%	24%

4. Discussion
Here, we compared gene expression in HUVECs exposed to 50-Hz magnetic fields with that in HUVECs subjected to sham exposure. We found that the expression of 43 genes changed by more than 2-fold or less than 0.5-fold ($p < 0.05$) due to LFMF exposure. PCA analysis demonstrated that the plots representing the Exp group were separated from those representing the Active-sham group. Also,

as was seen in a previous study (Piacentini et al., 2008), we observed an increase in the expression of a subunit of a calcium channel (CACNA1S; table 2). However, we did not see significant alterations in the expression of the specific genes that have been shown to have altered expression in response to LFMF exposure in previous studies (for example, HSP70 (Goodman and Blank, 1998), and α-amylase （Fedrowitz and Loscher, 2012)). The expression data from the entire set of Analyzed transcripts were not statistically significant when subjected to correction for multiple testing; FDR calculations left open the possibility that statistically significant extreme FC values of genes were false-positive results. In addition, there was a slight (0.2°C) difference in temperature between Exp and Active-sham samples; this temperature difference is small, but it may be unable to be ignored, because thermoregulatory behavior of monkeys exposed to microwave radiation changed upon an increase in the temperature of the hypothalamic region by only 0.2°C – 0.3°C (Adair et al., 1984). Thus, in the present study, the effect of a 50-Hz magnetic field on gene expression in HUVECs was not clear; the field strength (100 μT) might not be sufficient for causing a change. Further study is needed to evaluate the effect of 50 Hz magnetic field on gene expression in HUVEC.

The representation of ncRNAs, especially lincRNAs, was found to increase in the group of transcripts with extreme FC values (table 3); this increase in lincRNA representation also occurred within each treatment group (the Exp and the Active-sham group; table 4), indicating that the increase was generic and not due to LFMF or an associated side effect. The reason for the increased ncRNA representation among transcripts with extreme FC values remains unclear at present. However, our data show that the expression of individual lincRNAs *fluctuated* more readily than did that of coding RNAs. An important lesson here is that we should not ignore the non-trivial behavior of ncRNAs in the evaluation of statistical significance with multiple testing correction because the statistical significance may be affected by the existence of ncRNAs with large *fluctuation*s in expression. We note that the number of ncRNAs (transcripts) has increase on the latest microarrays. Thus, the analysis of gene expression in the presence of the intrinsic *fluctuation* of ncRNAs is a new challenge for microarray-based analyses, not only because of the behavior of ncRNAs themselves, but also because of their effect on the evaluation of the statistical significance of differentially expressed transcripts.

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