Spherical cell shape of FLC-4 cell, a human hepatoma cell, enhances hepatocyte-specific function and suppresses tumor phenotype through the integration of mRNA–microRNA interaction

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Summary

The induction mechanism of HNF-4α by spherical cell shape in human hepatoma cells, FLC-4, was investigated. To get insight into the induction mechanism of HNF-4α in three-dimensional FLC-4 cells, mRNA microarray analysis was performed. The gene expression related to drug metabolism and nuclear receptors, such as LXRα, was elevated in spherical FLC-4 cells. We found the first time that the expressions of genes related to malignancy of hepatoma cells, such as HIF-1α, c-Myc and VEGFC, were downregulated by spherical cell shape. Network analysis revealed that HNF-4α would both the enhancement of hepatocyte-specific gene expression and suppression of malignancy. Since HNF-4α gene expression was known to be regulated by microRNA, we inferred that spherical cell shape would induce HNF-4α gene expression through microRNA. To investigate the possibility of such a mechanism, mRNA–microRNA interactions were examined using microRNA microarray and bioinformatics analysis. The level of miR-24, a microRNA targeting HNF-4α, was reduced in spherical FLC-4 cells. On the other hand, spherical cell shape-induced miR-194 and miR-320c would directly downregulate SLC7A5 and E2F1 gene expression, respectively, which are both related to malignancy. Our study suggested that spherical cell shape itself would suppress malignancy in FLC-4 cells through microRNA, such as miR-194 and miR-320c.

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Key words: Differentiation, Human hepatoma, FLC-4, HNF-4, MicroRNA, Malignancy

Introduction

Three-dimensional culture of rat primary hepatocytes could restore cell polarity and upregulated many liver-specific gene expression including hepatocyte nuclear factor (HNF)-4α, albumin and apo A-I (Oda et al., 1995; Oda et al., 2008). Similarly, three-dimensional culture of human primary hepatocytes could improve the expression levels of drug metabolizing enzymes (Pampaloni et al., 2009). However, long term culture of human primary hepatocytes is difficult to achieve since these cells present limited growth activity and life-span, even in three-dimensional culture (Guillouzo and Guguen-Guillouzo, 2008). To overcome this issue, hepatoma cell lines are commonly used to study liver functions on the long term, though hepatoma cell lines generally lack a substantial set of liver-specific gene expression, especially major cytochrome P-450 (CYP) (Wilkening et al., 2003).

Functional liver cell (FLC) cell lines, human hepatoma cell lines, express and secrete liver-specific proteins at a high level (Hasumura et al., 1988; Homma et al., 1990). Spherical cell shape in FLC-4 cells enhanced liver-specific gene expression, including albumin, apo A-I, CYP3A7, at a similar level to that of human liver (Laurent et al., 2012). However, common hepatoma cell lines did not show higher liver-specific functions in three-dimensional culture (Laurent et al., 2012). We concluded that cell shape per se, but not single components of EHS-gel, enhanced this gene expression through HNF-4α, a crucial liver-enriched transcription factor (LETF) regulating a broad range of liver functions (Laurent et al., 2012). Up to now, it is unclear how cell shape induces LETF gene expression.

Recently, microRNA, which are 21–23 nucleotide non-coding RNA, were described as post-transcriptional regulators of gene expression through repressing many specific target mRNA (Ambros, 2004). miR-122, miR-192 and miR-194 are highly expressed in the liver and were shown to regulate liver functions (Giard et al., 2008; Xie et al., 2011). More recently, microRNA–LETF regulatory feedback loops have been found (Takagi et al., 2010; Zeng et al., 2010). In particular, miR-24 was shown to target directly HNF-4α and its downstream target gene expression, such as CYP7A1, in HepG2 cells (Takagi et al., 2010).
In this study, the mechanism of HNF-4α induction by three-dimensional cell shape in FLC-4 cells was explored by performing both mRNA and microRNA microarray analysis and integrating these data by bioinformatics. Gene expression analysis suggested that liver functions were increased and malignant phenotype was repressed in spherical FLC-4 cells. We found that spherical cell shape itself would repress malignancy-related gene expression through microRNA, including miR-194 and miR-320c.

Results and Discussion

Microarray of mRNA revealed that spherical cell shape enhanced drug metabolism and suppressed malignancy in FLC-4 cells

Spherical cell shape of FLC-4 cells on EHS-gel (Fig. 1A) induced HNF-4α gene expression in FLC-4 cells (Fig. 1B). Gene expression of HNF-4α was demonstrated to be regulated by many transcription factors, including LETF (Hatzis and Talianidis, 2001). To understand how spherical cell shape induced HNF-4α gene expression, we performed mRNA microarray and bioinformatics approach. Our results revealed that 83 genes were upregulated, and that 87 genes were downregulated in spherical FLC-4 cells. Various functions were altered by spherical cell shape in FLC-4 cells (Table 1). Among them, gene expression related to drug metabolism was upregulated according to pathway analysis (Table 1). Surprisingly, we found that cancer related gene expression was the most changed by spherical, and was downregulated by spherical cell shape of FLC-4 cells (Table 1).

Spherical cell shape enhanced hepatocyte-specific gene expression in FLC-4 cells

Since drug metabolism was upregulated by spherical cell shape in mRNA microarray (Table 1), we quantified this gene expression using qRT-PCR. Gene expression related to phase I drug metabolizing enzymes (CYP2C9, CYP3A7 and CYP4F2) was elevated in spherical FLC-4 cells (Fig. 1C). CYP2C9 and CYP3A are importantly expressed in the liver (Kamiyama et al., 2007; Rana et al., 2010). Spherical cell shape enhanced the gene expression related to phase II (glutathione S-transferase (GST) A3, sulfotransferase (SULT)1B1, UDP-glucuronosyltransferase (UGT)2B15) and drug efflux pump so-called phase III (ABC)C2, ABCC3 in FLC-4 cells (Fig. 1C). Drug metabolizing enzyme gene expression, such as CYP2C, CYP3A, SULT and ABC were shown to be induced by HNF-4α (Kamiyama et al., 2007). We hypothesized that spherical cell shape-dependent induction of HNF-4α would enhance the expressions of drug-metabolizing enzymes in FLC-4 cells.

The gene expression of several nuclear receptors was upregulated in microarray experiment (supplementary material Table S1), especially liver X receptor (LXR)α (NR1H3) (nuclear...
Table 1. Top biofunctions and pathways altered in spherical FLC-4 cells in microarray experiment. We compared gene expression between FLC-4 cells cultured on EHS-gel-coated dish or uncoated plastic dish using oligonucleotide microarray.

| Category                                      | P value | Upregulated genes | Downregulated genes |
|-----------------------------------------------|---------|-------------------|---------------------|
| Canonical pathway\(^a\)                      |         |                   |                     |
| Xenobiotic Metabolism Signaling               | 1.58E−20| 21                | 7                   |
| LPS/IL-1 Mediated Inhibition of RXR Function  | 2.51E−18| 21                | 2                   |
| Aryl Hydrocarbon Receptor Signaling           | 7.94E−17| 9                 | 10                  |
| Metabolism of Xenobiotics by Cytochrome P450  | 6.31E−12| 14                | 0                   |
| Aldosterone Signaling in Epithelial Cells     | 2.51E−11| 4                 | 11                  |
| NRF2-mediated Oxidative Stress Response       | 2.51E−11| 11                | 5                   |
| p53 Signaling                                 | 1.10E−10| 9                 | 10                  |
| Hereditary Breast Cancer Signaling            | 1.23E−10| 3                 | 10                  |
| Molecular Mechanisms of Cancer                | 1.26E−10| 6                 | 14                  |
| Role of CHK Proteins in Cell Cycle Checkpoint Control | 8.13E−10| 1 | 7 |
| Hepatic Cholestasis                          | 8.91E−10| 7                 | 6                   |
| Biofunctions\(^b\)                           |         |                   |                     |
| Cancer                                       | 4.92E−31| 45                | 61                  |
| Cell Death                                    | 8.95E−27| 31                | 58                  |
| Gastrointestinal Disease                     | 2.55E−22| 48                | 51                  |
| Hepatic System Disease                        | 2.55E−22| 31                | 33                  |
| Dermatological Diseases and Conditions        | 2.97E−22| 22                | 39                  |
| Cell Cycle                                   | 6.31E−21| 10                | 44                  |
| Genetic Disorder                             | 5.44E−19| 62                | 63                  |
| Respiratory Disease                          | 5.44E−19| 14                | 29                  |
| Cellular Growth and Proliferation             | 1.65E−17| 29                | 51                  |
| Tissue Morphology                            | 4.23E−17| 21                | 27                  |
| Cellular Development                         | 9.95E−16| 21                | 47                  |
| Cellular Movement                            | 6.34E−15| 23                | 31                  |
| Hematological Disease                        | 1.06E−14| 25                | 35                  |
| Reproductive System Disease                  | 1.44E−14| 30                | 44                  |
| Hematological System Development and Function | 1.07E−13| 20                | 40                  |
| Hematopoiesis                                | 1.37E−13| 14                | 30                  |
| Inflammatory Disease                         | 2.47E−13| 40                | 39                  |
| Cellular Function and Maintenance            | 6.19E−13| 13                | 28                  |
| Organismal Survival                          | 7.97E−13| 20                | 28                  |

\(^a\)Differentially expressed gene list from microarray experiment were imported in Ingenuity pathway analysis. Significance expressed as P values were calculated using the right-tailed Fisher’s exact test. Canonical pathways significantly changed were obtained (P < 10\(^{-5}\)).

\(^b\)Differentially expressed gene list from microarray experiment were imported in Ingenuity pathway analysis. Significance expressed as P values were calculated using the right-tailed Fisher’s exact test. Biofunctions significantly changed were obtained (P < 10\(^{-5}\)).
to repress the gene expression of malignancy-related genes, such as c-Myc and HIF-1α (Wang et al., 2011; Yin et al., 2008). From these results, it was supposed that cell shape-dependent induction of HNF-4α would mediate the induction of liver-specific gene expression and repression of malignancy-related gene expression (Fig. 2).

Changes in microRNA expression would mediate cell shape-dependent induction of HNF-4α and the suppression of malignant phenotype in spherical FLC-4 cells.

The purpose of this study was to get insight into the induction mechanism of HNF-4α gene expression by spherical cell shape in FLC-4 cells. To clarify this mechanism, we performed microRNA microarray analysis. The results indicated that 23 microRNA were upregulated, and 6 microRNA were downregulated significantly in spherical FLC-4 cells (Table 2).

We then analyzed integrated data of mRNA and microRNA microarray by bioinformatics to get insight into mRNA and microRNA interactions. Although 29 microRNAs were changed by spherical FLC-4 cells on EHS-gel, many of them were not related to changes in mRNAs. And only four microRNAs were selected to be associated with changes in mRNAs (Table 3). Therefore, we focused on these four microRNAs. None of them were not related to HNF-4α.

We also found that miR-24 level was slightly but significantly downregulated (Table 2), which directly suppressed HNF-4α.

Fig. 2. Hypothetic regulation of gene expression by HNF-4α by spherical cell shape in FLC-4 cells. Focusing on mRNA microarray data, ingenuity pathway analysis (IPA) was used to identify the HNF-4α signaling network in spherical FLC-4. Gray arrows and gray lines indicate regulation of gene expression and protein interactions respectively. Gray and white colors depict upregulation and downregulation of gene expression, respectively. Circles delimitate molecular functions of genes that were classified manually. The gene expression of SREBF1 and HNF4A were measured by real-time RT-PCR.

Table 2. MicroRNA differentially expressed in microRNA microarray experiments.

| Probe        | MicroRNA       | Log 2 ratio | FDR     |
|--------------|----------------|-------------|---------|
| A_25_P00012247 | hsa-miR-188-5p | 3.42        | 1.00E-05|
| A_25_P00010249 | hsa-miR-630   | 3.07        | 4.20E-04|
| A_25_P00015036 | hsa-miR-320c  | 2.79        | 1.00E-05|
| A_25_P00011703 | hcmv-miR-US4  | 2.59        | 1.40E-03|
| A_25_P00014861 | hsa-miR-483-5p| 2.46        | 1.00E-05|
| A_25_P00010800 | hsa-miR-663   | 2.44        | 4.40E-04|
| A_25_P00015195 | hsa-miR-1268  | 2.09        | 1.00E-05|
| A_25_P00011725 | hcmv-miR-UL70-3p| 1.91      | 8.00E-05|
| A_25_P00015087 | hsa-miR-1207-5p| 1.76        | 1.00E-05|
| A_25_P00010869 | hsa-miR-192   | 1.72        | 1.00E-05|
| A_25_P00012230 | hsa-miR-134   | 1.68        | 1.00E-05|
| A_25_P00014921 | hsa-miR-1225-5p| 1.64      | 8.00E-05|
| A_25_P00015075 | hsa-miR-1202  | 1.58        | 1.00E-05|
| A_25_P00010402 | hsa-miR-638   | 1.53        | 1.00E-05|
| A_25_P00015059 | hsa-miR-1181  | 1.49        | 3.40E-04|
| A_25_P00015317 | hsa-miR-1469  | 1.26        | 1.10E-04|
| A_25_P00012212 | hsa-miR-125a-3p| 1.23        | 7.00E-05|
| A_25_P00011006 | hsa-miR-194   | 1.19        | 7.00E-05|
| A_25_P00012698 | hsa-miR-455-3p| 1.18        | 2.40E-04|
| A_25_P00015209 | hsa-miR-1275  | 1.14        | 7.00E-05|
| A_25_P00010926 | hsa-miR-215   | 1.09        | 7.00E-05|
| A_25_P00012409 | hsa-miR-345   | 1.07        | 3.00E-05|
| A_25_P00015286 | hsa-miR-1471  | 1.03        | 3.14E-03|
| A_25_P00013116 | hsa-let-7b*   | 1.06        | 2.70E-04|
| A_25_P00014965 | hsa-miR-1238  | -1.14       | 3.60E-03|
| A_25_P00010054 | hsa-miR-29b   | -1.20       | 1.10E-04|
| A_25_P00014887 | hsa-miR-513a-5p| -1.79      | 1.86E-03|
| A_25_P00013789 | ebv-miR-BART19-3p| -2.12    | 9.10E-04|
| A_25_P00012257 | hsa-miR-193a-3p| -2.70      | 2.00E-05|
| A_25_P00010676*| hsa-miR-24    | -0.39      | 4.28E-03|

*MicroRNAs were declared significant when log 2 ratio was ≥ 1 or ≤ -1, and False Discovery rate (FDR) was below 0.05.

†Agilent Probe IDs are indicated.

‡Log 2 ratio (FLC-4 cells cultured on EHS-gel-coated dish versus FLC-4 cultured on plastic-coated dish) was calculated from three independent experiments.

§FDR was calculated.

‖miR-24 expression level was indicated though the absolute value of log 2 ratio was below 1.
Table 3. Putative mature microRNA–mRNA regulations in spherical FLC-4 cultured on EHS-gel. FLC-4 cells were plated at 40% density on uncoated or EHS-gel-coated plastic dishes and cultured for 48 hours. Total RNA or total RNA containing small RNA were extracted and submitted to mRNA microarray and microRNA microarray respectively.

| Mature miRNA* | Entrez gene id | Gene symbol | Description | Fold changeb |
|---------------|----------------|-------------|-------------|---------------|
| hsa-miR-125a-3p | 1871 | E2F3 | E2F transcription factor 3 | 2.40 |
| Log ratio: 1.23 | | | | |
| Targets: 139 mRNAs | | | | |
| hsa-miR-194 | 8140 | SLC7A5 | Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 | 1.90 |
| Log ratio: 1.19 | 1871 | E2F3 | E2F transcription factor 3 | 2.40 |
| Targets: 258 mRNAs | 7026 | NR2F2 | Nuclear receptor subfamily 2, group F, member 2 | 2.80 |
| hsa-miR-29b | 2941 | GSTA4 | Glutathione S-transferase A4 | 1.82 |
| Log ratio: −1.20 | 1376 | CPT2 | Carnitine palmitoyltransferase II | 1.98 |
| Targets: 850 mRNAs | 10437 | IFI30 | Interferon, gamma-inducible protein 30 | 1.92 |
| | 5295 | PIK3R1 | Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha) | 1.90 |
| | 9612 | NCOR2 | Nuclear receptor co-repressor 2 | 1.83 |
| | 1277 | COL1A1 | Collagen, type I, alpha 1 | 2.00 |
| | 3688 | ITGB1 | Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) | 4.11 |
| | 8503 | PIK3R3 | Phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma) | 2.29 |
| | 6059 | ABCE1 | ATP-binding cassette, sub-family E (OABP), member 1 | 1.94 |
| | 10058 | ABCB6 | ATP-binding cassette, sub-family B (MDR/TAP), member 6 | 2.38 |
| hsa-miR-320c | 2553 | GABPB2 | GA binding protein transcription factor, beta subunit 2 | 1.84 |
| Log ratio: 2.79 | | | | |
| Targets: 539 mRNAs | 1869 | E2F1 | E2F transcription factor 1 | 1.88 |
| | 5921 | RASA1 | RAS p21 protein activator (GTPase activating protein) 1 | 2.34 |
| | 2935 | GSPT1 | G1 to S phase transition 1 | 1.90 |
| | 1871 | E2F3 | E2F transcription factor 3 | 2.40 |
| | 7048 | TGFRB2 | Transforming growth factor, beta receptor II (70/80kDa) | 2.89 |
| | 5295 | PIK3R1 | Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha) | 1.90 |

aFor mature microRNA differentially expressed in microRNA microarray experiment, log 2 ratio (EHS-gel versus plastic) (n=3) and the number of putative target genes are indicated.

bFold change of gene expression is indicated for microRNA targeted genes that were differentially expressed in mRNA microarray experiment.

gene expression (Takagi et al., 2010). Other microRNA predicted to target HNF-4α in Targetscan database were not affected by spherical cell shape. Although the changes in miR-24 might partly explain the induction of HNF-4α by spherical cell shape, we supposed that the change in miR-24 was too small to explain the induction of HNF-4α.

Microarray of mRNA indicated that hepatocyte-specific gene expression, including drug metabolism and insulin signaling, was induced by spherical cell shape in FLC-4 cells (Table 1; supplementary material Table S1). In contrast, malignancy-related gene expression was repressed by spherical cell shape (Table 1; supplementary material Table S2). To understand how microRNA would regulate the gene expression changed by spherical cell shape, we integrated our microRNA and mRNA expression data using Targetscan database to predict mRNA–microRNA interactions. miR-29b was downregulated by spherical cell shape in FLC-4 cells, and was predicted to induce phosphatidylinositol 3-kinase regulatory subunit (PIK3R)1 and PIK3R3 gene expression (Table 3). Since these genes are involved in insulin signaling and malignancy (Anderson, 2010), spherical cell shape-dependent reduction of miR-29b level would regulate these functions in FLC-4 cells. In contrast, miR-194 was upregulated by spherical cell shape, and was predicted to suppress SLC7A5 gene expression, implicated in malignant phenotype (Table 3). These results suggested that spherical cell shape in FLC-4 cells would repress malignancy through miR-194, which was shown to suppress this gene expression in the liver (Meng et al., 2010). Three-dimensional cell shape induced miR-320c, which was predicted to inhibit E2F1 gene expression in FLC-4 cells (Table 3). miR-320 family and E2F1 were shown to inhibit and promote malignant progression respectively (Ladu et al., 2008; Wentz-Hunter and Potashkin, 2011). This indicated that miR-320c would also mediate the suppression of malignancy-related gene expression by spherical cell shape in FLC-4 cells. Based on these results, the enhancement of miR-194 and miR-320c level by spherical cell shape would suppress malignancy in FLC-4 cells.

In conclusion, the present study showed that spherical cell shape in FLC-4 cells greatly induced gene expression related to drug metabolism and lipid metabolism. The suppression of malignancy-related gene expression by spherical cell shape was put into evidence in FLC-4 cells. The induction of HNF-4α gene expression would have a central role in the enhancement of hepatocyte-specific gene expression and the suppression of the.
gene expression related to malignancy by spherical cell shape in FLC-4 cells. The integration of microRNA and mRNA microarray data indicated that spherical cell shape would elicit the repression of malignancy through enhancing miR-194 and miR-320c levels.

Materials and Methods

Cell culture

FLC-4 cells were maintained in ASF104 serum-free medium (Ajinomoto Co. Ltd., Tokyo, Japan). Cells were inoculated into 100-mm culture dishes (Corning Inc., Corning, NY, USA) at 40% confluency (number of cells: 2.7 × 10^6/cm^2) and cultured with medium changes every 3 days. Cell cultures were carried out in 100-mm dishes (Cat. no. 1029; Falcon, Becton Dickinson, Lincoln Park, NJ, USA) under a constant temperature of 37 °C with highly humidified 95% air and 5% CO2. Type I collagen (TIC)-coated dishes were prepared by adding 100 μg/mL TIC (Nitta Gelatin, Japan) to plastic dishes (Cat. no. 1029; Falcon, Becton Dickinson, Lincoln Park, NJ, USA). EHS-gel-coated dishes were prepared as described previously (Oda et al., 2008).

Total RNA and small RNA isolation and analysis

Total RNA was extracted according to the method of Chomczynski and Sacchi (1987). Total RNA containing small RNAs was extracted from spread FLC-4 cells or spherical FLC-4 cells using the mirVana™ microRNA isolation kit according to the manufacturer’s protocol (Cat. #AM1560, Ambion, Austin, TX, USA). Quality of RNA was determined using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and subsequently quantified using NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Cat. #4388884, Applied Biosystems, Foster City, USA) following the manufacturer’s instructions. Real-time RT-PCR amplifications were performed in ABI StepOne (Applied Biosystems, Foster City, USA) using 2× Power SYBR Master Mix (Cat. #4309155, Applied Biosystems) as described previously (Laurent et al., 2012). Primer sequences are indicated in supplementary material Table S3. Quantitative RT-PCR melting curve data were collected to check the specificity of PCR. Expression was calculated using the standard curve method. The expression of the chosen genes was normalized to that of 18S rRNA as a reference.

Nuclear protein extraction and electrophoretic mobility-shift assay (EMSA)

Nuclear proteins were extracted from FLC-4 cells and electrophoretic mobility-shift assay (EMSA) was performed as described previously (Laurent et al., 2012). EMSA was performed using the following double-stranded oligonucleotide: DR-4 (5′-agctTCAGGCTACCTACGGTAC-3′, 5′-tcgaGTGACCTGAAGTGACCT-3′) and 5′-tagcTTGGACCTGAAGTAGCTC-3′.

Microarray of mRNA and data analysis

Total RNA from spread and spherical FLC-4 cells were used for mRNA microarray analysis. We utilized a DNA oligonucleotide microarray containing duplicate cDNA spots of 1262 well annotated genes of various functional classes, including cytokines/growth factors and their receptors, oncogenes, drug metabolizing enzyme, transcription factors and housekeeping genes (Hitachi Life Science, Sattama, Japan). Five micrograms of total RNA isolated was in vitro amplified, and the antisense RNA (aRNA) from spread FLC-4 cells was labeled with a fluorescent dye Cy5, while aRNA from spherical FLC-4 cells was labeled with a fluorescent dye Cy3. The aRNA samples were hybridized to the Agilent Human microRNA Microarray V3 for 20 h at 20 rpm, 55 °C (Cat. #G4470C, Agilent, Santa Clara, CA, USA). Slides were washed and scanned according to the manufacturer’s instructions. Images were quantified using Feature Extraction (Agilent, Santa Clara, CA, USA). The miRNA array contained the complete content sourced from Sanger database 12.0, i.e. 851 probes for human and 88 probes for viral microRNA transcripts (Agilent, Santa Clara, CA, USA). The raw dataset was normalized and analyzed using the “AgiMicroRNA” package of the Bioconductor (http://www.bioconductor.org) suite of software for the R statistical programming language (http://www.r-project.org). Quantile normalization was then used to standardize the data across arrays, and a linear model was fitted to each microRNA using “AgiMicroRNA” package. The result P values were obtained using a moderated t-test statistics, adjusted for multiple testing by using the Benjamini–Hochberg correction of the false-discovery rate. MicroRNA were selected according to the following criteria: False Discovery Rate (FDR) < 0.05. The results are expressed in log 2 ratio (EHS-gel versus plastic). MicroRNA were selected according to the following criteria: log 2 ratio ≤ −1 or log 2 ratio ≥ 1. The miRNA target prediction was performed using TargetScan database (http://www.targetscan.org).

Statistical analysis

The significance of differences among values was analyzed by ANOVA and Student’s t-test. When P value was below 0.05, differences were considered significant. Values in the text are expressed as means ± s.e.m.

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Competing Interests

The authors have no conflicting interests to declare.

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