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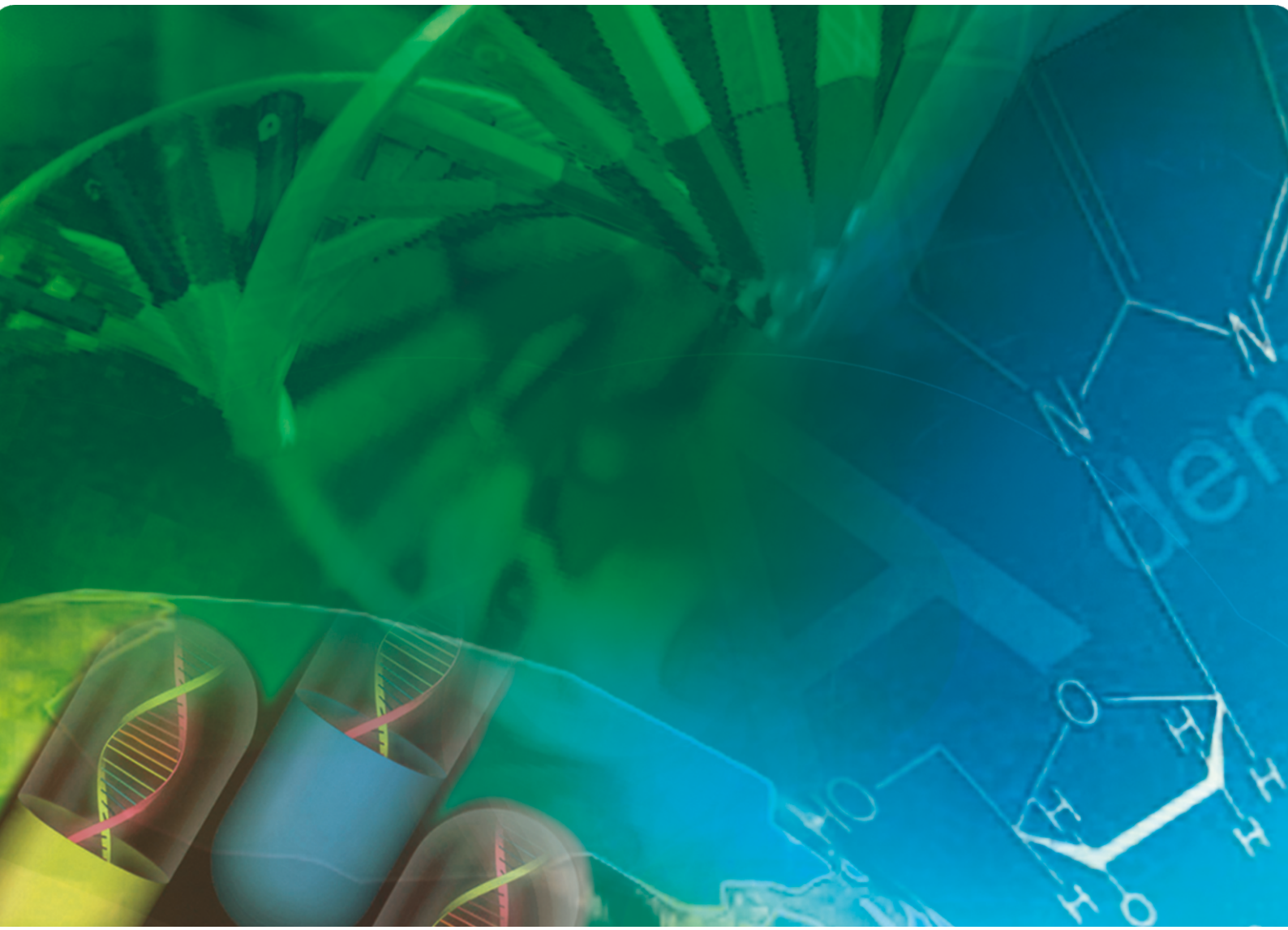

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Gene Expression Changes Associated with the Loss of Heterogeneous Nuclear Ribonucleoprotein M Function

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Abstract

Advanced glycation endproducts (AGEs) are formed by the nonenzymatic reaction of sugars with proteins. Glycation may adversely affect proteins, such as by inducing a loss of function. It has been shown that glyceraldehyde-derived AGEs (Glycer-AGEs) accumulate in the liver of patients with nonalcoholic steatohepatitis (NASH). Previously, we showed the formation of intracellular Glycer-AGEs upon exposure of hepatocytes to fructose *in vitro,* and identified an RNA-binding protein, heterogeneous nuclear ribonucleoprotein M (HNRNPM), as a target for glycation. However, the impact of glycated HNRNPM in NASH remains poorly understood. In this study, we examined gene expression changes caused by HNRNPM knockdown, and investigated the up- and down-regulated genes as noninvasive biomarker candidates for NASH. Microarray analysis after HNRNPM knockdown showed that the levels of 138 transcripts were increased, while those of 100 transcripts were decreased as compared with those in the control. Gene Ontology-based functional analysis showed that 14 upregulated and 9 downregulated genes were associated with the extracellular space, which may enable their detection using blood tests. Among these, six of the up- and down-regulated genes were associated with the extracellular exosome. These results suggest that the loss of HNRNPM function by glycation is reflected extracellularly. Therefore, the identified genes may serve as noninvasive biomarkers for Glycer-AGEs-related NASH.

Keywords

Glycation, Nonalcoholic Steatohepatitis, Heterogeneous Nuclear

Ribonucleoprotein M, Biomarkers

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), and is the most common liver disease worldwide [1] [2] [3] [4] [5]. Although simple steatosis is considered a benign and nonprogressive condition, NASH is a potentially progressive disease that can lead to fibrosis, cirrhosis, and hepatocellular carcinoma [6] [7]. Currently, only invasive liver biopsy can distinguish between these diseases, but may cause severe complications in patients. Therefore, the development of noninvasive biomarkers is needed [8].

Advanced glycation endproducts (AGEs) are formed by the Maillard reaction, a nonenzymatic reaction between the ketone or aldehyde groups of sugars and the amino groups of proteins [9] [10]. Recent studies have suggested that AGEs can additionally arise from carbonyl compounds derived from the autoxidation of sugars and other metabolic pathways [11] [12]. It is well known that glycation of proteins alters their function [13] [14]. Our recent study suggested that glyceraldehyde-derived AGEs (Glycer-AGEs) accumulate in the liver of NASH patients, but not in that of patients with simple steatosis [15]. Furthermore, we detected the formation of Glycer-AGEs in human hepatocyte Hep3B cells exposed to high fructose concentrations, and identified the heterogeneous nuclear ribonucleoprotein M (HNRNPM) as a target for glycation in these cells. HNRNPM plays an important role in the regulation of gene expression by processing heterogeneous nuclear RNAs into mature mRNAs [16]. However, the effects of glycated HNRNPM on the pathophysiology of NASH remain poorly understood.

In this study, we examined gene expression changes caused by HNRNPM knockdown, and investigated genes that may be further developed as noninvasive biomarkers for Glycer-AGEs-related NASH.

2. Materials and Methods

2.1. Chemicals

All chemicals were commercial samples of high purity, and were used according to the manufacturer's instructions. Fructose was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Cell Cultures

Hep3B and HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA), under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37˚C). Cells (Hep3B: 2×10^5 cells/mL, HepG2: 5×10^5 cells/mL) were seeded in culture dishes (BD Biosciences, Franklin Lakes, NJ, USA), and incubated for one day prior to use in experiments. Cells

were incubated with or without fructose for one day or five days, to form glycated HNRNPM.

2.3. Two-Dimensional Gradient Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Gradient SDS-PAGE

Cell lysates were prepared as described previously in [16], and protein concentrations were measured. In two-dimensional gradient SDS-PAGE, 100 μg cell lysates were resolved on an agar gel (pH range: 5 - 10) (ATTO, Tokyo, Japan), and a 5% - 20% SDS-polyacrylamide gradient gel (ATTO, Tokyo, Japan). In gradient SDS-PAGE, the cell lysates were resolved on a 4% - 12% SDS-polyacrylamide gradient gel (Life Technologies, Carlsbad, CA, USA). All processes were performed according to the manufacturer's instructions.

2.4. Western Blot Analysis

Western blot analysis was performed as described previously in [16], using the antibodies: rabbit anti-Glycer-AGEs, mouse anti-HNRNPM (Millipore Corporation, Billerica, MA, USA), mouse anti-β-actin (Santa Cruz Biotechnology, Inc., Billerica, CA, USA), anti-rabbit IgG (GeneTex, Irvine, CA, USA), and anti-mouse IgG antibody (Dako, Agilent Technologies, Santa Clara, CA, USA).

2.5. HNRNPM Knockdown

Two Silencer Select siRNAs targeting HNRNPM (si1 and si2) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used to downregulate the expression of HNRNPM in Hep3B cells. A nontargeting siRNA was used as control. siRNA transfection was performed using MISSION siRNA Transfection Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

2.6. Microarray Analysis

Total RNA was isolated using ISOGEN II (Nippon Gene, Tokyo, Japan), and labeled with CyDye, using the Low Input Quick Amp Labeling kit per manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Briefly, cDNA was synthesized from the isolated RNA using the T7 promoter primer. Then, cRNA was synthesized using T7 RNA polymerase in the presence of either Cy3-CTP or Cy5-CTP. The labeled cRNAs were hybridized to the DNA microarray (SurePrint G3 Human Gene Expression 8 × 60K version 2.0, Agilent Technologies, Santa Clara, CA, USA), and washed using the recommended washing buffer. Detection was performed using the Agilent Microarray Scanner (Agilent Technologies, Santa Clara, CA, USA), and the data were analyzed using the Agilent Feature Extraction software 10.7.3.1 (Agilent Technologies, Santa Clara, CA, USA). The differences were considered significant at $p \leq 0.05$ (two-sided). The dataset was summarized and normalized using the Gene Spring software (Agilent Technologies, Santa Clara, CA, USA). Gene Ontology (GO) functional classification was performed using AmiGO2 [17].

3. Results

3.1. Formation of Glycated HNRNPM Following Short- and Long-Term Exposure to Fructose

The effects of short-(one day) or long-term (five days) exposure to fructose were examined by western blot analysis, using the anti-Glycer-AGEs antibody, following two-dimensional gel electrophoresis. In Hep3B cells, glycated HNRNPM was detected upon long-term exposure to fructose, but not upon short-term (one day) exposure. In addition, it was not detected in either of the controls (Figure 1(a)). No significant differences were found in the HNRNPM expression levels between the four conditions (Figure 1(a)). This suggests that HNRNPM is not glycated by short-term exposure to fructose. Furthermore, glycated HNRNPM was also detected in HepG2 cells upon long-term fructose exposure (Figure 1(b)).

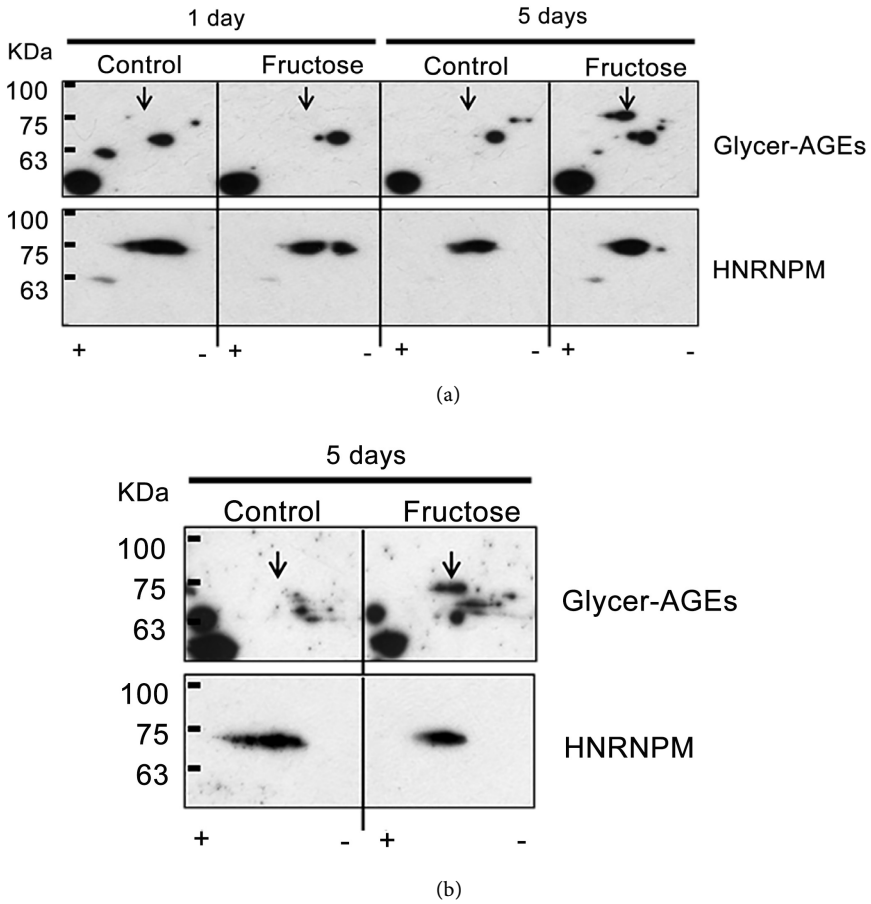


Figure 1. Western blot analysis of the glycated heterogeneous nuclear ribonucleoprotein M (HNRNPM). Cells were incubated with or without 10 mmol/L fructose for one day or five days. Cell lysates (100 μg protein/gel) were resolved using two-dimensional gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The blots were first probed with anti-glyceraldehyde-derived advanced glycation endproducts (Glycer-AGEs) antibody, and subsequently reprobed with anti-HNRNPM antibody. (a) Hep3B cells, (b) HepG2 cells. The arrows show the spots where glycated HNRNPM was identified.

3.2. HNRNPM Knockdown and Microarray Analysis

It has been suggested that HNRNPM glycation leads to a decrease in its function. Thus, we performed microarray analysis to investigate gene expression changes in response to HNRNPM knockdown. Western blot analysis showed that HNRNPM expression was remarkably depleted in cells transfected with siRNAs targeting HNRNPM, compared with that in the control (Figure 2). A similar result was provided with other cell line (data not shown).

We compared the RNA expression levels in the HNRNPM knockdown and control samples. We identified 138 and 100 transcripts with expression levels 1.5-fold higher and lower, respectively, compared with those in the control (Table 1, Table 2). Of these, 60 upregulated and 67 downregulated genes had GO terms.

3.3. Identification of Up- and Down-regulated Genes Associated with the Extracellular Space

We performed functional classification of the genes whose expression levels were influenced by HNRNPM knockdown. We extracted the GO terms that were associated with more than five genes in each of the up- or down-regulated gene set, and unified similar GO terms (Figure 3). We further analyzed the genes belonging to the category "cellular component," one of the three gene categories in GO. The ratio of genes classified as belonging to the cytoplasm, nucleus, membrane, endoplasmic reticulum, and extracellular space were no great difference in the upregulated genes and downregulated genes. We identified 14 upregulated and 9 downregulated genes annotated to the GO term "extracellular space," which may be further investigated as potential noninvasive biomarkers (Table 3, Table 4). Furthermore, among these genes, six each of the up- and down-regulated genes were annotated to the term "extracellular exosome" (Table 3, Table 4).

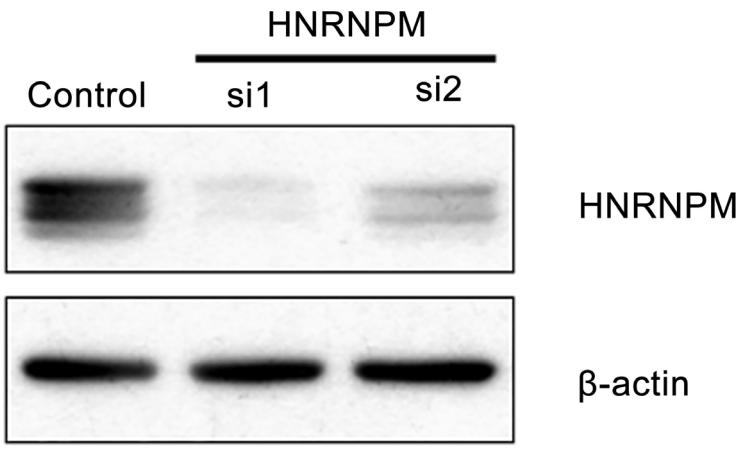


Figure 2. siRNA-mediated knockdown of heterogeneous nuclear ribonucleoprotein M (HNRNPM). Cell lysates were separated by SDS-PAGE and probed with anti-HNRNPM antibody. Equal protein loading was determined using anti-β-actin antibody.

Table 1. The transcripts upregulated (≥1.5-fold) by HNRNPM knockdown.

Rank	Gene symbol	FC†	Rank	Gene symbol	FC†	Rank	Gene symbol	FC†
1	*lnc-PARN-7*	10.1	47	*LINC01389*	2.4	93	*A_33_P3262022*	1.8
2	*lnc-NAALADL2-1*	8.5	48	*GNAS-AS1*	2.4	94	*LUZP1*	1.8
3	*ENST00000449119*	7.8	49	*lnc-CLIC3-1*	2.4	95	*ATP5E*	1.8
4	*lnc-COL18A1-2*	5.8	50	*A_21_P0014212*	2.3	96	*ZNF548*	1.8
5	*A_33_P3346643*	5.7	51	*WHSC1*	2.3	97	*LEAP2*	1.8
6	*lnc-RP11-539E17.5.1-1*	5.0	52	*ENST00000390298*	2.3	98	*AK130724*	1.8
7	*lnc-DCTD-8*	5.0	53	*LOC100996579*	2.3	99	*LOC100128242*	1.8
8	*MMP1*	4.2	54	*lnc-NDUFS8-1*	2.2	100	*LOC100289120*	1.7
9	*lnc-FOXF1-1*	4.0	55	*A_33_P3392350*	2.2	101	*PRR4*	1.7
10	*ENST00000590022*	3.9	56	*CADM2*	2.2	102	*FBXO36*	1.7
11	*CFAP57*	3.8	57	*ENST00000558690*	2.2	103	*HDAC9*	1.7
12	*XLOC_l2_010772*	3.8	58	*CDC6*	2.1	104	*MROH1*	1.7
13	*ASPHD2*	3.7	59	*PRH2*	2.1	105	*lnc-PRDM13-4*	1.7
14	*CA6*	3.7	60	*FXYD1*	2.1	106	*lnc-ATXN3L-2*	1.7
15	*lnc-CHTF8-2*	3.6	61	*ENST00000534505*	2.1	107	*PTGES2-AS1*	1.7
16	*KCNE5*	3.5	62	*PLA2G4F*	2.1	108	*C3orf52*	1.7
17	*SPON1*	3.5	63	*GS1-259H13.2*	2.0	109	*LINC00540*	1.7
18	*LOC441239*	3.4	64	*lnc-ALDH1A1-2*	2.0	110	*FLCN*	1.7
19	*ENST00000521016*	3.3	65	*ZFP14*	2.0	111	*lnc-C6orf221-1*	1.7
20	*lnc-MAEL-1*	3.3	66	*LOC100130502*	2.0	112	*BC009492*	1.7
21	*NAP1L4*	3.3	67	*CAPS2*	2.0	113	*ATXN7L1*	1.7
22	*HSD17B3*	3.3	68	*SARS*	2.0	114	*LOC102724910*	1.7
23	*LINC01491*	3.2	69	*GNGT1*	2.0	115	*ENST00000519159*	1.7
24	*CHN1*	3.2	70	*LOC388780*	2.0	116	*LINC01578*	1.7
25	*IGF2BP1*	3.2	71	*SLC38A4*	2.0	117	*ENST00000513405*	1.7
26	*GREB1*	3.1	72	*LOC101927571*	1.9	118	*lnc-PMM2-6*	1.7
27	*lnc-KBTBD6-1*	3.1	73	*DSE*	1.9	119	*ZNF396*	1.6
28	*OXNAD1*	3.0	74	*ENST00000332359*	1.9	120	*RECQL5*	1.6
29	*LOC102723456*	2.9	75	*ENST00000538869*	1.9	121	*FARP1*	1.6
30	*lnc-COPS4-1*	2.8	76	*lnc-RAB40AL-1*	1.9	122	*ZNF460*	1.6
31	*lnc-GABPA-4*	2.8	77	*CTBP2*	1.9	123	*SMIM7*	1.6
32	*lnc-MAGEA8-2*	2.7	78	*ENST00000566803*	1.9	124	*TMEM201*	1.6
33	*AOAH-IT1*	2.7	79	*A_33_P3298251*	1.9	125	*GOSR2*	1.6
34	*GIPR*	2.7	80	*LOC646938*	1.8	126	*FSTL5*	1.6
35	*RNF213*	2.7	81	*ITGBL1*	1.8	127	*GSAP*	1.6
36	*CTRL*	2.7	82	*POLA1*	1.8	128	*DNAJB6*	1.6
37	*PER2*	2.7	83	*SPI1*	1.8	129	*RPL28*	1.6
38	*XLOC_l2_003888*	2.6	84	*SUV420H1*	1.8	130	*ENST00000562341*	1.6
39	*lnc-SLC15A4-5*	2.6	85	*LOC646652*	1.8	131	*A_33_P3239102*	1.6
40	*LRRC9*	2.6	86	*LINC00476*	1.8	132	*EDIL3*	1.6
41	*YTHDC1*	2.5	87	*GPCPD1*	1.8	133	*MYLK-AS1*	1.6
42	*DPY19L2P3*	2.5	88	*LOC101929542*	1.8	134	*AF119870*	1.6
43	*NEAT1*	2.5	89	*THC2626681*	1.8	135	*LOC101927815*	1.5
44	*CFAP36*	2.5	90	*BDNF-AS*	1.8	136	*DDX31*	1.5
45	*RBM33*	2.4	91	*LEMD1*	1.8	137	*THOC7-AS1*	1.5
46	*PSMF1*	2.4	92	*A_33_P3238325*	1.8	138	*ENST00000412362*	1.5

FC: Fold change. †Fold change indicates 1.5 average or more in 2 analysis (control vs si1 or si2), and shaded cells indicate ≥3.0-fold.

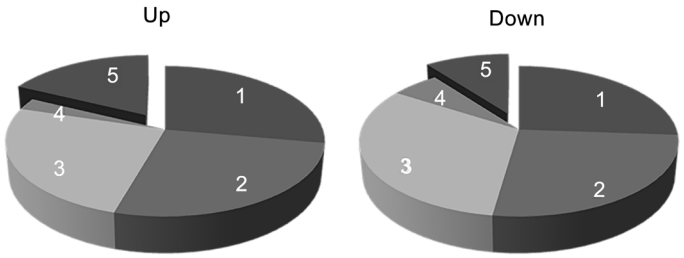
Table 2. The transcripts downregulated (≥1.5-fold) by HNRNPM knockdown.

Rank	Gene symbol	FC†	Rank	Gene symbol	FC†	Rank	Gene symbol	FC†
1	*NIPAL4*	3.9	35	*TFPI*	2.0	69	*A_33_P3262022*	1.7
2	*MIPEPP3*	3.8	36	*STK38L*	2.0	70	*LUZP1*	1.7
3	*ENST00000569087*	3.7	37	*lnc-C3orf52-1*	2.0	71	*ATP5E*	1.7
4	*FLJ45513*	3.6	38	*AUH*	2.0	72	*ZNF548*	1.7
5	*NUDCD3*	3.4	39	*CD59*	2.0	73	*LEAP2*	1.7
6	*XLOC_l2_012319*	3.4	40	*EPB41L5*	2.0	74	*AK130724*	1.7
7	*CCPG1*	3.2	41	*RNF112*	2.0	75	*LOC100128242*	1.7
8	*TYW3*	3.2	42	*ZDHHC11*	1.9	76	*LOC100289120*	1.7
9	*MBP*	3.1	43	*FARP2*	1.9	77	*PRR4*	1.6
10	*OBFC1*	2.9	44	*lnc-SKIV2L2-1*	1.9	78	*FBXO36*	1.6
11	*PHF7*	2.8	45	*PPP2R1B*	1.9	79	*HDAC9*	1.6
12	*LINC00400*	2.7	46	*ERICH1*	1.9	80	*MROH1*	1.6
13	*HMGB3*	2.5	47	*CDH26*	1.9	81	*lnc-PRDM13-4*	1.6
14	*QRICH2*	2.5	48	*HSF2*	1.9	82	*lnc-ATXN3L-2*	1.6
15	*RBM4*	2.4	49	*NDE1*	1.9	83	*PTGES2-AS1*	1.6
16	*SEPT8*	2.4	50	*SCLY*	1.9	84	*C3orf52*	1.6
17	*ENST00000527983*	2.3	51	*MTL5*	1.9	85	*LINC00540*	1.6
18	*RECQL4A*	2.3	52	*TM9SF4*	1.9	86	*FLCN*	1.6
19	*ZNF641*	2.3	53	*ZNF862*	1.9	87	*lnc-C6orf221-1*	1.6
20	*ENST00000550019*	2.3	54	*C6orf118*	1.8	88	*BC009492*	1.6
21	*LOC101927641*	2.2	55	*IL17RA*	1.8	89	*ATXN7L1*	1.6
22	*C16orf90*	2.2	56	*TMEM240*	1.8	90	*LOC102724910*	1.6
23	*lnc-EIF2AK4-4*	2.2	57	*DCBLD1*	1.8	91	*ENST00000519159*	1.6
24	*WDR4*	2.2	58	*TXNRD3*	1.8	92	*LINC01578*	1.6
25	*CRTAP*	2.2	59	*TMCO3*	1.8	93	*ENST00000513405*	1.6
26	*CNST*	2.2	60	*UBXN7*	1.8	94	*lnc-PMM2-6*	1.6
27	*AKR1E2*	2.2	61	*lnc-YIF1A-6*	1.8	95	*ZNF396*	1.6
28	*AK090765*	2.1	62	*TEX19*	1.8	96	*RECQL5*	1.6
29	*lnc-TXLNB-3*	2.1	63	*TMTC3*	1.7	97	*FARP1*	1.6
30	*WNK1*	2.1	64	*BLCAP*	1.7	98	*ZNF460*	1.5
31	*DCUN1D4*	2.1	65	*EIF4G3*	1.7	99	*SMIM7*	1.5
32	*ENST00000606907*	2.1	66	*ADAT2*	1.7	100	*TMEM201*	1.5
33	*lnc-ACTR6-1*	2.1	67	*DBNDD2*	1.7			
34	*ZHX3*	2.1	68	*DNAJB6*	1.7			

FC: Fold change. †Fold change indicates 1.5 average or more in 2 analysis (control vs si1 or si2), and shaded cells indicate ≥3.0-fold.

4. Discussion

NASH is recognized as a component of the metabolic syndrome and is closely associated with insulin resistance and disorders of glucose and lipid metabolism [18] [19] [20] [21]. We recently demonstrated that in NASH patients, Glycer-AGEs are elevated in the sera and simultaneously accumulated in the liver [15]. The accumulation of Glycer-AGEs in the liver is caused by the glycation of



No.	Function description	Genes%	
		Up	Down
1	cytoplasm	28	26
2	nucleus	27	26
3	membrane	25	32
4	endoplasmic reticulum	3	6
5	extracellular spaces	18	10

Figure 3. Gene Ontology (GO) classification of genes up- or down-regulated by HNRNPM knockdown. The gene functional annotations were classified on the basis of the GO term "cellular component."

Table 3. List of the up-regulated genes associated with the extracellular space.

Gene symbol	Gene title
*MMP*1	*matrix metallopeptidase* 1 (*interstitial collagenase*)
CA6††	*carbonic anhydrase VI*
*SPON*1	*spondin 1, extracellular matrix protein*
*GREB*1††	*Growth Regulation By Estrogen In Breast Cancer* 1
CTRL	*chymotrypsin-like*
*PRH*2	*proline-rich protein HaeIII subfamily* 2
SARS††	*seryl-tRNA synthetase*
*ITGBL*1	*integrin, beta-like* 1 (*with EGF-like repeat domains*)
*LUZP*1††	*leucine zipper protein* 1
*LEAP*2	*liver expressed antimicrobial peptide* 2
*PRR*4	*proline rich* 4 (*lacrimal*)
*FSTL*5	*follistatin-like* 5
*RPL*28††	*ribosomal protein L*28
*EDIL*3††	*EGF-like repeats and discoidin I-like domains* 3

††indicates gene associated with the extracellular exosome.

intracellular proteins [22]. Previously, we showed that in Hep3B cells, intracellular Glycer-AGEs increased in response to fructose, and HNRNPM was one of the target proteins for glycation [16]. In this study, we showed that HNRNPM was glycated after long-term exposure to fructose, but not after short-term exposure (**Figure 1(a)**). The generation of glycated HNRNPM after long-term exposure to fructose was also confirmed in HepG2 cells (**Figure 1(b)**). This is consistent with studies showing that Glycer-AGEs are formed by a nonenzymatic reaction dependent on the accumulation of intracellular glyceraldehyde, which is produced by fructose metabolism. Moreover, HNRNPM is suggested to be more easily glycated than other hepatocyte proteins [16].

Table 4. List of the down-regulated genes associated with the extracellular space.

Gene symbol	Gene title
CRTAP	*cartilage associated protein*
AKR1E2††	*aldo-keto reductase family* 1, *member E2*
TFPI	*tissue factor pathway inhibitor* (*lipoprotein-associated coagulation inhibitor*)
CD59††	*CD59 molecule, complement regulatory protein*
PPP2R1B††	*protein phosphatase* 2, *regulatory subunit A, beta*
IL17RA	*interleukin* 17 *receptor A*
ATP6V1C1††	*ATPase, H+ transporting, lysosomal* 42*kDa, V*1 *subunit C*1
GNAI3††	*guanine nucleotide binding protein* (*G protein*), *alpha inhibiting activity polypeptide* 3
N4BP2L2††	*NEDD*4 *binding protein* 2*-like* 2

††indicates gene associated with the extracellular exosome.

During glycation, the lysine and arginine side chains in the protein are modified by reducing sugars, and this causes functional changes in the protein [23] [24]. HNRNPM has an unusual hexapeptide-repeat region, rich in methionine and arginine residues (MR repeat motif), which may participate in the formation of AGEs [25]. HNRNPM plays an important role in the processing of mature mRNAs from heterogeneous nuclear RNAs to regulate gene expression. HNRNPM directly binds to nascent RNA polymerase II transcripts [25]. In RNA-binding proteins, the basic residues lysine and arginine are important for the RNA-protein interactions [26] [27] [28] [29] [30], suggesting that the function of HNRNPM is suppressed by glycation.

Microarray analysis showed that multiple genes were up- or down-regulated after HNRNPM knockdown compared with those in the control (**Table 1**, **Table 2**). Among the genes regulated by HNRNPM knockdown, *MMP1* is associated with the pathophysiology of NASH. Sookoian *et al.* reported that the expression of *MMP1* is significantly higher in NASH compared with that in simple steatosis, and this may contribute to an increased risk of atherosclerosis [31]. We suggest that this effect of NASH on MMP1 expression might be mediated via the loss of HNRNPM function.

We demonstrated that the expression of 23 genes associated with the extracellular space (including the extracellular exosome) was altered upon HNRNPM knockdown (**Table 3**, **Table 4**). Because the protein products of these genes are released or secreted into the blood, they may be easily detected by noninvasive methods. We thus plan to analyze the changes in expression levels of these proteins to develop a noninvasive method to measure the formation and accumulation of intracellular Glycer-AGEs. Among these genes, *MMP1* is known to be closely associated with NASH, but the roles of the other genes are not well known. Therefore, further investigations, including protein expression analysis in the sera of NASH patients, will be necessary to elucidate their roles.

To summarize, we have demonstrated that HNRNPM knockdown causes gene expression changes, and our work identifies the possibility that the loss of HN-

RNPM function can be noninvasively measured. Our results suggest that the identified extracellular region-associated genes have the potential to serve as noninvasive biomarkers for Glycer-AGEs-related NASH.

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Isolation and Identification of Lactic Acid Bacteria with Probiotic Potential from Fermented Cow Milk (Nono) in Unguwar Rimi Kaduna State Nigeria

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Abstract

Lactic acid bacteria (LAB) strains from fermented cow milk (nono) sold in-Unguwar Rimi, Kaduna markets were isolated on lactic bacteria specific medium (De Man Rogosa Sharpe MRS media). Isolated strains were identified and characterized using morphological, biochemical test and carbohydrate fermentation system (API-50 CHL). Six (6) pure colonies were distinctly obtained and identified as *Lactobacillus* strains. Out of the 6 isolated Lactobacilli, 5 were further identified as *Lactobacillus delbrueckii* ssp. lactis 2, *Lactobacillus bulgaricus*, *Lactobacillus salivarius*, *Lactobacillus acidophilus* 2 and *Lactobacillus rhamnosus*. They were further screened for antimicrobial activity and antibiotic sensitivity. *Lactobacillus acidophilus* had higher resistance to all but one of the antibiotics used (Chloramphenicol 30 µg) with no zone of inhibition to Ampicillin, Amoxicillin, Gentamycin, Penicillin, Streptomycin and Tetracycline. The antimicrobial activity against *Escherichia coli* exhibited varying degree of inhibitory activity. It can be concluded that the presence of these microorganisms in fermented cow milk (nono) is of great benefits to humans and animals either as supplements or food production processes.

Keywords

Lactobacillus, Morphological, Antimicrobial, Supplements

1. Introduction

Lactic acid bacteria (LAB) are common inhabitants of fermented products (milk, meat and vegetables) and gastrointestinal tracts, most of which are responsible for maintaining a balance of the micro-biota of healthy host. This group of bac-

teria has the ability to colonize the gastrointestinal tract and hence ferment carbohydrate which produces lactic acid as the major metabolic end product that aids digestion. Also as part of its host benefits, it prevents the action of pathogenic microorganism through the production of inhibitory substances (metabolites) and a formation of a bio-film to protect the intestinal mucosal membrane [1] [2].

Consumption of milk and its products as food substances dates back to thousands of years. Although it was lacking any scientific basis until when it was observed that Bulgarian peasants who often consumed large quantities of soured milk as part of their daily diet had a longer survival age [3]. He believed that consumption of fermented milk would reverse the adverse effect of the lower gut micro flora on the host animal. Fermented milk has a beneficial effect on the host's health [4]. Milk products containing viable LAB have been used as prophylactics for treatment of intestinal infections in adults infected with *Salmonella* and lactose intolerance [5] [6].

Probiotics are live microorganisms that confer health benefits to the hosts by improving intestinal microbial balance [7] [8]. Some LAB species (*Lactobacillus*, *Streptococcus, Enterococcus* and *Pediococcus*) have been reported as active candidates for probiotic use in humans and animals by several researchers [4].

Selection of lactobacilli as potential health-promoting probiotic in food and pharmaceutical preparations entails *in vitro* screening for certain criteria, which include antibiotic tolerance, bile tolerance, inhibiting the growth of other microorganisms and gastric juice which allow them to be established in the intestinal tract. Therefore the present study was undertaken with the objective of isolating and identifying *Lactobacilli* from fermented cow milk (nono) and *in vitro* determination of tolerance to antibiotic, bile and microbial inhibition.

2. Materials and Methods

A total of five (5) nono samples were collected in sterile bags from different locations around Unguwan Rimi Market, Kaduna State. Samples were transported to the laboratory in a cold box and stored in a refrigerator at −4˚C for isolation of LAB.

MRS (De Mans, Rogosa and Sharpe) agar and broth medium in powdered form were weighed and reconstituted using sterile water according to manufacturer's instruction (TITAN Biotech Ltd., India) and sterilized at 121˚C and 15PSI for 15 minutes before cooling.

25 ml each of the collected samples were homogenized in sterile normal saline. Serial dilution up to 10^{-6} was made using a sterile pipette by transferring 1ml from 10 ml of the normal saline culture into 9 ml of diluent in sterile test tubes. Enumeration of LAB was done by pipetting and plating out appropriate dilutions using MRS agar. Plates were incubated in an inverted manner at 37˚C for 48hours. After incubation, colonies were counted and recorded as colony forming units (CFU). Morphologically distinct colonies were sub-cultured and purified by streaking on agar plates repeatedly. LAB cultures were maintained on

MRS agar slant at 4˚C and sub-cultured every 4 weeks.

Isolates were characterized after 48 hours of incubation using: macroscopic examination for shape, elevation, size and pigmentation; microscopic examination by gram staining; growth at 15˚C and 45˚C and; biochemical methods [9] [10]. Further identification of LAB strains by sugar fermentation using API 50 CHL system (Biomerieux® France).

Smear fixation was carried out by spreading loopful of isolate on a glass slide and passing it over low flame 3 times. Smear was covered with 1% crystal violet, Lugol's iodine solution and washed with 95% ethanol and stained with 2% safarin before being observed under light microscope.

A drop of 3% hydrogen peroxide (H_2O_2) was added to a loopfulLAB culture.

The LAB was inoculated in 5ml of tryptone broth and incubated at 37˚C for 24hrs. Five (5) drops of 0.5% Kovac's reagent was added after incubation and mixed by gently shaking.

Test culture was inoculated on slants of Simmon's citrate agar then incubated at 37˚C for 24 hours.

24 hours LAB cultures were inoculated in 5 ml glucose phosphate peptone water and incubated at 37˚C for 24 hours. Following incubation, drops of 0.02% methyl red solution were added.

The rapid identification of different strains using the API 50 CH kit (Biomerieux) which is a standardized system was used to differentiate LAB isolates at strains level. Wells in the incubation trays were filled with sterile distilled water to create a humid atmosphere, strips were placed on the trays accordingly. Pure culture incubated for 24 hours were harvested into ampoules containing sterile peptone water. Bacterial suspension in the ampoule (2.0 McFarland) was dispensed into the mediathen into the strip's microtubules using pipette avoiding bubbles formation. Wells were covered with sterile mineral oil to achieve anaerobiosis and incubated at 37˚C for 48 hours. Reaction based on changes in color of each well was studied and interpreted as negative, positive or doubtful. Identification was obtained after result patterns were analyzed with the numerical profile using api*web*™ (Version 5.1).

The antibiotic sensitivity test was performed as described by [11]. MRS agar plates were prepared with 0.1 ml for each of the identified *Lactobacilli* strains. Standard antimicrobial susceptibility test discs (Sigma-aldrich) were prepared and applied to the surface of the plates and incubated for 18 hour at 37˚C. Following incubation, zones of inhibition surrounding the discs were measured.

The antimicrobial effect of all isolated *Lactobacilli* species against *Escherichia coli* (indicator bacteria) was determined by the disc diffusion method.

The effects of bile on the growth of the probiotic strains were determined using methods from [12]. Bile salt solutions (0.3% and 1.0% conc.) were prepared by dissolving 0.3 g and 1.0 g of sodium desoxycholate in 100 ml distilled water each. Turbidity from cell-lysis was examined after incubating for 4 hours and Gram-staining.

3. Results

Five (5) species of genus *Lactobacillus* were successfully isolated from samples of fermented cow milk (nono) using MRS media. The isolates were identified using conventional bio-chemical methods as presented in Table 1. All the isolates were found to be Gram positive and lack the ability to utilize citrate (negative reaction) and catalase test indicated that all isolates were non-catalase producing bacteria. Further identification was carried out using standard API-50 CHL system Table 2. The first micro tube lacks any active carbohydrate substrate and served as negative control. Entire isolated microorganisms fermented glucose, fructose and lactose which were indicated by the change of color from purple to pale yellow. However, there was variation in fermentation pattern of other substrates. LAB 1 was identified as *Lactobacillus acidophilus* after fermenting Melibiose and raffinose. LAB 2 hydrolyzed most of the carbohydrate substrates and was profiled as *Lactobacillus rhamnosus*. LAB 3 was identified to be *Lactobacillus lactis 2.* Esculin hydrolysis revealed by a change to a darker colour or black was represented by all isolates except LAB 4 which was identified to be *Lactobacillus bulgaricus*. Identified isolates were maintained at 4˚C on MRS agar slants.

The result of the antibiotic sensitivity of selected *Lactobacilli* to commonly used antibiotics is presented in Table 3. They were expressed as sensitive (S) or resistant (R) [13]. *Lactobacillus acidophilus* has higher resistance to all but one of the antibiotics used (Chloramphenicol 30 µg). This is because there was no

Table 1. Morphological and Biochemical Characteristics of Isolated Microorganisms.

Isolate	Characteristics on Agar Plates	Microscopic Characteristics	Growth @ 15˚C	Growth @ 45˚C	Methyl Red Test	Citrate Test	Catalase Test	Indole Test
Lab1	Small, flat, smooth, fuzzy	Gram positive, singly and tapering end	-	+	-	-	-	-
Lab2	Rough, convex, off-white, colonies	Gram positive, rods, straight, singly, non-spore	-	+	-	-	-	-
Lab3	Small, flat, crenated, creamy colour	Gram positive, rods, singly and short chains	-	+	-	-	-	-
Lab4	Circular, irregular, off-white	Gram positive, rods, chained, non-spore	-	+	-	-	-	-
Lab 5	White, smooth, convex	Gram positive, rods, round ends, singly and chains	-	+	-	-	-	-

KEY: (+): Positive Reaction (−):Negative Reaction.

Table 2. Identification of isolated microorganisms using apiweb (v5.1) system.

Isolate	Specie Identified	Identification (%)
LAB 1	*Lactobacillus acidophilus*	93.7
LAB 2	*Lactobacillus rhamnosus*	99.9
LAB 3	*Lactobacillus delbrueckii* ssp. Lactis 2	86
LAB 4	*Lactobacillus delbrueckii* ssp. *bulgaricus*	99.7
LAB 5	*Lactobacillus salivarius*	99.9

zone of inhibition to Ampicillin, Amoxicillin, Gentamycin, Penicillin, Streptomycin and Tetracycline. *Lactobacillus rhamnosus and L. bulgaricus* showed resistance to Amoxicillin and Streptomycin both administered at 25 μg and 10 µg respectively.

The result of the antimicrobial activity of *Lactobacilli* against *Escherichia coli* exhibited varying degree of inhibitory activity against enteric *Escherichia coli* as shown in Table 4.

The result of the bile tolerance test of the isolate is presented in Table 5. Observed values show that all of the 4 isolates are resistant to 0.3% bile salt with gradual decrease of viable cells in 1.0% bile salt. *Lactobacillus bulgaricus* and *L. acidophilus* were more tolerant.

Table 3. Diameter of inhibition zone of Lactobacilli sensitivity to antibiotic.

Isolates	Antibiotics/ Zone of inhibition (mm)						
	AMP 10 µg	AMO 25 µg	GEN 25 µg	PEN 25 µg	STR 25 µg	TET 25 µg	CHL 30 µg
L. rhamnosus	2.5 (R)	5.6 (R)	-	-	4.3 (R)	-	-
L. acidophilus	-	-	-	-	-	-	2.5 (R)
L. bulgaricus	-	5.5 (R)	-	-	3.5 (R)	-	-
***L. lactis* 2**	12.3 (S)	-	-	-	1.5 (R)	-	-
L. salivarius	3.2 (R)	-	13.0 (S)	1.5 (R)	5.0 (R)	-	-

AMP: Ampicillin, AMO: Amoxicillin, GEN: Gentamycin, PEN: Penicillin, STR: Streptomycin, TET: Tetracycline, CHL: Chloramphenicol, R: Resistant, I: Intermediate, S: Susceptible, (−): No inhibition

Table 4. Mean inhibition zone for antimicrobial activity against Escherichia coli.

Isolate	*E. coli* Inhibition Diameter (mm)
L. rhamnosus	9
L. acidophilus	17.5
L. bulgaricus	3.5
***L. lactis* 2**	9.4
L. salivarius	10.5

Table 5. Bile Tolerance Test of Isolated Lactobacilli.

ISOLATE	Concentration/Result	
	0.30%	1.00%
L. rhamnosus	++	+
L. acidophilus	+++	+
L. bulgaricus	+++	+
***L. lactis* 2**	++	+
L. salivarius	++	+

Key: +++ maximum resistance, ++ Moderate resistance, + Minimum resistance.

4. Discussion

Lactobacilli known to be affiliated to lactic acid bacteria (LAB) commonly found in the gastrointestinal tract of animals and humans can also be found in fermented food such as milk and milk product. The creamy or whitish appearance of the isolated *Lactobacillus* species on MRS agar confirms that Lactobacilli have dominance in fermented milk products when compared to other lactic bacteria [14] [15]. One of the characteristics of an ideal probiotics is resistance against antibiotics mostly after antibiotic administration. The results of antibiotic susceptibility of isolated Lactobacilli which showed that almost all the species were found resistant to commonly used antibiotics except *Lactobacillus lactis* 2 and *L. salivarius* that were only susceptible to ampicillin and gentamycin agree with the report of [16]. Other authors reported multidrug resistance [17] [18] [19]. It is unreasonable to use susceptible probiotics in combination with antibiotics in case of bacterial infections [20]. Resistant probiotic bacteria do not have the genes which can be transferred to other bacterial population by conjugation [16].

Antimicrobial activity against entero-pathogens is another important character of probiotic bacteria. All isolated species were tested for antimicrobial activity against *E. coli*. Zone of inhibition (mm) was used as basis to measure antimicrobial activity in this study. Maximum activity (17.5 mm) was shown by *L. acidophilus* while minimum activity (3.5 mm) was shown by *L. bulgaricus*. The average diameter of inhibition zone from the duplicate test clearly showed that all *Lactobacilli* have antimicrobial effect inhibiting the growth of *E. coli*. However, *L. bulgaricus* showed lowest antimicrobial effect against both indicator strains. The strongest antimicrobial effect was shown by *L. acidophilus*. This activity may have been attributed to bacteriocins produced by the antagonistic activity of lactic acid bacteria as observed by [21] [22]. Many investigations have confirmed the antagonistic activities of lactic acid bacteria in humans and animals.

Bacteria must tolerate bile salts concentration for their colonization and metabolic activity in the gastrointestinal tract [23]. However, there was no consensus about the precise concentration *Lactobacilli* strain should tolerate. It is necessary to evaluate the resistance ability of isolated *Lactobacilli* to bile acids before using them as probiotics [24]. This study shows that isolated *Lactobacilli* were tolerant to high concentration (1.0%) of bile salt (Sodium desoxycholate). *L. lactis* 2, *L. rhamnosus* and *L. salivarius* showed minimum resistance at 0.3% and 1.0% concentration of bile salt. These results of resistance against bile salt are supported by other authors who reported that *Lactobacilli* which were isolated from milk products showed resistance to bile salt [25] [26]. Although, research has shown that intestinal strains have more resistance [27]. Resistance ability varies among *Lactobacillus* species as well as among different strains [28]. Because resistance to bile salt is due to the presence of bile salt hydrolase (BSH), an enzyme that reduces toxic effects by conjugating bile. BSH activity is mostly found in the species of *Lactobacillus* which are isolated from feces or intestines of animals [29].

In conclusion, lactic acid bacteria were successfully isolated from fermented cow milk (nono). The characterization of isolates on the basis of microscopic analysis and biochemical properties (phenotypic characterization) is very useful being the most widely recognized and accepted method. Molecular approach based on 16S rDNA restriction analysis should be used for specificity of species because it is believed that identification using carbohydrate is ambiguous and unreliable. *Lactobacillus* species from cow milk are all excellent candidates for further *in vitro*/*in vivo* characterization for application as probiotics in animals to increase production, degrade gluten and cholesterol lowering due to presence of bile salt hydrolase (BSH); bio-preservation of food using bacteriocin produced and gastrointestinal tract delivery vector in both animals and humans.

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Microarray Analysis of the Effects of Amelogenin on U937 Monocytic Cells

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Abstract

Periodontal diseases are chronic inflammation caused by particular types of bacteria and have been recognized as a cause of tooth loss in adults. These bacteria which invade periodontal tissue are phagocytosed mainly by monocytes and macrophages in this immune response, and will be presented to lymphocytes. Recently, therapies for regenerating periodontal tissues have been used extensively to treat periodontal disease, and in particular, enamel matrix derivative (EMD) is commonly used for such therapies in Japan. Amelogenin is a type of the extracellular matrix protein that accounts for 90% of the constituents of EMD. In this study, we carried out a detailed microarray analysis in order to evaluate a gene group involved in amelogenin stimuli in the human monocytic cell line U-937. Microarray analysis revealed that statistically significant changes were apparent in 273 genes (163 up-regulated and 110 down-regulated) subsequent to 4 h of amelogenin stimulation. The most highly enriched categories included "cell cycle", "DNA replication", and "DNA repair" in up-regulated annotation terms. On the other hand, "type I diabetes mellitus", "allograft rejection", and "graft versus host disease" were observed in down-regulated annotation terms. Specifically, the gene expression of major to compatibility complex (MHC) class I/II and CD80/86 was impaired in U937 cells after stimulation with amelogenin. In addition, the results of heat-map showed that the gene expression of inflammatory cytokine such as tumor necrosis factor (TFN), interleukin-18 (IL-18), and CXCL16 was markedly decreased after stimulation of monocytes with amelogenin. In conclusion, the findings of our study showed that by inducing monocyte growth through the suppression of the antigen-presenting ability of U937 cells, amelogenin may affect the immune responses of periodontal tissues originating from monocytes. Examining the effects of amelogenin on the transformation of macrophages differentiating from monocytes may establish a molecular ba-

sis for the anti-inflammatory effect of amelogenin in periodontal tissues.

Keywords

Human Monocyte, Periodontal Disease, Amelogenin, Microarray, Heat-Map

1. Introduction

Periodontal diseases are chronic inflammation caused by particular types of periodontal disease-causing bacteria. Because they cause resorption of the alveolar bone, they have been recognized as a cause of tooth loss in adults aged 40 years or older [1]. Teeth are supported by surrounding tissues known as periodontal tissues and are composed of four types of tissues: gingival soft tissue; periodontal ligament around the dental root; cementum, comprising the hard tissue at the surface of the dental root; and alveolar bone [2]. Treatment of periodontal disease has mainly consisted of removing the causative lesion, such as tooth-brushing instruction aimed at improving the condition of the oral cavity through cleaning, or removal of subgingival calculi by periodontal scaling and root planing or flap surgery. The main purpose of these treatments is to suppress the progression of periodontal disease [3]. However, recently developed regenerative therapies aimed at regenerating periodontal tissues have been used extensively to treat periodontal disease, and some success has been achieved [4]. In Japan, enamel matrix derivative (EMD) extracted from the tooth germs of young pigs is commonly used for such therapies. EMD has been commercialized under the name Emdogain® gel [5]. Among periodontal tissue regeneration materials that contain bioactive proteins such as growth factors, Emdogain® gel is currently the only treatment approved by the Japanese Ministry of Health, Labour and Welfare. However, although numerous studies have attempted to determine the mechanism of action of EMD, the molecular aspects of periodontal tissue regeneration at the signal transduction level remain unclear. Additionally, periodontal surgical treatment using EMD has been empirically shown to promote healing and is associated with reduced pain and swelling [6]. Previous reports have shown that amelogenin had an anti-inflammatory effect from the perspective of pro-inflammatory and anti-inflammatory cytokine production [7].

Amelogenin is a type of the extracellular matrix protein that accounts for 90% of the constituents of EMD and is a primary molecule in periodontal tissue regeneration using EMD [8]. In the field of medicine, extracellular matrix has been applied to wound healing and regenerative therapy, and amelogenin has been used to treat refractory pressure ulcers under the trade name Xelma® [9]. The practical application of amelogenin in periodontal tissue regeneration was developed based on the biological imitation of the dental development environment [10]. Amelogenin is secreted by ameloblasts during the formation of tooth germs and is thought to be involved in periodontal tissue formation, beginning with cementum which is deposited in the dentin [11]. Amelogenin-knockout

mice exhibited cementum hypoplasia and abnormal differentiation of osteoclasts in their oral cavity [12]. Recombinant amelogenin has been reported to cause periodontal tissue regeneration in laboratory animals [13]. In periodontal ligament cells and osteoblasts, which are important for periodontal tissue regeneration, amelogenin-associated molecules should be identified in order to establish the molecular basis of their effects. By proteomic analysis focusing on amelogenin, which is a major protein in EMD, we identified a new group of amelogenin-associated molecules such as Grp78 in osteoblasts [14]. In addition, the association between amelogenin (rM180) and Grp78 has been reported to be involved in the cellular migratory capacity of periodontal ligament stem cells [15].

When periodontal disease-causing bacteria invade periodontal tissues, oral bacteria are phagocytosed by neutrophils in a non-specific manner in order to confer protection to the host. However, if this is the only protection and if it is insufficient, the organism will develop a full immune response. In this immune response, antigens are phagocytosed mainly by monocytes and macrophages and will be presented to lymphocytes (antigen-presenting reaction). This explains why monocytes are considered the basis of the immune responses. In our study, we examined the effects of amelogenin on immune responses. We carried out a detailed microarray analysis in order to evaluate a gene group involved in amelogenin stimuli in the human monocytic cell line U-937.

2. Materials and Methods

2.1. Cell Culture

U937 human monocytic cell line was purchased from RIKEN BioResouce Center (Ibaraki, Japan). U937 cells were cultured in RPMI-1640medium containing 10% heat-inactivated fetal bovine serum (FBS), penicillin, and streptomycin at 37˚C in a 5% CO_2 incubator. Cells were sub-cultured every 48 - 72 h, inoculum being 5 × 10^5/ml and cell viability (>95%) was confirmed by trypan blue exclusion.

2.2. Recombinant Protein

Cloning and expression of a glutathione *S*-transferase (GST) full-length recombinant amelogeninfusion (GST-rM180), and the purification of rM180 were previously described [14]. Removal of endotoxin from rM180 was confirmed by Limulus Amebocyte Lysate Assay (Endotoxin Level: 10 μg of rM180 < 0.03 EU). U937 cells were stimulated with rM180 (10 μg/mL) for 4 h, 8 h, 12 h, and 24 h.

2.3. Gene Expression Profiling by Microarray

Total RNA was isolated with Isogen 2 (Nippon Gene, Tokyo, Japan) and purified from U937 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). Subsequent hybridization and microarray analysis were conducted at the Research Support Center, Kyushu University Graduate School of Medical Sciences according to the manufacture's recommended protocols. Briefly, RNA integrity was verified

using BioRad Experion (Bio-Rad Laboratories, Hercules, CA) by measuring the ratio of band intensity between the 28 S and 18 S rRNA. The RNA integrity number (PRN) was close to 10 for all samples. Total RNA (200 ng) was converted to biotinylated cRNA using an Illumina TotalPrep RNA Amplification Kit (Life Technologies, Foster City, CA, USA). Next, a total of 750 ng of biotinylated-cRNA was overlaid onto individual array spots of the HumanHT-12 v4 BeadChip (Illumina, San Diego, CA, USA). The chip was hybridized at 58˚C for 18 h, washed, stained with fluorescent reagents, and scanned using a BeadArray Reader (Illumina). Relative hybridization intensities and background hybridization values were calculated using Genome Studio v1.8 (Illumina). Using procedures recommended by Illumina, the raw signal intensities of five samples were normalized using quantile algorithms from the "lumi" [16] and "preprocess Core" library packages [17] on the Bioconductor software [18]. Probes flagged as "Detection P-value < 0.05" in at least one sample were selected for analysis. To identify genes that were up regulated or down regulated, we calculated the intensity-based Z-scores [19] and the ratios (non-log scaled fold-change) of the normalized signal intensities for each probe, comparing the control and experiment samples. Next, we established criteria for differentially expressed genes (DIGs): upregulated genes, Z-score ≥ 2.0 and ratio ≥ 1.5-fold; down regulated genes, Z-score ≤ −2.0 and ratio ≤0.66.

2.4. Microarray Analysis

To determine the cellular effects of these DIGs and significant pathway enrichment, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/home.jsp) [20]. To characterize the biological processes affected by DIGs, we used Gene Ontology (GO) and the functional annotation-clustering feature of DAVID. The functional annotation clustering tool measures the similarities between GO terms based on the extent of their associated genes, and assembles similar and redundant GO terms into annotation clusters. Each clustered GO term is assigned a Fisher Exact *P*-value representing the degree of enrichment of the GO term within the DIGs. Each cluster is assigned an enrichment score signifying its biological significance. A biologically significant cluster (high enrichment score) is generated only when the majority of the GO term members have significant enrichment values. Functional annotation clusters with an enrichment-score of >1.3 were considered significant [21], and the resulting clusters were further filtered to retain only those GO terms with *P* values of <0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was also performed to analyze the significance of biological functions identified in large-scale transcriptome data. In addition, we generated a heat-map using Multi Experiment Viewer (MeV) software (http://www.tm4.org/mev.html) [22] and a hierarchical clustering (HCL) method for gene sorting. The inflammation-associated genes were identified as GO terms containing "inflammatory response". Color indicates the distance from the median of each column. "Pearson correlation" and "average linkage

clustering" were used to determine distance and linkage, respectively. Microarray data analysis was supported by Cell Innovator (http://www.cell-innovator.com/).

3. Results

3.1. Amelogenin Up-Regulates the Expression Profiles of Cell Proliferation-Associated Genes in U937 Cells

To characterize the biological effects of amelogenin in monocytes, we performed transcriptional profiling of U937 cells using microarray analysis. Samples of U937 cells were stimulated with rM180 for 4 h, 8h, 12h, or 24 h. U937 cells in the absence of rM180 stimulation were used as the control sample (unstimulated). Scatter plot analyses of global gene expression compared with the control (unstimulated) demonstrated that 4 h of rM180 stimulation (unstimulated vs. rM1804h) resulted in greater changes to gene expression than 24 h of stimulation (unstimulated vs. rM18024h) (**Figure 1**). Although amelogenin induced relatively early changes in transcriptional regulation, most of these were dissipated after 24 h. Statistically significant changes were apparent in 273 DIGs (163 up-regulated and 110 down-regulated) subsequent to 4 h of rM180 stimulation. We performed functional annotation clustering of the DIGs following 4 h, 8 h, 12h, and 24h of amelogenin treatment to identify significantly up-regulated annotation terms (**Table 1** and **Figure 2**). The most highly enriched categories included "cell cycle", "DNA replication", "DNA repair", and "ribosome". These results indicate that amelogenin induces the expression levels of genes encoding cell proliferation in U937 cells.

3.2. Amelogenin down-Regulates the Expression Profiles of Antigen Presentation-Associated Genes in U937 Cells

On the other hand, we also performed functional annotation clustering of the DIGs following 4, 8, 12 h, and 24 h of rM180 treatment to identify significant down-regulated annotation terms (**Table 2** and **Figure 3**). The most highly enriched categories included "type I diabetes mellitus", "allograft rejection", and "graft versus host disease". Specifically, the gene expression of major his to compatibility complex (MHC)class I/II and CD80/86 were impaired in U937 cells after stimulation with rM180 (**Figure 3**).

Our results showed that amelogenin inhibits the expression levels of genes encoding antigen presentation in U937 cells.

3.3. Amelogenin Down-Regulates the Expression Profiles of Inflammation-Associated Genes in U937 Cells

Since amelogenin plays an inhibitory role in the antigen presentation of U937 cells, we analyzed the effects of rM180 on the transcriptional profiles of inflammation-related genes. Five samples of U937 cells were categorized according to the period of rM180 stimulation (0 h, 4 h, 8 h, 12 h, and 24 h); and a clustered heat-map of the 106 inflammation-related genes was generated (**Figure4(a)**).

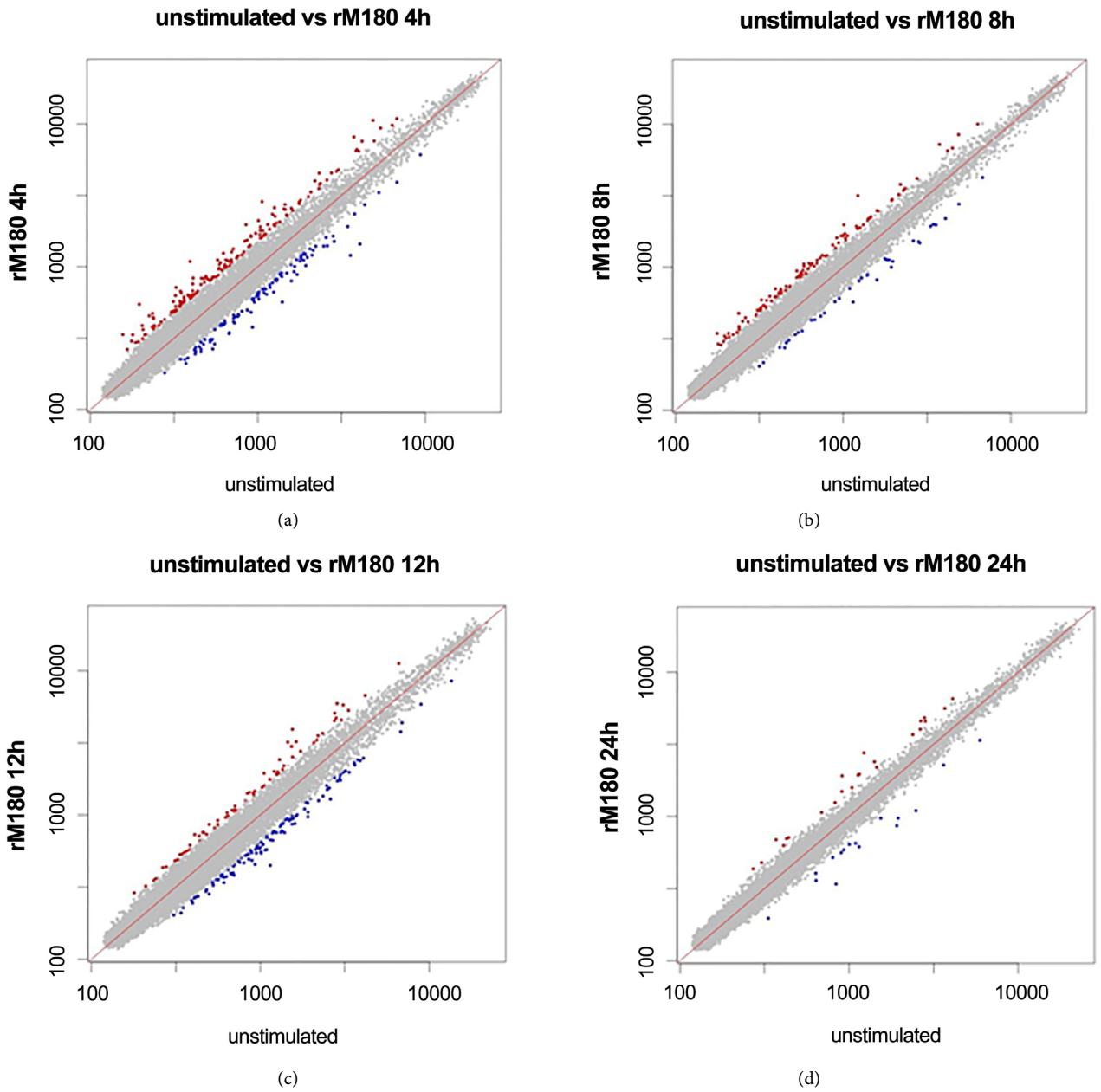
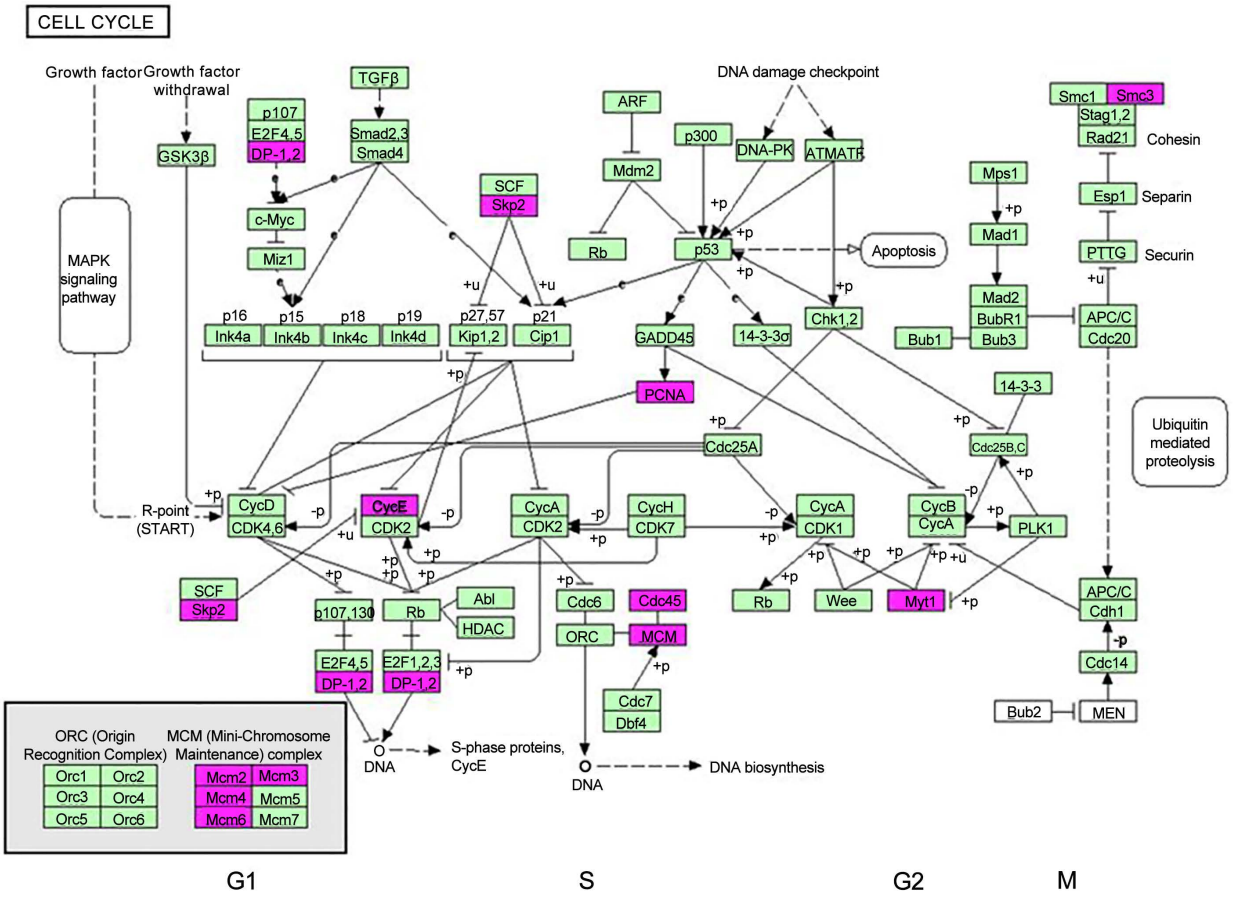


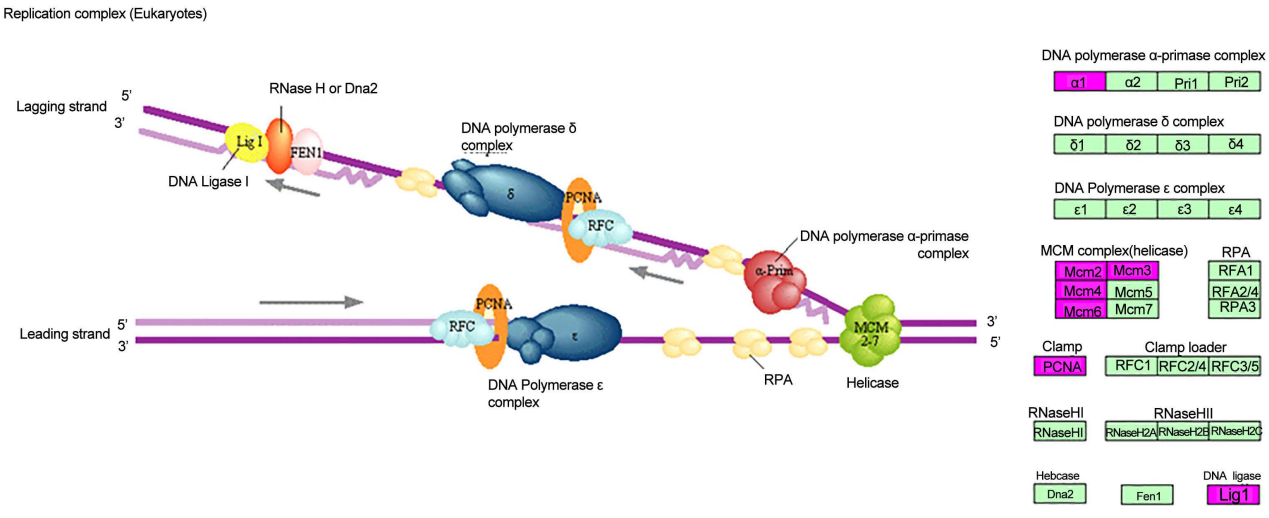
Figure 1. Scatter plots comparing global gene expression profiles confirming the effects of amelogenin treatment in U937 cells. (a)-(d) U937 cells were stimulated with rM180 (10 g/mL) for4 h (a); 8h (b), 12h (c); or 24h (d). The X-axis indicates the relative normalized log2-signal intensity of the unstimulated, and the Y-axis indicates the normalized log2-signal intensity of rM180 stimulated. Red dots denote upregulated genes (Z-score ≥ 2.0 and ratio ≥ 1.5-fold), and blue dots indicate downregulated genes(Z-score ≤ −2.0 and ratio ≤ 0.66).

Subsequently, the 20 genes exhibiting a >2-fold difference between the four parameters at 4 h, 8 h, 12 h, and 24 h were validated (**Figure 4(b)**). The gene expression of inflammatory cytokine and chemokine such as tumor necrosis factor (TFN), interleukin-18 (IL-18), and CXCL16 was markedly decreased at 4 h and 8 h after stimulation of monocytic cells with amelogenin. On the other hand, the gene expression of IL-8 peaked 4 h after the addition of rM180 and then gradually decreased in U937 cells. These findings indicated that amelogenin enhances

the expression levels of genes encoding anti-inflammatory response in U937 cells.



(a)



(b)

Figure 2. Representative KEGG Pathway Maps of cell cycle (a) and DNA replication (b) signaling which include up-regulated genes after stimulation of amelogenin for 4 h. The grid with the round edge represents another pathway; the pink-marked nodes are associated with up-regulated genes (Z-score ≥ 2.0 and ratio ≥ 1.5-fold); the green nodes have no significance.

Table 1. Functional annotation clustering predicted to be up-regulated after stimulation of rM180.

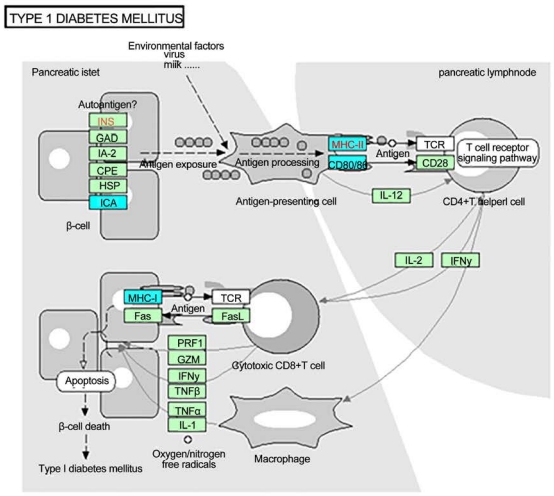
Stimulation Time (h)	Term	Gene count	*P*-Value
4	Cell cycle	11	5.4E−7
	DNA replication	7	1.6E−6
	Spliceosome	7	2.0E−3
	Mismatch repair	3	2.4E−2
	Base excision repair	3	5.3E−2
	Nucleotide excision repair	3	7.9E−2
	Proteasome	3	8.8E−2
8	Spliceosome	3	2.1E−3
	Cell cycle	4	1.8E−2
12	Non-small cell lung cancer	3	4.5E−3
	Cell cycle	5	3.5E−2
24	Ribosome	3	1.7E−2

DAVID v6.7 functional annotation bioinformatics microarray analysis software was used to obtain the functional annotation clustering. Only GO pathway terms that showed statistically significantly differences in the amount of genes (compared unstimulated vs. rM180 4h, 8h, 12h, and 24h) are shown (P-value ≤ 0.05).

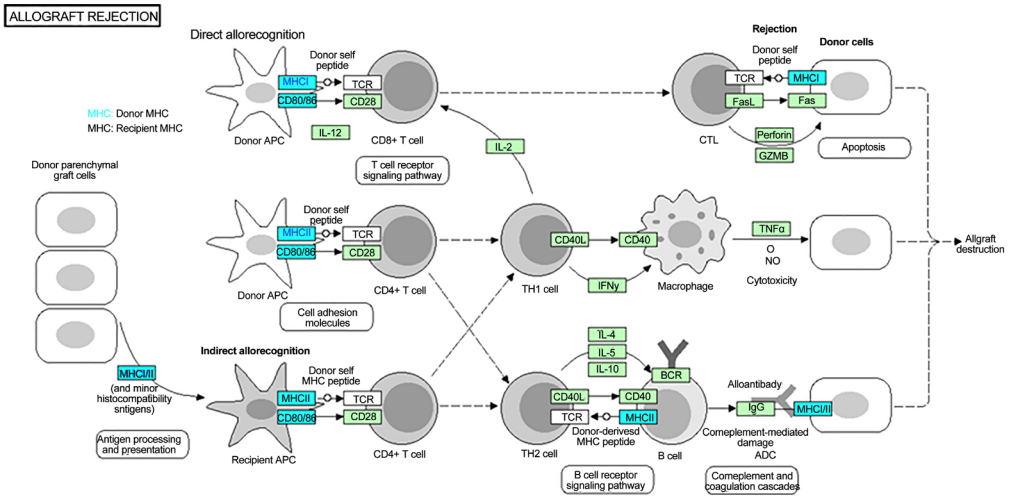
4. Discussion

When considering the onset of periodontal diseases, it may appear that oral bacteria act as antigens and initiate an inflammation reaction; however, the constituents of oral bacteria, namely their outer membrane proteins, pili, and lipopolysaccharides, have a diverse range of antigenicity [23]. In addition, they activate living cells through various receptors in the cells. When oral bacteria invade the periodontal tissue, neutrophils protect the host organism by nonspecific phagocytosis of the oral bacteria; when this does not confer sufficient protection, a full-fledged immune response develops. In the immune response, the antigens are mainly phagocytosed by monocytes and macrophages, followed by antigen presentation to lymphocytes. Thus, monocytes are considered the origin of immune responses. Additionally, the lipopolysaccharides of gram-negative bacteria have a strong activating effect on monocytes [24] and are important for determining the etiopathology of periodontal diseases involving inflammation and infection due to gram-negative bacteria. The amelogenin used in this study was a recombinant protein from *Escherichia coli*, but because Triton X was used in the purification process, the level of endotoxin was confirmed to be 0.03 EU/L or lower when 10 mg amelogenin was used; therefore, the stimulatory effect of amelogenin on monocytes with lipopolysaccharides from *E. coli* was negligible.

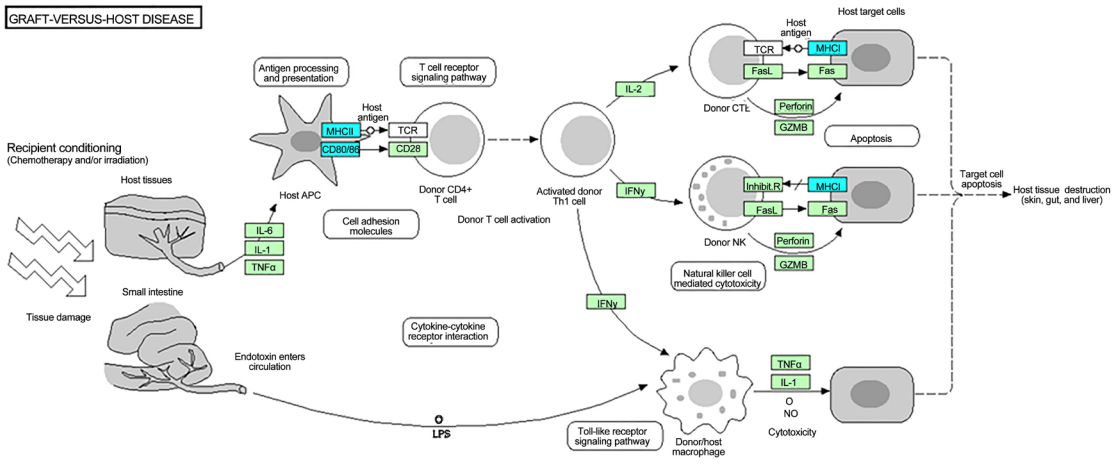
In scatter plot analysis aimed at confirming the temporal changes in gene expression caused by amelogenin stimuli, our findings showed that unlike cytokines, amelogenin did not induce notable variations in gene expression; our findings also confirmed that the effects of amelogenin generally developed at a



(a)

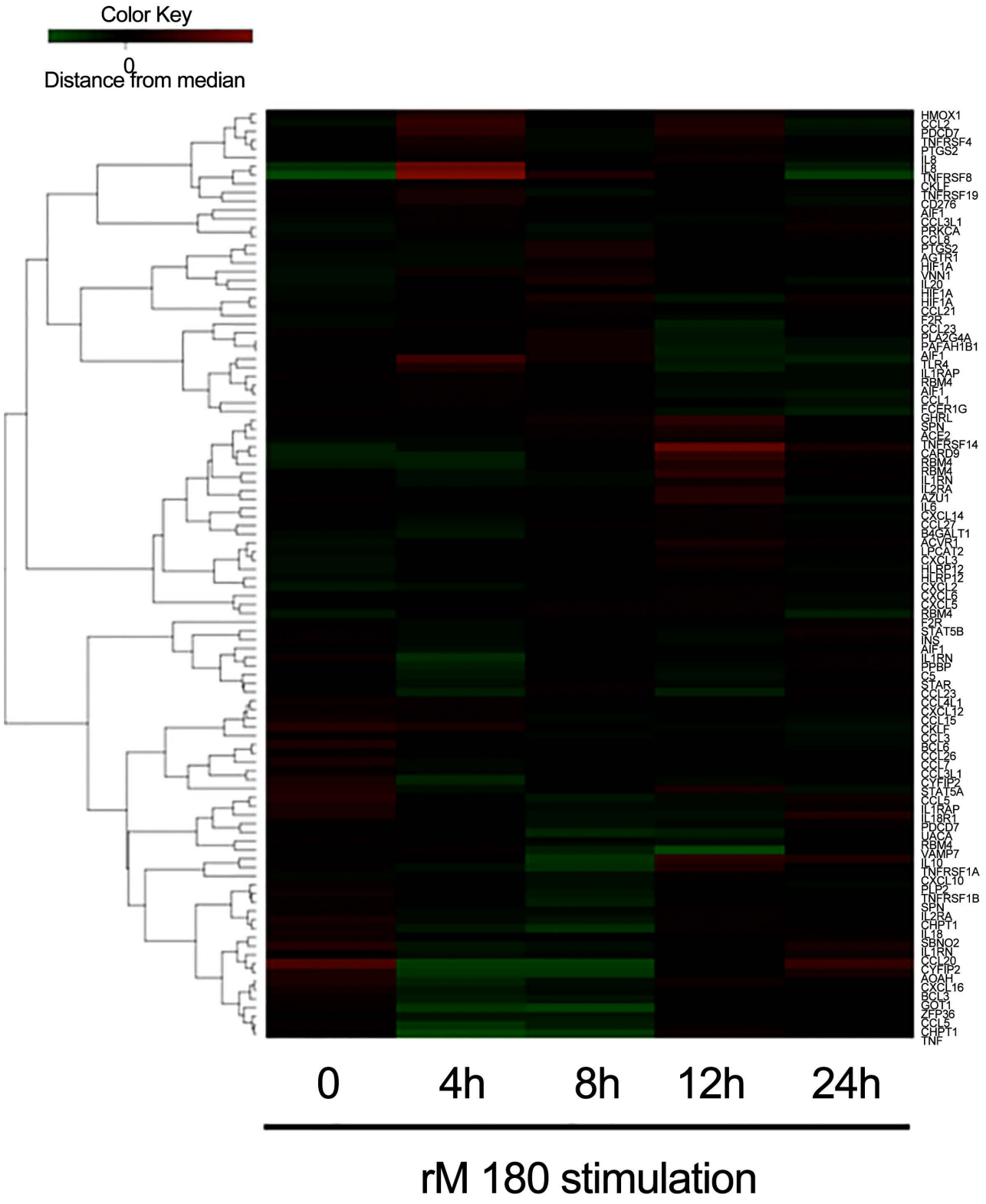


(b)

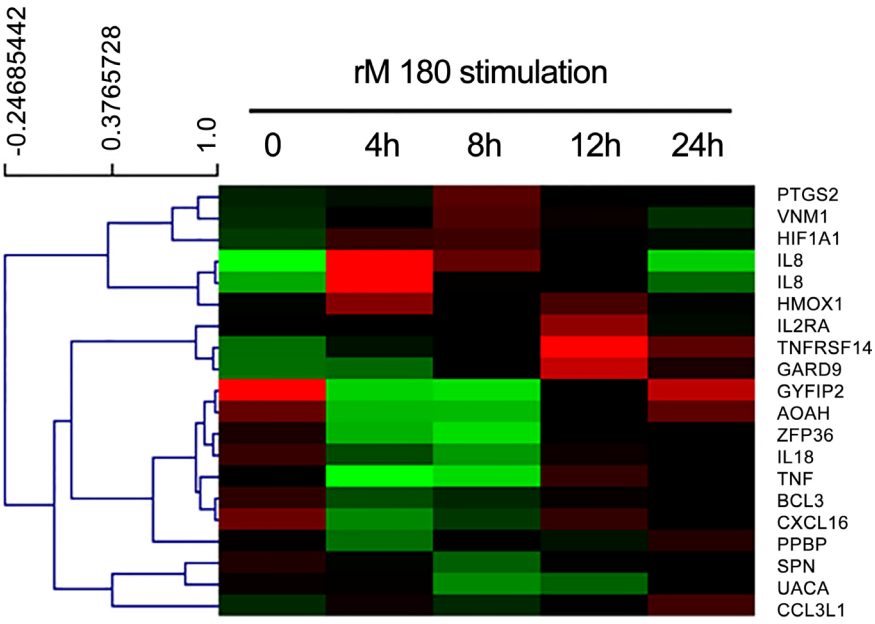


(c)

Figure 3. Representative KEGG Pathway Maps of type 1 diabetes mellitus (a), allograft rejection (b), and graft versus host disease (c) signaling which include down-regulated genes after stimulation of amelogenin for 4 h. The grid with the round edge represents another pathway; the blue-marked nodes are associated with downregulated genes (Z-score ≤ −2.0 and ratio ≤ 0.66); the green nodes have no significance.


Color Key
0
Distance from median
HMOX1
CCL2
PDCD7
TNFRSF4
PTGS2
IL8
IL8
TNFRSF8
CXCL
TNFRSF19
CD276
AIF1
CCL3L1
PRKCA
CCL8
PTGS2
AGTR1
HIF1A
VNN1
IL20
HIF1A
HIF1A
CCL21
F2R
CCL23
PLA2G4A
PAFAH1B1
AIF1
TLR4
IL1RAP
RBM4
AIF1
CCL1
FCER1G
GHRL
SPN
ACE2
TNFRSF14
CARD9
RBM4
RBM4
IL1RN
IL2RA
AZU1
IL6
CXCL14
CCL27
B4GALT1
ACVR1
LPCAT2
CXCL3
HLRP12
HLRP12
CXCL2
CXCL6
CXCL5
RBM4
F2R
STAT5B
INS
AIF1
IL1RN
PPBP
C5
STAR
CCL23
CCL4L1
CXCL12
CCL15
CKLF
CCL3
BCL6
CCL26
CCL7
CCL3L1
CYFIP2
STAT5A
CCL5
IL1RAP
IL18R1
PDCD7
UACA
RBM4
VAMP7
IL10
TNFRSF1A
CXCL10
PLP2
TNFRSF1B
SPN
IL2RA
CHPT1
IL18
SBNO2
IL1RN
CCL20
CYFIP2
AOAH
CXCL16
BCL3
GOT1
ZFP36
CCL5
CHPT1
TNF
0
4h
8h
12h
24h
rM 180 stimulation

(a)



(b)

Figure 4. The expression profiles of amelogenin-induced cell function genes in U937 cells. (a) The clustering diagram and heatmap of gene trees were generated using MeV software. Five samples of U934 cells were categorized according to the period of amelo-genin stimulation (0h, 4h, 8h, 12h and 24h). Hierarchical clustering (HCL) was employed to sort the genes (using "Pearson correlation", and "average linkage clustering"). Each row represents a sample and each column represents a gene. Inflam-mation-associated genes were extracted using a Gene Ontology (GO) term containing "inflammatory response". The color gradient indicates the distance from the median of each column. Red blocks represent high expression, and green blocks, low expression relative to the unstimulated sample; black blocks indicate similar expression; (b) Heatmap illustrating the expression of selected genes in the four U937 cell groups. Discernible genes ($|\log FC| \geq 0.5$) were extracted by converting the ratio of inflam-mation-related genes. A red band indicates high relative expression and a green band indicates low relative expression.

relatively early stage (4h) (**Figure 1**). We previously reported that the growth of osteoblasts and the migration of periodontal ligament stem cells were enhanced by amelogenin [14] [15]. In other studies, amelogenin was reported to enhance the differentiation of cementoblasts as well as that of periodontal ligament cells; however, the results of the present study suggest that amelogenin promotes cell growth mainly by enhancing the gene expression of proliferating cell nuclear antigen, CycE, and Cdc45 in monocytes (**Figure 2**). Amelogenin has been reported to exert various effects depending on the cell type and stage of differentiation; in addition, Emdogain Gel has been reported to enhance the growth and differentiation of osteoblasts, cementoblasts, and periodontal ligament cells, and to inhibit the in growth of gingival epithelial cells toward bony defects [25]. The U-937 cells used in this study were monocytic cells; the effects after differentiation of these cells into macrophages should be examined in future studies. Previous clinical reports have shown that while the periodontal tissue regeneration effect of Emdogain persisted over a few years, the microarray analyses results revealed that the stimulatory effect of amelogenin on monocytes was limited to the early stages. This is thought to be because monocytes were floating cells and that amelogenin was not affected as a scaffold for the extracellular matrix.

Table 2. Functional annotation clustering predicted to be down-regulated after stimulation of rM180.

Stimulationtime (h)	Term	Gene count	*P*-Value
4	Type I diabetes mellitus	5	3.0E−4
	Ribosome	6	5.4E−4
	Viral myocarditis	5	2.2E−3
	Allograft rejection	4	2.7E−3
	Graft-versus-host disease	4	3.4E−3
	Autoimmune thyroid disease	4	7.2E−2
	Cell adhesion molecules	5	1.9E−2
	Intestinal immune network for IgA production	3	5.6E−2
	Natural killer cell modified cytotoxicity	4	8.6E−2
8	Terpenoid backbone biosynthesis	2	3.2E−2
	Allograft rejection	2	7.5E−2
	Graft-versus-host disease	2	8.1E−2
	Type I diabetes mellitus	2	8.7E−2
12	Ribosome	4	1.4E−3
	Valine, leucine and isoleucine degradation	3	2.7E−2
	Terpenoid backbone biosynthesis	2	8.5E−2
24	n.p	-	-

DAVID v6.7 functional annotation bioinformatics microarray analysis software was used to obtain the functional annotation clustering. Only GO pathway terms that showed statistically significantly differences in the amount of genes (compared unstimulated vs. rM180 4h, 8h, 12h, and 24h) are shown (*P*-value ≤ 0.05).

The pathways inhibited by amelogenin may include antigen presentation pathways such as MHC class I/II and CD80/86 (**Figure 3**). Generally, the process of cell growth and differentiation includes a number of conflicting issues, and our findings showed that because of the proliferative effect of amelogenin on monocytes, amelogenin may have inhibited the latter's differentiation into macrophages responsible for antigen presentation. In addition, candidate pathways for the stimulation of monocytes by amelogenin include intracellular uptake through phagocytosis and endocytosis and receptor-mediated signal transduction. However, because of the impaired antigen-presenting ability, the phagocytic pathway is highly likely to be excluded. We previously reported that GRP78 on the cell membrane of periodontal ligament stem cells served as a receptor for amelogenin [15]. GRP78 is a ubiquitous chaperone protein present mainly in the endoplasmic reticulum [26], but in undifferentiated stem cells, it is also expressed on the cell membrane [27] and has been reported to function as a receptor for Cript [28] and DMP1 [29]. GRP78 is also thought to be expressed in the U-937 cell line composed of monocytes, but no previous studies have evaluated the expression of GRP78 on the cell membrane during the differentiation process. Amelogenin is likely phagocytosed by macrophages; however, further studies are needed to examine the expression of GRP78 on the cellular mem-

brane of monocytes and other cells involved in immune responses, as well as the possibility that GRP78may be a receptor for amelogenin.

Clinical reports of the use of Emdogain suggested that amelogenin has an anti-inflammatory effect [7] [30]; however, whether the inhibitory effect of amelogenin on the antigen-presenting ability of monocytes is directly linked to its immunosuppressive effect remain unclear. The clustered heat-map assay revealed that the inflammation-related gene expression of IL-8 was enhanced and that of TNF-α was reduced at the early stage (4 h) after amelogenin stimulation (**Figure 4(b)**). It is interesting that inflammatory cytokine TNF-α was suppressed by amelogenin in U937 cells whileIL-8 was also known as neutrophil chemotactic factor. The early stages of the inflammatory process are essential for tissue repair and regeneration; if the subsequent series of events, including the resolution of the inflammation, does not occur, the condition will persist as chronic inflammation. Because amelogenin exerted a growth-enhancing effect and chemotaxis/angiogenesis induced by IL-8 on monocytes [31], an increasing number of macrophages leaked out of blood vessels and accumulated in tissues; thus, shortening the period until the resolution of inflammation with the inhibition of TNF-α should be examined. Additionally, we found that amelogenin stimulation suppressed the expression of interleukin-18 (IL-18) [32] which induces the production of interferon-γ (IFN-γ) at an early stage (4h). IL-18 inhibitionmay be involved in the down-regulation of MHC class II by amelogenin stimuli since the expression of MHC class II is induced by IFN-γ [33]. The effects of amelogenin on the transformation and functions of macrophages require further analysis because monocytic cell lines before differentiation were used in this study. In addition, gingival fibroblasts are host cells involved in the construction of periodontal connective tissues, but around monocytes, they control the environment of periodontal tissues. Therefore, while the extracellular matrix is produced in healthy periodontal tissues, the matrix produces substrate-degrading enzymes such as collagenases while regulating its own synthesis and degradation, thereby constantly restructuring the gingival tissues [34]. However, when an inflammatory substance spreads in the connective tissues and causes periodontal disease, gingival fibroblasts become the target cells of various cytokines produced by immunocompetent cells such as monocytes [35] [36]. In addition, gingival fibroblasts produce various cytokines and play a role in some cytokine networks [37]. Thus, at the site of the inflammation in the connective tissue, gingival fibroblasts control and regulate the periodontal tissue environment to efficiently establish immune responses.

In conclusion, the findings of our study showed that by inducing monocyte growth through the suppression of the antigen-presenting ability of these cells, amelogenin may affect the immune responses of periodontal tissues originating from monocytes. Examining the effects of amelogenin on the transformation of macrophages differentiating from monocytes, as well as on gingival fibroblasts, may establish a molecular basis for the anti-inflammatory effect of amelogenin in periodontal tissues.

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Disclosure

The authors have no conflicts of interest to disclose.

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