

Activation of cancer-related and mitogen-activated protein kinase signaling pathways in human mature osteoblasts isolated from patients with type 2 diabetes



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ABSTRACT

Diabetes mellitus is a disease of glucose metabolism, and it adversely affects bone metabolism and increases the risk of cancer development. Previously, we reported a method for the direct isolation of human mature osteoblasts and indicated that osteoblasts were associated with type 2 diabetes mellitus-related signaling pathways. In addition, a recent report suggested that osteoblasts are involved in glucose metabolism. Thus, we sought to examine the effects of diabetes on osteoblast signaling *in vivo*. We recruited eight patients with type 2 diabetes and eight non-diabetic individuals. We isolated human mature osteoblasts from the resected femoral heads during orthopaedic surgery and extracted their RNA. We compared the gene expression between the two groups by RNA microarray and pathway analyses. Microarray analysis showed significant differences in 885 of 19,463 genes between the two groups ($p < 0.05$), and pathway analysis revealed that pathways related to cancer and the mitogen-activated protein kinase signaling pathway were significantly activated in the diabetes group ($p < 0.01$). These preliminary findings suggest that diabetes affects intracellular signaling in human mature osteoblasts and that osteoblasts might not only play a key role in the regulation of bone and glucose metabolism, but might also be related to cancer metabolism. We plan to conduct further studies to examine signaling in diabetic osteoblasts and to further investigate the genes and pathways identified here.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia accompanied by microangiopathy and macrovascular disorders. DM also disturbs bone metabolism (Caliaperoumal et al., 2018; Hamann et al., 2012; Heath et al., 1980; Katz et al., 1990; Khosla and Hofbauer, 2017) and increases the risk of cancer development (Johnson and Gale, 2010; Schrijnders et al., 2018), which is thought to be due to insulin signaling (Vestergaard et al., 2005; Yan and Li, 2013). In recent years, positive feedback between insulin and osteocalcin secretion from osteoblasts has been well studied (Ferron et al., 2010; Fukumoto and Martin, 2009); furthermore, it has been reported that osteocalcin enhances glucose uptake in insulin-stimulated oxidative muscle (Lin et al., 2018). These reports indicate that osteoblasts are vitally important in

regulating glucose metabolism. Nevertheless, although DM has a substantial effect on insulin secretion, it remains unclear how DM affects the intracellular signaling in osteoblasts.

Previously, we reported a method for the direct isolation of human mature osteoblasts from a small bone biopsy and demonstrated a relationship between osteoblasts and type 2 DM (Fujita et al., 2014a). There are some reports on the effects of diabetes on osteoblasts *in vitro*; however, *in vitro* culture affects intracellular signaling in osteoblasts (Garcia-Hernandez et al., 2012; Katayama et al., 1997). However, there are no *in vivo* reports on this topic. As our method can isolate *in vivo* osteoblasts without culture, we sought to examine the effects of diabetes on osteoblasts *in vivo*, thus we preliminarily investigated the changes in osteoblast signaling in patients with DM using RNA microarray and pathway analyses.

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2. Materials and methods

2.1. Participants

The Tokyo Medical and Dental University institutional review board approved this study, and all participants provided written informed consent. All the participants underwent elective total hip arthroplasty (THA) for hip osteoarthritis at Tokyo Medical and Dental University Hospital and were divided into a type 2 DM group (DM group, $n = 8$) and an age and sex-matched non-DM control group (control group, $n = 8$). The inclusion criterion for the DM group was a preoperative hemoglobin A1c (HbA1c) value $\geq 6.5\%$ (Bloomgarden, 2009; Kilpatrick et al., 2009). The exclusion criteria were THA for osteonecrosis and tumor, revision THA, and a history of treatment with corticosteroids. Four of the eight participants in the DM group were taking oral anti-diabetic drugs, including two taking metformin.

2.2. Determination of patient characteristics

Each patient's age, DM history, and medication history were recorded. Body height, weight, blood HbA1c, fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), bone mineral density (BMD), and bone turnover markers were measured. Body mass index (BMI, kg/m^2) was calculated using the standard formula (WHO Expert Consultation, 2004). For reference, low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) data were collected in the DM group.

2.3. Osteoblast isolation

Mature osteoblasts were isolated using our previously reported method to isolate highly enriched populations of mature osteoblasts from human bone biopsy samples (Fujita et al., 2014a). We obtained resected femoral heads during THA and excised cortical and cancellous bone chips from the medial femoral neck away from the cut surface. We removed attached soft tissues from the bone samples, washed them thoroughly with PBS to reduce blood cell contamination, and chopped them into small fragments with scissors. As an initial digestion step, these fragments were incubated with highly purified, endotoxin-free collagenase (0.6 WU/mL, Liberase DL solution, Roche Applied Science, Bavaria, Germany) for 30 min at 37°C . The solution was centrifuged for 10 min at $440g$ at 4°C , and the collected suspended cells constituted the 1st fraction. The digested bone fragment was incubated in fresh Liberase solution for 60 min at 37°C for the second digestion, and the suspended cells following centrifugation constituted the 2nd fraction. We used 1×10^6 cells from the 1st and 5×10^5 cells 2nd fractions for RNA extraction with QIAzol Lysis Reagent (QIAGEN, Hilden, Germany). The remaining cells from the 2nd fraction were incubated with a human alkaline phosphatase (AP) biotinylated antibody (Anti-Alkaline Phosphatase, Human/Mouse/Rat, Mouse-Mono(B4-78), Biotin, R&D Systems, Minneapolis, MN, USA) for 30 min, followed by incubation with Anti-Biotin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min and positive selection on an autoMACS Pro Separator (Miltenyi Biotec). We collected the suspended cells in AP-positive and AP-negative fractions for further extraction.

2.4. RNA extraction

Total RNA was extracted from the cells in each fraction using an RNeasy Mini Kit (QIAGEN), and its quality and purity were confirmed on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

2.5. Two-step real-time PCR

We synthesized cDNA from the total RNA using ReverTra Ace®

qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) and diluted the cDNA template solutions to a total volume of $50 \mu\text{L}$ with autoclaved MilliQ water. Osteoblastic and osteocytic genes were selected with reference to the previous report (Fujita et al., 2014b) and these primers were designed as follows: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) forward 5'-GACCTGACCTGCCGTCTAGAAA-3' and reverse 5'-CCTGCTTCACCACCTTCTTGA-3', alkaline phosphatase, liver/bone/kidney (*ALPL*) forward 5'-TCGTGACACCTGGAAGAGCTT-3' and reverse 5'-CGTGCGGTTCAGATGAAGT-3', bone gamma-carboxyglutamic acid-containing protein (*BGLAP*) forward 5'-CTCACA CTCTCGCCCTATT-3' and reverse 5'-TTGGACACAAAGGCTGCACC-3', runt-related transcription factor 2 (*RUNX2*) forward 5'-CAAACAGCCTCTTCAGCACAGT-3' and reverse 5'-GTGCTCGGATCCCCAAAGAA-3', collagen type I, alpha 1 chain (*COL1A1*) forward 5'-TGTACCCCACTCAGCCAGT-3' and reverse 5'-GAACAGACATGCCTCTTGTCC-3', sclerostin (*SOST*) forward 5'-GTACACACAGCCTCCGTGTAG-3' and reverse 5'-GATTTCCGTGGCATCTTCT-3', phosphate regulating endopeptidase homolog, X-linked (*PHEX*) forward 5'-AACCTTAGCAGGCCTTTCAG-3' and reverse 5'-AGTTGTGGTCCCTGGATTA-3', and fibroblast growth factor 23 (*FGF23*) forward 5'-AGGCTCTGGGTCTGTGCTT-3' and reverse 5'-GAGGCATTGGGATAGGCTCT-3'.

Real-time PCR was performed using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO) in a Mx3000P QPCR System (Stratagene, San Diego, CA, USA) using the settings described in the instruction manual. Normalization for variations in input RNA was performed using *GAPDH* as a housekeeping gene.

2.6. RNA microarray assay

We evaluated the RNA integrity number (RIN) of cells from AP-positive fractions using an Agilent 2100 Bioanalyzer system. We selected three RNAs with a relatively high RIN value from each group and performed complementary RNA (cRNA) labeling and amplification using a Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). We refined the cRNA labeling using RNeasy Mini spin columns (QIAGEN) and hybridized the cRNAs using a Gene Expression Hybridization Kit (Agilent Technologies). The glass slide was cleaned with Gene Expression Wash Buffer (Agilent Technologies). Hybridized cRNAs were scanned on a Microarray Scanner (Agilent Technologies), and the spots were quantified.

2.7. Statistical and bioinformatic analysis

Participant data are presented as the median and interquartile range (IQR), and Mann-Whitney *U* tests were used for comparisons. Real-time PCR data are presented as the mean and standard deviation (SD), and Student's *t*-tests were used for comparisons. Statistical significance was set at $p < 0.05$. RNA microarray data were analyzed to detect variable genes with variable expression between groups using the Westfall-Young method. Gene expression analysis and quality control were performed in Gene Spring 14.8 (Agilent Technologies). For quality control, lower signal data (threshold: 20.0%) were filtered from the raw data. We analyzed probes (19,463 in total) for which all samples had values within the quality control threshold. Hierarchical clustering analysis was applied to the DM and control groups, using all probes (similarity measure: Euclidean, linkage rule: complete). To extract differentially expressed genes (DEGs) for further analysis, Student's *t*-test was used ($p < 0.05$, by Benjamini-Hochberg and Westfall-Young). Pathway analysis was performed on DEGs using the Kyoto Encyclopedia of Genes and Genomes database. We estimated that a sample size of three samples per group would be required to achieve 80% power to detect a 0.6-point difference in the gene expression ratio between the two groups, assuming an overall SD of 0.2 points, with reference to a previous study (Gogg et al., 2009).

Table 1
Descriptive characteristics of the two groups.

	DM (n = 8 ^a)	Control (n = 8 ^b)	p-value [*]
Sex (female)	8	8	
Age (years)	74.5 (68.3–79.3)	70 (65–80)	0.6
BMI (kg/m ²)	22.9 (19.9–24.3)	24.5 (23.2–25.7)	0.21
HbA1c (%)	7.5 (6.7–7.5)	5.9 (5.7–6)	< 0.001
FBG (mg/dL)	119 (111–132)	106 (103–106)	0.007
TC (mg/dL)	199 (185–233)	199 (182–219)	0.92
TG (mg/dL)	102 (89–132)	115 (74–208)	0.56
LDL-C (mg/dL)	65.5 (50.3–90.5)		
HDL-C (mg/dL)	117 (111–128)		
Bone mineral density (g/cm ²)	0.69 (0.68–0.74)	0.90 (0.75–1.11)	0.18
Total PINP (ng/mL)	37.2 (32.5–77.7)	66.7 (57.7–71.6)	0.54
TRACP 5b (mU/dL)	440 (399–722)	623 (576–794)	0.33
Homocysteine (nmol/mL)	8.2 (6.1–10.6)	8.4 (7.5–9.5)	0.79

Data are presented as the median (interquartile range). DM, diabetes mellitus; BMI, body mass index; HbA1c, hemoglobin A1c; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; PINP, procollagen type I N-terminal propeptide; TRACP, tartrate-resistant acid phosphatase.

^{*} Statistical significance was determined by the Mann–Whitney *U* test.

^a N = 5 in PINP.

^b N = 6 in PINP, BMD, LDL-C and HDL-C.

2.8. Microarray validation

Several osteoblast marker genes (*ALPL*, *BGLAP*, *RUNX2*, and *COL1A1* (Fujita et al., 2014b)) were chosen from the microarray dataset and validated by real-time PCR using a previously reported method (Roforth et al., 2015).

3. Results

3.1. Characteristics of the patients, bone samples, and extracted RNA

The clinical characteristics of each group are shown in Table 1. All participants were female. No patients were undergoing treatment for cancer or had a history of cancer. The median HbA1c value and FBG level were significantly higher in the DM group than those in controls. However, there were no significant differences between groups detected for the other variables; moreover, LDL and HDL levels were nearly within the normal range in the DM group (Table 1). The amount of bone sample collected, the number of cells obtained in each fraction, and RINs of the extracted RNA are shown in Table 2. There were no significant differences between the two groups.

3.2. Expression of osteoblast marker genes in isolated cells

Compared with cells in the 1st fraction, cells in the 2nd fraction were enriched for osteoblast marker genes (*BGLAP*, 2.8-fold; *RUNX2*, 1.4-fold; and *COL1A1*, 3.6-fold) and compared with AP-negative cells. Subsequently, the AP-positive cells were further enriched for osteoblast marker genes (*ALPL*, 49-fold; *BGLAP*, 6.6-fold; *RUNX2*, 2.9-fold; and *COL1A1*, 3.3-fold; Fig. 1). The AP-positive cells expressed very low to

Table 2
Characteristics of specimens from the two groups.

	DM (n = 8)	Control (n = 8)	p-Value [*]
Bone weight (mg)	1024 (739–1096)	1038 (831–1147)	0.38
Number of cells (× 10 ⁵)	2.3 (1.5–2.8)	2.7 (1.7–3.8)	0.32
Ratio of number of cells ^a	1.3 (0.8–2.0)	1.4 (0.5–1.7)	0.57
RNA integrity number	8.2 (7.4–8.6)	7.7 (7.2–7.7)	0.49

Data are presented as the median (interquartile range). DM, diabetes mellitus.

^{*} Statistical significance was determined by the Mann–Whitney *U* test.

^a The ratio of the number of AP-negative and AP-positive cells.

undetectable levels of osteocyte marker genes, including *SOST*, *PHEX*, and *FGF23* (Table S1).

3.3. Pathway analysis of DEGs from the RNA microarray analysis

The raw data obtained from the RNA microarray analysis are shown in Table S2 and Texts S3–S8. Overall, 885 of 19,463 genes (Fig. 2), including phosphoinositide 3-kinase (*PI3K*), serine/threonine kinase 1 (*AKT1*), resistance to audiogenic seizures (*RAS*), were significantly differentially expressed between the DM and control groups ($p < 0.05$). Pathway analysis revealed that components of pathways in cancer and the mitogen-activated protein kinase (MAPK) signaling pathway were significantly enriched in the DM group compared with the control group ($p < 0.01$). Of the genes involved in the target pathways, 13 of 327 pathways in cancer genes and 17 of the 257 MAPK signaling pathway genes were identified as DEGs (Table 3). Furthermore, expression levels of inflammation-related genes such as *IL17* and *IL32* and senescence-related genes such as *TNFRSF8* and *IGFBP3* were also increased significantly in the DM group.

3.4. Validation of microarray data by real-time PCR

A high correlation between microarray data and real-time PCR data was observed ($r = 0.95$, $p < 0.001$; Fig. 3).

4. Discussion

First, we isolated human mature osteoblasts from resected femoral heads during THA, as described in our previously reported method (Fujita et al., 2014a). The cells from AP-positive fraction showed higher expression of osteoblastic genes. Second, we compared the gene expression in DM and non-DM control osteoblasts using RNA microarray analysis by using method previously (Roforth et al., 2015; Farr et al., 2015; Fujita et al., 2014c; Roforth et al., 2014). Osteoblasts from the DM group showed significant activation of pathways in cancer and MAPK signaling pathways.

Activation of MAPK signaling by high glucose or DM has been previously reported *in vitro* in isolated human peritoneal mesothelial cells and microvascular endothelial cells (Gogg et al., 2009; Xu et al., 2003). However, MAPK pathway activation by DM has been reported only in osteoblast-like cell lines *in vitro* (Wang et al., 2010; Zayzafoon et al., 2002), and not in osteoblasts. This study demonstrated that DM activates MAPK signaling in human mature osteoblasts *in vivo*, without

Expression of osteoblast marker genes

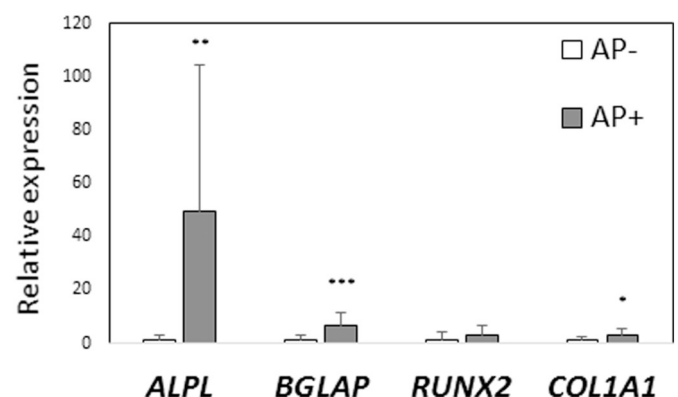


Fig. 1. Expression of osteoblast marker genes in AP-negative and AP-positive cells.

AP-positive cells expressed significantly higher levels of *ALPL*, *BGLAP* and *COL1A1* (N = 8/group). Data represent mean $\pm$ SD (error bars). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Mann–Whitney *U* tests).

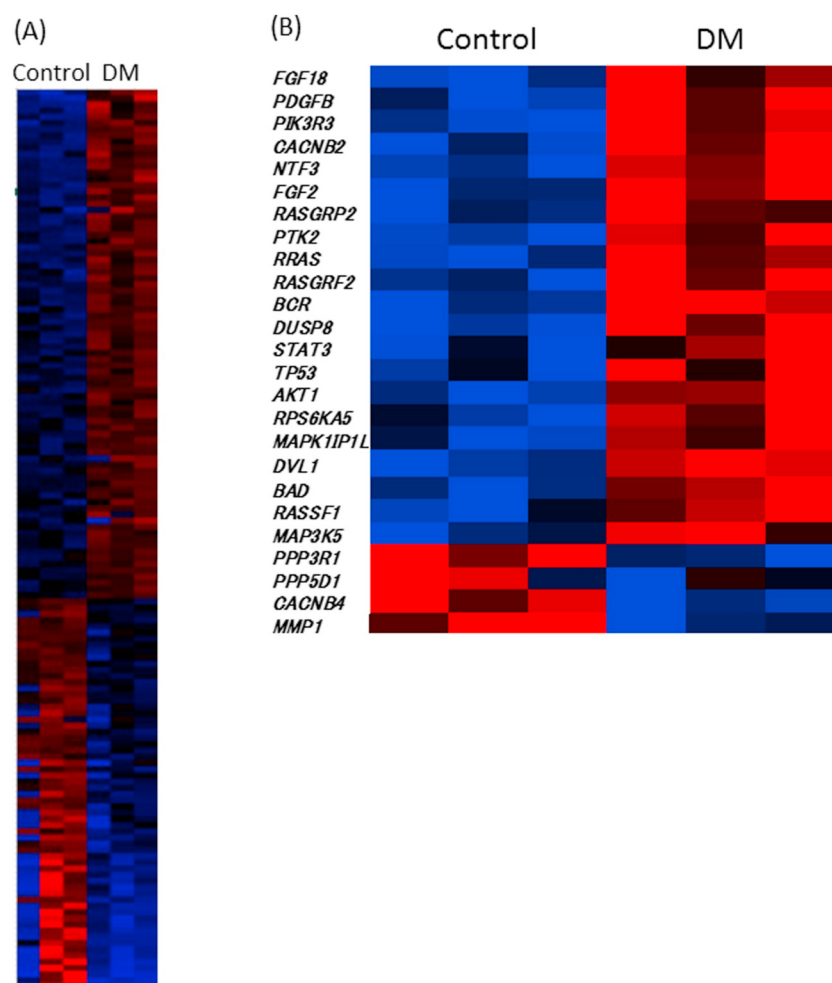


Fig. 2. Heat map representation of differentially expressed genes in the DM group.

(A) All genes that changed significantly between the DM and control group. (B) Only genes that were included in significantly changed pathways. Each column represents the expression profile of individual samples. Red and blue color indicate high and low gene expression, respectively. Color scale represents relative expression levels of the genes.

Table 3

Pathways and associated genes with significant changes in the DM group.

Pathway	DEGs between the DM and control groups	p-Value*
Pathways in cancer	Up <i>AKT1</i> , <i>BAD</i> , <i>BCR</i> , <i>DVL1</i> , <i>FGF2</i> , <i>FGF18</i> , <i>PDGFB</i> , <i>PIK3R3</i> , <i>PTK2</i> , <i>RASSF1</i> , <i>STAT3</i> , <i>TP53</i>	< 0.001
	Down <i>MMP1</i>	
MAPK signaling	Up <i>AKT1</i> , <i>CACNB2</i> , <i>DUSP8</i> , <i>FGF2</i> , <i>FGF18</i> , <i>MAP3K5</i> , <i>MAPK1IP1L</i> , <i>NTF3</i> , <i>PDGFB</i> , <i>RASGRF2</i> , <i>RASGRP2</i> , <i>RASSF1</i> , <i>RPS6KA5</i> , <i>RRAS</i> , <i>TP53</i>	0.004
	Down <i>PPP3R1</i> , <i>PPP5D1</i>	

DEG, differentially expressed gene; MAPK, mitogen-activated protein kinase.

* Statistical significance was determined by Student's *t*-test.

cell culture using fetal bovine serum and other growth factors, which could alter intracellular signaling and gene expression. It is well known that DM increases the risk of various cancers (Giovannucci et al., 2010) and some studies have also suggested a relationship between DM and osteosarcoma (Adamson et al., 1980; Goodman et al., 1978); moreover, MAPK signaling promotes the proliferation of many types of cancers (Burotto et al., 2014; Dhillon et al., 2007; Grimberg, 2003). Consistently, DM also activated pathways in cancer in human mature osteoblasts in this study.

Although this study was preliminary, the results were interesting in that they demonstrated the relationships between DM, MAPK signaling, and carcinogenesis in mature osteoblasts, which are essential for bone and glucose metabolism. A positive feedback loop between mature osteoblasts and lung tumors was recently demonstrated (Engblom et al., 2017), and our results are consistent with this report. Furthermore, MAPK signaling involves two signaling pathways: the PI3K-AKT1 pathway, which is essential for osteoblast differentiation (Mukherjee and Rotwein, 2009), and the RAS pathway, which promotes cancer development. Hence, our findings suggest that the inhibition of MAPK signaling in order to decrease cancer risk due to DM might, conversely, promote the amelioration of DM by inhibition of osteoblast differentiation and decreasing the secretion of osteocalcin. We plan to conduct further *in vitro* studies to validate these results. In particular, we are planning to culture isolated human mature osteoblasts from DM patients and healthy controls, and to compare the change of expression profiles for genes in these signaling pathways and cell mineralization between the two groups.

Another notable finding of this study was an increase in the expression levels of inflammation- and senescence-related genes in the diabetic group. Since these genes are known to be involved in carcinogenesis (Kuilman and Peeper, 2009; Miyahara et al., 2008; Numasaki et al., 2005; Yousif et al., 2013), diabetes-induced senescence (Palmer et al., 2015), and senescence-induced mineralization failure of osteoblasts (Farr et al., 2017), the present results further suggest that these

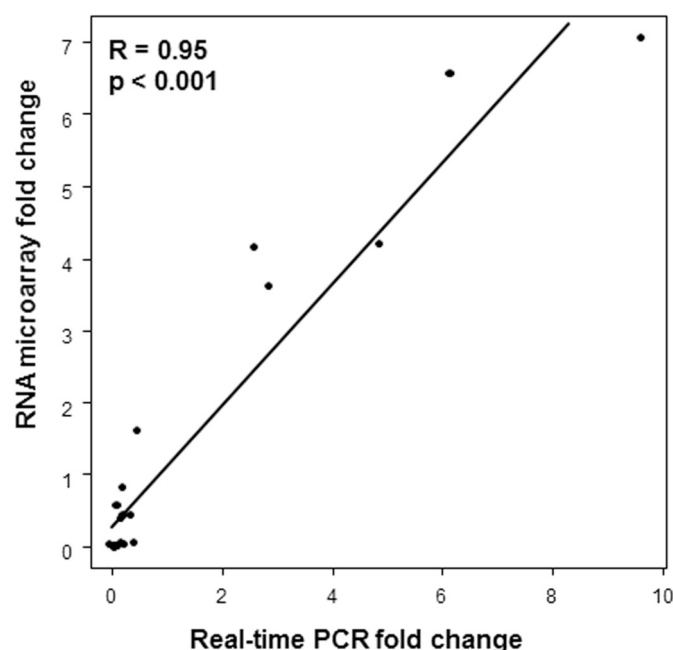


Fig. 3. Real-time PCR confirmation of a subset of differentially regulated genes identified in the RNA microarray analysis.

Correlation plot of osteoblast marker genes in the RNA microarray dataset and real-time PCR validation. A high correlation was observed between the two methods.

genes may play a role in the mechanism of the increased risk of fracture due to diabetes.

Several limitations of this study should be acknowledged. First, the degree of DM in the patients was relatively mild. Second, the evaluation of the degree of diabetes, such as the extent of insulin resistance, was insufficient. Third, the patients with DM were prescribed oral metformin, which affects MAPK signaling. However, metformin has a suppressive effect on cancer development, and our findings demonstrated cancer-promoting effects even in the presence of metformin. Therefore, we believe that metformin exerted minimal effects on the influence of DM on cancer signaling.

5. Conclusions

We demonstrated significant changes in the expression of genes involved in pathways in cancer and MAPK signaling pathways in osteoblasts isolated from patients with DM. These preliminary findings suggest that diabetes affects mature osteoblasts through these pathways and will be useful in elucidating the relationships between DM, osteoblasts, and cancer. We plan to conduct further studies including a larger number of patients and investigate the mechanisms that altered the expression of the genes identified here.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bonr.2019.100199>.

Declarations of interest

None.

Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

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