The improved performance of mesenchymal stem cell cultures by fibronectin and mixed self-assembled monolayers under serum-free conditions

Ph.D. Thesis

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ABSTRACT

Mesenchymal stem cells (MSCs) are a promising cell source for regenerative medicine. For cell-based therapy applications, MSCs must be maintained in chemically defined, xeno-free culture system. However, it is difficult to support proliferation of MSCs with the high differentiation potential in serum-free cultures. Recently, serum-free media for MSCs and embryonic stem cells became commercially available, but the conventional tissue culture plates (TCP) are not suitable for serum-free cultures. The aim of this study is to examine the effects of fibronectin (FN)-coated plates and self-assembled monolayers (SAM) expressing functional groups on adhesion, spreading, proliferation, and differentiation of MSCs under serum-free conditions. Human bone marrow-derived MSCs were maintained on TCP, FN, and a mixed SAM expressing hydroxyl and carboxylic acid groups at 60:40 with a serum-free medium. Cell spreading was estimated by measuring cell area using Image-J software at 2 and 24 h. The cell number was determined on days 6 - 18. DNA microarray analysis was performed using Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarray at 24 h. Metabolomic analysis was performed using liquid chromatography and mass spectrometry. In some studies, MSCs were incubated with a serum-free osteogenesis-induction medium after the serum-free cultures became confluent. The expression levels of bone-related genes were quantified by real time RT-PCR, and calcification was evaluated by alizarin red staining. FN increased cell size/area at 2 and 24 h after seeding, and enhanced proliferation on days 6 - 18 compared with TCP.

However, FN had little effect on a global gene expression profile at 24 h. MSCs on FN, as well as those on TCP, maintained the osteogenic potential as indicated by the expression of bone-related genes and the formation of the calcified matrix after exposure to the serum-free osteogenesis induction medium. The mixed SAM also enhanced MSC proliferation compared with TCP, and maintained the osteogenic potential at high levels. However, unlike FN, the mixed SAM markedly altered the global gene expression profile, although it had little effect on cell size/area. In Gene Ontology (GO) analysis, many (158) gene groups, including Mitochondrion, were down-regulated on the mixed SAM, whereas no gene groups were down-regulated on FN. Furthermore, metabolome analysis using liquid chromatography and mass spectrometry showed the lactate/pyruvate ratio and a 2-oxoglutaric acid level were increased on the mixed SAM. In conclusion, FN and the mixed SAM improved the performance of MSC cultures under serum-free conditions, although these surfaces elicited distinct effects on cell size/area and the gene expression profile of MSCs.

CHAPTER 1 (INTRODUCTION)

Mesenchymal stem cells (MSCs) have the great potential for regenerative medicine and cell therapy applications, as these can differentiate into various types of cells, including osteoblasts, chondrocytes, and adipocytes. Furthermore, MSCs have the anti-inflammatory, immunomodulatory, and paracrine actions [1]. Many clinical trials using autologous and allogeneic MSCs for various diseases, including bone fracture, are currently underway.

However, we have to expand MSCs in culture to obtain enough numbers of transplantable cells. Fetal bovine serum (FBS) has been used for ex vivo expansion of MSCs, but FBS has the potential risks of infection with viruses and prion, and allergic reactions upon transplantation [2]. Moreover, culture performance is inconsistent with the different FBS batches.

Therefore, serum-free media are desired for regenerative medicine and stem cell-based therapy. Numerous serum-free media that maintain self-renewal and multi-potency of MSCs, embryonic stem cells (ES cells), and induced pluripotent stem cells (iPS cells) have been developed, and some of them are commercially available [3, 4]. In this study, we used serum-free media STK2 and STK3, which contain ~80 compounds, including several growth factors, but not undefined materials [5, 6]. These media can support proliferation and osteogenic differentiation of MSCs, respectively [7].

Plastic tissue culture plates (TCP) have been widely used in cell culture. However, TCP are surface modified using corona discharge or gas-plasma to allow adhesion and proliferation of various cells [8, 9]. Upon this treatment, the polystyrene surface becomes hydrophilic by addition of oxygen-containing chemical groups or opening of the benzene ring. However, fine structure of the ionized surface is unstable and gradually changes with time. Therefore, TCP surface cannot be chemically defined. Ideally, chemically defined culture surfaces stably exposing certain functional groups should be used in serum-free cultures.

Fibronectin (FN) has been widely used in cell culture and tissue engineering. However, in most studies, the effects of FN on proliferation and differentiation were examined in the presence of FBS, which contains various adhesion molecules [10]. The author examined here the effects of FN on proliferation and differentiation of MSCs with serum-free media, which do not contain adhesion molecules. We also investigated the effect of FN on cell spreading, because cell size/area may influence proliferation and differentiation of MSCs. Previous studies have shown that cell shape/area modulates proliferation and differentiation of MSCs under various experimental settings [11, 12]. In addition, we determined a gene expression profile of MSCs 24 h after seeding, because cell size/area may also affect the gene expression profile.

Self-assembled monolayers (SAM) of alkanethiols form stable organic layers on a gold surface, and binding of thiol group of alkanethiols to gold, along with van der Waals

interactions between their alkyl chains, allows formation of chemically defined surfaces exposing terminal functional groups (Fig. 1) [13, 14]. In pilot studies, we found that, among various SAMs, a mixed SAM exposing hydroxyl and carbonic acid groups at 60:40 can support proliferation of MSCs under serum-free conditions. Therefore, we examined here whether this mixed SAM may improve the performance of serum-free MSC cultures compared with TCP.

We also compared the effects of FN and the mixed SAM on serum-free MSC cultures, as adhesion molecules and functional groups should have distinct actions. Results showed that both surfaces enhanced proliferation of MSCs compared to TCP at similar levels. In addition, both surfaces supported osteogenic differentiation of MSCs at high levels. Although FN increased cell size/area to a greater extent than TCP, FN had little effect on a gene expression profile of MSCs.

Interestingly, the mixed SAM altered the gene expression prolife and down-regulated many gene groups, including mitochondrion, but it had little effect on cell size/area. The down-regulation of mitochondria-related genes in MSCs was associated with changes in glucose metabolism, and may relate to low mitochondria activity of stem cells [15]. The information obtained in this study provides new insights into cell-culture/material surface interactions, and may be useful for development of high performance MSC cultures under serum-free conditions.

CHAPTER 2 (MATERIALS AND METHODS)

2.1. Preparation of FN-coated surface

The 60 mm and 24 well tissue culture plates (TCP) (Corning, Nagog Park Acton, MA, USA) were incubated with human FN at 10 μg/ml (BD Bioscience, Osaka, Japan) in distilled water at room temperature for 1 h. The FN-coated plates were washed three times with phosphate buffered saline (PBS) (Life Technologies Japan Ltd, Tokyo, Japan).

2.2. Preparation of mixed SAM

The glass plates (thickness: 1 nm, diameter 15 mm and 60 mm) (Matsunami Glass Ind. Ltd., Osaka, Japan) were prepared by first depositing thin chromium (Cr) and gold (49 nm) under 23.0 x $10^{-}3$ Pa, by using vacuum deposition system (VTR-350M/ERH, ULVAC KIKO Inc., Miyazaki, Japan).

To prepare alkanethiol solutions of 1 mmol/L of 11-mercapto-1-undecanol (OH-SH) and 11-mercapto-1-undecanoic acid (COOH-SH) (Sigma, St. Louis, MO, USA), nitrogen gas was bubbled through deoxidized ethanol for 1 h. These two alkanethiol solutions were then mixed at 6:4 (OH:COOH) volume ratios.

The gold deposited glass surface was immersed in the mixed alkanethiol solution at 25 °C for 24 h to allow the formation of the mixed SAM on the gold surface. After washing 3 times by ethanol, the mixed SAM plates were kept in ethanol at -20 °C until the experiment. The mixed SAM plates were washed with PBS three times before cell seeding.

2.3. Isolation and culture of MSCs

MSCs derived from human iliac bone marrow were collected after written informed consent was obtained with approval by Hiroshima University Ethics Committee. Human MSC lines were purchase from Lonza Japan Ltd (Tokyo, Japan). These cells were cultured and grown on 100 mm TCP in a serum-free medium STK2 (DS Pharma Biomedical, Osaka, Japan). After cultures became near-confluent, Accutase (Funakoshi Co. Ltd, Tokyo, Japan) was used to harvest the cells. Cells in the third to the eighth passage cultures were used in this study.

2.4. Cell adhesion assay

MSCs were seeded at 1 x 10^5 cells/cm² on TCP, FN-coated surface, and the mixed SAM surface in a 24-multi-well plate with STK2. At 30, 60, and 120 min, non-adherent cells were removed by washing with PBS, and the number of adherent cells was estimated using the

themethylthiazole tetrazolium, thiazolyl blue (MTT) assay with Cell Counting Kit-8 (CCK-8) (Wako, Osaka, Japan). The formation of formazan was determined at absorbance 490 nm using VersaMax ELISA microplate reader (Molecular Devices, Tokyo, Japan).

2.5. Cell proliferation

MSCs were seeded at 4 x 104 cells/well on TCP, FN-coated surface, and the mixed SAM in 60 mm dishes, and incubated with STK2 at 37 °C in a 5% CO2 incubator. After cultures became near-confluent, cells were harvested with Trypsin-EDTA (Sigma, St. Louis, MO, USA). To inactivate the enzyme, Soybean trypsin inhibitor (Sigma, St. Louis, MO, USA) was added to the cell suspension. The dispersed cells were transferred into passage cultures with STK2. The number of cells was determined with Burker-Turk on days 6, 12, and 18. The assay was performed by continuously culture, cell number measurement and reseeding on day 6, 12, and 18, respectively.

2.6. Cell size/area

MSCs were seeded at a density of 4 x 104 cells/well on various surfaces in 60 mm plates and incubated with STK2 for 2 and 24 h. Cell area/size was determined using ImageJ analysis software (National Institutes of Health, Bethesda, USA) [16].

2.7. Osteogenic differentiation

MSCs were seeded at 2.5 x 10^5 cells/cm² on various surfaces in 24-well plates with STK2. After cultures became confluent (day 0), the cells were exposed to a serum-free osteogenesis induction medium STK3 (DS Pharma Biomedical, Osaka, Japan) at 37 °C in 5% CO2. Alkaline phosphatase activity was determined on day 14 by an established colorimetric assay as described previously [17]. Briefly, osteogenic cell monolayers were washed with PBS and lysed with 0.5 ml of 0.2% Triton-X100 for 5 min. The lysates were homogenized using a sonicator (Vibra Cell™ 130; Sonix & Materials Inc., Newtown, CT, USA) (amplitude = 30; time = 10 s) on ice. Aliquots of 10 µl of the lysates were mixed with 90 µl of 16 mM p-nitrophenyl phosphate (Calbiochem, Darmstadt, Germany) in a solution (pH 10.4) containing 350 mM 2-amino-2-metyl-1-propanol, 2 mM MgSO4, 1 mM ZnSO4, and 2 mM EDTA for 10 min at 37 °C. Thereafter, 100 µl of 1N NaOH was added to each well to stop the reaction. The absorbance was read at 410 nm using VersaMax ELISA microplate reader (Molecular Devices,

Tokyo, Japan). The assay was performed in triplicate and compared against p-nitrophenol standards.

Calcification was evaluated by alizarin red staining (A5533-25G, Sigma). In studies with the mixed SAM, the calcium content was determined on day 14 using the Calcium C Test Wako (Wako Pure Chemical Industries, Ltd.) [18]. Briefly, monolayer cultures were washed with 800 μl of 10 mM Tris-HCl buffer (pH 7.0) and lysed in 0.2 ml of 10% formic acid for 30 min at room temperature. Aliquots of 20 μl of 2- or 10-fold diluted lysates were used according to the manufacturer's instructions. The absorbance was read at 570 nm using VersaMax ELISA microplate reader (Molecular Devices, Japan).

To determine mRNA levels for bone-related genes, total RNA was prepared using Trizol (Invitrogen Life Technologies Corp., Carlsbad, CA, USA) and RNeasy mini-column kits (QIAGEN, Tokyo, Japan). The concentration of RNA was measured by Nanodrop instrument (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). The RNA extraction was carried out at day 0, 4, 8 and 12 after osteogenic induction medium STK3. For cDNA synthesis, ReverTra Ace (TOBOYO, Osaka, Japan) reagent was used in this study. Real time PCR analysis was performed by using an ABI Prism 7900HT Sequence Detection System and software (Applied Biosystems Life Technologies Corp., Carlsbad, CA, USA). The primers and

probes were purchased from Roche Applied Science, Japan (Table 1). Human GAPDH was used as internal control (Taqman Gene Expression Assays, Applied Biosystems, Japan).

2.8. DNA microarray analysis

MSCs were seeded at 8 x 105 cells/well on the mixed SAM or TCP in 60 mm plates and incubated with STK2 for 24 h. Total RNA was extracted from MSCs cultured on TCP, FN coated surface, and the mixed SAM gold surface 24 h after seeding using Trizol and RNeasy mini-colum kits. The analysis of gene expression was performed using SurePrint G3 Human Gene Expression 8x60K v2 Microarray Kit (Agilent Technologies, Inc., Tokyo, Japan). GeneSpring version 12 (Silicon Genetics, Redwood, USA) was used for gene filtering and statistical analysis. To validate DNA microarray data, we performed real-time PCR analysis using an ABI Prism 7900HT Sequence Detection System and software. The primers and probes were purchased from Roche Applied Science (Tokyo, Japan) (Table 1). Human GAPDH was used as internal control.

2.9. Metabolomics

MSCs were seeded at 1 x 10^6 cells on the mixed SAM or TCP in 60 mm plates and incubated with STK2 for 24 h. The metabolites in the cells were extracted according to the manufacturer's protocol. Liquid chromatography and mass spectrometry (LC-MS) were performed at Human Metabolome Technologies Inc., Tsuruoka, Japan. For normalization of metabolite concentrations, the number of cells in these cultures was determined with Burker-Turk.

2.10. Statistical analysis

Statistical analyses were performed using a two-tailed unpaired Student's t-Test Gene Ontology term and WIKI gene pathway were performed using Fisher's exact test analysis.

CHAPTER 3 (RESULTS)

3.1 Cell adhesion and proliferation

To examine the effect of culture surfaces on cell adhesion, MSCs were seeded on TCP, FN, and the mixed SAM with STK2. FN did not enhance MSC adhesion compared to TCP at 30, 60, and 120 min (Fig. 2A). The mixed SAM delayed MSC adhesion at 30 min, but not at 60 and 120 min.

To examine the effect of the surfaces on cell proliferation, MSCs were seeded on TCP, FN, and the mixed SAM and incubated with STK2. On days 12 and 18, both FN and the mixed SAM increased the cell number 3- and 6-fold, respectively compared with TCP (Fig. 2B).

3.2 Cell morphology and cell size/area at 2 and 24 h

Fig. 3 and 4 show the effects of FN and the mixed SAM on cell size/area at 2 and 24 h. At 2 h after cell seeding, MSCs on the mixed SAM showed similar cell size/area with those on TCP. Most of the cells on the mixed SAM and TCP were round compared with the elongated and fibroblast like-shape on FN. Accordingly, MSCs on FN but not on the mixed SAM showed an

increase of cell size/area compared with TCP. This increase in cell size/area on FN was also observed at 24 h.

3.3 Osteogenic differentiation

MSCs were seeded on TCP, FN coated surface, or the mixed SAM gold surface and incubated with the growth medium STK2. When cultures became almost confluent, the cells were exposed to a serum-free osteogenesis induction medium STK3. After the onset of differentiation, alkaline phosphatase activity increased on FN and TCP at a similar level, and calcification was induced in both surfaces at a similar level (Fig. 5). In studies with the mixed SAM, we quantified the calcium content, since the mixed SAM on gold is stained with alizarin red. In the calcium assay, the calcium content increased on the mixed SAM and TCP at a similar level after exposure to STK3.

Fig. 6 shows the up-regulation of bone-related genes on the TCP, FN, and the mixed SAM after exposure to the serum-free osteogenesis induction medium. MSCs cultured on both FN and mixed SAM, as well as those on TCP, maintained the osteogenic differentiation potential. On these surfaces, mRNA levels of alkaline phosphatase, liver/bone/kidney (ALPL), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), and osteopontin/secreted phosphoprotein 1 (OPN/SPP1) markedly increased after exposure to STK3. The expression of

these genes started to increase on day 4 or 8, and the increased levels were sustained until day 8 or 12. The time course and the extent of gene expression were similar among TCP, FN, and the mixed SAM.

However, FN increased the expression level of ALPL on day 8, but decreased on day 12 compared with TCP: FN increased the expression of RUNX2 on day 8, but not day 12: FN increased the expression of OCN on days 4 and 12, but not day 8: FN increased the expression of OPN on day 4, but not on days 8 and 12. In other words, FN enhanced the expression of bone-related genes to some extents in some, but not all, time points in the differentiation process.

3.4 Global gene expression profile

To investigate the initial effect of these surfaces on MSCs, we performed DNA microarray analysis of MSCs on these surfaces at 24 h. Fig. 7 shows the scatter plots of the DNA microarray data of MSCs on FN and the mixed SAM. In these plots, each dot shows expression levels of a transcript on FN or the mixed SAM compared with TCP. The scatter plot for FN showed a very narrow distribution of dots, indicating a marginal effect of FN on the global gene expression (Fig. 7A). In contrast, the scatter plot for the mixed SAM showed a wider

distribution of dots, indicating a greater effect of the mixed SAM on the global gene expression (Fig. 7B).

Fig. 8 shows changes in the gene expression profile in MSCs on FN and the mixed SAM compared with those on TCP at 24 h. Many (1333 and 957) genes were down-regulated and up-regulated, respectively, with a 1.5-fold change cutoff and $p < 0.05$ on the mixed SAM, whereas only 146 and 101 genes were down-regulated and up-regulated, respectively, on FN. Table 2 and 3 show the list of top 20 genes down-regulated and up-regulated on FN and the mixed SAM. According to the microarray data, no common genes were found in Table 2 and 3. The top 20 did not include genes directly involved in cell cycle or integrin-mediated signaling pathways.

In Gene Ontology (GO) analysis, we found 158 and 5 term groups were down-regulated and up-regulated, respectively, on the mixed SAM, whereas no groups were down-regulated or up-regulated on FN (Fig. 8). Subsequently, we focused on the down-regulated GO groups, as the number of the down-regulated groups was much greater than that of the up-regulated ones. Interestingly, the top 20 GO groups down-regulated on the mixed SAM included 10 mitochondria-related and 3 ribosome/translation-related gene groups (Table 4).

In WIKI pathway analysis, osteoclast signaling pathway alone was significantly ($P < 0.001$) down-regulated on FN (3 in 10 genes), whereas 4 pathways, including electron transport chain,

oxidative phosphorylation, proteasome degradation, and cytoplasmic ribosomal protein pathways, were down-regulated on the mixed SAM (Table 5). The two gene-set approaches indicated that mitochondria and ribosome-related genes were down-regulated on the mixed SAM, whereas FN had a marginal effect on the gene expression profile, although some osteoclast-related genes were down-regulated on FN.

DNA microarray data showed lower expression levels of MAPK8 and OPN involved in osteoclast signaling pathway in MSCs on FN than on TCP (Fig. 9). These data were validated by real time PCR analysis (Fig. 10). The DNA microarray data also showed lower expression levels of several genes involved in the electron transport chain in MSCs on the mixed SAM than on TCP (Fig. 11). These microarray data were also validated by real time PCR analysis (Fig. 12).

3.5 Metabolomic profile

The down-regulation of mitochondria-related genes in MSCs on the mixed SAM may alter metabolomic profiles. To explore this possibility, we analyzed concentrations of metabolites in MSCs from three donors using LC-MS. Unexpectedly, there were no significant differences in almost all examined metabolites involved in glycolysis, tricarboxylic acid (TCA) cycle, and metabolism of amino acids and lipids between the mixed SAM and TCP.

However, the 2-oxoglutarate (alpha-ketoglutarate) content and the lactate/pyruvate ratio were higher on the mixed SAM than on TCP (Fig. 13 and 14). The P-value for the 2-oxoglutarate content was 0.044, and that for the lactate/pyruvate ratio was $P = 0.078$, although the content and ratio were consistently higher on the mixed SAM than on TCP in all examined MSCs from different donors.

CHAPTER 4 (DISCUSSION)

Culture surfaces suitable for serum-free cultures are essential for safe and reliable stem cell-based therapies. FN-coated plates are suggested to be useful in serum-free cultures of MSCs, since FN enhances MSC proliferation in media with FBS [19-20]. FN is the abundant component of the extracellular matrix in connective tissues. FN coating of culture dishes may compensate the shortage of adhesion molecules in serum-free media and mimic the extracellular matrix.

FN did not affect the number of adherent cells at 0.5-2 h, but markedly increased cell size/area at 2 and 24 h compared with TCP. In addition, FN enhanced MSC proliferation on days 6 - 18 under serum-free conditions, and supported osteogenic differentiation of MSCs at high levels. These results suggest that FN coating improves the performance of serum-free MSC cultures. In general, the increase in cell size/area is associated with low growth rate and cell senescence [21]. However, FN enhanced proliferation of MSCs, despite marked increase of cell size/area by FN. Therefore, FN-induced overspreading did not lead to acceleration of MSC senescence.

By the contrary, FN facilitated the progression of cell cycle, partly because stable spreading triggers entry into cell cycle [22]. No cell cycle-related genes were enhanced on FN

in GO and the pathway analyses. It is thus unlikely that FN enhances the proliferation by induction of certain cell cycle-related genes at 24 h. Since cell shape and area have been reported to be crucial for proliferation and the lineage determination of MSCs [11, 12, 22, 23], we speculated that the marked increase in cell size/area could affect a gene expression profile of MSCs.

Contrary to our expectation, scatter plots of DNA microarray data showed marginal changes in the gene expression profile on FN compared with TCP. GO analysis revealed that no gene groups were selectively regulated by FN. In the pathway analysis, the osteoclast signaling pathway alone was affected by FN. The physiological relevance of this pathway is unknown, since MSCs cannot differentiate into osteoclasts that are derived from the monocyte-macrophage lineage [24]. In any case, FN did not substantially alter the global gene expression profile of MSCs. These findings suggest that the enhanced MSC spreading by FN is mediated by integrin-mediated intracellular signaling pathways at a post-translational level but not at a transcriptional level.

The role of FN in osteogenic differentiation of MSCs is also unclear, although the FN receptor integrin alpha5 is required for osteogenic differentiation of MSCs [25]. FN and RGD containing peptides enhance or have little effect on osteogenic differentiation of MSCs in the presence of 10% FBS after exposure to the osteogenesis induction medium [23, 26, 27]. In the

present study, MSC cultures on FN showed extensive calcification after osteogenic differentiation under serum-free conditions. The extent of calcification estimated by alizarin red staining was similar between FN and TCP. However, FN increased the expression levels of bone-related genes compared to TCP at some time points after exposure to the serum-free osteogenesis induction medium. In a previous study, FN increased the expression of bone-related genes in MSCs after exposure to osteogenesis induction medium containing 10% FBS [23]. These findings suggest that FN enhances osteogenic differentiation of MSCs to some extents.

Ideally, culture/material surfaces should enhance adhesion, proliferation, and differentiation without affecting the intrinsic property of stem cells. MSCs grown on FN maintained the MSC gene expression profile and the osteogenic potential. Therefore, FN can be applied to tissue engineering for bone defects.

The mixed SAM expressing hydroxyl and carboxylic acid groups may mimic the extracellular matrix, as glycosaminoglycans and extracellular matrix proteins, along with glycoproteins, expose hydroxyl and carboxylic acid groups abundantly. In previous studies, SAMs supported proliferation of various cells depending upon the composition of functional groups [13, 14]. We demonstrated here that a mixed SAM expressing hydroxyl and carboxylic acid groups at 60:40 enhances MSC proliferation compared with TCP under serum-free

conditions through sequential passages. This composition of functional groups appears to be permissive for MSCs, as compared with TCP expressing the benzene ring at a high ratio.

The mixed SAM and FN had distinct effects on cell size/area and the gene expression profile. These findings suggest that the enhanced MSC proliferation by the mixed SAM is not mediated by adhesion molecules that are secreted by MSCs in culture, although certain SAM surfaces may bind adhesion molecules. Unexpectedly, the mixed SAM markedly altered the gene expression profile of MSCs. This is the first observation of changes in global gene expression profiles by surface functional groups. In other words, the composition of surface functional groups seems to be crucial in the regulation of gene expression. We intend to examine the effect of different functional group compositions on the gene expression profile in separate studies.

In GO and pathway analyses, many genes of mitochondrion and electron transport chain pathway were selectively down-regulated on the mixed SAM. The physiological relevance is unknown, but it is generally known that the mitochondria state is crucial for energy metabolism and reactive oxygen species (ROS) generation. In addition, it has been reported that low mitochondrial activity is required for the maintenance of the stemness of ES cells, iPS cells, and hematopoietic stem cells [15, 28 - 30]. These stem cells show anaerobic glycolysis, as well as reduced ROS generation, compared to differentiated cells. In the present study, all examined

MSCs from different donors showed increased the lactate/pyruvate ratio and increased 2-oxoglutarate content, along with down-regulation of mitochondria-related genes, on the mixed SAM. These findings suggest that functional groups on culture surfaces modulate mitochondria functions and glucose metabolism in MSCs.

The two culture surfaces have advantages and disadvantages. FN-coated plates can easily be prepared, whereas preparation of the mixed SAM is laborious. TCP coated with FN may not be homogenous and are biological, whereas the mixed SAM is a homogenous and chemically defined surface. FN-coated plates are expensive, as recombinant FN fragments should be used for clinical applications. On the other hand, alkanethiols are not expensive, although gold is expensive. FN induces overspreading of MSCs, whereas the mixed SAM does not do so. The mixed SAM alters the global gene expression profile of MSCs, whereas FN does not do so. Whether a combination of adhesion molecules and surface functional groups further enhances MSC proliferation and differentiation is the issue that should be addressed in a next step.

CHAPTER 5 (CONCLUSION)

In conclusion, FN and the mixed SAM had distinct effects on cell size/area and the gene expression profile of MSCs, although both surfaces enhanced MSC proliferation at similar levels. The FN-induced increase in cell size was not associated with changes in proliferation, osteogenic differentiation, and the global gene expression profile in the present experimental setting. In any case, FN and the mixed SAMs expressing functional groups will be useful in development of high performance MSC cultures, which facilitates therapeutic applications of MSCs.

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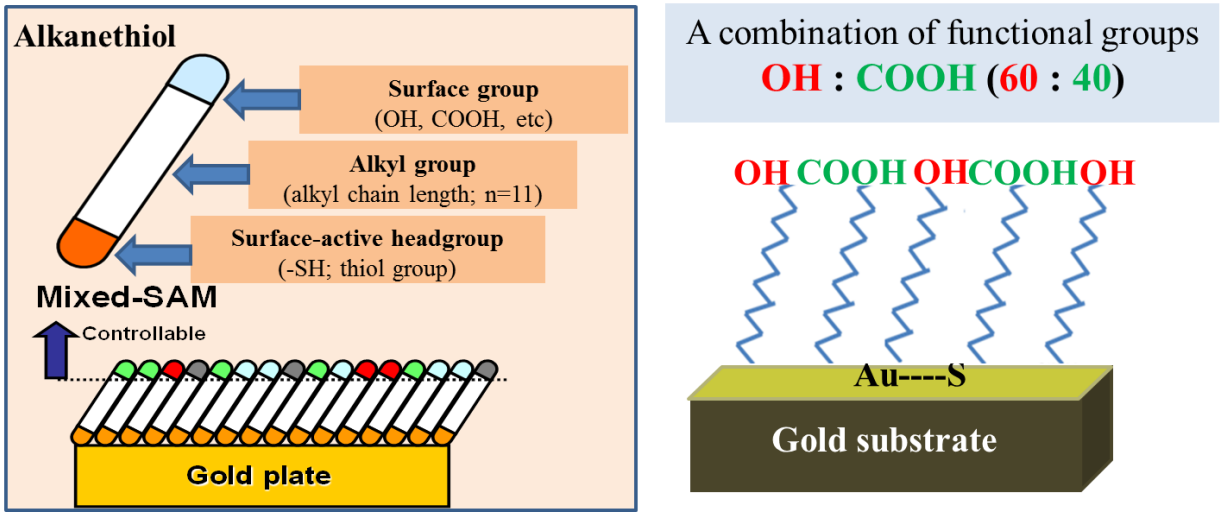


Fig. 1. Gold plates and structure of mixed SAMs. In this study, we used a mixed SAM expressing hydroxyl and carboxylic acid group at 60:40. The surface functional groups are randomly mixed on the surface, as shown in this figure.

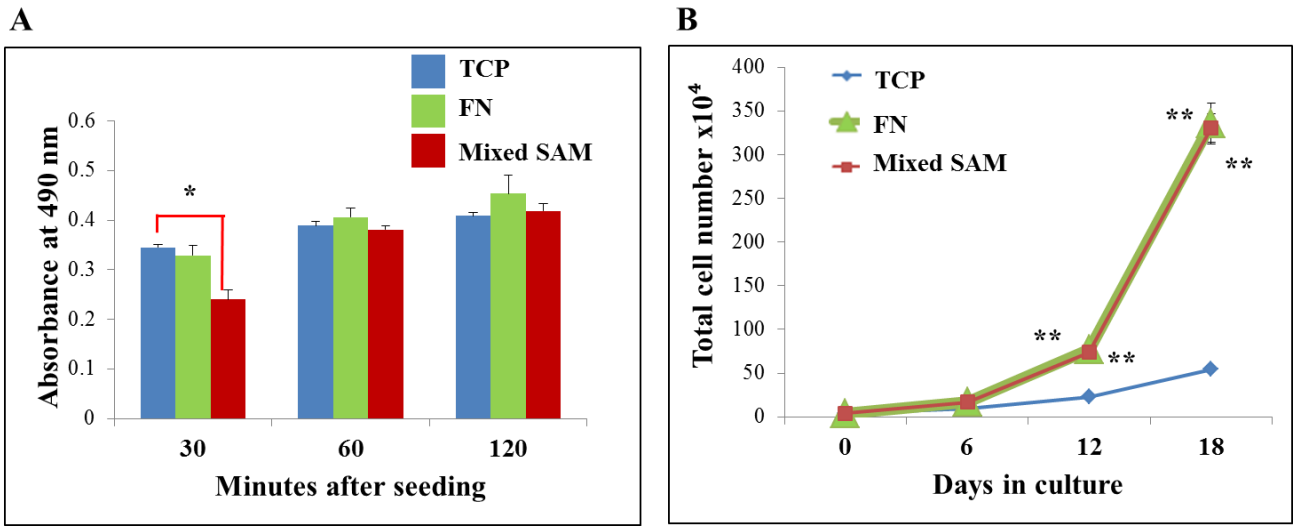


Fig. 2. Effects of FN and the mixed SAM on adhesion (A) and proliferation (B) of MSCs under serum-free conditions. A) MSCs were seeded at 1 x 10^5 cells/cm² on TCP, FN and the mixed SAM in a 24-multiwell plate with STK2. At 30, 60 and 120 min, floating cells were removed by washing with PBS, and the number of adherent cell was estimated in MTT assays. B) MSCs were seeded at 4 x 10^4 cells/well on TCP, FN and the mixed SAM in 6 cm diameter plates and incubated with STK2 until 18 days. The cell number was determined on the indicated days. Values are average ± SD for three cultures. *$P < 0.05$, **$P < 0.01$.

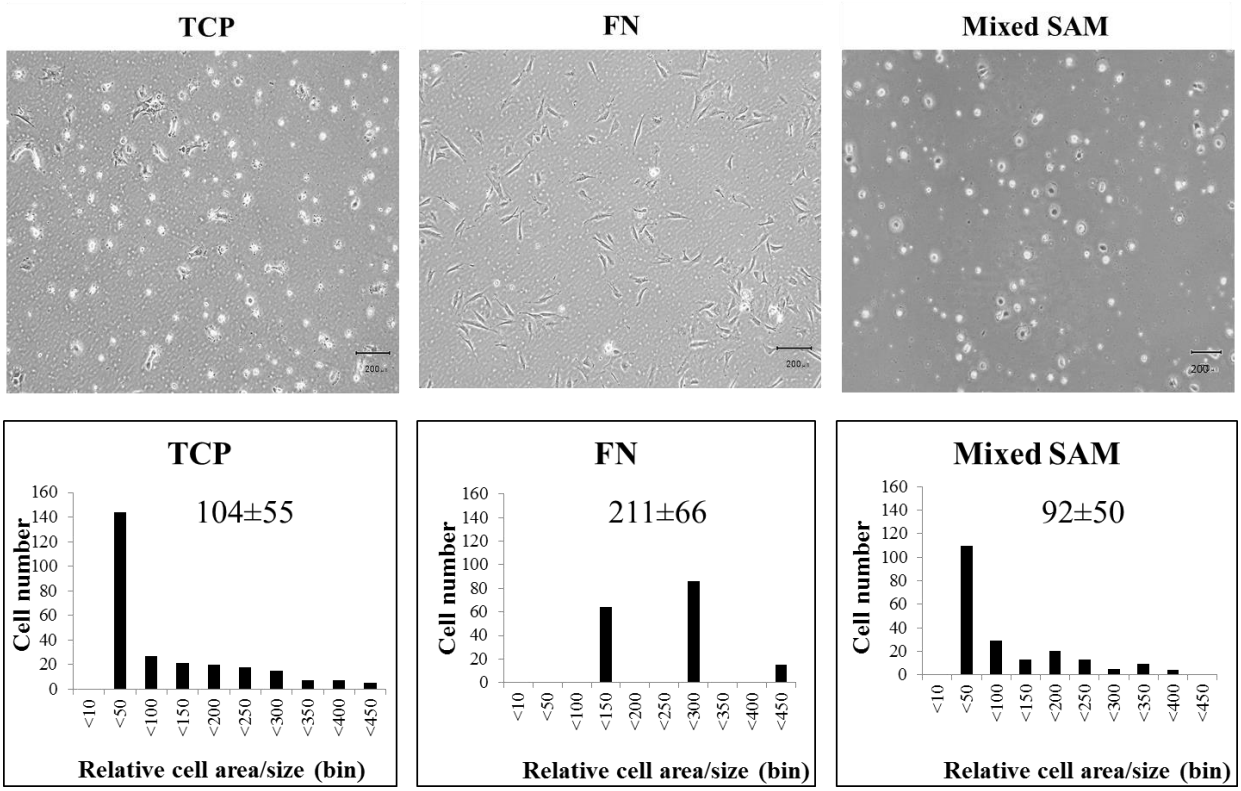


Fig. 3. Effects of FN and the mixed SAM on cell size/area at 2 h. Values are average ± SD for three cultures. $^{**}P < 0.01$ (FN versus TCP). No significant difference between the mixed SAM and TCP.

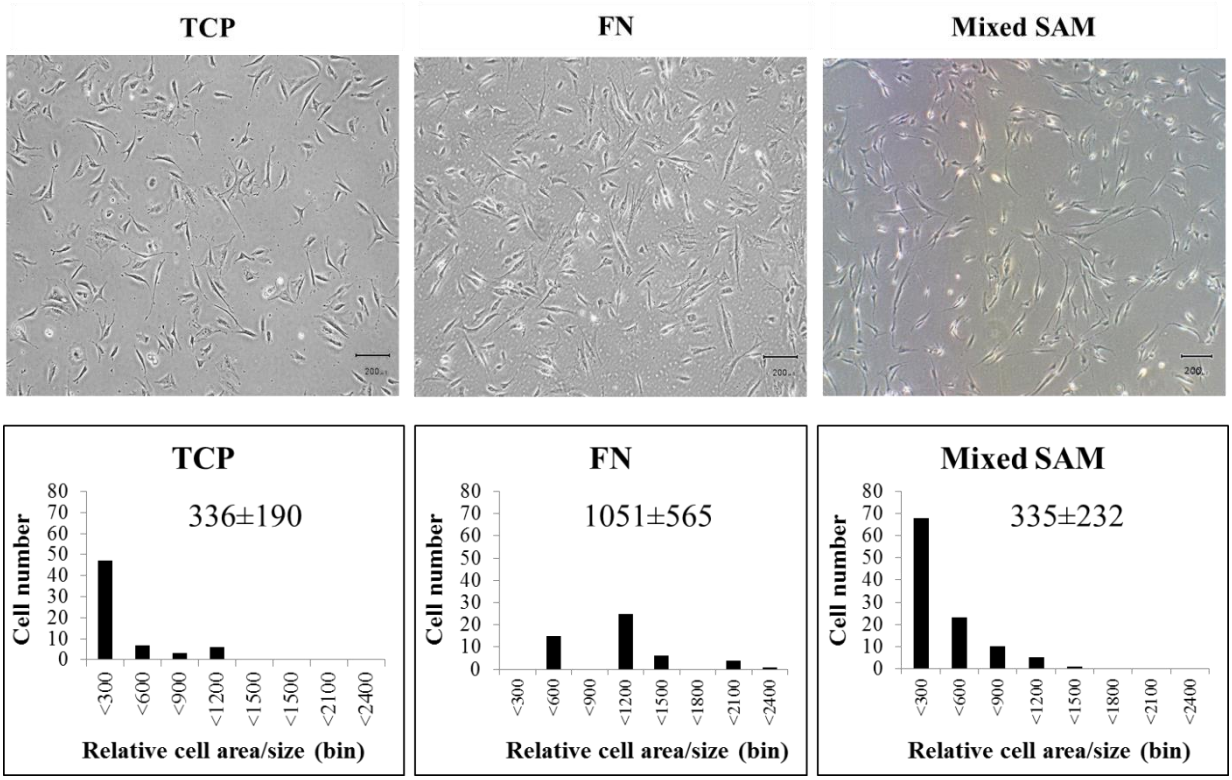


Fig. 4. Effects of FN and the mixed SAM on cell size/area at 24 h. Values are average ± SD for three cultures. $^{**}P < 0.01$ (FN versus TCP). No significant difference between the mixed SAM and TCP.

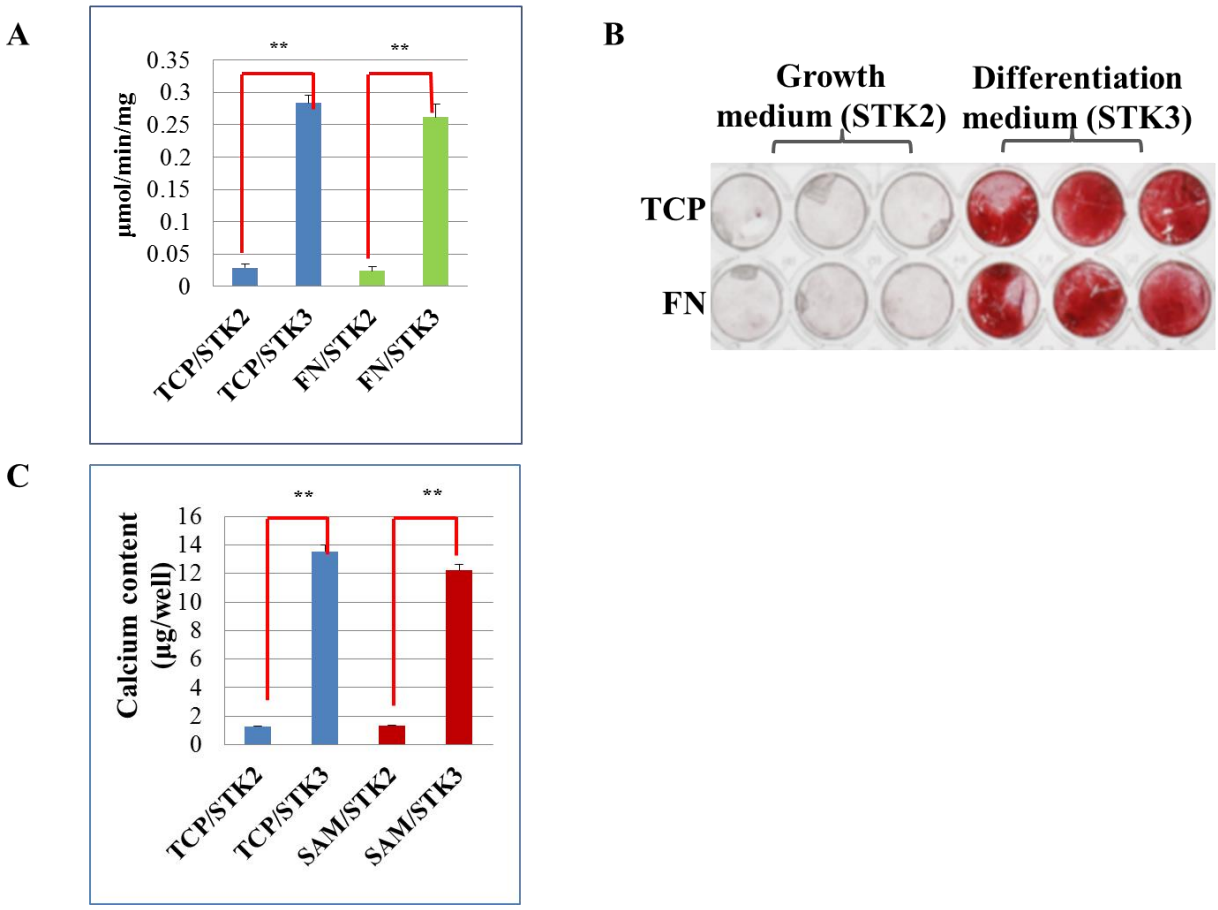


Fig. 5. Effects of FN and the mixed SAM on osteogenic differentiation of MSCs at day 14. A) Alkaline phosphatase activity B) Alizarin red staining C) Calcium content. Values are average ± SD for three cultures. $^{**}P < 0.01$.

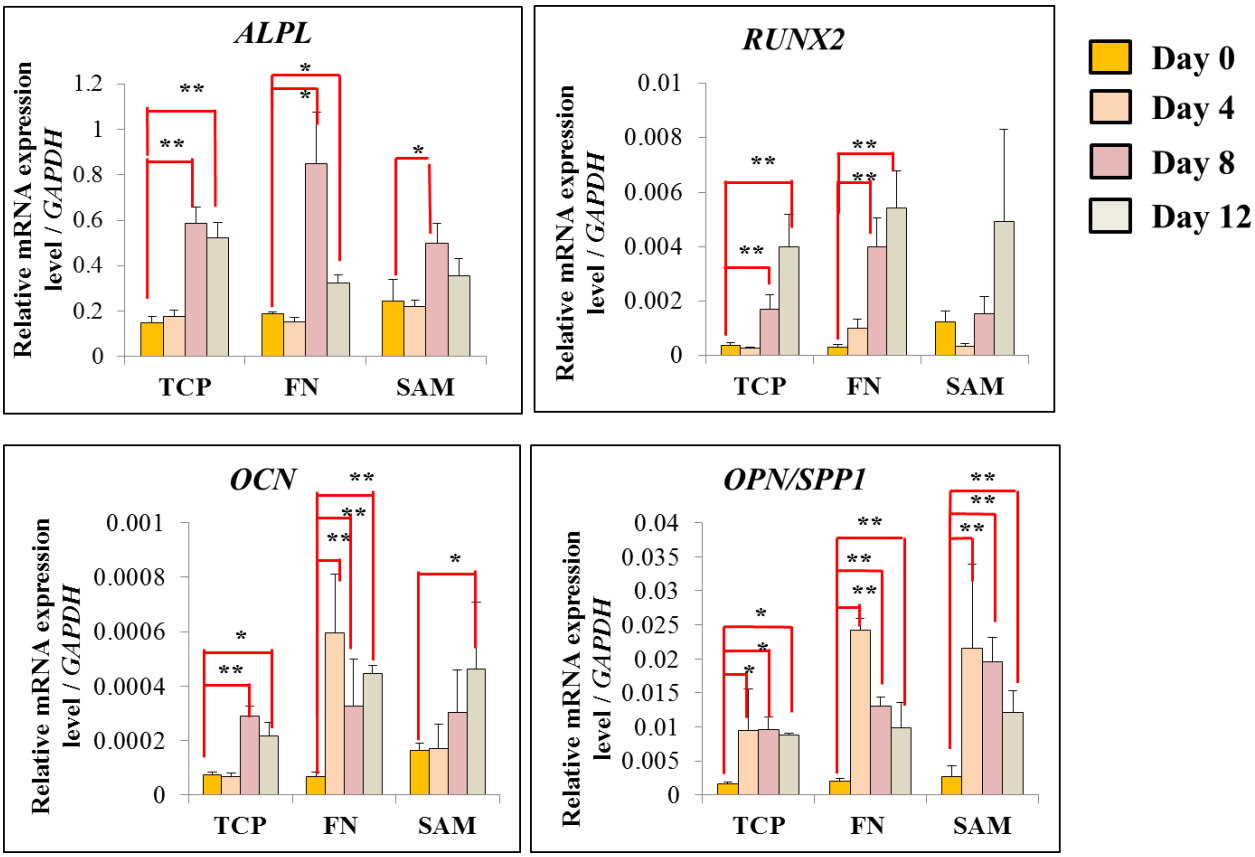


Fig. 6. The up-regulation of bone-related genes on TCP, FN and the mixed SAM after exposure to a serum-free osteogenesis induction medium STK3. *$P < 0.05$, **$P < 0.01$.

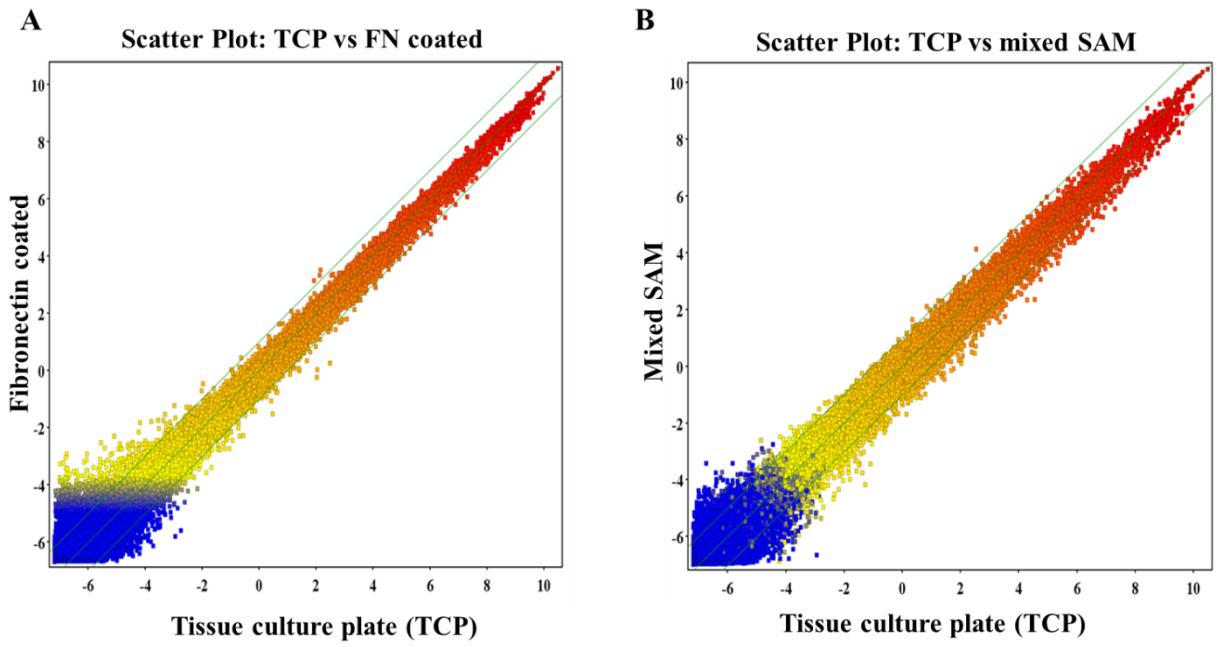
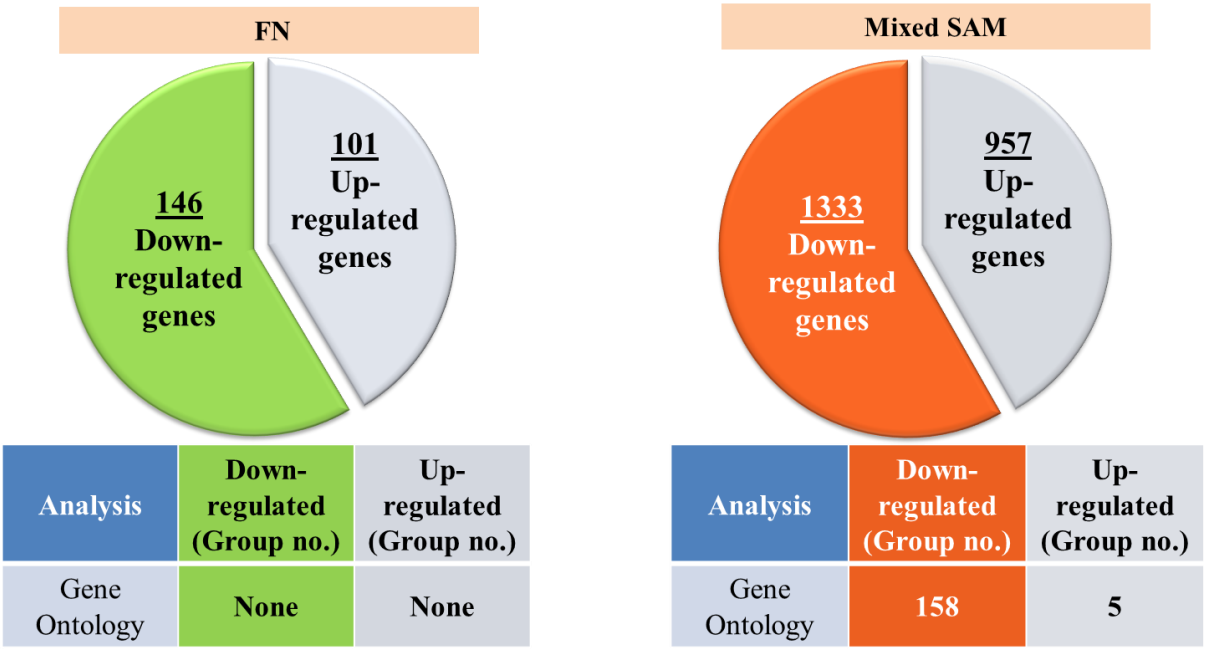


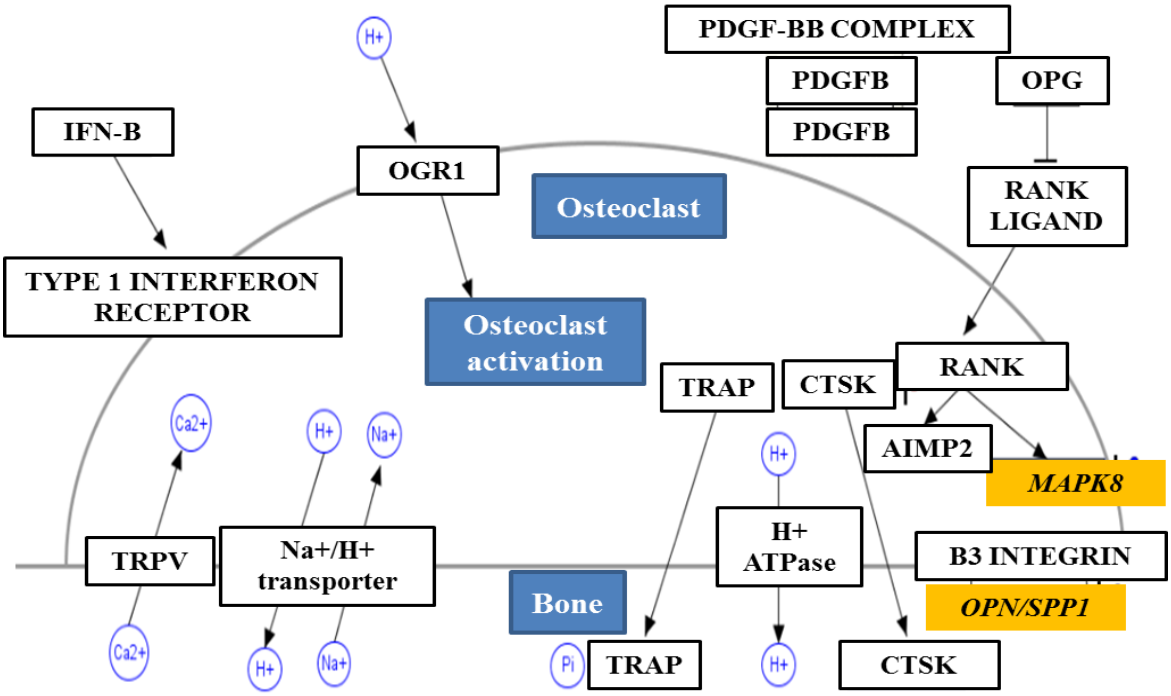
Fig. 7. DNA microarray data of MSCs on FN and the mixed SAM at 24 h. Scatter plots show distribution of down-regulated genes and up-regulated genes on FN and the mixed SAM versus TCP as a control.



Analysis	Down-regulated (Group no.)	Up-regulated (Group no.)
Gene Ontology	None	None

Analysis	Down-regulated (Group no.)	Up-regulated (Group no.)
Gene Ontology	158	5

Fig. 8. DNA microarray data of MSCs on FN and the mixed SAM at 24 h. Genes that showed >1.5-fold changes with $P < 0.05$ (n=3) were selected using GeneSpring software. Gene Ontology groups that showed significant differences with $P < 0.05$ were selected by Fisher's exact test analysis.



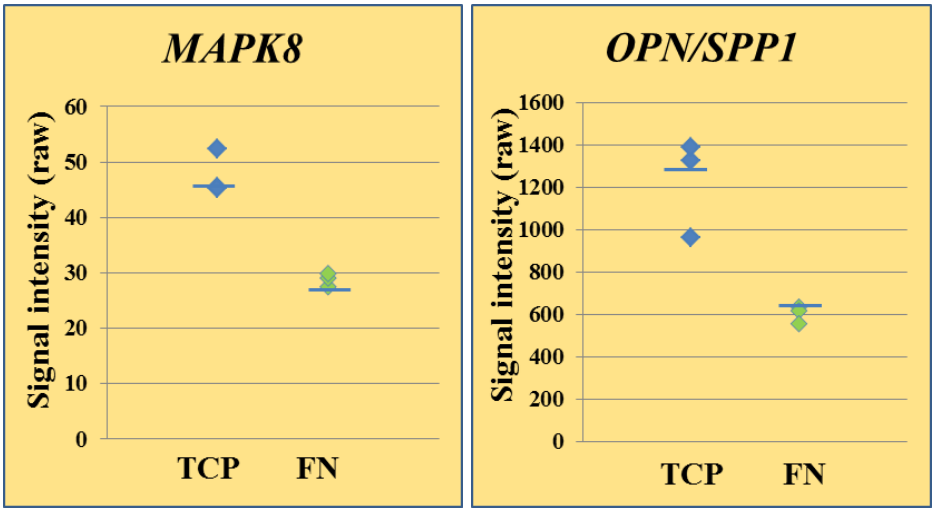


Fig. 9. DNA microarray analysis of mitogen-activated protein kinase 8 (*MAPK8*) and osteopontin (*OPN/SPP1*) on TCP and FN at 24 h. Values show signal intensity for three MSCs cultures.

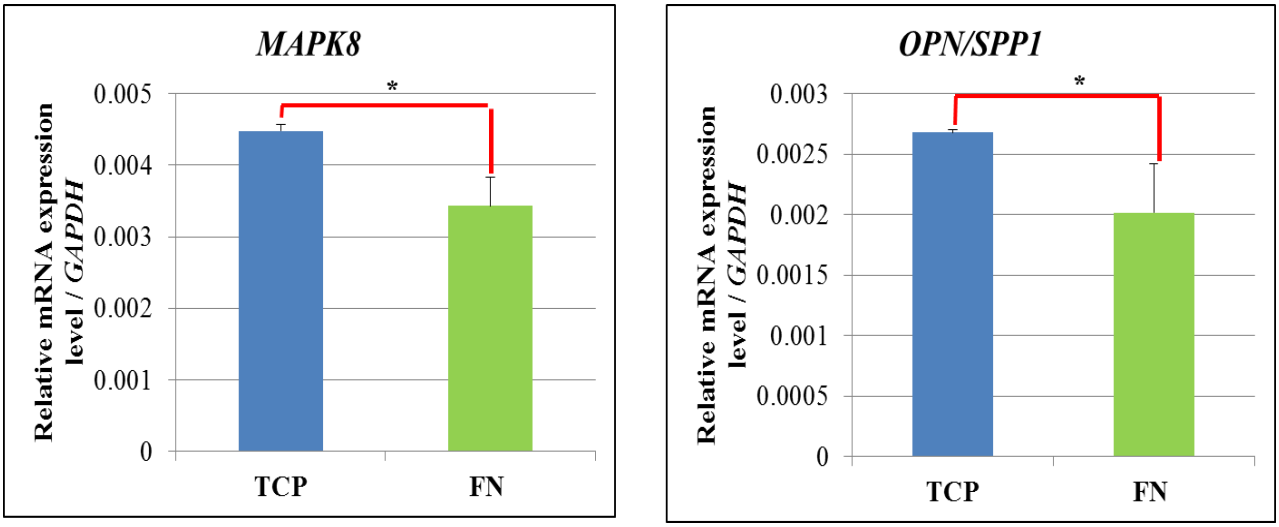


Fig. 10. Validation of DNA microarray analysis by real-time PCR on TCP and FN at 24 h. Values are average±SD for three cultures. *$P < 0.05$.

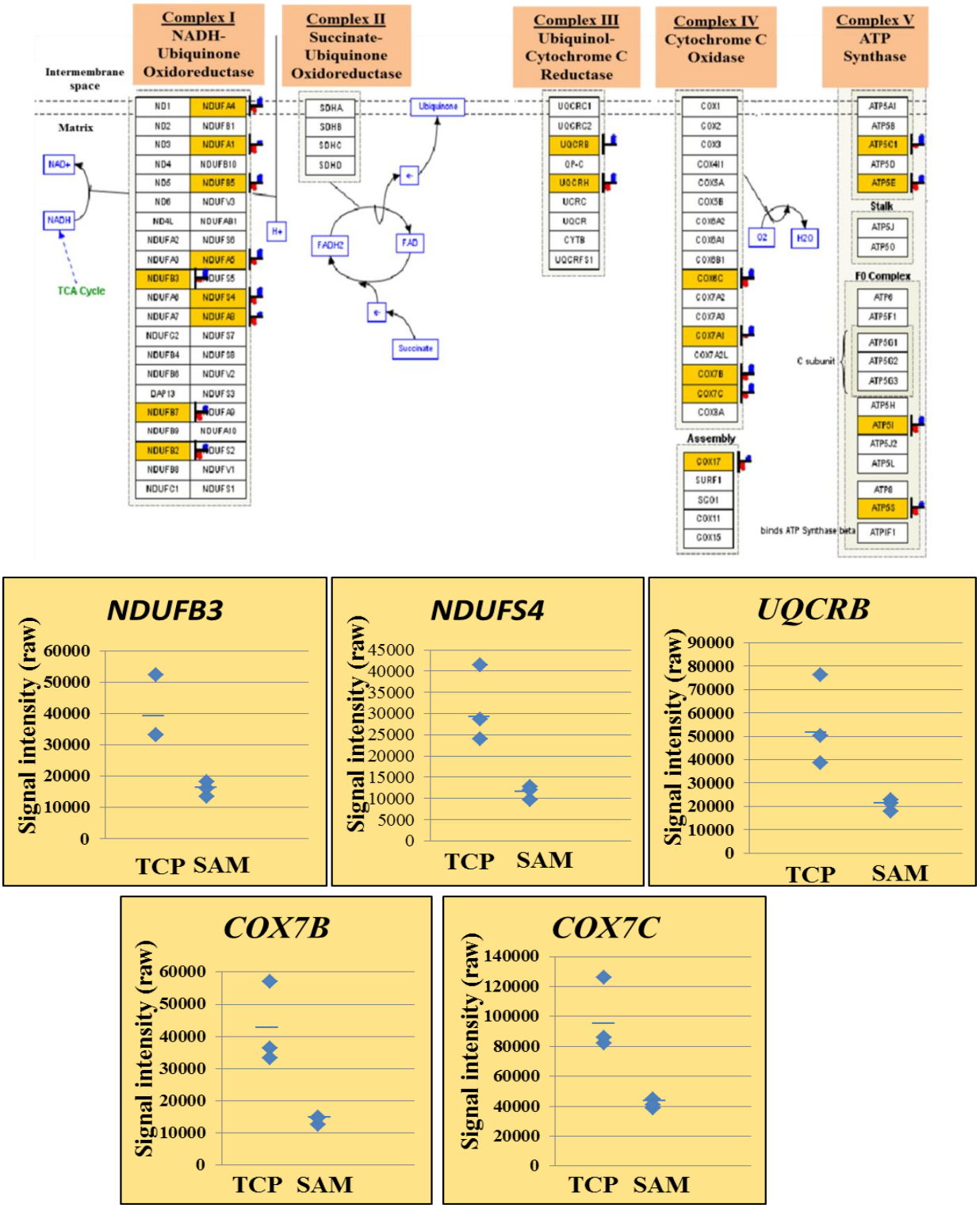


Fig. 11. DNA microarray analysis of the mitochondria-related genes expression on TCP and mixed SAM at 24 h. Values show signal intensity for three MSCs cultures.

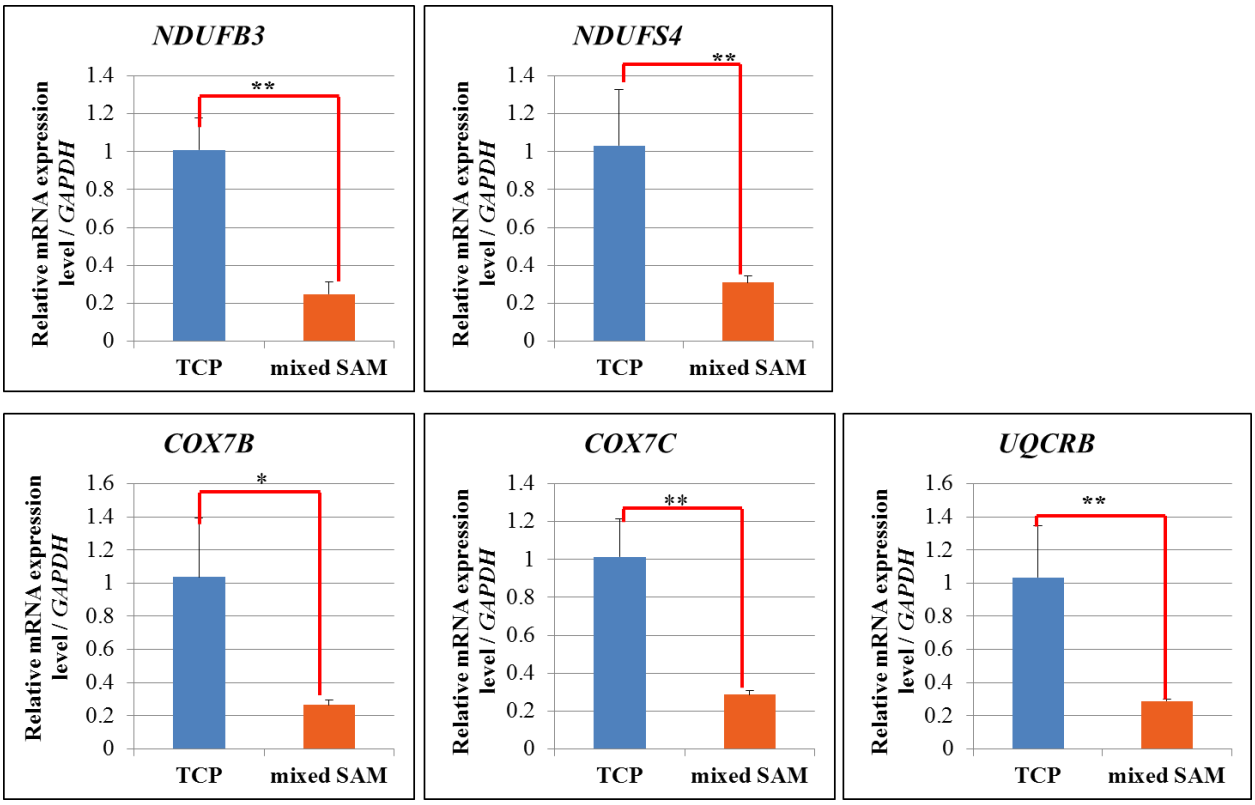


Fig. 12. Validation of DNA microarray analysis by real-time PCR on TCP and mixed SAM at 24 h. Values are average±SD for three cultures. *$P < 0.05$, **$P < 0.01$.

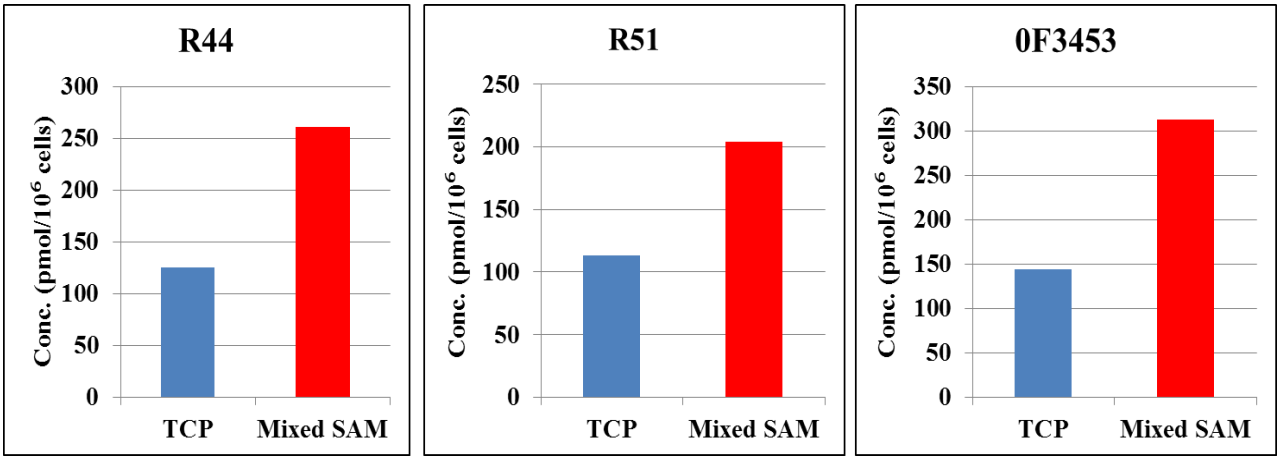


Fig. 13. The 2-oxoglutarate/α-ketoglutarate content in MSCs on TCP and mixed SAM at 24 h. The concentration of the 2-oxoglutarate was determined by LS-MS in MSCs from three donors (R44, R51 and 0F3453).

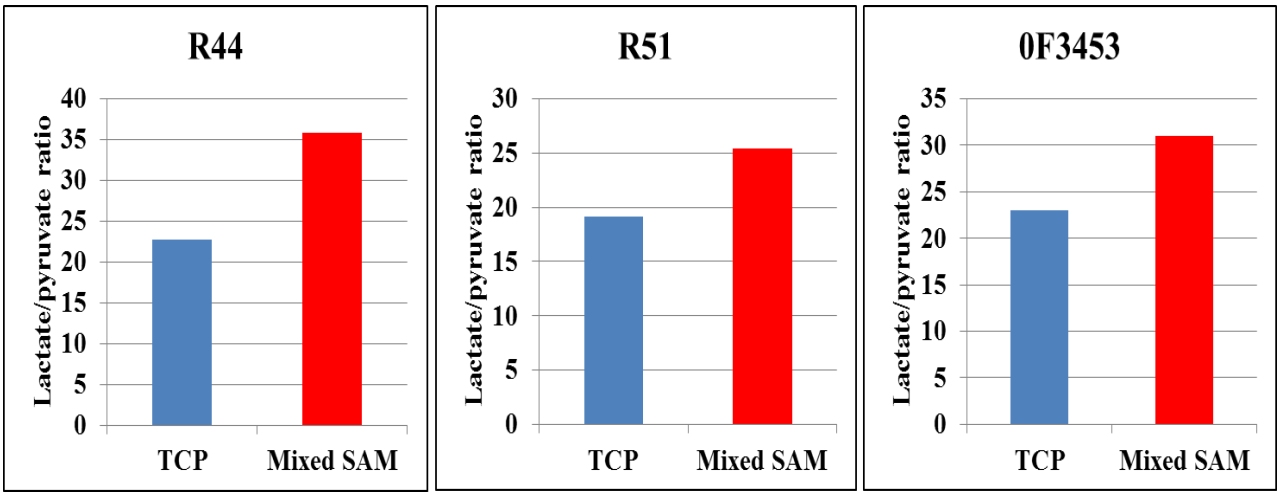


Fig. 14. The lactate/pyruvate ratio in MSCs on TCP and mixed SAM at 24 h. The concentration (pmol/10^6 cells) of lactate and pyruvate were determined by LS-MS in MSCs from three donors (R44, R51 and 0F3453).

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Table 1
List of primers and probes used in this study.

Target genes	Primer sequences (5' to 3')	Probe no. (Cat no.)
OCN (Osteocalcin)	F: TGAGAGCCCTCACACTCCTC R:ACCTTTGCTGGACTCTGCAC	#81 (Roche Applied Science)
Runx2 (runt-related transcription factor 2	F: CAGTGACACCATGTCAGCAA R: GCTCACGTCGCTCATTTTG	#66 (04688651001) (Roche Applied Science)
ALPL (alkaline phosphatase, liver/bone/kidney)	F: TCACTCTCCGAGATGGTGGT R: GTGCCCGTGGTCAATTCT	#12 (04685113001) (Roche Applied Science)
OPN/SPP1 (Osteopontin/secreted phosphoprotein 1	F: TTTCGCAGACCTGACATCC R:GGCTGTCCCAATCAGAAGG	#61 (Roche Applied Science)
NDUFB3 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa)	F: GGGACACCATTAGAAACTATCCA R: CCACCCATGTATCTCCAAGC	#71 (04688945001) (Roche Applied Science)
NDUFS4 (NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	F: GGCTGATCCCTTATCCAACA R: GGACTTGGGTTTTGGAACCT	#58 (04688554001) (Roche Applied Science)
COX7B (Cytochrome c oxidase subunit VIIb)	F: AGCGCACTAAATCGTCTCCA R: GAAAATCAGGTGTACGTTTCTGG	#13 (04685121001) (Roche Applied Science)
COX7C (cytochrome c oxidase subunit VIIc)	F: TGGTCCGTAGGAGCCACTAT R: CGACCACTTGTTTTCCACTG	#81 (04689046001) (Roche Applied Science)
UQCRB (ubiquinol-cytochrome c reductase binding protein)	F: GGTCAAAATGGCTGGTAAGC R: GCAGCATTGTAATACCATTTTCG	#42 (04688015001) (Roche Applied Science)
MAPK8 (mitogen-activated protein kinase 8)	F: GGGCAGCCCTCTCCTTTA R: CATTGACAGACGACGATGATG	#89 (04689143001) (Roche Applied Science)

Table 2
List of genes down-regulated and up-regulated on FN (top 20).

Down-regulated on FN				Up-regulated on FN			
Rank	**Gene symbol**	**Fold change**	**Regulated genes (Control)**	**Rank**	**Gene symbol**	**Fold change**	**Regulated genes (Control)**
1	MAPK8	5.20	5.46(34.42)	1	PLA2G2A	4.0	11.52(2.88)
2	MSL3	4.48	5.71(27.06)	2	DGKB	3.5	15.27(4.80)
3	C17orf69	4.00	4.82(21.31)	3	CD48	3.5	24.91(7.59)
4	FAM87B	3.93	379.77(1549.60)	4	HTR4	3.1	19.87(6.43)
5	PHF20L1	3.92	4.73(20.19)	5	C22orf24	3.0	21.32(7.58)
6	CHI3L2	3.82	6.34(31.68)	6	C1orf86	2.9	20.4
7	SNORD42A	3.55	11.94(43.18)	7	NRXN1	2.9	15.46(5.18)
8	SNCA	3.49	6.22(24.17)	8	FAM22F	2.7	21.82(8.38)
9	ARG2	3.40	6.32(22.78)	9	TXNL1	2.7	35.77(13.31)
10	SEPSECS	3.39	6.79(23.23)	10	PDZK1IP1	2.5	15.66(7.14)
11	CDKN2B-AS	3.33	4.77(16.72)	11	ITPRIPL1	2.4	20.94(9.40)
12	S100A4	3.01	5(15.94)	12	ANO9	2.2	20.34(9.81)
13	ZNF624	3.00	75.39(234.25)	13	CHD6	2.2	39.93(19.44)
14	PHEX	2.91	9.45(28.65)	14	ZNF236	2.1	17.75(9.08)
15	MGP	2.80	5.4(16.22)	15	LENG8	2.1	26.58(13.03)
16	RSPH1	2.75	3.88(11.71)	16	C2CD3	2.1	26.63(14.26)
17	CRISPLD1	2.72	4.98(14.02)	17	LGALS7	2.1	367.92(188.02)
18	KHDRBS3	2.71	20.09(56.37)	18	CNKSR2	2.0	13.08(6.64)
19	BEX5	2.62	13.03(36.71)	19	CXCL12	2.0	60.98(31.25)
20	RRAGB	2.61	9.01(24.05)	20	GGTLC1	1.9	38.84(21.76)

Table 3
List of genes down-regulated and up-regulated on the mixed SAM (top 20).

Down-regulated on the mixed SAM				Up-regulated on the mixed SAM			
Rank	**Gene symbol**	**Fold change**	**Regulated genes (control)**	**Rank**	**Gene symbol**	**Fold change**	**Regulated genes (control)**
1	CYP4Z1	4.86	7.82(40.21)	1	CHD2	5.1	17.78(3.55)
2	OMD	3.73	389.12(1548)	2	FLJ42393	5.1	26.51(5.87)
3	EVI2A	3.99	505.09(2147.71)	3	FKBP5	4.8	61.28(12.86)
4	CENPQ	3.28	419.37(1466.15)	4	KLK10	4.6	15.49(3.47)
5	PMCH	3.34	5.19(18.38)	5	GPR113	4.3	17.47(4.25)
6	IL20RA	3.16	5.03(16.63)	6	RAB44	4.2	12.72(3.07)
7	JAKMIP3	3.52	347.14(1310.96)	7	DGKB	3.4	15.87(4.80)
8	DEFB109P1B	4.93	4.12(21.31)	8	HTR4	3.3	19.52(6.43)
9	ZNF829	3.88	5.37(19.20)	9	EGR1	3.1	6504.86(2131.86)
10	PIGK	3.53	4.66(17.44)	10	CEACAM1	2.9	62.67(22.03)
11	HSPB8	3.41	3.98(13.36)	11	MLL	2.8	28.97(10.72)
12	SPHKAP	4.62	3.20(15.66)	12	HERC2	2.7	36.89(14.01)
13	ADAMTS18	4.69	4.10(21.71)	13	DNM1P46	2.6	83.16(32.02)
14	KLRC1	3.16	54.82(187.29)	14	BREA2	2.6	40.62(16.01)
15	IL8	3.11	4.82(14.36)	15	DNM1P46	2.6	149.89(58.41)
16	LY96	3.79	1853.93(7412.22)	16	AMHR2	2.6	13.97(5.89)
17	KIAA0825	4.26	6.67(29.16)	17	HSPG2	2.6	130.00(51.05)
18	MKL2	3.40	431.94(1569.44)	18	RNF213	2.6	62.03(25.56)
19	SNAR-D	3.21	10327.80(34287.35)	19	CD244	2.5	30.89(13.04)
20	TXNDC9	3.32	1233.00(4359.03)	20	SH3BP2	2.5	18.26(7.75)

Table 4
List of gene ontology groups down-regulated on the mixed SAM (top 20).

GO terms of down-regulated genes on the mixed SAM			
Rank	**GO terms**	**p-value**	**Regulated genes (all)**
1	Mitochondrion	1.88E-19	246(1395)
2	Intracellular	1.79E-15	1257(11768)
3	Intracellular part	3.99E-15	1231(11488)
4	Intracellular membrane-bounded organelle	5.63E-15	1005(8954)
5	Membrane-bounded organelle	5.63E-15	1006(8963)
6	Cytoplasmic part	7.4E-15	728(6045)
7	Ribonucleoprotein complex	1.22E-14	116(532)
8	Cytoplasm	3.02E-14	956(8472)
9	Ribosome	2.54E-13	60(197)
10	Intracellular organelle	2.54E-13	1081(9899)
11	Organelle	2.82E-13	1082(9916)
12	Structural constituent of ribosome	1.39E-12	51(155)
13	Translation	2.42E-11	76(315)
14	mRNA metabolic process	8.79E-10	110(577)
15	Ribosomal subunit	1.56E-08	40(129)
16	Mitochondrial membrane part	1.42E-07	39(132)
17	Gene expression	2.01E-07	219(1543)
18	Mitochondrial part	2.01E-07	118(692)
19	Macromolecular complex	3.10E-07	439(3605)
20	Intracellular organelle part	3.25E-07	676(5980)

Table 5
List of gene pathways down-regulated on FN and the mixed SAM ($P < 0.001$).

FN			
Rank	Pathway	p-value	Regulated genes (all)
1	Osteoclast signaling	6.61E-05	3(16)

Mixed SAM			
Rank	Pathway	p-value	Regulated genes (all)
1	Electron transport chain	0	20(104)
2	Oxidative phosphorylation	5.25E-06	10(62)
3	Proteasome degradation	6.28E-05	9(65)
4	Cytoplasmic ribosomal proteins	7.24E-04	9(88)