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Transcriptomic change of human gingival cells during cultivation on gelatin composite hydroxyapatite and pig brain extract

Fahsai Kantawong[1*] Yasumin Chaiyasert[1] Nichanun Bungkhuan[1] Kanyamas Choocheep[1] Warunee Kumsaiyai[1] Penpitcha Wanachantararak[2] Thasaneeya Kuboki[3]

[1]Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand
[2]The Dental Research Center, Faculty of Dentistry, Chiang Mai University, Chiang Mai Province, Thailand
[3]Laboratory of Biomedical and Biophysical Chemistry, Institute for Materials Chemistry and Engineering, Kyushu University, Fukuoka, Japan

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ABSTRACT

Background: Biomaterials that contain mechanical and biochemical properties similar to neural tissue will provide the environment that support neuronal survival and development.

Objectives: This study assessed the effects of three gelatin-based biomaterials on gene expression of primary human gingival fibroblasts.

Materials and methods: Human gingival cells were cultured on 3 types of biomaterials; 10% gelatin, 10% gelatin with hydroxyapatite and 10% gelatin with hydroxyapatite and pig's brain extract. These biomaterials were used in cell culture to investigate that they could support long-term culture of adult somatic cells like human gingival cell or not.

Results: Human gingival cells were cultured on biomaterials for 21 days then, RNA sequencing showed up-regulation of 259 genes and down regulation of 210 gene in human gingiva cells cultured on Gel+HA+Brain compared to cells on tissue culture plates. RNA sequencing showed up-regulation of antioxidant genes, solute-carrier gene (*SLC*) superfamily, histone and cell cycle gene. Down-regulation of ECM and cytoskeletal protein were observed. The further study by reverse transcription real time – PCR was performed to confirm the result of *Klf4*, *Tuj1*, *OCN, ACAN* and *VCAN* gene expression in human gingival cells. Moreover, some neuronal related genes in human gingiva cells cultured on Gel+HA+Brain compared to cells on tissue culture plates were detected.

Conclusion: The biomaterials (Gel+HA+Brain) affected gene expression in many aspects at 21 days culture. It was possible that 10% gelatin with nano-hydroxyapatite and pig's brain extract could be used to support cell differentiation of the human gingival cells. In conclusion simple fabrication of the biomaterial from 10% gelatin with nano-hydroxyapatite and pig's brain extract could be used for modulation of gene expression in adult somatic cells.

* **Corresponding author.**
Author's Address: *Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.*
** **E-mail address:** fahsai.k@cmu.ac.th
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Introduction

Clinically, gingival tissue is easy to collect by biopsy thus it is feasible to isolate cells from gingival tissue based on their highly proliferative nature.[1] The gingival tissues were containing mesenchymal stem cells (GMSCs). Around 90% of GMSCs are derived from cranial neural crest cells (CNCC) and 10% from the mesoderm.[1] GMSCs show remarkable tissue reparative and regenerative potential.[2] Human GMSCs showed the ability of differentiation into neuronal lineages make it a promising tissue construct for translational application. Recently, there has been an emergence of research groups offering new concepts focused on using human gingival cells to treat neurodegenerative disorders.[3] Moreover, human gingiva-derived mesenchymal stem cells (GMSC) have shown anti-inflammatory and immunomodulatory effects on modulating inflammatory monocytes/macrophages and alleviating atherosclerosis.[4] However, in the present study, there was no attempt to isolate stem cells because this study aimed to test the supportive role of the fabricated biomaterial before moving on to the bioinductive role in the future. Human gingival cells from healthy gingival tissue without inflammation were used in this study.

Biomaterials that contain mechanical and biochemical properties similar to neural tissue will provide the environment that support neuronal survival and development.[5] Previous studies by Kantawong *et al*. indicated that biomaterials produced from 10% gelatin, nano-hydroxyapatite and pig brain extract (Gel+Ha+Brain) enhanced increase expression of reprogramming factor *Klf4* and transcription factor (*Nfia*, *Nfib* and *Ptbp*) in NIH/3T3 at 9 days culture, which indicated the possibility of astrocytic phenotype.[6]

This study assessed the effects of three gelatin-based biomaterials on gene expression of primary human gingival fibroblasts. The biological evidence supporting the use of hydroxyapatite for neuronal differentiation was previously presented [6, 7] and the study of Lambrichts *et al.* also indicated that hydroxyapatite scaffolds containing peptide hydrogels enhanced blood vessel ingrowth.[8]

In the present study, human gingiva cells cultured on 10% gelatin with nano-hydroxyapatite and pig's brain extract were subjected for the further investigation by RNA sequencing (Next-Gen sequencing, Illumina) to observe change in global gene expression for predicting and validating roles of biomaterials in regulatory mechanisms underlying human gingival cell development.

Materials and methods

Brain extract

Brain extract preparation was modified from the previous study. [6] Pig brain was purchased from local market and preserved in frozen condition before use. Frozen pig brain was thawed at room temperature then 100 grams of pig brain was washed in sterile water before blending in a blender with 100 mL of sterile phosphate buffer saline (PBS) containing 1% Pen-Strep using liquidify mode for 1 min, then the liquidified brain was poured into 50 mL centrifuge tube and spun at 2,500 rpm for 10 min in the centrifuge (KUBOTA Model 5200). The supernatant was discarded and the white brain precipitant was kept at -20 °C until used.

Biomaterial preparation

Biomaterial preparation was modified from the previous study.[6] Gelatin (10% w/v) in distilled water was incubated in water bath 50 °C for 45 min until gelatin was dissolved. Then gelatin solution was stirred at 50 °C for 30 min. For Gel+HA, the gelatin solution was stirred for 15 min at room temperature before adding 100 mg of nano-hydroxyapatite and continued stirring for 30 min. For Gel+HA+Brain, add 1 mL of brain extract together with 100 mg HA then 25% glutaraldehyde 5-6 µL/mL was added and mixed with each type of gelatin solution before pouring into 6-well plate. The biomaterial was prepared well-by-well using 2 mL of solution per well. The solution was left overnight then polymerized gelatin was washed with sterile distilled water three times for 30 min each. After that polymerized gelatin was soaked in sterile distilled water overnight. Water was discarded and polymerized gelatin was then gently tapped on tissue paper to absorb the rest of water on surface of gelatin. Polymerized materials (Figure 1S) were frozen at -20 °C before freeze drying was performed in the lyophilizer (LYOLAB; LYOPHILIZATION SYSTEMS, INC, USA) at 9 mTorr with condenser temperature 84.1 °C for 24 hr.

Preconditioning of biomaterials

Biomaterials were immerged in 70% ethanol for 30 min. After 70% ethanol was discarded, biomaterials were immerged in sterile water for 30 min 3 times. Complete DMEM (3 mL) was added to each biomaterial and incubated in 5% CO_2 incubator at 37 °C for at least 24 hr. Then the biomaterials were ready for cell culture.

Human gingival cells culture

Ethical approval by the Ethics Committee of the Faculty of Dentistry, Chiang Mai University (21.1/2018), was obtained for the study of human gingival cells. Human gingiva tissue was rinsed in sterile phosphate-buffered saline and transferred to a Petri dish containing DMEM. The tissue was minced with a scalpel. The obtained suspension of tissue fragments and gingival cells were condensed by centrifugation (200 × g for 5 min). The resuspended pellet was placed in a tissue culture flask with culture medium (DMEM) containing 10% FCS penicillin/streptomycin. A monolayer culture was incubated at 37°C in an incubator containing 5% CO_2. The medium was renewed every 2 days. The human gingival cells were confluent after 5-7 days of culture. The human gingival cells were seeded onto each type of preconditioned biomaterials at the density $1x10^5$ cells per gel. The cells were cultured in an incubator with 5% CO_2 at 37 °C, medium was changed every 5-7 days until 3 weeks.

Sample preparation and RNA sequencing

Human gingival cells ($1x10^5$ cells per gel) were grown in tissue culture plate and Gel+HA+Brain for 21 days. Total RNAs were purified by NucleoSpin RNA isolation kit. The total RNA was collected from 42 gelatin biomaterials (7 plates of 6-well plate) and pooled together. The purity of the RNA preparations was qualified by nanodrops and gel electrophoresis as shown in supplementary data (Figure 2S). Transcriptome sequencing was performed using a service from Next Gen with the Illumina platform. Sequencing data processing. Reads are mapped to human reference genome.

Cell staining

Cell morphology was obtained by staining the cells with Alcian blue and Coomassie blue R. Briefly, the cells were fixed with 4% formaldehyde for 5-10 min and stained with 0.5% Alcian blue for 5 min. The stain was removed and the cells were rinsed with tap water before visualizing under an inverted microscope (Nikon ECLIPSE TS 100).

Real-time PCR

RNA extraction was performed using Nucleospin (MACHEREY-NAGEL) and cDNA synthesis was performed using ReverTra Ace cDNA synthesis kit (Toyobo) following the protocols of manufacturers. cDNA sample was diluted to the desired dilution, then 4.0 µL of the cDNA was mixed with SYBR Green Mastermix (Bioline) and the primer at a final concentration of 0.25 µM in the total volume of 10 µL. Real time-PCR was performed in LightCycler® 480. The PCR program contain 3 steps: (1) Pre-incubation step at 95 °C for 2 min (2) PCR step (denature at 95 °C, annealing at 60 °C and extension at 72 °C) for 40 cycles. (3) Melting curve analysis was performed at 95, 65 and 97 °C. The experiments were repeated at least 3 times. Target genes were normalized with reference genes *GAPDH* and *18s rRNA* using LightCyCler 4.0 software. Sequences of primers used in real-time PCR were shown in Table 1S.

Statistical analysis

For real-time PCR, three independent assays were performed. The fold change of gene expression in Gel+HA and Gel+HA+Brain was compared with Gel using independent t-test by SPSS 17.0 software. The statistical significance was determined at $p<0.05$.

Results

RNA sequencing

Differential gene expression found 259 genes up-regulation and 210 genes down-regulation in human gingival cells cultured on Gel+HA+Brain compared to those cultured on tissue culture plate (Figure 1).

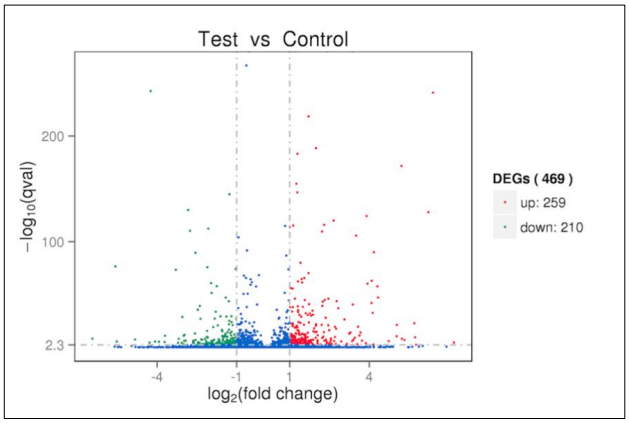

Figure 1. *Volcano plots showed differential gene expression between human gingival cells cultured on tissue culture plate (Control) and human gingival cells cultured on 10% gelatin with nano-hydroxyapatite and pig brain extract for 3 weeks (Test) with an adjusted P value of < 0.01. Genes with log 2-fold change ≥1 were highlighted in red while genes with log 2 fold-change ≤1 were highlighted in green.*

Table 1 Expression cytoskeletal proteins, ECM proteins and receptor-related proteins.

Genes	log2.Fold change*	Note
Collagen, type XII	-1.9662	A component of human skeletal muscle
Collagen, type XI	-2.1506	Broadly distributed in articular cartilage, testis, trachea, tendons, trabecular bone, skeletal muscle, placenta, lung, and the neoepithelium of the brain
Collagen, type XIV	-2.4678	Skin, tendon, cornea, and articular cartilage
Collagen, type IV	-2.7612	Found in the skin within the basement membrane zone
Collagen, type XV	-2.9963	Located in the basement membrane zones of microvessels and cardiac or skeletal myocytes
Collagen, type V	-3.0496	Bone matrix; corneal stroma; and the interstitial matrix of muscles, liver, lungs, and placenta
Laminin, alpha 1	-1.7556	Extracellular matrix glycoprotein
Fibulin 1	-2.8291	Elastic fiber–associated protein
Hyaluronan and proteoglycan link protein 1	-1.8867	A key molecule in the formation and control of hyaluronan-based condensed perineuronal matrix in the adult brain
Versican	-1.5414	Extracellular matrix (ECM) component
Biglycan	-1.8302	Extracellular space proteins
Podocan	-1.8867	Extracellular space proteins
Aggrecan	-2.1317	Major proteoglycan component of the extracellular matrix in the growth plate and articular cartilage
Fibronectin	-1.8995	Extracellular matrix glycoprotein
Tenascin C	-1.9473	Extracellular matrix glycoprotein

Table 1 Expression cytoskeletal proteins, ECM proteins and receptor-related proteins. (continues)

Genes	log2.Fold change*	Note
Nidogen 2	-1.0058	Basement membrane glycoprotein
Elastin	-3.2948	Extracellular matrix protein
Integrin, alpha 6	-1.0029	Transmembrane glycoproteins
Integrin, alpha 11	-1.5099	Transmembrane glycoproteins
Integrin, alpha 8	-1.7528	Transmembrane glycoproteins
Integrin, alpha 4	-1.9703	Transmembrane glycoproteins
Integrin, alpha 10	-2.9821	Transmembrane glycoproteins
Integrin, alpha 7	-3.6994	Transmembrane glycoproteins
Calponin 1	-1.0096	Actin-binding protein
Spectrin	-1.0127	Actin-bundling and membrane-anchoring proteins
Cadherin 13	-2.3413	Cell adhesion molecules
Tensin 1	-2.1974	Cytoplasmic phosphoprotein that localized to integrin-mediated focal adhesions
Matrix-remodeling associated 5	-1.3526	Secreted extracellular protein
Matrix metallopeptidase 10	-6.4405	Played roles in macrophage inflammation
Matrix metallopeptidase 14	1.2899	Transmembrane-type MMP, digests the triple helical portions of interstitial collagen types I, II, and III and other ECM components, including fibronectin, laminin, aggrecan, and gelatin
Matrix metallopeptidase 15	2.0475	Transmembrane-type MMP, digests fibronectin, tenascin, nidogen, aggrecan, perlecan, and laminin
Matrix metallopeptidase 3	2.4599	Progelatinase; involved in joint destruction in rheumatoid arthritis
Matrix metallopeptidase 7	5.768	Expressed in endothelial cells, cardiomyocytes, and macrophages
Nestin	-1.0175	Intermediate filament
Actin filament associated protein 1	-1.1859	Cytoskeletal associated protein
Actin, alpha 2	-1.7938	Cytoskeletal protein
Actin, gamma 2, smooth muscle	-3.934	Cytoskeletal protein
Myosin ID	-1.4094	Actin motor protein
Myosin X	-1.4293	Actin motor protein
Myosin, heavy chain 11	-5.524	Actin motor protein
Microtubule-associated protein 1B	-1.2792	Cytoskeletal associated protein
TPX2, microtubule-associated	-1.8495	Cytoskeletal protein
Anillin	-2.0966	Cctin binding protein
Kinesin family member 11	-2.0579	Motor proteins
Calponin 1	-1.0096	Calponin is an actin filament-associated regulatory protein
Lamin B2	-1.2096	Type V intermediate filaments
Syntrophin, beta 1	-1.1385	Role in cytoskeletal organization
Keratin 19	-2.6364	Low-molecular-weight cytoskeletal protein

Note: **adjusted p<0.001*

Table 2 Increased expression of antioxidant genes.

Antioxidant protein	log2.Fold change*	Gene expression
Thioredoxin reductase 1	1.1221	increased
Glutathione peroxidase 3	1.3424	increased
Thioredoxin	1.4955	increased
Microsomal glutathione S-transferase 1	1.9204	increased
Oxidative stress induced growth inhibitor family member 2	2.0575	increased
Heme oxygenase (decycling) 1	1.1758	increased

Note: **adjusted p<0.001*

Table 3 Increased expression of histone and cell cycle-related genes.

Histone	log2.Fold change*	Gene expression
H2A histone family, member Y2	1.2871	increased
Histone cluster 1, H2ac	1.8861	increased
Histone cluster 1, H2bk	1.9536	increased
Histone cluster 2, H2be	2.3063	increased
Histone cluster 1, H1c	2.4874	increased
Cyclin-dependent kinase inhibitor 1A (CDKN1A)	1.4043	increased
Cyclin L1	1.59	increased
Cyclin-dependent kinase inhibitor 2B (CDKN2B)	1.6196	increased
Cyclin D2	2.9397	increased
Regulator of cell cycle	1.2656	increased

Note: **adjusted p<0.001*

Table 4 Expression of genes related to neuronal function.

Gene	log2.Fold change*	Note
1. Neural proliferation and differentiation control protein-1 (NPDC-1)	1.0051	Control neural proliferation and differentiation [9-11]
2. Solute carrier family 6 (neurotransmitter transporter) (SLC6A8)	1.0865	GABA transporter [12, 13]
3. Leucine rich repeat neuronal 3 (LRRN3)	1.6603	Brain development [14] and the synaptic connection [15]
4. Neuromedin B (NMB)	1.7012	Neuropeptide found in the pituitary, gastrointestinal tract and the central nervous system [16]
5. CD44 molecule (CD44)	1.0214	Surface markers of neural lineage [17]
6. GABA(A) receptor-associated protein like 1 (GABARAPL1)	1.082	Maintaining normal autophagic flux [18, 19]
7. Amyloid beta (A4) precursor-like protein 1 (APLP1)	1.0749	Upregulated during postnatal development coinciding with synaptogenesis [20]
8. Semaphorin3C (SEMA3C)	1.2086	Growth of axon in dopamine neuron [21]
9. B-cell translocation gene 2 (Btg2)	2.5507	Played important roles in cholinergic neuronal differentiation of BMSCs [22]
10. Transmembrane protein 132A (TMEM132A)	2.3203	Considered as novel IG domain containing proteins of the CNS [23]
11. Transient receptor potential ankyrin 1 (TRAP1)	1.551	Played roles in matrix homeostasis and inflammation [24]
12. Activating transcription factor 3 (ATF3)	2.0147	CNS inflammatory responses [25]

Table 4 Expression of genes related to neuronal function. (continues)

Gene	log2.Fold change*	Note
13. Prostaglandin D2 synthase 21kDa (brain) (PTGDS)	4.093	Catalyzes the conversion of prostaglandin H2 (PGH2) to postaglandin D2 (PGD2). PGD2 functions as a neuromodulator as well as a trophic factor in the central nervous system [26]
14. Inhibitory synaptic factor 1 (INSYN1)	1.5057	Dampen neuronal activity through postsynaptic hyperpolarization [27]
15. Synuclein alpha (SNCA)	1.2324	Neuronal protein that plays several roles in synaptic activity such as regulation of synaptic vesicle trafficking and subsequent neurotransmitter release [28]
16. Neuronal pentraxin receptor (NPTXR)	1.0894	Synapse formation [29, 30]
17. Neuronal pentraxin 1 (NPTX1)	1.000	Synapse formation [29, 30]

Note: **adjusted p<0.001*

KEGG pathway & GO analysis

KEGG pathway analysis in human gingival cells cultured on Gel+HA+Brain compared to those on tissue culture plate were shown in Table 4S. The most significant KEGG pathway was lysosome (hsa04142) which might relate to ECM remodeling/degradation. Gene-ontology (GO) compared between human gingival cells cultured on Gel+HA+Brain and normal control was shown in Figure 2. No significant GO term was down-regulated and 11 GO terms was significantly up-regulated. The most significant GO terms were chemokine activity and chemokine receptor binding which related to up-regulation of increase expression of chemokines and cytokines was presented in Table 3S. Every biomaterial was carefully observed under the microscope and no microorganism infection was found. The researchers confidently believed that up regulation of all chemokines and cytokines (*CXCL1, CXCL5, CXCL14, CXCL10, CCL2, CXCL3, CXCL6, CX3CL1* and *CXCL2*) was the response of cells to biomaterials.

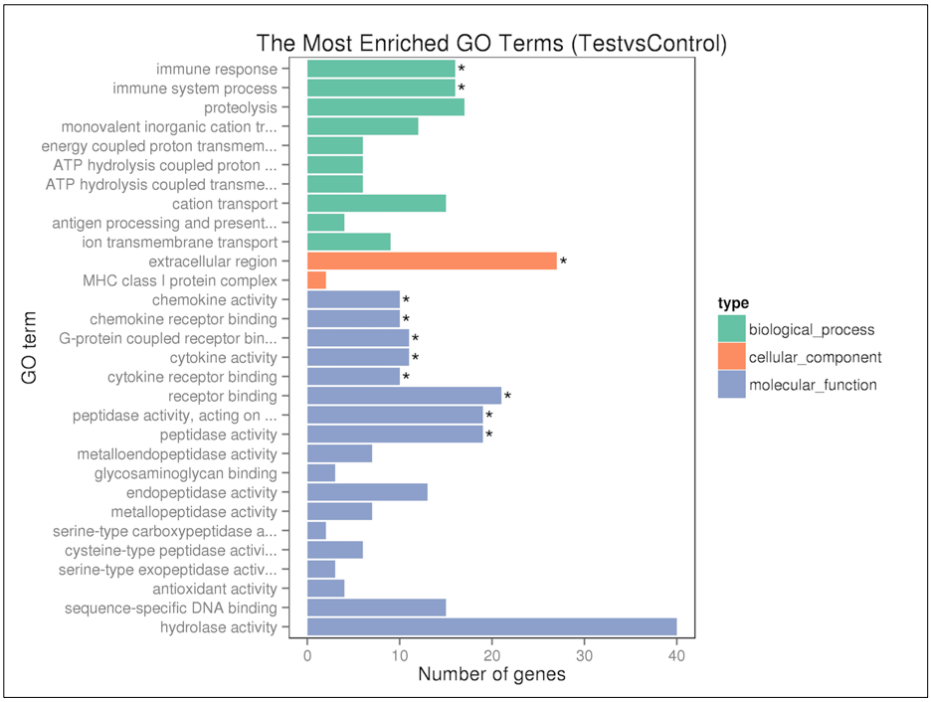

Figure 2. *GO enrichment of genes that were upregulated after cultivation on 10% gelatin with nano-hydroxyapatite and pig brain extract for 3 weeks. Vertical axis: GO terms, Horizontal axis: number of the differentially expressed genes annotated in the GO term. Total 11 GO terms were significantly up-regulated (p≤0.05). No GO term was significantly down-regulated.*

Cells staining

Human gingival cells were seeded on top of the biomaterials and penetrated into hydrogel. Human gingival cells appeared healthy and elongated on Gel+HA+Bain compared to cells cultured on tissue culture plate (Figure 3). Positive Alcian blue staining in human gingival cells was concordant with the differential gene expression that showed up-regulation of *lumican* (2.2299 folds) and *tsukushi* (1.6405 folds); small leucine rich proteoglycans. The images of human gingival cells indicated non-infection with any microorganism.

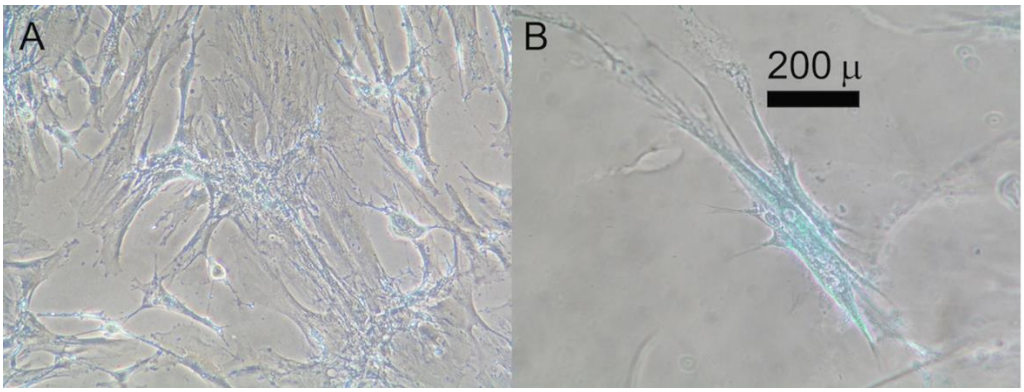

Figure 3. *Alcian blue staining. A: human gingival cells cultured on tissue culture plate for 3 weeks, B: human gingival cells cultured on 10% gelatin with nano-hydroxyapatite and pig brain extract for 3 weeks.*

Human gingival cells gene expression by real-time PCR

Figure 4 showed increased expression of *Klf4* gene and decreased expression of *βIII-tubulin* (*Tuj1*), *aggrecan (ACAN)*, *versican (VCAN)* and *osteocalcin (OCN)* using RT-PCR technique. The results of *Klf4*, *ACAN* and *VCAN* were correlated to the result from RNA sequencing that presented up-regulation of *Klf4* transcription factor and decreased ECM-cytoskeletal protein and proteoglycans.

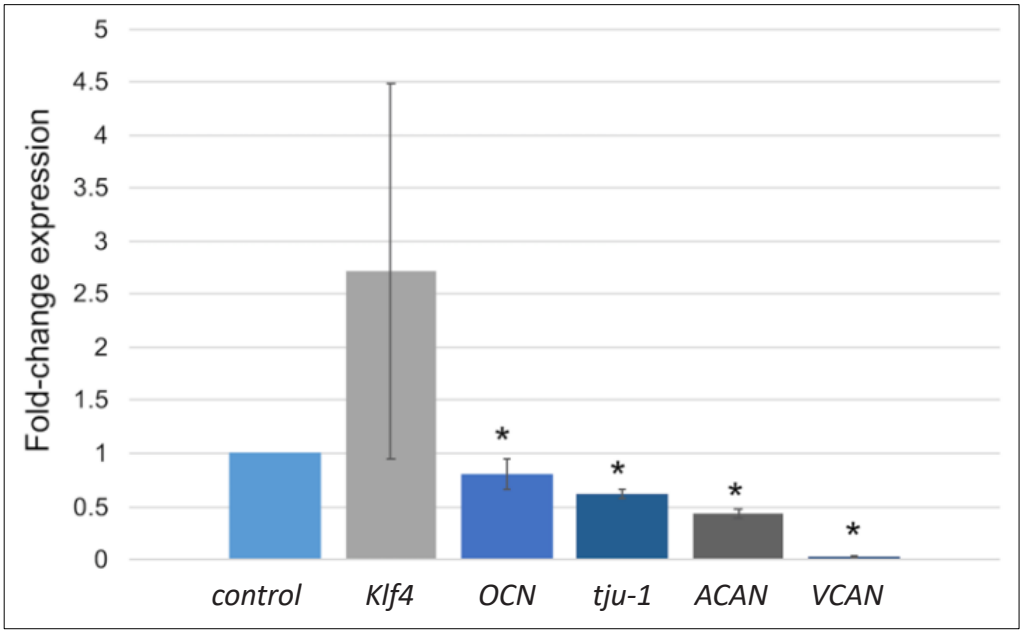

Figure 4. *Increased expression of Klf4 gene and decreased expression of βIII-tubulin (Tuj1), aggrecan (ACAN), versican (VCAN) and osteocalcin (OCN) when the expression of each gene in the control group was set as 1.*

Effect of nano-hydroxyapatite and pig brain extract

The comparison between each type of gelatin biomaterial was investigated to observe the effect of the addition of nano-hydroxyapatite and pig brain extract into gelatin. Since some neuronal related gene were detected in RNA sequencing so some neural-specific genes like *Glial fibrillary acidic protein (GFAP), Neurofilament light polypeptide (NFL), βIII-tubulin (Tuj1), Microtubule associated protein2 (MAP2)* and *CD44* were analyzed by real-time PCR. The expression of *MAP2* and *CD44* were changed. The level of *MAP2* gene expression in human gingival cells cultured in Gel+HA was 1.92 folds compared to plain 10% gelatin and not statistically significant (p=0.224587). The level of *MAP2* gene expression in human gingival cells cultured in Gel+HA+Brain was 10.7 folds compared to plain 10% gelatin and not statistically significant (p=0.136431) (Figure 5A). *CD44* gene expression was 1.57 folds and not significantly increased in human gingival cells cultured in Gel+HA compared to plain 10% gelatin (p=0.113239). The level of *CD44* gene expression in human gingival cells cultured in Gel+HA+Brain was 1.6 folds and not statistically significant compared to plain 10% gelatin (p=0.241992) (Figure 5B). The expression of *Tuj1* gene was decreased in human gingival cells cultured on Gel+HA+Brain without statistically significant (Figure 3S). The expression of *GFAP* and *NFL* genes were not detected.

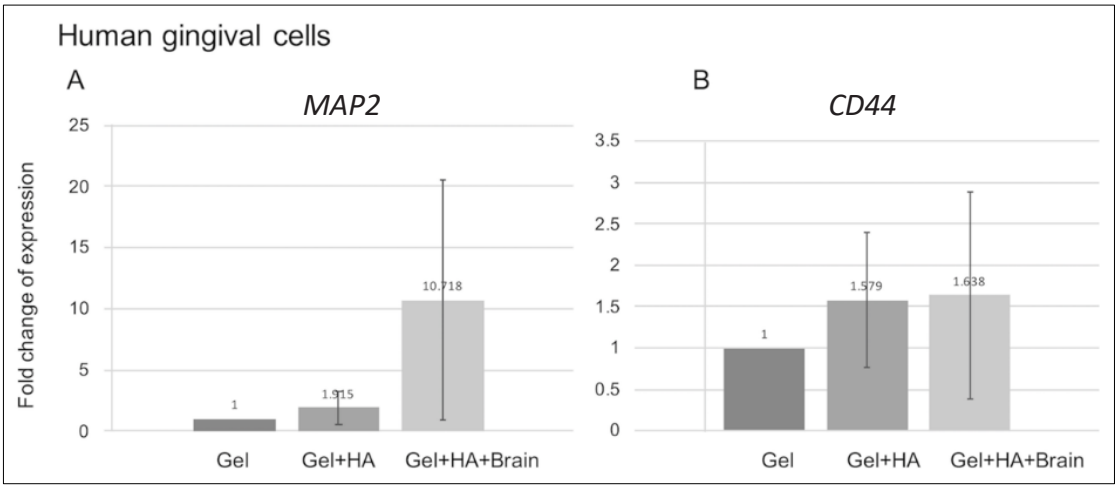

Figure 5. *Human gingival cells cultured on Gel+HA and Gel+HA+Brain showed up-regulation of MAP2 (A) and CD44 genes without statistically significance compared to 10% gelatin (B).*

Discussion

In the first step, the researchers aimed to screen for the impact of biomaterial compared with conventional tissue culture plate using RNA sequencing. In the next step, the researchers investigated the effects of the add up components such as nano-hydroxyapatite and pig brain extract.

Human gingival cells culture on Gel+HA+Brain presented dramatically change of transcriptome compared to human gingival cells cultured on tissue culture plate using RNA sequencing. The expression of antioxidant protein in Table 2 indicated that the cells were protecting themselves from oxidative stress and trying to survive. Increased expression of thioredoxin reductase 1 helped to maintain the sufficient amount of reduced thioredoxin for oxidative stress resistance.[31, 32] Microsomal glutathione S-transferase 1 protected the intracellular macromolecules from reactive electrophiles by accelerating the conjugation of glutathione with electrophilic compounds.[33] Glutathione peroxidase 3 in combination with reduced glutathione eliminated hydrogen peroxide in cells.[34]

Expression of histone genes and cell cycle-related genes were shown in Table 3. Histone gene expression dramatically occurs during the synthetic (S) phase of the eukaryotic cell cycle.[35] Increase chromatin during S phase occurs in DNA replication.[36] Somehow, increased histone expression might be related to increased lifespan and slowing the aging process of cells.[37] Gene that involved with self-renewal such as histone *H2A.2, KLF2, KLF4, NKX3-1* were also up-regulated in this study. Increased expression of the cell cycle proteins was also shown in Table 3. The regulator of cell cycle was associated with stimulation of cell cycle progression. The CDKN1A or p21 was related to cell growth, cell differentiation and apoptosis. In addition, p21 plays an important role in the cell cycle arrest at G1/S checkpoint.[38] When the expression of CDKN1A was suppressed, increases the cell division was occurred.[39] CDKN2B maintained the cells in a state of growth-arrested premalignant state.[40] The expression of Cyclin D2 related to self-renewal properties that controlled stem cells to differentiate without becoming cancer cells.[41] Down-regulation of *CDC20* and centrosome proteins were detected. *CDC20* is important as spindle assembly checkpoint protein and a key component of the mammalian cell cycle mechanism that activates the anaphase-promoting complex.[42] Some centrosomal proteins were down-regulated during DNA synthesis.[43]

Most of the top upregulated KEGG pathways was lysosome (Table 4S). Lysosome activity helped to maintain the youthfulness of cells.[44] Increased expression of lysosomal protein could also be found in the neuronal development because it reflected the increase degradation by lysosomal pathway.[45] Key pro-inflammatory cytokines and chemokines were upregulated in this study (Table 3S). A group of innate immune cells that synthesized and secreted various cytokines and chemokines were macrophages, neutrophils, mast cells, eosinophils, dendritic cells, and epithelial cells.[46] KEGG phagosome had increased significantly. Normally, the phagosome could be found in professional phagocytic cells, including macrophage monocyte neutrophil and dendritic cell. Cells engulfed certain substances that into cells such as amino acid by phagocytosis.[47] It could be possible that the hydrogels were in the biodegradable process and the cells engulfed the degraded particles into cells because the KEGG pathway showed decreased in ECM-receptor interaction and focal adhesion.

Upregulation of some neural-related genes was found i.e. *leucine-rich repeat neuronal 3 (LRRN3)* was relatively specific gene expressed in brain. LRRN3 plays an important role in brain development.[15] The expression of *NPDC-1* was very specific in the brain and controlled neural proliferation and differentiation.[9] Increased expression of solute carrier family 6 (neurotransmitter transporter) indicated that human gingival cells were trying to develop into a functional neuron because SLC6A8 acts as a GABA transporter.[12] Semaphorin 3C (Sema3C) was secreted from nerve cells and had a stimulating effect on the growth of axon in dopaminergic neurons.[21] GABARAPL1 was highly expressed in the brain, and restricted

to neurons which was required for maintaining normal autophagic flux.[18] *APLP1* were upregulated during postnatal development coinciding with synaptogenesis.[20] Neuronal pentraxin receptor was related to the synapse formation.[29] *B-cell translocation gene 2 (Btg2)* played important roles in cholinergic neuronal differentiation of BMSCs.[22] Down-regulation of *Btg2* inhibited neurite outgrowth.[48] TMEM132 molecules were considered as novel immunoglobulin-like domain containing proteins of the CNS23 which were important for cell survival in regulating certain ER stress-related gene expression in neuronal cells.[49] TRPA1 was predominantly expressed in peripheral nerve terminals, dorsal root ganglia, and central nerve terminals of primary sensory neurons in the spinal dorsal horn. TRPA1 was up-regulated in developing intervertebral disc and played roles in matrix homeostasis and inflammation.[24] Increase expression of solute-carrier gene (*SLC*) superfamily SLC was involved in the uptake of small molecules into cells such as amino acids, urea, nucleotide sugars, vitamin, neurotransmitters and etc.[50] Although the neuronal-related genes were found, neuronal differentiation could not be concluded. The gelation biomaterials used in this study showed the supportive roles for cultivation of adult somatic cells. The improvement of gelatin biomaterial properties might be useful for the application in induction of cell differentiation and the further study was continued in our laboratory.

Conclusion

Human gingival cells cultured on 42 gelatin biomaterials for 21 days showed increased oxidative stress protection and cells were in S-phase of cell cycle. Decreased cell-ECM interaction and ECM remodeling were occurred together with expression of some neuronal-related genes but neuronal-specific genes, *GFAP* and *NFL*, were not detected by RT-PCR. Immunomodulation was presented by up-regulation of chemokine and cytokine genes in human gingival cells cultured on gelatin biomaterials without microorganism infection under microscopic observation. This study aimed to investigate the biocompatibility of biomaterial. However, the physical properties such as pore size is very important and should be study in the further study.

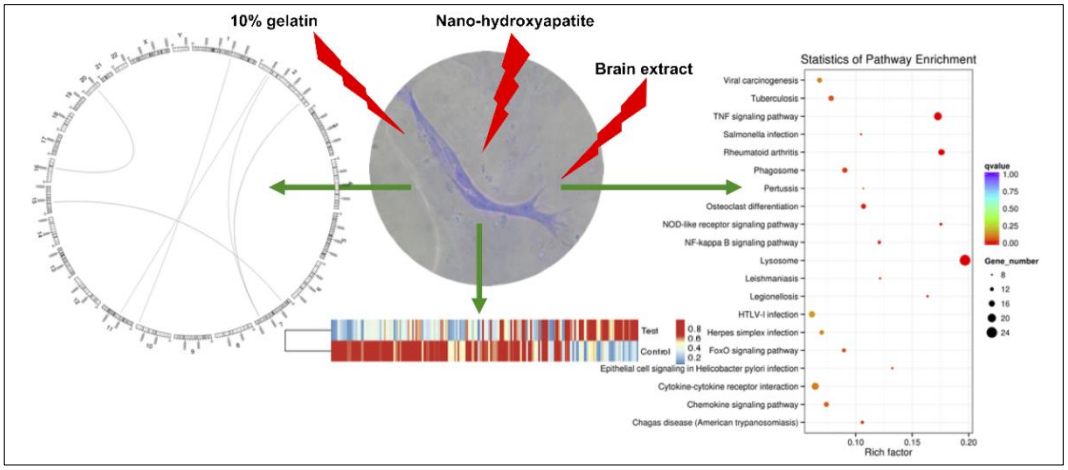

Figure 6. *Highlights of this study. Gelatin with hydroxyapatite and pig brain extract can be used in the modulation of somatic cells. Up-regulation of neuronal and antioxidant genes were found upon cultivation on the scaffold. Decreased of genes related to ECM remodeling were occurred upon cultivation on the scaffold.*

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Conflicts of Interest

The authors declare that there is no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethic approval

Human gingival cells were obtained according to Ethical approval by the Ethics Committee of the Faculty of Dentistry, Chiang Mai University (21.1/2018).

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Supplementary data

Figure 1. *Lyophilized biomaterials made from 10% gelatin, 1 mg/mL nano-hydroxyapatite and pig brain extract (Gel + Ha + Brain).*

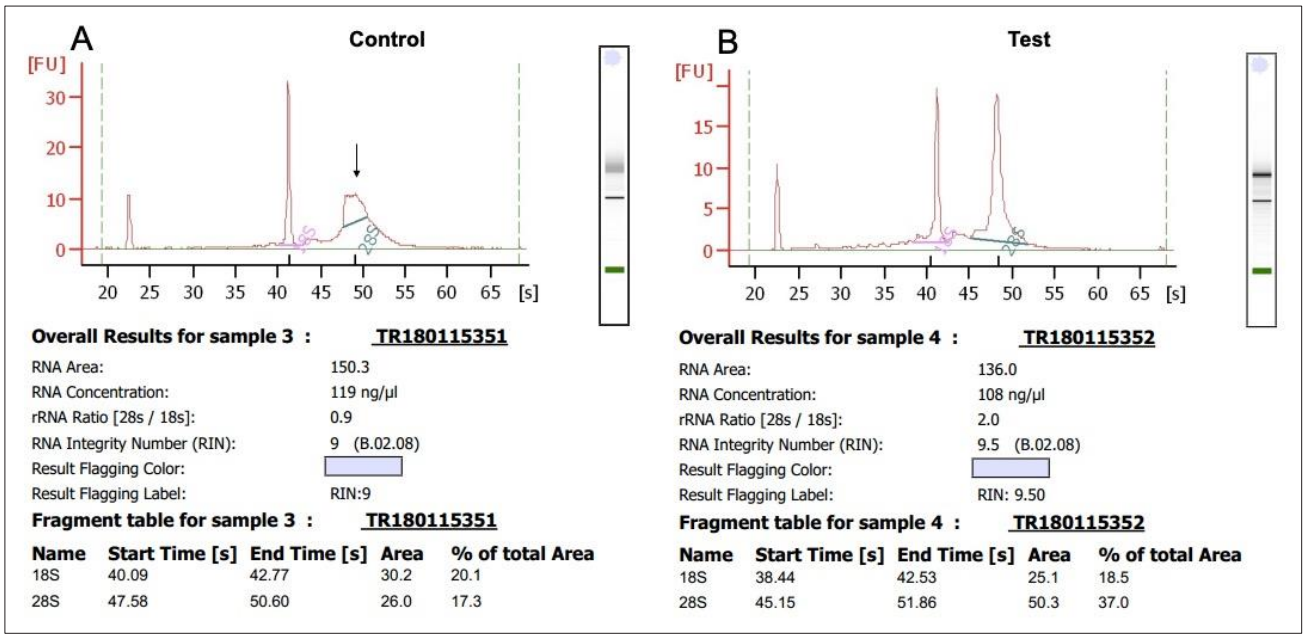

Figure 2S. *RNA QC report showed no degradation of 28 srRNA. A: control group showed a degradation of 28 srRNA, B: cells cultured on hydrogel.*

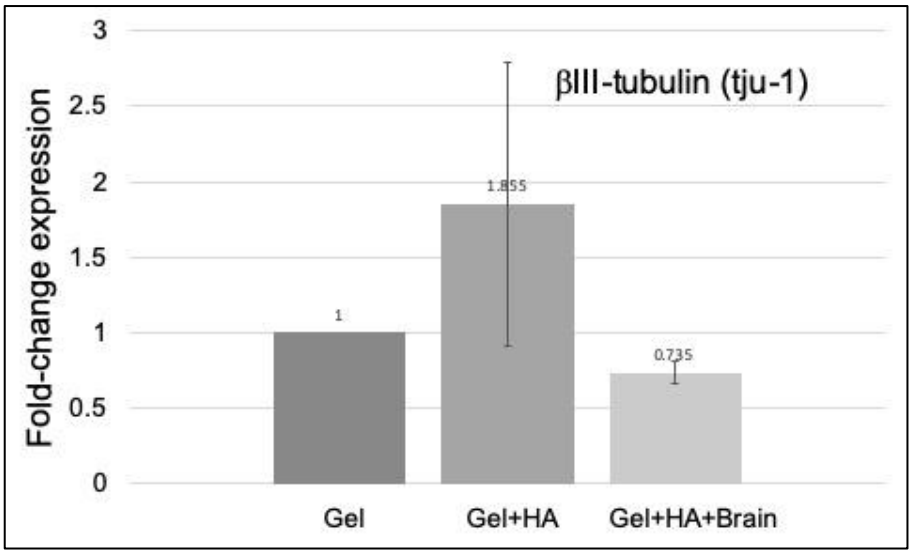

Figure 3S. *Expression of tju-1 gene was not significant between each type of biomaterials.*

Table 1S Sequences of primers used in real-time PCR.

Target genes	Primer sequences (5'->3')
1. Klf4	F: GAAATTCGCCCGCTCCGATGA
	R: CTGTGTGTTTGCGGTAGTGCC
2. βIII-tubulin	F: CGCACGACATCTAGGACTGA
	R: TGAGGCCTCCTCTCACAAGT
3. CD44	F: AGCAGCGGCTCCACCATCGAGA
	R: TCGGATCCATGAGTCACAGTG
4. MAP2	F: CCAAGGAGTCTGATTGCAGGA
	R: CCTCAACCACAGCTCAAATGC
5. ACAN	F: CTACGACGCCATCTGCTACA
	R: ACGAGGTCCTCACTGGTGAA
6. VCAN	F: AACATTTTTCAGGTGGTGAG
	R: GATGCAGAACTTGGAACTAT
7. OCN	F: GGACAGCCAGGACTCCATTG
	R: TGTGGGGACAACTGGAGTGAA
8. 18s rRNA	F: GGCCCTGTAATTGGAATGAGTC
	R: CCAAGATCCAACTACGAGCTT
9. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	F: AAATCCCATCACCATCTTCCAGGAGC
	R: CATGGTTCACACCCATGACGAACA

Table 2S Solute-carrier gene (SLC) superfamily.

SLC gene superfamily	log2.Fold change*	Note	Function
1. Solute carrier family 6	1.0865	increased	Na^+- and Cl^--dependent neurotransmitter symporter family
2. solute carrier family 3	1.1069	increased	Heavy subunits of the heteromeric amino acid transporters
3. Solute carrier family 11	1.3954	increased	Proton-coupled metal ion transporter family
4. solute carrier family 40	1.5438	increased	Basolateral iron transporter family
5. Solute carrier family 7	1.6839	increased	Cationic amino acid transporter/glycoprotein- associated amino acid transporter family
6. Solute carrier family 25	1.7743	increased	Mitochondrial carrier family
7. Solute carrier family 43	1.8382	increased	Na+-independent system-L-like amino acid transporter family
8. Solute carrier family 12	2.5553	increased	Electroneutral cation/Cl- co-transporter family
9. Solute carrier family 39	2.7085	increased	Metal (M_2^+) ion transporter family
10. Solute carrier family 16	3.6315	increased	Monocarboxylate transporter family

**** adjusted p<0.001***

Table 3S Key pro-inflammatory cytokines and chemokines.

Gene	log2.Fold_ change*	Function
1 Chemokine (C-X-C motif) ligand 5 (CXCL5)	7.1949	*Chemokine which recruits and activates leukocytes*
2. Chemokine (C-X-C motif) ligand 1 (CXCL1)	6.4068	*Plays a role in inflammation and acts as a chemoattractant for neutrophils*
3. Interleukin 8 (IL8)	6.2294	*A major mediator of the inflammatory response serves as a chemotactic factor by guiding the neutrophils to the site of infection* *Direct regulation of adaptive T cell reactivity*
4. Chemokine (C-X-C motif) ligand 2 (CXCL2)	5.7015	*Chemotactic for neutrophils and elevated expression of CXCL2 is associated with many inflammatory and autoimmune diseases*
5. Chemokine (C-X3-C motif) ligand 1 (CX3CL1)	5.3254	*A membrane-bound chemokine* *Has a dual function as an adhesion molecule and a chemoattractant* *A neuronal cytokine that acts on microglial CX3CR1 receptor involved in the maturation of GABAergic synapses*
6. I6 (IL6)	5.2299	*Cytokine that functions in inflammation and the maturation of B cells*
7. Complement component 3 (C3)	5.2134	*Plays a central role in the activation of complement system*
8. Chemokine (C-X-C motif) ligand 6 (CXCL6)	5.0382	*Chemokine expressed by macrophages and epithelial and mesenchymal cells during inflammation*
9. Chemokine (C-X-C motif) ligand 3 (CXCL3)	5.0317	*Plays a role in inflammation and acts as a chemoattractant for neutrophils*
10. Tumor necrosis factor, alpha-induced protein 3 (TNFAIP3)	4.3074	*Inhibit NF-kappa B activation as well as TNF-mediated apoptosis*
11. Chemokine (C-C motif) ligand 2 (CCL2)	4.174	*Displays chemotactic activity for monocytes and basophils but not for neutrophils or eosinophils*
12. Chemokine (C-X-C motif) ligand 10 (CXCL10)	4.1312	*Stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression*
13. Prostaglandin D2 synthase 21kDa (brain) (PTGDS)	4.093	*Catalyzes the conversion of prostaglandin H2 (PGH2) to postaglandin D2 (PGD2).* *PGD2 functions as a neuromodulator as well as a trophic factor in the central nervous system*

Table 3S Key pro-inflammatory cytokines and chemokines. (continues)

Gene	log2.Fold_change*	Function
14. Intercellular adhesion molecule 1 (ICAM1)	4.0847	*Typically expressed on endothelial cells and cells of the immune system*
15. Kynureninase (KYNU)	3.9303	*Biosynthesis of NAD cofactors from tryptophan*
16. Prostaglandin-endoperoxide synthase 2 (PTGS2 or COX-2)	3.9002	*Prostaglandin biosynthesis*
17. v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB)	3.5467	*Transcription factor that plays an important role in the regulation of lineage-specific hematopoiesis*
18. Interleukin 32 (IL32)	3.3573	
19. Tumor necrosis factor, alpha-induced protein 6 (TNFAIP6)	3.2914	*Extracellular matrix stability and cell migration*
20. Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA)	2.8315	*Involved in inflammatory responses*
21. Interferon regulatory factor 1 (IRF1)	2.7041	*Involved in both innate and acquired immune responses*
22. Retinoic acid receptor responder 2 (RARRES2)	2.6768	*Chemotactic protein that initiates chemotaxis*
23. POU class 2 homeobox 2 (POU2F2)	2.5773	*Transcriptional activator of immunoglobulin (Ig) in B lineage cells*
24. BTG family, member 2 (BTG2)	2.5507	*Involved in the regulation of the G1/S transition of the cell cycle*
25. Interleukin-1 receptor-associated kinase 2 (IRAK2)	2.4458	*Participate in the IL1-induced upregulation of NF-kappaB*
26. Chemokine (C-X-C motif) ligand 14 (CXCL14)	2.3953	*Regulation of immune cell migration, also executes antimicrobial immunity*
27. Major histocompatibility complex, class I, B (HLA-B)	2.2969	*Express on most of the somatic cells in the body* *Play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen* *Display peptides that result from the degradation of cytosolic proteins to T lymphocytes and natural killer (NK) cells*
28. Beta-2-microglobulin (B2M)	1.1334	*Serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells*
29. Interferon gamma receptor 2 (IFNGR2)	1.1464	*Non-ligand-binding beta chain of the gamma interferon receptor*
30. MHC class I polypeptide-related sequence A (MICA)	1.2047	*A membrane-bound protein acting as a ligand to stimulate an activating receptor expressed on the surface of essentially all human natural killer (NK), T and CD8(+) T cells*
31. Sema domain, immunoglobulin domain (Ig) (SEMA3C)	1.2086	*Soluble proteins that are well recognized for their role in guiding axonal migration during neuronal development.* *In the immune system, Sema3A has been shown to influence murine dendritic cell (DC) migration by signaling through a neuropilin (NRP)-1/plexin-A1 coreceptor axis*

Table 3S Key pro-inflammatory cytokines and chemokines. (continues)

Gene	log2.Fold_change*	Function
32. Major histocompatibility complex, class I, C (HLA-C)	1.2341	*Express on most of the somatic cells in the body* *Play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen* *Display peptides that result from the degradation of cytosolic proteins to T lymphocytes and natural killer (NK) cells*
33. Major histocompatibility complex, class I, A (HLA-A)	1.5698	*Express on most of the somatic cells in the body* *Play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen* *Display peptides that result from the degradation of cytosolic proteins to T lymphocytes and natural killer (NK) cells*
34. Interferon gamma receptor 1 (IFNGR1)	1.5842	*IFN-gamma and its receptor subunit, IFNGR1, in direct contact with the promoter region of IFN-gamma-activated genes*

**** adjusted p<0.001***

Table 4S KEGG pathway.

KEGG up	Corrected *p* value	Cell type
1. Lysosome (hsa04142)	7.34×10^{-9}	Any
2. TNF-signaling pathway (hsa04668)	2.53×10^{-6}	Any
3. Rheumatoid arthritis (hsa05323)	1.69×10^{-5}	Synovial macrophages
4. NOD-like receptor signaling pathway (hsa04621)	0.0022	Macrophages Dendritic cells Peripheral blood Leukocytes Interstitial epithelial cells Paneth cells
5. Legionellosis (hsa05134)	0.0068	Macrophages Epithelial cells
6. Osteoclast differentiation (hsa04380)	0.0069	Bone marrow derived monocyte/macrophage precursor cells
7. NF-kappa B signaling pathway (hsa04064)	0.0107	Any
8. Epithelial cell signaling in Helicobacter pylori infection (hsa05120)	0.0172	Epithelial cells
9. Phagosome (hsa04145)	0.0206	Phagocytic cells
10. Chagas disease (American trypanosomiasis) (hsa05142)	0.0206	Macrophage Fibroblast Skeletal muscle cell Cardiac muscle cell
11. Leishmaniasis (hsa05140)	0.0215	Macrophage
12. FoxO signaling pathway (hsa04068)	0.0385	Any
13. Salmonella infection (hsa05132)	0.0466	Interstitial epithelial and M cells
14. Tuberculosis (hsa05152)	0.0479	Macrophages Dendritic cells
15. Pertussis (hsa05133)	0.0604	Phagocytic cells
16. Chemokine signaling pathway (hsa04062)	0.0653	Eosinophil Neutrophil Macrophages T lymphocytes

Table 4S KEGG pathway. (continues)

KEGG up	Corrected *p* value	Cell type
17. ECM-receptor interaction (hsa04512)	0.000000195	Any
18. Focal adhesion (hsa04510)	0.00000743	Any
19. PI3K-Akt signaling pathway (hsa04151)	0.0000497	Any
20. Arrhythmogenic right ventricular cardiomyopathy (ARVC) (hsa05412)	0.000650596	Cardiac myocyte
21. Protein digestion and absorption (hsa04974)	0.001761442	Small intestinal epithelial cell
22. Hypertrophic cardiomyopathy (HCM) (hsa05410)	0.006059177	Cardiac myocyte
23. Dilated cardiomyopathy (hsa05414)	0.000423057	Cardiac myocyte
24. Regulation of actin cytoskeleton (hsa04810)	0.022123169	Any