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**Establishment and application of an *in vitro* method to
investigate the intercellular communication between cells
involved in orthodontic tooth movement**

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Abbreviations used

μm	Micrometer
ANOVA	Analyses of variance
ATP	Adenosine triphosphate
B2M	Beta-2-microglobulin
BMP	Bone morphogenetic protein
cDNA	complementary DNA
cm^2	Square centimeter
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal bovine serum
g	<i>Constant of gravity; 9.81 m/s²</i>
g/cm^2	Gram per square centimeter
GCF	Gingival crevicular fluid
h	Hour
hOB	human osteoblasts
hPDLF	human periodontal ligament fibroblasts
IL1A	Interleukin 1 α
IL1B	Interleukin 1 β
IL6	Interleukin 6
LCD	liquid-crystal display
MEM	Minimum Essential Medium
Mg	Magnesium
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
mm^2	Square millimeter
Na	Sodium
OPG	Osteoprotegerin
OTM	Orthodontic tooth movement
P2RX7	Purinergic receptor P2RX7
PCR	Polymerase chain reaction
PDL	Periodontal ligament
PGE ₂	Prostaglandin E ₂
PTGS2	Prostaglandin-endoperoxide synthase 2 (= cyclooxygenase 2)
RANKL	Receptor activator of NF-kappaB ligand

RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SD	Standard deviation
SaOS-2	Osteosarcoma cell line
TNF	Tumor necrosis factor
TRAF	TNFR-associated factor
U/ μ l	Units per Microliter
μ L	Microliter

1 Introduction

Orthodontic tooth movement (OTM) is accomplished by application of external forces on a tooth or a group of teeth and is accompanied by extensive bone remodeling: bone resorption on the compression side and bone formation on the tension side of force application (Kim et al. 2006; Liu et al. 2009; Long et al. 2001; Mayahara et al. 2012).

Three main cell types, osteoblasts, osteoclasts and periodontal ligament fibroblasts, are involved in this remodeling process. In orthodontic therapy, mechanical forces are loaded onto teeth. These forces are transmitted primarily to periodontal ligament fibroblasts (PDLF). The PDLF response to this force and interact with other cell types within the periodontal tissue to regulate the remodeling of the bone matrix (Kanzaki et al. 2002; Lekic and McCulloch 1996). Without PDLF tooth cannot be moved in the alveolar bone under orthodontic mechanical force (Mitchell and West 1975). Osteoblasts are responsible for bone matrix formation relevant to bone differentiation and resorption (Garlet et al. 2007; Grimm et al. 2015).

Numerous studies evidenced that cytokines play pivotal roles in bone remodeling and bone homeostasis (Jacobs et al. 2014; Kanzaki et al. 2002; Mah et al. 2014). Specifically, either physical or chemical factors stimulate periodontal tissue cells to produce cytokines and growth factors which then regulate the micro environment surrounding the tooth (Li et al. 2013a; Ren and Vissink 2008).

2 Literature Review

2.1 Orthodontic tooth movement

Orthodontic tooth movement (OTM) is achieved by sequential bone remodeling (Figure 1): bone resorption on the compressive side and bone formation on the tension side during orthodontic therapy (Wang et al. 2012). Periodontal tissues including periodontal ligament, gingival tissues and alveolar bone are involved in this process. Inflammatory response in the periodontal tissue occurs upon mechanical force stimulation. The inflammatory reaction varies depending on the force magnitudes and force types (Koyama et al. 2008), and it finally regulates bone reconstruction (Koyama et al. 2008; Krishnan and Davidovitch 2006).

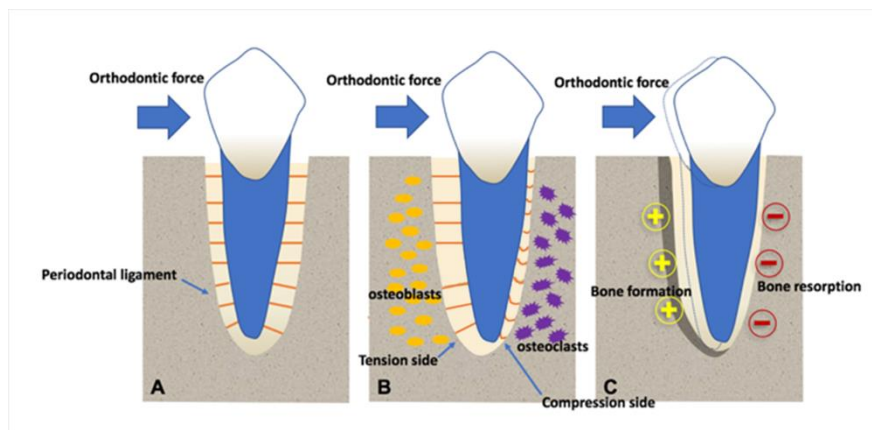


Figure 1. Bone remodeling during orthodontic force application: (A) orthodontic force is loaded on a tooth. (B) Osteoblasts accumulate on the tension side and osteoclasts on the compression side. (C) Bone remodeling occurs by a sequential bone resorption on the compressive side and bone formation on the tensile side (redrawn and modified after Kitaura et al. 2014).

According to Krishnan and Davidovitch (2006, p. 469.e17), OTM includes the following sequence of events:

1. *“Movement of PDL fluids from areas of compression into areas of tension.*
2. *A gradual development of strain in cells and ECM in involved parodontal tissues.*
3. *Direct transduction of mechanical forces to the nucleus of strained cells through the cytoskeleton, leading to activation of specific genes.*

4. *Release of neuropeptides (nociceptive and vasoactive) from parodontal afferent nerve endings.*
5. *Interaction of vasoactive neuropeptides with endothelial cells in strained parodontal tissues.*
6. *Adhesion of circulating leukocyte to activated endothelial cells.*
7. *Plasma extravasation from dilated blood vessels.*
8. *Migration by diapedesis of leukocytes into the extravascular space.*
9. *Synthesis and release of signal molecules (cytokines, growth factors, and CSFs) by the leukocytes that have migrated into the strained parodontal tissues.*
10. *Interaction of various types of parodontal cells with the signal molecules released by the migratory leukocytes.*
11. *Activation of the cells to participate in the modeling and remodeling of the parodontal tissues.”*

2.2 Periodontal tissues involved in orthodontic tooth movement

2.2.1 Periodontal ligament fibroblasts

In periodontal tissue, the periodontal ligament (PDL), a connective ligament tissue, is located between cementum and alveolar bone. It can be regarded as a specialized connector joining tooth and bone (Berkovitz 1990; Li et al. 2013b; Sodek and Limeback 1979). To maintain integrity and stability of tooth, PDL distributes and buffers force loaded by masticatory or external forces (Lekic and McCulloch 1996).

Orthodontic tooth movement relies on biological reactions in the PDL and alveolar bone to mechanical force. Without PDL, tooth cannot be moved during orthodontic therapy (Mitchell and West 1975), a situation found in “ankylosed teeth” for example. These are teeth that are fused with the alveolar bone due to missing PDL.

The first step in tooth movement is the transduction of orthodontic forces applied to the tooth. Due to its anatomical location situated between teeth and alveolar bone, PDL acts like a transmitter of external forces from the teeth to the surrounding tissue (Reitan 1960). Here, osteoblasts, osteoclasts and other cells in the surrounding tissue react regulating the resorption and formation of bone matrix (Kanjamekanant et al. 2013; Kanzaki et al. 2002; Lekic and McCulloch 1996). Upregulation of several inflammatory factors associated with osteogenesis and bone degradation are detectable

in the PDL fibroblasts (PDLF) after mechanical force stimulations (Kanzaki et al. 2002; Kook et al. 2009). This is closely linked to physiological changes of osteoblasts and osteoclasts during orthodontic tooth movement (Davidovitch 1991; Long et al. 2001; Maeda et al. 2007; Shimizu et al. 1994).

2.2.2 Osteoblasts

Bone plays an important supportive role of the whole body. In the oral cavity, alveolar bone maintains teeth in their positions to drive their normal physiological function. On the other hand, alveolar bone shows plasticity under mechanical force stimulation (Katagiri and Takahashi 2002). Previous studies showed that with mechanical forces applied osteoblasts produce various cytokines. This contributes to the mechano-transduction process (Ducy et al. 2000; Li et al. 2007) leading to the remodeling of periodontal tissues. Osteoblasts also play a role in the balance of the PDL and its surrounding cells during alveolar bone remodeling (Beertsen et al. 1997; Yamazaki et al. 2014).

However, osteoblast regulation after mechanical force application is not yet fully understood; in particular, how different magnitudes of force influence this process and how PDL is involved in this.

2.3 Factors involved in orthodontic tooth movement

The significance of the mechanical loading for bone resorption and bone formation is undisputed. Extracellular matrix molecules, ion channels, signal molecules and integrin play active roles in mechanic transduction (Kanzaki et al. 2002; Maeda et al. 2007). On the other hand, the mechanisms of transduction on cellular and subcellular level in bone remodeling are still not fully understood (Nakao et al. 2007).

During orthodontic tooth movement (OTM), different inflammatory cytokines, including interleukin 6 (IL6) (Jacobs et al. 2014), interleukin 1 β (IL1B) (Shimizu et al. 1994), prostaglandin-endoperoxide synthase 2 (PTGS2; previous name: cyclooxygenase-2, COX2) (Shimizu et al. 1998), tumor necrosis factor (TNF) (Kim et al. 2013), receptor activator of NF- κ B ligand (RANKL) (Yamaguchi 2009), the runt-related

transcription factor 2 (RUNX2) (Baumert et al. 2004; Ziros et al. 2002) and the purinergic receptor/ion channel P2RX7 in periodontal tissue (Kanjanamekanant et al. 2013; Vecilli et al. 2009).

The cytokines mentioned above are either released by periodontal tissue cells (i.e. PDL, osteoblasts and osteoclasts) or were shown to be regulated (i.e., RUNX2, P2RX7) by mechanical force stimulation and thus play regulatory roles in alveolar bone reconstructions during tooth movement (Kanjanamekanant et al. 2013; Li et al. 2013a; Mayahara et al. 2012).

2.3.1 Prostaglandin-endoperoxide synthase 2 (PTGS2) and Prostaglandin E₂ (PGE₂)

The isoenzymes of prostaglandin-endoperoxide synthase (PTGS), PTGS1 (cyclooxygenase 1, “COX1”) and PTGS2 (cyclooxygenase 2, “COX2”), are widely studied as key enzymes in prostaglandin biosynthesis (O'Neill and Ford-Hutchinson 1993). Prostaglandins are part of several pathways leading to the activation and secretion of pro-inflammatory mediators, like cytokines, mitogens, and lipopolysaccharides (Hla and Neilson 1992; Jones et al. 1993; Lee et al. 1992), and were shown to accelerate bone resorption in the alveolar bone during orthodontic therapy (Klein and Raisz 1970). In the initial stage of orthodontic therapy, PDLF produce prostaglandin-related acute inflammatory factors in response to orthodontic mechanical stimulation. PTGS2 is induced in periodontal tissue cells, including osteoblasts, osteoclasts, gingival fibroblasts, cementoblasts and PDLF (Römer et al. 2013; Yucel-Lindberg et al. 2006), leading to prostaglandin E₂ (PGE₂) secretion (Kraemer et al. 1992; Kujubu and Herschman 1992). *PTGS2* gene expression is closely correlated with PGE₂ biosynthesis during inflammatory reactions. PGE₂ is detectable after orthodontic force loading in periodontal tissues; it is known as a pro-inflammatory intermediary, an effective factor for inducing osteoclasts (Mayahara et al. 2012; Römer et al. 2013; Saito et al. 1991). Additionally, it was shown that PGE₂ secretion in response to mechanical force changes along with PTGS2 enzyme activity under mechanical load (Shimizu et al. 1998).

2.3.2 Interleukin 6 (IL6)

Interleukin 6 (IL6) is a cytokine associated with changes of bone mass (Bakker et al. 2014). Overexpression of IL6 accelerates bone reconstruction by decreasing bone resorption and increasing bone formation (Baker et al. 1999). Differentiation of progenitor cells to osteoblasts is also facilitated by IL6 (Bellido et al. 1997). In contrast, lack of IL6 lowers bone mass via reducing the numbers of osteoblasts (Balto et al. 2001; Yang et al. 2007). Periodontal tissue remodeling during inflammatory response and orthodontic tooth movement is affected by IL6 (Alhashimi et al. 2001). Differential expression of IL6 were detected in gingival crevicular fluid and PDLF after orthodontic force loading, indicating IL6 might play a role in the bone remodeling process after mechanical stimuli (Madureira et al. 2015; Ren et al. 2007).

2.3.3 Runt-related transcription factor 2 (RUNX2)

Runt-related transcription factor 2 (RUNX2) promotes the differentiation of progenitor cells into mature osteoblasts. In general, RUNX2 plays an important role in bone growth equilibrium and bone remodeling. *RUNX2* knockout mice show a complete lack of bone formation (Komori et al. 1997) and heterozygous *RUNX2* mutations lead to cleidocranial dysplasia (Baumert et al. 2005). RUNX2, thereby, is considered as a “master gene” or “pacemaker gene” for bone development and is part of several signaling pathways in osteoblastic differentiation (Ducy et al. 1999).

RUNX2 itself is induced by bone morphogenetic proteins (BMP) in cells of the osteoblast lineage in advance of osteoblast differentiation (Ducy et al. 1997). In the promoter region of many osteogenic specific genes (i.e. osteocalcin, alkaline phosphatase, type-I collagen) RUNX2 binding sites exist (Colden et al. 2017; Kern et al. 2001; Zhang et al. 2017).

2.3.4 Purinergic receptor/ion channel P2RX7

P2RX7 receptor is an adenosine triphosphate (ATP)-dependent ion channel and plays a crucial role in bone biology and inflammation (North 2002). Mechanical forces induce the increase of extracellular ATP concentration, which is considered a cellular stress signal (Kanjanamekanant et al. 2013). Upon binding extracellular ATP, the

P2RX7 ion channel opens and Ca^{2+} enter the cell (Kariya et al. 2015; Schneider et al. 2006; Trubiani et al. 2014; Viecilli et al. 2009). This leads to accumulation of intracellular calcium and liberation of fundamental inflammatory mediators, like TNF, IL6, IL1A, and IL1B, which regulate bone physiology (Ferrari et al. 2006; Lister et al. 2007). Another major function of P2RX7 is to accelerate necrotic tissue metabolism. However, it is still unclear concerning the effects of P2RX7 on bone remodeling and tooth movement (Viecilli et al. 2009).

2.3.5 Tumor Necrosis Factor (TNF)

The tumor necrosis factor (TNF) is a pro-inflammatory cytokine which can induce apoptosis in PDLF and osteoblasts (Alikhani et al. 2004; Pavalko et al. 2003). Increased levels of TNF were discovered in osteoporosis and periodontitis patients (Ralston et al. 1990), as well as in PDL on tension or compression side after mechanical force load during orthodontic tooth movement (Uematsu et al. 1996). This leads to a stimulation of osteoclastogenesis and an inhibition of bone formation and thus to an adjustment in bone remodeling (Bertolini et al. 1986; Tan et al. 2006).

2.4 Experimental designs to simulate orthodontic tooth movement *in vitro*

In vivo studies on orthodontic tooth movement are conducted in human and animal models (e.g. rat, beagle dogs, and mice). Analysis of tissue reactions are mostly limited to the screening of cytokines released to the gingival crevicular fluids, and histologic dissections of tissue reactions due to force application (Thilander et al. 2005; von Böhl et al. 2004). Due to the complexity of the tissues involved in orthodontic tooth movement, subcellular investigations specific to individual cell types is performed using *in vivo* studies. These exclude non-experimental tissues and cell interference thus alleviating the examination of specific intra- and intercellular signal transduction pathways (Kim et al. 2006).

2.4.1 Influence of mechanical force

Mechanical force is considered not only a main element in regulating bone homeostasis, but also a morphological determinant in bone development and reconstruction (Kook et al. 2009; Meazzini et al. 1998; Yokoya et al. 1997). Various aspects, such as osteogenic differentiation, bone morphology, gene expression, osteocyte proliferation and apoptosis are analyzed (Hughes-Fulford and Lewis 1996; Li et al. 2007; Meazzini et al. 1998).

During orthodontic tooth movement, mechanical forces are transmitted to the periodontal tissue (see also Section 2.1). In this process, spatial and temporal changes occur in parallel in different cell types of periodontal tissue. For example, cell damage due to tissue injury happens along with cellular reactions based on force stimulation. Additionally, sequential intra- and intercellular changes induce bone resorption (Chambers et al. 1993; Lanyon 1992; Rubin et al. 1995). A suitable mechanical force is essential for inducing physiological bone changes instead of damaging teeth or periodontal tissues (Koyama et al. 2008; Mayahara et al. 2012).

To simulate the condition during orthodontic force loading as near as possible to the *in vivo* situation, experimental studies used different setups to produce mechanical force on adherent cells *in vitro* (Figure 2). Clinically, two types of force, tension and pressure, are mainly involved in orthodontic tooth movement. Pressure narrows the width of the PDL. This induces the expression of bone remodeling markers. Histological alteration on the compressive side in the periodontal tissues are detectable (Otero et al. 2016).

Concerning sampling, two different sampling procedures are applied in studies related to compressive force application: “direct sampling” and “pulse-chase sampling”. In “direct sampling” biological samples (i.e. supernatant, protein lysate, cell lysate) are drawn directly after force application stopped (e.g. Morikawa et al. 2016; Redlich et al. 2004a). The procedure of “pulse chase sampling” involves the application of a force (i.e. the “pulse”) to the specimen for a defined period of time. Then, samples are drawn at defined time intervals after force application stopped (e.g. Baumert et al.

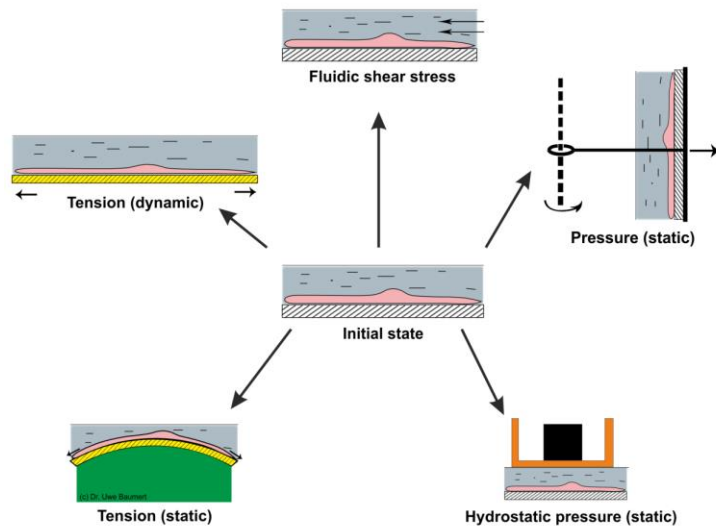


Figure 2. Summary of experimental methods to apply different types of force related to OTM on adherent cells *in vitro* (image contributed by Dr. Baumert).

2004). Both sampling procedures in common is the “pulse” step; the difference is the time point when samples are drawn for further analysis. With “direct sampling” only a small “time window” is available for analysis. The position of this “window” in relation to the time point of force release is not stated in any publication. “Pulse chase sampling”, on the other hand, enables to study the effect of a stress application over a longer period of time. Gene expression and translation take time and this time differs between different genes. Some genes are rapidly transcribed after stimulation (“primary” or “early” response genes) and some genes are slowly induced (“secondary” or “late” response genes) (Sandoval et al. 2016). “Pulse chase sampling” is therefore suitable for screening purposes.

2.4.2 *In vitro* culture systems

A variety of methods to study the mechanical responses of periodontal tissue cells *in vitro* were applied using cell type-specific experimental designs (Baumert et al. 2004; Redlich et al. 2004a; Römer et al. 2013; Ziros et al. 2002). In the *in vivo* situation, intercellular communication between the different cells and cell types of the periodontal tissues is taking place. Adaptation of the *in vivo* situation to *in vitro* models would therefore involve force application not only on one cell type but on two

or more different cell types simultaneously, thus enabling communication between these different cell types during force application.

Currently, intercellular communication cannot be fully elucidated during mechanical loading *in vivo*. A possible way to investigate intercellular communication between cells is a special kind of *in vitro* cell culture system that is called “co-culture” (Figure 3).

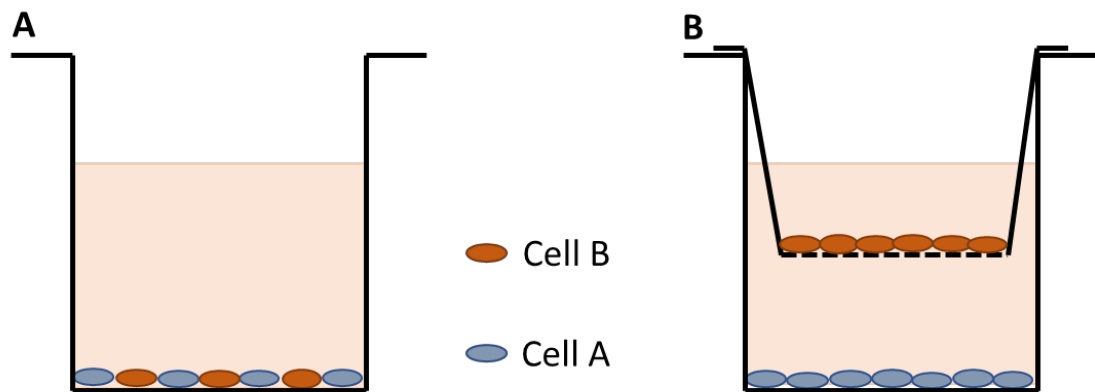


Figure 3. The two types of “co-culture”: (A) “Direct contact co-culture”: cell types A and B share the same space and grow next to each other. (B) “Indirect contact co-culture”: cell type B is growing on a permeable membrane sharing the same culture medium as cell type A without touching each other.

“Co-culture” describes various techniques where different types of cell populations are cultivated near to each other in the same cell culture environment (Figure 3). At present, two types of co-culture systems are used: “direct contact co-culture” and “indirect contact co-culture”. In “direct contact co-culture”, two types of cells are cultivated directly next to each other (Figure 3A). Direct cell-cell contacts can be established as well as paracrine signaling pathways (Jones et al. 2009). However, some drawbacks exist with this mixed co-culture model:

1. If one cell type outgrows the other one and thus occupying most of the limited growth space, it might cause massive cell death.
2. The analysis of gene expression in both cell types separately is technically demanding in this totally mixed environment.

Taken together, with direct-contact co-culture it is not possible to analyze the effects of long-term force application or to conduct gene expression studies on each cell type separately.

These issues can be solved in the “indirect contact co-culture system”. In this system, two types of cells share the same growth environment without contacting each other directly (Figure 3B). Intercellular communication via the release of cytokines and growth factors is still possible. The indirect contact co-culture system is established using a double-chamber that is formed by inserting cell culture inserts into suitable cell culture plates. The separation is achieved by a porous membrane that forms the bottom of the cell culture inserts. Its pore size inhibits cell migration through the membrane but enables molecules (i.e. from the cultivation medium, signaling molecules or metabolites) to pass through. Using this setup, two different kinds of cells can be cultivated using the identical medium without physical contact to each other. Afterwards, these cell types can be analyzed and evaluated separately by moving the inserts to another suitable plate (Hatherell et al. 2011).

In summary, previous studies have several technical drawbacks: (1) Force is applied mostly on cells in mono-culture. (2) Co-culture models are used but not with force applied. (3) During force application, physiological conditions like temperature are not controlled. (4) “Direct sampling” procedures are used and the duration of sampling is not reported.

3 Aim of the study

Aim of this study, therefore, was to establish an *in vitro* method to investigate the intercellular communication between cells during orthodontic tooth movement meeting the following prerequisites:

- Compressive force application to hPDLF and hOB in co-culture;
- Access to both cell types after centrifugation without cross-contamination;
- Application of force at physiological conditions;
- Application of a stringent “pulse chase” sampling regime.

To meet the prerequisites, compressive force was applied onto both hPDLF and hOB in indirect co-culture using a laboratory centrifuge for 1, 2 and 4 h. Due to the technical limitations of the available centrifuge force application took place at a reduced temperature at 30 °C instead of 37 °C. Reproducibility and validity of the setup was evaluated by testing cell viability and apoptosis induction. The temperature conditions were monitored in all experimental conditions (force magnitude, force duration, culture condition) for both experimental groups and negative control groups. Genes or metabolites known to be regulated in intercellular communication during orthodontic tooth movement were further analyzed in hPDLF and hOB at 2, 4, 8 and 16 min after compressive force application, i.e. *IL6*, *TNF*, *PTGS2*, *RUNX2*, *P2RX7* and *PGE₂*. The experiments were repeated with mono-cultured cells for direct comparison.

To make the results of the “pulse chase” sampling more comparable with previous published studies applying “direct” sampling, the results of both the gene expression analysis and the ELISA testing are presented in two different ways: (1) “direct” sampling: measurements from 2 and 4 min chase are combined; (2) “pulse chase” sampling as is.

4 Materials and Methods

4.1 Materials

4.1.1 Chemicals

All chemicals were purchased at the highest available purity from the companies listed.

Reagent kits are listed below and are described in the relevant methods section.

Chemicals name	Supplier
Agarose	LE Agarose (Biozym, Hessisch Oldendorf)
Apoptosis kit	Multi-Parameter Apoptosis Assay Kit (Cayman, Ann Arbor, USA)
cDNA synthesis kit	SuperScript [®] IV First-Strand Synthesis System (Thermo, Munich)
Cell culture medium	<ul style="list-style-type: none">MEM-Alpha-Medium (Thermo, Munich)Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma Aldrich, Steinheim)Dulbecco's MEM (Biochrom, Berlin)
Dimethylsulfoxid (DMSO)	Cell culture grade (AppliChem, Darmstadt)
Dithiothreitol (DTT)	1 M solution (AppliChem, Darmstadt)
DNA ladder	Thermo, Munich: <ul style="list-style-type: none">GeneRuler 100bp Plus DNA LadderGeneRuler Low Range DNA Ladder
DNA loading buffer	6× Loading Buffer with orange G (Genaxxon, Ulm)
ELISA kits	R&D Systems, Minneapolis, USA: <ul style="list-style-type: none">Human IL6 Duo SetHuman IL6 Quantikine KitPGE₂ Parameter AssayHuman TNFα Duo Set
Ethanol	For disinfection: 70% denatured (CLM GmbH, Niederhummel) For Zymo kit application: 99.8%, denatured (Carl Roth, Karlsruhe)
Ethidium bromide	Ethidium bromide solution (0.07%) "Dropper-Bottle" (Appllichem, Darmstadt)
FBS	Fetal Bovine Serum Low in Endotoxin (Sigma Aldrich, Steinheim)
Glutamine	200 mM L-Glutamin solution, sterile-filtered (BioXtra, Sigma Aldrich, Steinheim)
Incidin	Incidin [®] Liquid (Ecolab, Monheim am Rhein)
MEM-Vitamines	MEM-Vitamine Solution (100×) (Biochrom, Berlin)
Mycoplasma Test	PCR Mycoplasma Test Kit I/C (Promokine, Heidelberg)
PBS-Buffer	PBS Dulbecco without Ca ²⁺ w/o Mg ²⁺ (Biochrom, Berlin)

Chemicals name	Supplier
PCR primers	Synthesis by Metabion, Planegg: <i>IL6, PTGS2, RUNX2, P2RX7, B2M, TNF</i>
PCR water	Aqua ad iniectabilia (Braun, Melsungen)
qPCR-Mastermix	Luminaris Color HiGreen qPCR-Mastermix (Thermo, Munich)
Resazurin	Resazurin sodium salt (Sigma Aldrich, Steinheim)
RNA isolation kit	Quick-RNATM MicroPrep (Zymo Research, USA)
RNA lysis buffer	RNA Lysis Buffer (Zymo Research, Irvine, USA)
RNase inhibitor	Recombinant RNasin® Ribonuclease Inhibitor (Promega, Madison, USA)
TAE-Buffer	TAE buffer (50×) Molecular biology grade (AppliChem, Darmstadt)
Trypan blue	Trypan Blue Solution (0.4%) (Sigma Aldrich, Steinheim)
Trypsin	Trypsin/EDTA-Solution (0.05% / 0.02%) (Biochrom, Berlin)

4.1.1 Apparatus

Type of apparatus	Exact apparatus name, company
Agarose electrophoresis chamber	EasyPhor Horizontal Agarose Gel System (Biozym, Hess. Oldendorf): <ul style="list-style-type: none"> • EasyPhor Midi • EasyPhor Maxi
Autoclave	Tuttnauer-Laborautoklav 3150 EL (Tuttnauer, Gießen)
Balance	Satorius, Göttingen: <ul style="list-style-type: none"> • TE 1502S • MSA2203P-000-DE
Cell culture safety cabinet	HERA safe KS (ThermoScientific, Langenselbold)
Centrifuge	<ul style="list-style-type: none"> • Refrigerated Centrifuge 4-16K (Sigma Aldrich, Osterode am Harz) • Centrifuge Mikro 200R (Hettich, Tuttlingen) • Sprout mini centrifuge with SnapSpin™ Rotors (Healthrow Scientific®, Vernon Hills, USA)
CO ₂ cell culture incubators	CellIncubator 120 & CellIncubator 250 (Thermo, Munich)
Dishwasher	Laboratory Dish Washer G7883 (Miele, Gütersloh)
ELISA reader	Varioscan (Thermo Electron Corporation, Vantaa, Finland)
Fluorescence microscope	EVOS® <i>fl</i> Color (Invitrogen, Carlsbad, CA)
Image Analysis System	LIAS (Avegene Life Science, Taiwan, China)
Magnetic hotplate stirrer	RCT classic IKAMAG® (IKA®-Werke GmbH & Co. KG, Staufen)
Microbiological incubator	Incubator BD23 (Binder, Tuttlingen)
Microwave oven	Microwave R-939-A (Sharp Electronics, Hamburg)
Mixing Block	Mixing Block MB-107 (Biozym, Hessisch Oldendorf)

Type of apparatus	Exact apparatus name, company
PCR machines	<ul style="list-style-type: none"> • TProfessional Thermocycler (Biotra, Göttingen) • LightCycler480 (Roche, Basel, Switzerland)
pH Meter	HI 2210 pH Meter (Hanna Instruments, Vöhringen)
Pipetman	Eppendorf Easypet® 4432 (Eppendorf, Hamburg)
Pipettes	<p>Eppendorf, Hamburg:</p> <ul style="list-style-type: none"> • Eppendorf Research® • Eppendorf Reference® • Multipette® stream <p>Proline® Plus (Biohit, Helsinki, Finland)</p>
Power supply	EV202 (Consort, Turnhout, Belgium)
Refrigerator	<ul style="list-style-type: none"> • 4 °C Refrigerator (Liebherr, Ochsenhausen) • -20 °C Refrigerator (Siemens, Munich) • -80 °C Refrigerator (Thermo Fisher Scientific, Munich)
Shaker	Lab dancer (vwr, Darmstadt)
Spectrophotometer	Nanodrop 1000 (PqLab, Erlangen)
Sterilization	APT.line™ ED (E2) (Binder, Tuttlingen)
Temperature data logger	<p>From Elektronik Fuchs (Weingarten):</p> <p>DS1921H-F5 Thermochron iButton®</p> <p>1-Wire Network Cable</p>
Thermo printer	Video Graphic Printer (Sony, Tokio, Japan)
Ultra-pure water machine	Arium® 611VF (Sartorius, Göttingen)
Vortexer	Vortex-Genie® 2 (Si Scientific Industries, Inc., Bohemia, USA)
Water bath	LAUDA Aqualine (Lauda, Lauda-Königshofen)

4.1.2 Consumables

Consumable item	Supplier
“Eppendorf” tips	<p>Sarstedt, Nümbrecht:</p> <ul style="list-style-type: none"> • Biosphere® Fil. Tip: 20 µL, 100 µL • Biosphere® Fil. Tip 1250 µL, Long • Pipette tips 1250 µL, Long • Pipette tips 250 µL
Adhesive film	Optical clear adhesive sealing tape for PCR (Sarstedt, Nümbrecht)
Cell counting chamber	G-Slide Neubauer (C-Chip) (Kisker, Steinfurt)
Cell culture flask	<p>From Sarstedt (Nümbrecht):</p> <ul style="list-style-type: none"> • Cell culture flask T-25 • Cell culture flask T-75
Cell culture inserts	ThinCert cell culture inserts for 12-well plates (Greiner Bio-One, Frickenhausen)

Consumable item	Supplier
Labels	From Diverified Biotech (Dedham, USA): <ul style="list-style-type: none"> • Teeny Tough-Tags® • Cryo-Babies® • Microtube Tough-Tags®
Multi-pipette tip	From Eppendorf (Hamburg): <ul style="list-style-type: none"> • Combitips advanced®, 5.0 mL, Farbcode blue • Eppendorf Quality™ Combitips 10 mL
Multiwell plates	<ul style="list-style-type: none"> • Cell culture multiwell plate (12 well) (Greiner Bio-One, Frickenhausen) • 384 Well Lightcycler plate (Sarstedt, Nümbrecht)
Pipette	Serological Pipette (2 mL, 5 mL, 10 mL, 25 mL) (Sarstedt, Nümbrecht)
QIA shredder	QIA shredder (Qiagen, Hilden)
Reaction tubes	Sarstedt, Nümbrecht: <ul style="list-style-type: none"> • Micro Tube: 0.5 mL, 1.5 mL, 2.0 mL • 15 mL centrifuge tube (“Falcon” type) • 50 mL centrifuge tube (“Falcon” type) Cryo.s™ 2 ml (Greiner Bio-One, Frickenhausen) Quali-PCR-Tubes 0.2 mL (Kisker, Steinfurt)
Syringe	BD, Heidelberg: <ul style="list-style-type: none"> • BD Discardit™ II Syringe 5 mL • BD Microlance™ 3 Needle

4.2 Cell culture

4.2.1 Cells used

Human periodontal ligament fibroblasts (HPDLF) and human osteoblasts (hOB) were obtained from healthy tissue of patients undergoing surgery for orthodontic reasons with informed consent (Ethics Committee LMU Munich Medical Center, registration number: 045-09). The following in-house established human primary cells were used:

Type of cell	Patient Id.	Age	Gender	Orthodontic treatment	Medium
hPDLF	170946	31	female	Unknown	DMEM, 10% FBS, 2% L-glutamine
hOB	379583	20	male	Pretreatment	DMEM/F12 (1:1), 10% FBS, 2% MEM vitamins

The primary cells were prepared using explant culture techniques according to Ng and Schantz (2010) with the following modifications. Freshly extracted teeth or bone biopsies were collected separately in sterile 50 mL centrifuge tubes (“Falcon tubes”) containing transport medium: PBS with 2× antibiotics/antimycotic mixture (Life TechnologiesTM, Darmstadt, Germany) and 2× gentamycin (Biochrom, Berlin, Germany). The samples were processed directly after collection.

hPDLF: The teeth were washed with transport medium. The middle third of the tooth root surface was scraped off using a sterile surgical blade and the periodontal ligament explants were placed in 6-well plates and cultivated with Dulbecco’s Modified Eagle’s medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Steinheim, Germany), 2% L-glutamine (Sigma-Aldrich, Steinheim, Germany) and 1× antibiotics/antimycotic mixture (Life TechnologiesTM, Darmstadt, Germany).

Bone: Bone biopsies from alveolar bone were diced into small pieces and washed three times with transport medium. Afterwards, they were three times digested with collagenase II, each time for 30 min at 37 °C. Cells from the last

digestion step were cultivated using Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich, Steinheim, Germany) with 10% FBS and 2% MEM-vitamins (Biochrom, Berlin, Germany) and 1× antibiotics/antimycotic mixture (Life TechnologiesTM, Darmstadt, Germany). The bone pieces were washed again in transport medium, placed in 6 well plates and covered with the same medium.

Both explant cultures, from hPDLF and hOB were placed in the CO₂ incubator with 5 % CO₂ at 37 °C in humidified atmosphere and incubated until the cells started outgrowing ("E1"). Upon reaching confluency, they were passaged (split ratio 1:3) and cultivated in the corresponding medium using 0.05 % trypsin-EDTA solution (Biochrom, Berlin, Germany). Cells at the fifth passage were used for processing the experiments.

The human osteosarcoma cell line SaOS-2 (bought from DSMZ) was used for evaluation and testing purposes. Cultivation was done using the same medium and procedures as described for human osteoblasts.

4.2.2 Mycoplasma assay

Regularly, cell culture supernatants are tested for mycoplasma infection. For mycoplasma testing, cell culture supernatants were collected from growing cell cultures at confluency. The supernatants were processed using the PCR Mycoplasma Test Kit I/C (PromoKine, Heidelberg, Germany) according to the manufacturer's instructions.

Specifically, testing samples (sample tube, 23 µl rehydration buffer and 2 µl sample), positive control (positive tube, 23 µl rehydration and 2 µl DNA-free water) and negative control (negative tube, 23 µl rehydration and 2 µl DNA-free water) were prepared. Then PCR was performed according to the manufacturers's protocol. Tubes were placed in the TProfessional Thermocycler (Biometra, Göttingen, Germany) machine. The PCR program consisted of the following steps: denaturation for 2 min at 95 °C; 40 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 55 °C, 40 s primer extension at 72 °C, and cooling to 4 - 8 °C.

PCR products were separated on a 1.5% standard agarose as described in Section 4.4.5: 8 μ l of each PCR product and 5 μ l of molecular weight marker per lane were loaded. Separation was done using agarose electrophoresis for 45 min at 90 V in 2% agarose, 1 \times TAE buffer.

4.2.3 Cell counting

When cells reached confluence, they were digested using 0.05 % trypsin-EDTA solution at 37 °C and 5% CO₂ for 5 min. Digestion was stopped using the respective cell culture medium. Cell counting was done using single-use Neugebaur counting chamber (Kisker Biotech, Steinfurt, Germany) and an inverted microscope (EVOS^{fl}, Invitrogen, Carlsbad, USA). Cell suspension and trypan blue solution (0.4%) (Sigma-Aldrich, Steinheim, Germany) were mixed 1:1. Ten microliters of this mixture was pipetted into each side of the counting chamber. Cells were counted at 10 \times magnification under the phase contrast microscope. Cells at four corner quadrants of each side of the chamber were counted as described in the documentation of the counting chamber used.

4.3 Force application

After cell preparation, compressive force (CF) was applied to hPDLF and hOB respectively using centrifugation according to Redlich et al. (1998), with modifications from Baumert et al. (2004). This model resembled clinical orthodontic force.

4.3.1 Pretesting temperature stability and apoptosis induction

In pre-experiments the temperature stability during centrifugation was examined and a workflow for CF application was established. To test the effect of temperature (30 °C vs 37 °C) an assay was used to test apoptosis induction.

4.3.1.1 Temperature stability during centrifugation

Long-term temperature stability during centrifugation was monitored using the Thermochrom iButton[®] data logger DS1921H-F5 (Maxim Integrated, San Jose, USA).

These data logger measure the temperature within a range of 0 to 46 °C with an accuracy of ± 1 °C and measuring intervals from 1 to 255 minutes.

The data logger was programmed and read-out using the OneWireViewer program, version 0.3.17.44 (Maxim Integrated, San Jose, USA) together with the 1-Wire Network Cable, DS1402D-DR8 (Maxim Integrated, San Jose, USA) (Figure 4).

Force application was done using the SIGMA 4-16K centrifuge (Sigma, Osterode am Harz, Germany). Speed, time and temperature are adjustable and read via a LCD display in front of the centrifuge. In parallel to the centrifugation cells, cells serving as negative controls are incubated in a microbiological incubator (Binder Incubator BD 23, Binder, Tuttlingen, Germany).

To establish a stable temperature during centrifugation, pretesting was done measuring by iButton devices. One iButton device was put on bench for measuring the room temperature, one was installed in the microbiological incubator (set at 30 °C) and another two were placed into the centrifuge at beginning (one for temperature measurement, the other for equilibrium). Different combinations of centrifugation speed, temperature preset and (with the aid of the technical service of the manufacturer) temperature offset (in the manufacturer settings of the centrifuge) were evaluated to achieve a constant and reproducible temperature regime.

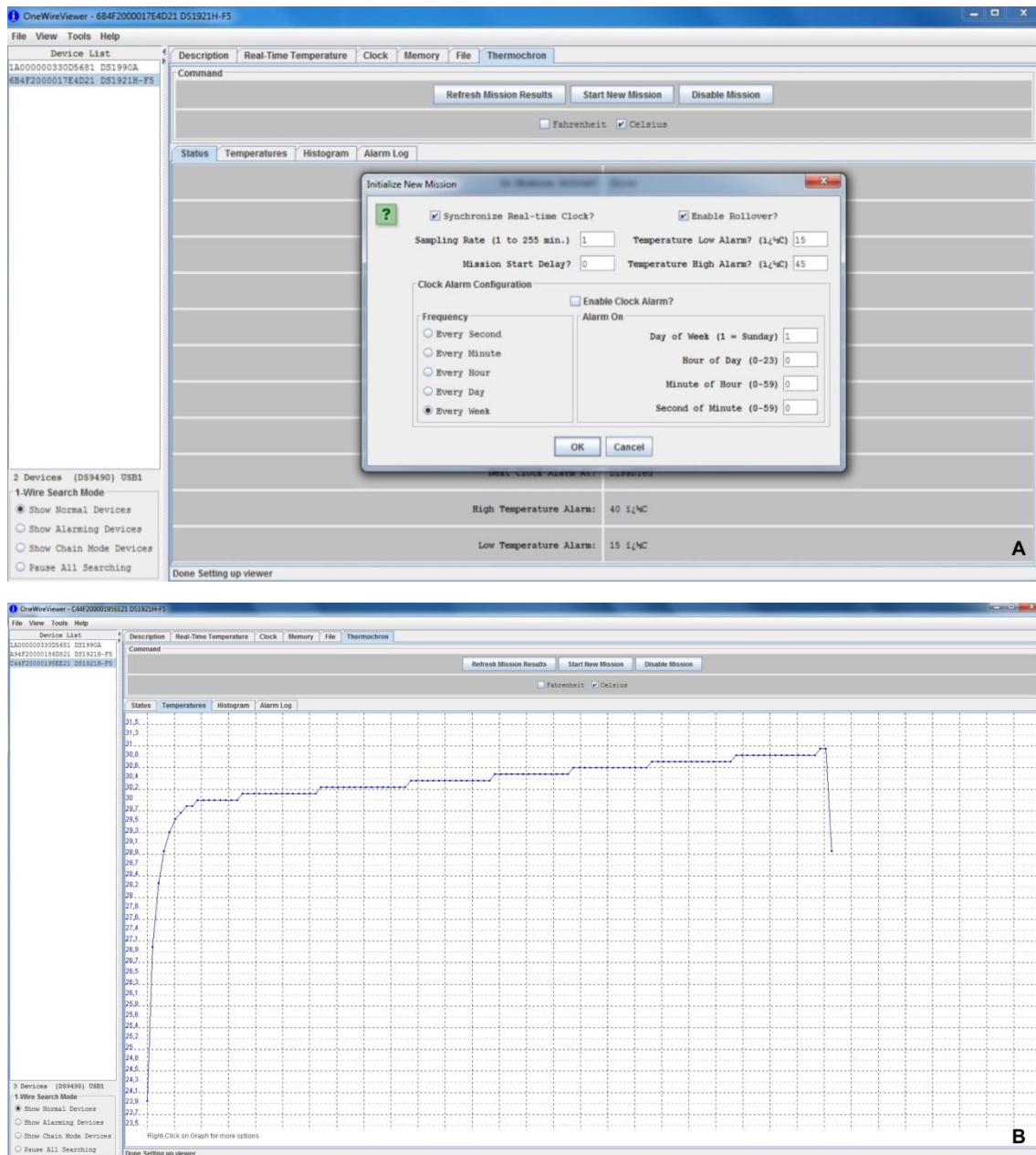


Figure 4. The interface of OneWireViewer program, version 0.3.17.44. (A) Programming for iButton device. (B) Read-out of iButton device.

4.3.1.2 Apoptosis assay

Cellular apoptosis was examined to test cell viability after incubation at 30 °C for up to 4 h in comparison to 37 °C. HPDLF and hOB were seeded in 12-well plates with 1×10^5 cells/ well, followed by overnight incubation at 37 °C with 5% CO₂. Plates representing the experimental group were incubated at 30 °C in a microbiological incubator. Control plates were kept at 37 °C and 5 % CO₂. After 1, 2 and 4 h of incubation, the cells were stained using the Multi-Parameter Apoptosis Assay Kit

(Cayman, Ann Arbor, USA) according to the manufacturer's protocol. Nuclear fragmentation was detected by Hoechst Dye staining, reversal of the mitochondrial membrane potential with TMRE and the flipping of the membrane lipids using Annexin V-FITC. Immediately after staining, the cells were examined using a fluorescence microscope (EVOS fl , Invitrogen, Carlsbad, USA). These assays were repeated three times for both cell types.

4.3.1.3 Procedure for compressive force application

All CF applications were monitored using iButton devices as follows: two iButton devices were used to monitor the room temperature and the temperature of the negative controls placed into the microbiological incubator. The centrifugation temperature was set to 30 °C and the temperature offset to -2.3. After positioning a third iButton device into the centrifuge, the centrifuge was pre-run at 800× g until the centrifuge reached the pre-set temperature of 30 °C shown on the centrifuge's display. Cells seeded in 12-well plates were placed in the centrifuge and the fourth iButton device was placed in the counter-weight plate. All centrifugations were done on the same day at 200× g and all centrifugations (1, 2, and 4 h) were monitored using iButton devices. The negative controls were kept in the microbiological incubator at 30 °C for the same durations as centrifugation (Figure 5).

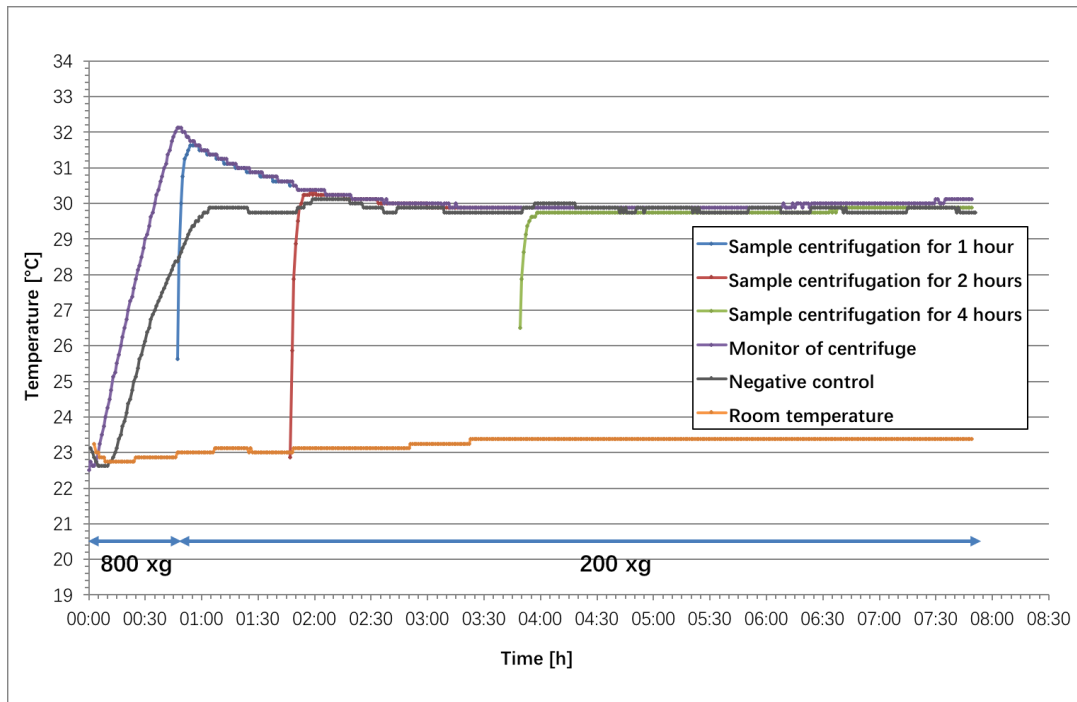


Figure 5. Temperature profile of a typical experiment running in total over 8 h. The temperatures (centrifuge, biological incubator and room temperature) were monitored using iButton[®] temperature data loggers. The centrifuge was pre-run at 800×g to reach the required temperature (30 °C) quickly. Then the experiments were performed at 200×g.

The cells were centrifuged at 200×g, 30 °C for 1, 2 and 4 h, respectively. Different levels of force (47.4 and 73.7 g/cm²) were adjusted by adjusting the volume of cell culture medium within each well according to the following formula (Redlich et al. 2004a; Redlich et al. 2004b):

$$P = \frac{m \times r \times rpm^2 \times \pi^2}{A \times 9.8 \times 900}$$

Force (P):	47.4 g/cm²	73.7 g/cm²
Mass of medium [m]:	0.9 g (~900 μL)	1.4 g (~1,400 μL)
Radius of centrifuge rotor (r):	0.16 m	0.16 m
Speed of centrifuge [rpm]:	970 rpm	970 rpm
Growth area of cells (A):	3.8 cm ²	3.8 cm ²

Cells in the microbiological incubator at 30 °C served as negative controls. For each cell type/culture combination six samples were used. Thereafter, CF was applied to the co-cultured hPDLF and hOB in the same way as mono-cultured, described previously.

4.3.2 Seeding of mono-culture setup

HPDLF and hOB were cultured in their specific culture media with 37 °C and 5% CO₂ in humidified atmosphere. Both cell types, hPDLF and hOB, were seeded the same way: in each well of a 12-well plate 10⁵ cells/well were seeded with 900 or 1400 μL culture medium. For 1, 2 and 4 h experimental durations, three plates were prepared as described above: one will be placed into the centrifuge for CF application (“experimental group”), one in the biological incubator (30 °C) as negative control and one will be used as blank control (T₀) (Figure 6). After attachment (about 6 h) in 37 °C and 5% CO₂, cell culture medium was replaced with cell culture medium containing 1% FBS (“serum starvation”). The plates were placed back to the 37 °C/ 5% CO₂ and incubated overnight for 10 h.

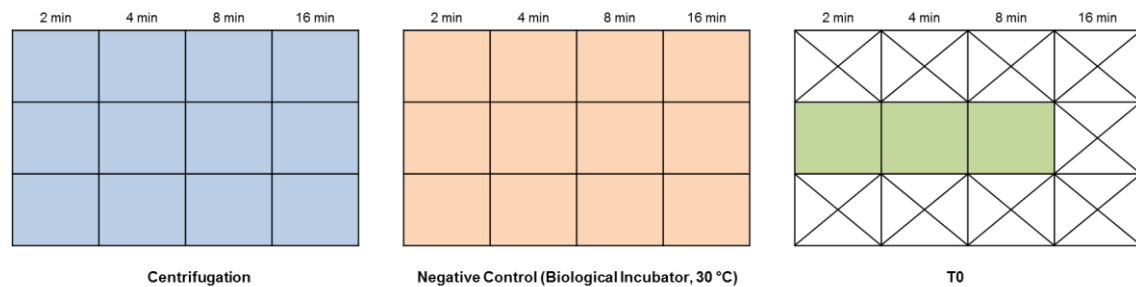


Figure 6. Cells were seeded in 12-well plates with 1×10^5 cells/well. The same seeding condition for both cell types (hPDLF and hOB). (A) Plates were seeded for CF application in the centrifuge, (B) negative control in biological incubator and (C) blank control (T₀) in cell culture incubator, respectively. This seeding was proceeded for each duration period (1, 2 and 4 h).

4.3.2.1 Cell culture inserts

ThinCert™ cell culture inserts (Greiner Bio-One, Frickenhausen, Germany) (Figure 7) is a device for indirect contact co-culture; different cell populations can be cultivated in the same cell culture environment. The advantage of this insert is that it keeps the two co-cultured cell populations separated from each other via a porous membrane made from a polyethylene terephthalate capillary pore membrane tightly sealed to the polystyrene housings. The pore size of the optical transparent membrane is 1.0 μm with 2×10^6 pores/cm². Due to its physical and chemical properties, the cell culture

inserts are applicable for light microscopy, electron microscopy, fluorescence microscopy, immunocytochemistry and co-culture experiments. The cell culture surface of a 12-well insert is 113.1 mm^2 with an inner diameter 13.85 mm, an outer diameter 15.85 mm and an overall height of 16.25 mm. The working volume is 0.3 - 1.0 ml per insert.

Using the cell culture inserts, two cell populations can be co-cultivated, one in the insert and the other one in the well. They are physically separated sharing the same culture environment. Paracrine communication is possible via the pores of the membrane.

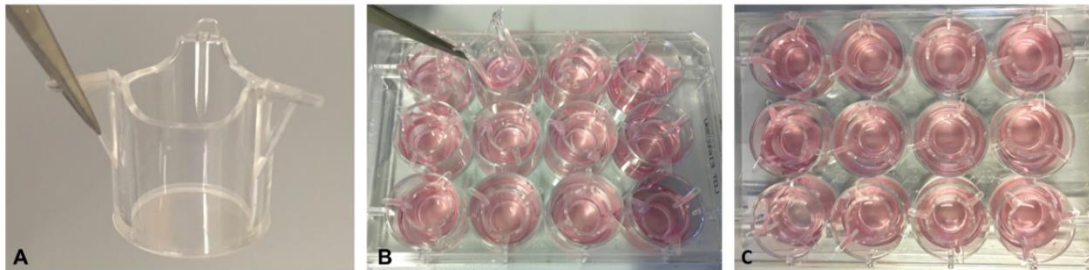


Figure 7. Cell culture inserts used: (A) Greiner ThinCert™ cell culture insert; (B, C) Inserts in 12-well plate.

4.3.2.2 Co-culture of human PDL fibroblasts and human osteoblasts

HPDLF and hOB were co-cultured. In hPDLF setup, hPDLF was seeded in 12-well plates at a density of 1×10^5 cells per well (Figure 8A). HOBs were seeded in ThinCert™ cell culture inserts (Greiner Bio-One, Frickenhausen, Germany) at a density of 5×10^4 cells per insert (Figure 8B). After attachment (about 6 h), cell culture media were removed. DMEM/F-12 containing 1% FBS was added to the wells. The seeded inserts were placed into the corresponding wells (Figure 8C) and also filled with DMEM/F-12 giving a total volume of 900 μL or 1400 μL . They were co-cultivated for at least 10 h. Then, CF was applied to co-cultured hPDLF and hOB in the same way as mono-cultured force loading. In hOB setup, the positions of cell types were switched (Figure 8D-F). Same amount of plates was seeded as mono-culture.

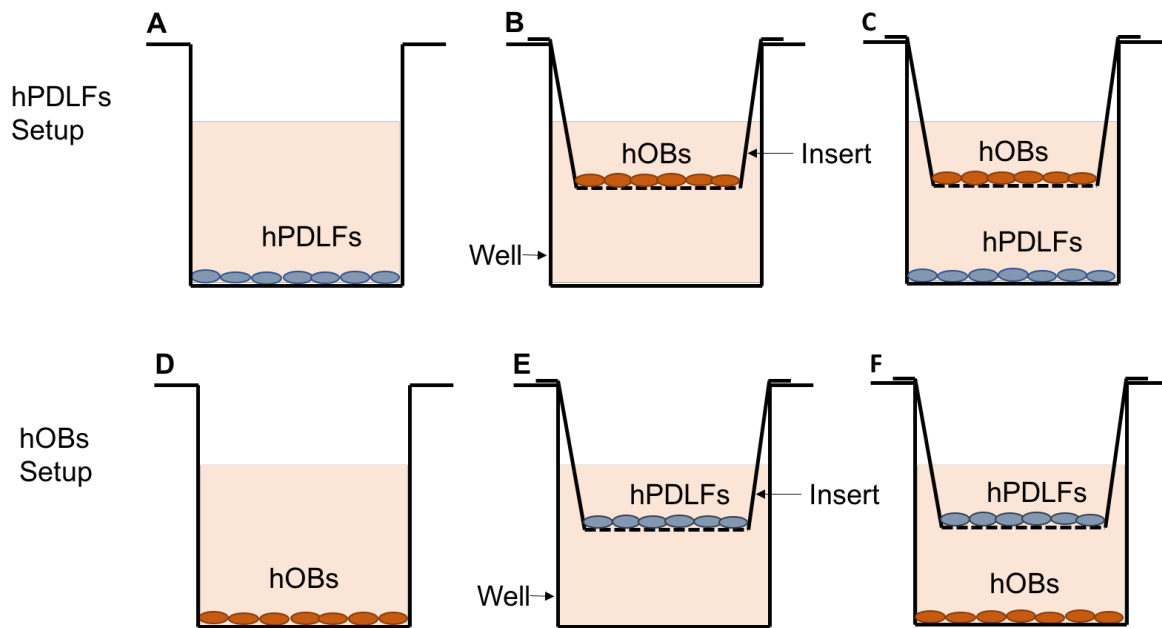


Figure 8. Co-culture setup. (A) hPDLF was seeded in the wells of 12-well plates. (B) hOB was seeded in the cell culture inserts. (C) Cell culture inserts were placed into wells at least 10 h before CF application. (D) hOB was seeded in the wells of 12-well plates. (E) hPDLF was seeded in the cell culture inserts. (F) Cell culture inserts were placed into wells at least 10 h before CF application.

4.3.2.3 Pulse chase multi-sampling

After each centrifugation and incubation, samples were collected at 2, 4, 8, and 16 min. Time was counted strictly with timer and three biological replications were performed for every chase point. From each sample 800 μ l of cell culture supernatant was collected, centrifuged at 15,000 rpm, 4 $^{\circ}$ C for 10 min and then transferred to a fresh Eppendorf tube. These samples were used for ELISA analysis of PGE₂, IL6 and TNF.

After removal of the cell culture supernatants from each well, hPDLF and hOB were lysed using a total amount of 750 μ L RNA lysis buffer (Zymo Research, Irvine, America) with 40 mM dithiothreitol (DTT) (AppliChem, Darmstadt). The cell lysates from each well were transferred to fresh, sterile 1.5 ml Eppendorf tubes and stored at -80 $^{\circ}$ C until further processing. These lysates were used for gene expression analysis of *IL6*, *TNF*, *RUNX2*, *P2RX7*, and *PTGS2*. After rigorously testing several potential genes, *B2M* was selected as the reference gene (see below).

4.4 Gene expression analysis

All samples from one experimental condition (cell type, force magnitude, force duration, culture condition) were processed and analyzed in parallel.

4.4.1 RNA Purification

The required RNA lysates were defrosted. Before continuing RNA isolation, all cell lysates were vortexed and spun down briefly. All further procedures were carried out at room temperature. First, the cell lysates were passed through individual QIAshredder columns (Qiagen, Germany) to fragment high molecular weight genomic DNA and other high molecular cellular components. This is fundamental for a successful RNA isolation. Afterwards, total RNA was isolated using the Quick-RNA™ MicroPrep Kit (Zymo Research, Irvine, America) according to the instruction manual as following:

1. An equal volume of ethanol (95-100 %) was added to the sample and mixed.
2. The mixture was transferred to a Zymo-Spin IC Column placed into a collection tube and centrifuged for 30 seconds. The flow-through was discarded. To process samples >800 µL, samples were stepwise loaded onto the same Zymo-Spin columns.
3. RNA Wash Buffer was added to the column and centrifuged for 30 seconds. Flow-through was discarded.
4. For in-column DNase I treatment the DNase I reaction mix provided by the manufacturer was added directly to the column matrix, incubated at room temperature for 30 minutes and then centrifuged for 30 seconds.
5. RNA Prep Buffer was added to the column and centrifuged for 30 seconds. Flow-through was discarded.
6. RNA Wash Buffer was added to the column and centrifuged for 30 seconds. Flow-through was discarded.
7. RNA Wash Buffer was added and centrifuged the column for 2 minutes to ensure complete removal of the wash buffer.

8. The column was placed into an RNase-free tube. DNase/RNase-Free Water (15 μ L, preheated to 95 °C) was added directly to the column matrix and centrifuged at 15,000 rpm for 30 seconds.
9. RNAsin[®] ribonuclease inhibitor (Promega, Madison, USA) was added to the total RNA preparation to give a final concentration of 1U/ μ L: for 15 μ L total RNA solution 0.375 μ L RNAsin[®] was added.
10. The processed RNA samples were stored at -80°C until further use.

4.4.2 Quality and quantification of total RNA

Total RNA yields were quantified using the NanoDrop1000 photometer (Peqlab, Erlangen, Germany) directly after purification. All samples were vortexed and spun down for a few seconds. One microliter of each sample was used for RNA quality and quantification examination using the absorbance readings at 230 nm, 260 nm and 280 nm. Pure RNA preparations have a A_{260}/A_{280} in the range of 2.0-2.2 (Green and Sambrook 2012, pp. 365-366). Ratios below indicate contamination with proteins or phenol. The ratio A_{260}/A_{230} is used to evaluate possible contamination with organic compounds or chaotropic salts. For pure RNA a A_{260}/A_{230} ratio of 2.0 should be expected (Green and Sambrook 2012, pp. 365-366).

4.4.3 Complementary DNA (cDNA) synthesis

From each RNA sample 600 ng total RNA was reverse transcribed into cDNA using the SuperScript[®] IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, USA) in a TProfessional Thermocycler (Biometra, Göttingen, Germany). cDNA synthesis process was performed according to the instruction manual:

1. To remove possible secondary structures in the RNA and to ease primer annealing, 600 ng total RNA (volume depending on RNA concentration), random hexamers (50 ng/ μ L), 10 mM dNTP mix in a total volume of 13 μ L are heated to 65 °C for 5 min and then rapidly cooled down on ice.
2. After addition of SSIV buffer (5 \times), DTT (100 mM), 2.0 U/ μ L RNase inhibitor and 200 U/ μ L reverse transcriptase, incubation continues according to the following temperature profile: 10 min at 23 °C; 10 min at 50 °C; 10 min at 80 °C

3. After completion, RNA was eliminated from the reaction mixture with the addition of 1 μ L RNase H and further incubation for 20 min at 37 °C.
4. The cDNA was stored at -20 °C and diluted 1:5 for gene quantification using quantitative real-time PCR.

4.4.4 PCR primer design

Potential PCR primers derived from the literature or bought from Realltimeprimers.com (with purchase of a primer pair the relevant primer sequences were made available) were evaluated using the following workflow adapted from Thornton and Basu (2015) exemplified for human *PTGS2* in the appendix:

- “HomoloGene”¹ at NCBI was used for selection of the current version of the mRNA reference sequence and checking for splice variants.
- “Electronic PCR”² (Rotmistrovsky et al. 2004) was used to test the specificity of mRNA/cDNA amplification and possible genomic DNA co-amplifications due to genomic DNA contamination of the samples. The size deviation of transcriptome was chosen up to 200 base pairs and genome up to 500 base pairs. A maximum of 2 mismatches and 2 gaps were allowed. “Electronic PCR” is now defunct and replaced by “Primer Blast”³ (Ye et al. 2012).
- Energy considerations of PCR products were checked using the “UNAFold” implementation at IDTdna⁴ (maximum sequence length allowed: 255 bp) using the following settings: nucleotide type “DNA”; sequence type “linear”; temperature at 60 °C; Na⁺ concentration at 50 mM; Mg²⁺ concentration at 3 mM; all the others were left with default.
- “Beacon Designer Free Edition”⁵ (Premier Biosoft, Palo Alto, America) was used for energy evaluation. The web service was used with the default settings.

¹ URL: <http://www.ncbi.nlm.nih.gov/homologene/> (Date accessed: 06-12-2017)

² URL: <http://www.ncbi.nlm.nih.gov/projects/e-pcr/reverse.cgi> (not available anymore!)

³ URL: <http://www.ncbi.nlm.nih.gov/BLAST/> (Date accessed: 06-12-2017)

⁴ URL: <http://eu.idtdna.com/UNAFold> (Date accessed: 06-12-2017)

⁵ URL: <http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1> (Date accessed: 06-12-2017)

- *De novo* PCR primer design was applied using “Primer3plus”⁶ with the following settings: reference mRNA sequence file with GenBank accession number; product size ranges from 90 to 200; primer size with 18-20-27; primer T_m with 58-60-62; Maximum T_m difference with 2; Mispriming/Repeat library “HUMAN”.

Forward and reverse primers (Table 1) were synthesized by Metabion (Planegg, Germany) and delivered separately as 100 μ M solutions in 10 mM Tris-EDTA (TE) buffer. Primer pairs from Realltimeprimers.com were delivered already diluted to 0.1 μ M.

4.4.5 Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)

The sequences of the gene-specific primers are listed in Table 1. Quantitative real-time RT-PCR was carried out using the Luminaris Color HiGreen qPCR-Mastermix (Thermo Scientific, Schwerte, Germany) in a LightCycler 480 PCR (Roche, Basel, Switzerland) according to the instructions of manufacturer. Each PCR reaction (final volume: 20 μ L) consisted of

- 2 μ L of diluted cDNA,
- 10 μ L of qPCR-MasterMix,
- 0.3 μ M of each forward and reverse primer (synthesized primers) or 0.2 μ M of Realltimeprimers.com primer solution,
- 6.8 μ L of deionized water.

The RT-PCR was started with a pretreatment step for 2 min at 50 °C and followed by an initial denaturation step for 10 min at 95 °C. Then amplification of 45 PCR cycles had been done, which contained 15 s denaturation at 95 °C, 30 s primer annealing at primer specific temperatures (Table 1), 30 s primer extension at 72 °C, and additional data acquisition step at 5 °C below specific gene melting point (Table 1) for 5 s. The

⁶ URL: <http://primer3plus.com/cgi-bin/dev/primer3plus.cgi> (Date accessed: 06-12-2017)

PCR products of each gene were further tested by electrophoresis on a 2% agarose gel to ensure the specificity of each primer and PCR fragment.

Table 1: The gene-specific primer sequences for the forward (f) and reverse (r) primers and the specific reaction conditions of *B2M*, *PTGS2*, *IL6*, *RUNX2*, *P2RX7* and *TNF*.

Gene	Primer sequence or order information	Reference sequence [GenBank accession number]	Amplification length [bp]	Annealing temp. [°C]	Data acquisition temp. [°C]	Primer efficiency
<i>B2M</i>	Realtimerprimers.com: HHK-1	NM_004048.2	86	58	77	1.887
<i>PTGS2</i>	f: AAG CCT TCT CTA ACC TCT CC r: GCC CTC GCT TAT GAT CTG TC	NM_000963.3	234	58	77	1.921
<i>IL6</i>	f: TGG CAG AAA ACA ACC TGA ACC r: TGG CTT GTT CCT CAC TAC TCT C	NM_000600	168	58	76	1.85
<i>RUNX2</i>	f: GCG CAT TCC TCA TCC CAG TA r: GGC TCA GGT AGG AGG GGT AA	NM_001015051	176	58	81	1.875
<i>P2RX7</i>	f: AGT GCG AGT CCA TTG TGG AG r: CAT CGC AGG TCT TGG GAC TT	NM_002562	143	58	78	1.916
<i>TNF</i>	Realtimerprimers.com: VHPS-9415	NM_000594	173	58	79	1.933

Primer efficiency was evaluated for all primer pairs as follows: total RNA preparations from SaOS-2 cells generated during pre-tests of the force application protocol were used. Serial dilutions of cDNA were prepared: undiluted, 1:10, 1:100, 1:1000, and 1:10000. Quantitative real-time RT-PCR was carried out as described above. Standard curves were generated using the LightCycler® 480 software (version 1.5.1) and the primer efficiency was calculated. This is exemplified for *B2M* primer efficiency determination in Figure 9.

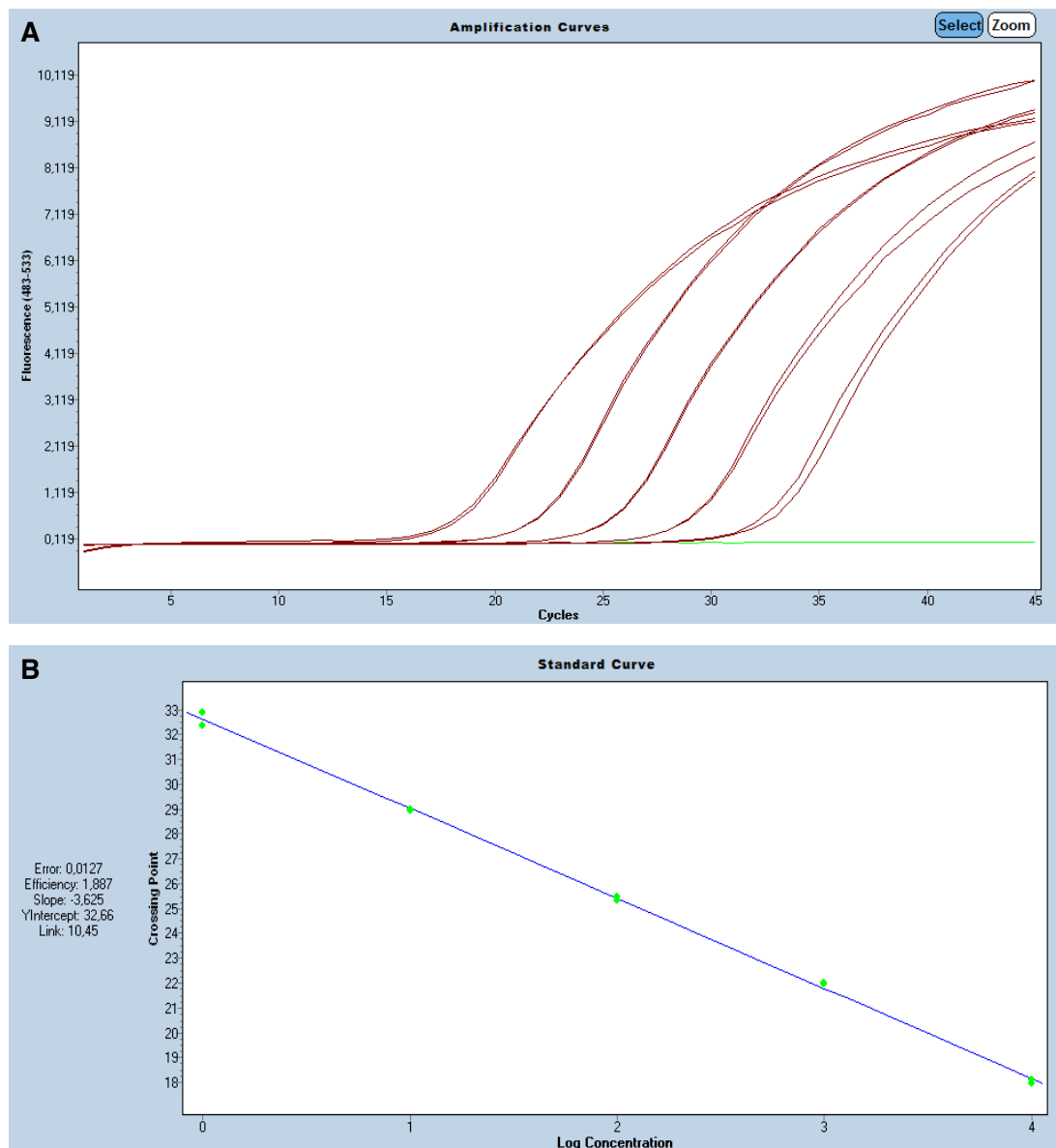


Figure 9. *B2M* primer efficiency was determined after quantitative real-time RT-PCR. (A) Amplification curves of serial dilutions of cDNA were shown. (B) Standard curve was calculated.

Five potential genes (*PGK1*, *PPIA*, *RPL*, *B2M* and *TFRC*) from realtimeprimers.com “housekeeping panel” HHK-1 were examined to serve as a potential reference gene for relative quantitative real-time PCR. To do so, cDNA from both negative control and experimental condition, drawn during the validation of the centrifugation model, were prepared as described above. Quantitative real-time PCR was done as shown above and the results were evaluated. A “reference gene” (also called “housekeeping gene”) is a gene that is neither regulated in the negative controls

nor in the experimental condition. Therefore, it has to be established for each experimental condition separately. *B2M* was selected as the reference gene, because it showed the most stable gene expression within an experiment independently of being negative control, T_0 or experimental condition (Figure 10).

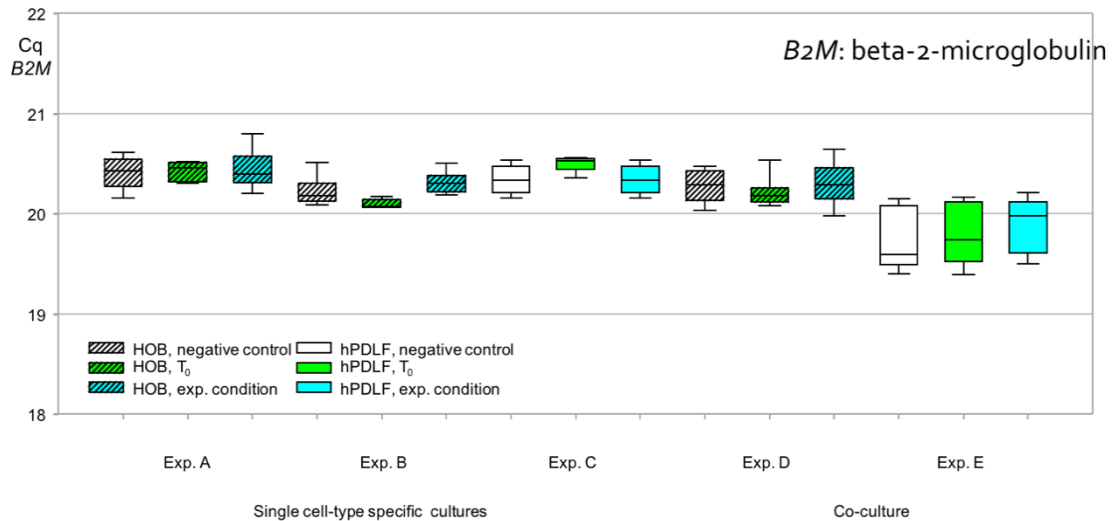


Figure 10. The boxplots show the C_q raw values generated by LightCycler480 software in automatic modus. Each experiment consisted of three sample sets: samples from negative control, T_0 and experimental condition. Experiments A to C were mono-cultures. Experiments D and E were co-cultures with changed cell types in well and cell culture insert.

After each PCR, the specificity and size of the PCR products was evaluated using 2 % agarose gels. These gels were prepared according to the manufacturer of the agarose gel apparatus (EasyPhor Midi, Biozym, Hess. Oldendorf, Germany):

1. The running buffer was prepared from 50×TAE buffer by dilution to 1×TAE buffer using distilled water.
2. To prepare a 2 % agarose solution, 1 g agarose was dissolved in 50 mL 1×TAE with boiling in the microwave oven. This amount is enough, to pour one agarose gel of 10×10 cm² size.
3. After cooling down to ca. 50 °C, two drops of 0.07 % ethidium bromide (Applichem, Darmstadt, Germany) were added.
4. The gel was poured according to the manufacturer's instructions and the sample loading combs were inserted. After solidification, the gel was transferred to the tank and prepared according to the manufacturer's instructions.

5. From each individual PCR reaction 10 μ L PCR product and 2 μ L DNA loading buffer (containing Orange G) were mixed.
6. After the gel was solidified, 8 μ L of each prepared sample and 5 μ L of DNA molecular weight marker (“GeneRuler 100bp Plus DNA Ladder”, “GeneRuler Low Range DNA Ladder”) were loaded onto the gel.
7. Then the gel was run at 90 V for 45 minutes.
8. Visualization of the DNA pattern was done using the LIAS image analysis system (Avegene Life Science, Chinese Taipei). Printouts were prepared using a thermos printer (Video Graphic Printer, Sony, Tokyo, Japan).

4.5 Analysis of cell culture supernatants using enzyme-linked immunosorbent assays

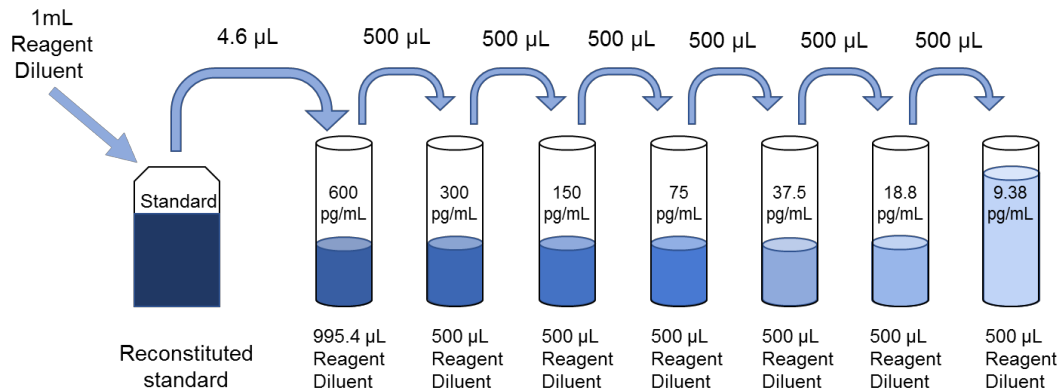
HPDLF and hOB cells were subjected to CF at 30 °C for 1, 2 and 4 h in two different setups (mono- and co-culture). After each experimental condition, 800 μ l cell culture supernatant was collected at 2, 4, 8 and 16 min. To remove cell debris and other particular material all samples were centrifuged at 15,000 rpm for 10 minutes at 4 °C. The clear cell culture supernatant was then transferred into a fresh tube and stored at 80 °C. These samples were used for enzyme-linked immunosorbent assay (ELISA) for IL6, TNF, and PGE₂ concentration determination.

4.5.1 Measurement of IL6 protein concentration

Samples of cell culture medium supernatants stored at -80 °C were defrosted. IL6 protein concentration in the supernatants was measured using the “Human IL6 Duo Set ELISA kit” (R&D Systems, Minneapolis, USA) according to the manufacturer’s instruction. In short, the specific procedure was performed as follows:

1. The 96-well plate was coated with diluted capture antibodies overnight at room temperature.
2. The next morning, the 96-well plate was washed three times with diluted wash buffer.

3. Then, the plate was blocked with reagent diluent for 1.5 h at room temperature.
4. Afterwards, the plate was washed again three times with diluted wash buffer.
5. The IL6 standard provided with the kit was prepared according to the manufacturer's instructions by serial dilution using reagent diluent solution:



6. Sample and standards were added in to defined wells and incubated at room temperature for 2 h.
7. The plate was washed three times with diluted wash buffer.
8. Diluted detection antibody was added to each well and incubated at room temperature for 2 h.
9. The plate was washed three times with diluted wash buffer.
10. Streptavidin-HRP was diluted as stated in the protocol. The diluted Streptavidin-HRP was then added to each well and incubated for 20 min at room temperature
11. The plate was washed three times with diluted wash buffer.
12. Substrate Solution was added to each well and incubated for 20 min at room temperature.
13. Stop Solution was added to each well.
14. Optical density of each well was determined immediately using a microplate reader at 450 nm and 540 nm.
15. IL6 concentration was determined using a four-parameter logistic (4-PL) curve using the IL6 standards included in each measurement.

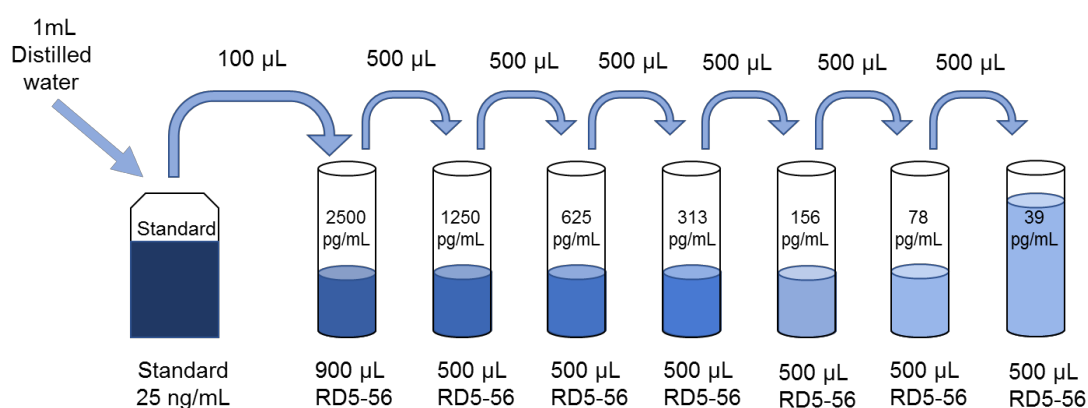
4.5.2 Measurement of TNF protein levels

TNF protein concentrations in cell culture medium was measured using the “Human TNF α Duo Set ELISA kit” (R&D Systems, Minneapolis, USA) according to the manufacturer’s instruction. The procedure follows the same steps as exemplified with the “IL6 Duo Set ELISA kit” from the same manufacturer (see above).

4.5.3 Measurement of PGE₂ levels

Samples of cell culture medium supernatants stored at -80 °C were defrosted. PGE₂-ELISA was carried out for all samples from both negative controls and experimental conditions. The “PGE₂ Parameter Assay kit” (R & D Systems, Minneapolis, USA) was used according to the manufacturer’s protocol:

1. Wash buffer and substrate solution were prepared in advance.
2. PGE₂ standards were prepared according to the manufacturer’s instructions:



3. Calibrator Diluent RD5-56 (200 µL) was added to the non-specific binding (NSB) wells. Calibrator Diluent RD5-56 (150 µL), standards (150 µL) and samples (150 µL) were added to the zero standard (B0) and remaining wells respectively.
4. Primary antibody solution was added into each well excluding the NSB and incubated for 1 h at room temperature on a shaker (IKA[®]-Werke GmbH &Co. KG, Staufen, Germany).
5. PGE₂ conjugate was added to each well and followed by 2 h of incubation at room temperature on the shaker.

6. The whole plate was washed four times with wash buffer.
7. Substrate solution was added to each well and incubated 30 min at room temperature in the dark.
8. Stop solution was added to each well.
9. Optical density was determined at 450 nm and 540 nm using a microplate reader.
10. PGE₂ concentration was determined using a four-parameter logistic (4-PL) curve using the standards included in each measurement.

4.6 Statistical analysis

To evaluate significant changes between negative control and experimental condition the independent-samples Mann-Whitney U test was applied using IBM SPSS Statistics 24 (IBM Corp., Armonk, N.Y.). The $p < 0.05$ was considered as a statistically significant difference.

5 Results

An experimental setup was established to enable compressive force (CF) application on cells in co-culture enabling variations in force magnitude and force duration. This new model differed from previously established models in two ways: (1) force was applied to cells in mono- and co-culture using a temperature adjusted centrifuge running at 30 °C and (2) for durations of 1, 2 and 4 h.

The performance of this model was controlled using a set of temperature data loggers to monitor temperature stability and a microscopic apoptosis assay to evaluate the influence of temperature (30 °C vs. 37 °C) on cell viability. These results are presented in Section 5.1.

Human periodontal ligament fibroblasts (hPDLF) and human primary osteoblasts (hOB) were cultivated in mono- and co-culture and subjected to centrifugation forces for different durations. Gene expression for *TNF*, *IL6*, *PTGS2*, *RUNX2* and *P2RX7* was evaluated using quantitative real-time PCR (qPCR), protein secretion into the cell culture supernatant was determined using ELISA for PGE₂, IL6 and TNF. Results from mono- and co-culture were compared. To mimic “direct sampling”, measurements from 2 and 4 min chase are combined and presented in Section 5.2. “Pulse chase” sampling results are shown as is in Section 5.3.

5.1 Validation of the *in vitro* model for compressive force application

To evaluate the effect of the reduced temperature during centrifugation, apoptosis assays were used. HPDLF and hOB seeded in 12-well plates were incubated at 30 °C (experimental groups) and at 37 °C (control groups) for 4 h. The assay was a multi parameter assay detecting different stages of apoptosis in the same sample. Defragmentation of nuclei was assessed using Hoechst Dye staining, mitochondrial membrane potential break-down was detected using TMRE staining and flipping of membrane lipids was detected using annexin V-FITC antibody labeling. The results of a representative experiment are shown in Figure 11. Both, the experimental group at

30 °C as well as the control group at 37 °C present identical findings: after up to 4 h of incubation at 30 °C in comparison to 37 °C no signs of apoptosis were detectable. Hoechst staining showed that all the cells presented round and intact nuclei (Figure 11, a4-d4), TMRE staining revealed that cells had undisrupted mitochondrial membrane (Figure 11, a3-d3) and all the cells were annexin V negative (Figure 11, a2-d2).

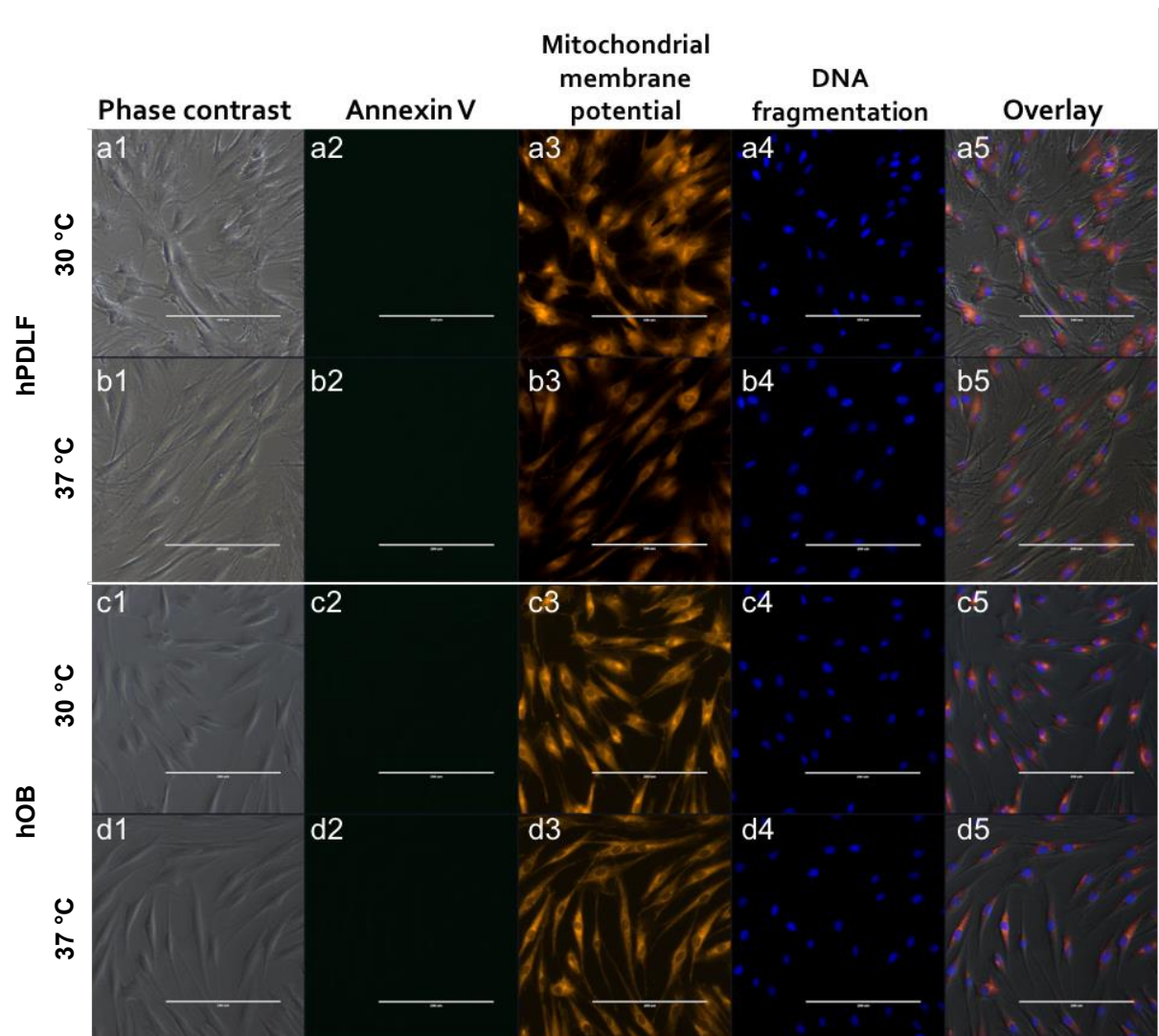


Figure 11: Apoptosis staining of hPDLF (a, b) and hOB (c, d) incubated at 30 °C (a, c) or 37 °C (b, d) for 4 h. Cells were stained with TMRE/Hoechst Dye and annexin V-FITC. Phase contrast (a1-d1), annexin V (a2-d2), mitochondrial membrane potential (a3-d3), DNA fragmentation (a4-d4) and overlay (a5-d5) images are shown. Scale bar: 200 μ m.

To evaluate temperature stability during force application with centrifugation, temperature data loggers were used to monitor the temperatures during centrifugation

in the centrifuge and in the microbiological incubator used for the controls. Additionally, the room temperature during centrifugation was measured. The temperature was recorded throughout all experiments. Figure 5 shows the measurements during an experimental cycle: force applications for 1, 2 and 4 h were applied in order on the same day. The room temperature was constant at 23 ± 1 °C during the experiments. To shorten total centrifugation time and to reach a stable temperature of 30 °C, the centrifuge was set to $800\times g$ for 47 min. Afterwards, the centrifuge was set to $200\times g$ and this setting was then used for all three consecutive experimental force applications. The data shows, that the temperature within the centrifuge was stable at 30.5 ± 1 °C (Figure 5). Identical temperature profiles were recorded in all experiments.

5.2 “Direct sampling”

5.2.1 Gene expression in mono-cultured cells after low mechanical force application

Tumor necrosis factor (TNF) gene expression was slightly upregulated in human periodontal ligament fibroblasts (hPDLF) (Figure 12A) after 1, 2 and 4 h of CF application. Prostaglandin-endoperoxide synthase 2 (*PTGS2*) gene expression increased significantly 2.25-fold ($p = 0.001$) after 1 h of CF and this increase was even more pronounced (4.86-fold) ($p < 0.001$) after 2 h of CF (Figure 12C). Interleukin 6 (*IL6*) gene expression showed a significant 1.34-fold ($p < 0.001$) upregulation after 1 h, 1.41-fold ($p < 0.001$) upregulation after 2 h and 1.13-fold upregulation after 4 h of CF application (Figure 12E). Runt-related transcription factor 2 (*RUNX2*) gene expression increased significantly after 1, 2 and 4 h of CF durations in hPDLF (Figure 12G). Gene expression of the purinergic ion channel/receptor *P2RX7* increased after 2 ($p = 0.005$) and 4 h ($p = 0.012$) of CF in hPDLF (Figure 12I).

In human osteoblasts (hOB), *TNF* gene expression significantly increased 4-fold ($p < 0.001$) after 1 h of CF but remained unchanged after 2 and 4 h CF application (Figure 12B). *PTGS2* gene expression showed a 2.5-fold upregulation ($p < 0.001$) after

1 and 2 h of CF application, and continuously increased to 4.37-fold ($p < 0.001$) after 4 h of CF application (Figure 12D). A similar pattern was observed for *IL6* gene expression: only a small but significant increase in *IL6* gene expression after 1 ($p < 0.001$) and 2 h ($p < 0.001$) of CF but 5.15-fold ($p < 0.001$) after 4 h of CF application (Figure 12F). *RUNX2* gene expression was upregulated after 1 ($p < 0.001$) and 2 h ($p < 0.001$) of CF (Figure 12H), and *P2RX7* gene expression increased after 1 h ($p = 0.01$) but decreased after 4 h ($p < 0.001$) of CF (Figure 12J).

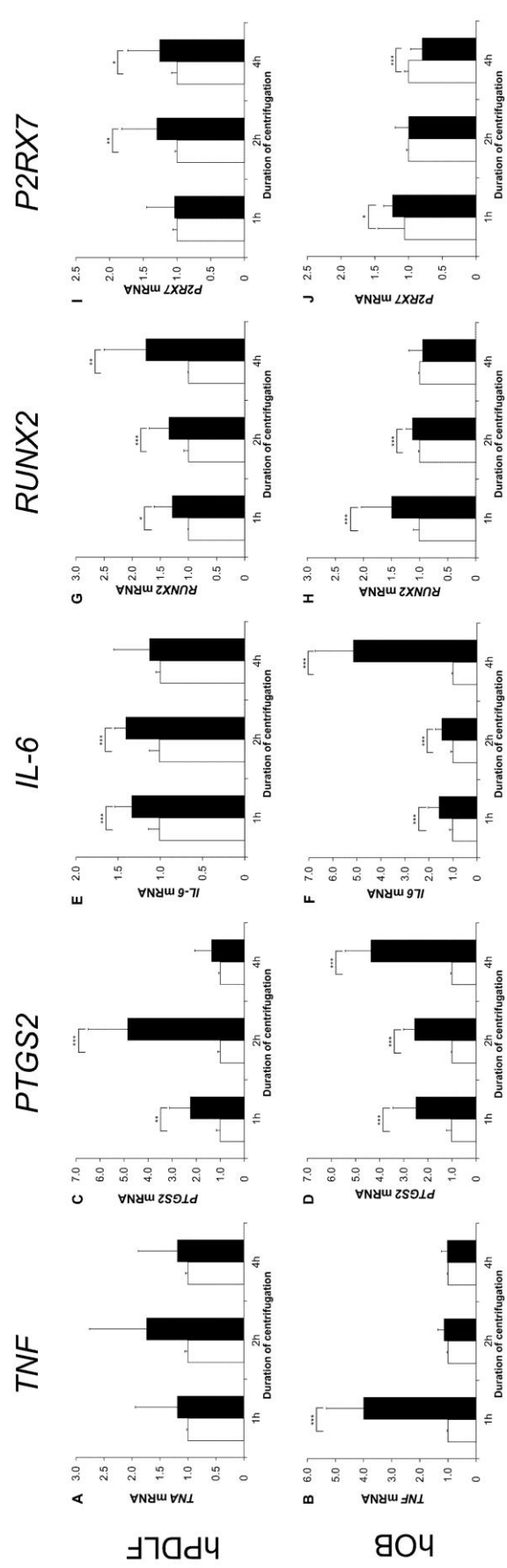


Figure 12. Gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* in mono-cultured hPDLF and hOB after 1, 2 and 4 h of CF at 47.4 g/cm². The $\Delta\Delta C_q$ method was applied and B2M was used as the reference gene. Expression level of control was defined as 1. Values are presented as mean \pm SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). □: Control ■: CF.

5.2.2 Gene expression in mono-cultured cells after higher mechanical force application

HPDLF and hOB were also subjected to 73.7 g/cm^2 CF in mono-cultured setup. After 1, 2 and 4 h force application, gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* in hPDLF and hOB were measured using quantitative real-time PCR. Presented in Figure 13, *TNF* was upregulated after 1 ($p < 0.001$) and 4 h of CF, but slightly decreased after 2 h of CF in hPDLF (Figure 13A). *PTGS2* and *IL6* gene expression showed significant increase after 1, 2 and 4 h of CF durations ($p < 0.001$) in hPDLF (Figures 13C, 13E). As shown, *RUNX2* ($p = 0.003$) (Figure 13G) and *P2RX7* ($p = 0.002$) (Figure 13I) gene expression were increased remarkable after 1 h of CF in hPDLF.

In hOB, the gene expression of *TNF* was no regulated after 1 h of CF in hPDLF. Whereas it was decreased after 2 h ($p < 0.001$) and increased significantly after 4 h ($p < 0.001$) of CF in hPDLF (Figure 13B). Both *PTGS2* and *IL6* gene expression were significantly upregulated after 1, 2 and 4 h of CF durations ($p < 0.001$) (Figures 13D, 13F). No significant regulation of *RUNX2* and *P2RX7* gene expression was observed after 1, 2 and 4 h of CF durations (Figures 13H, 13J).

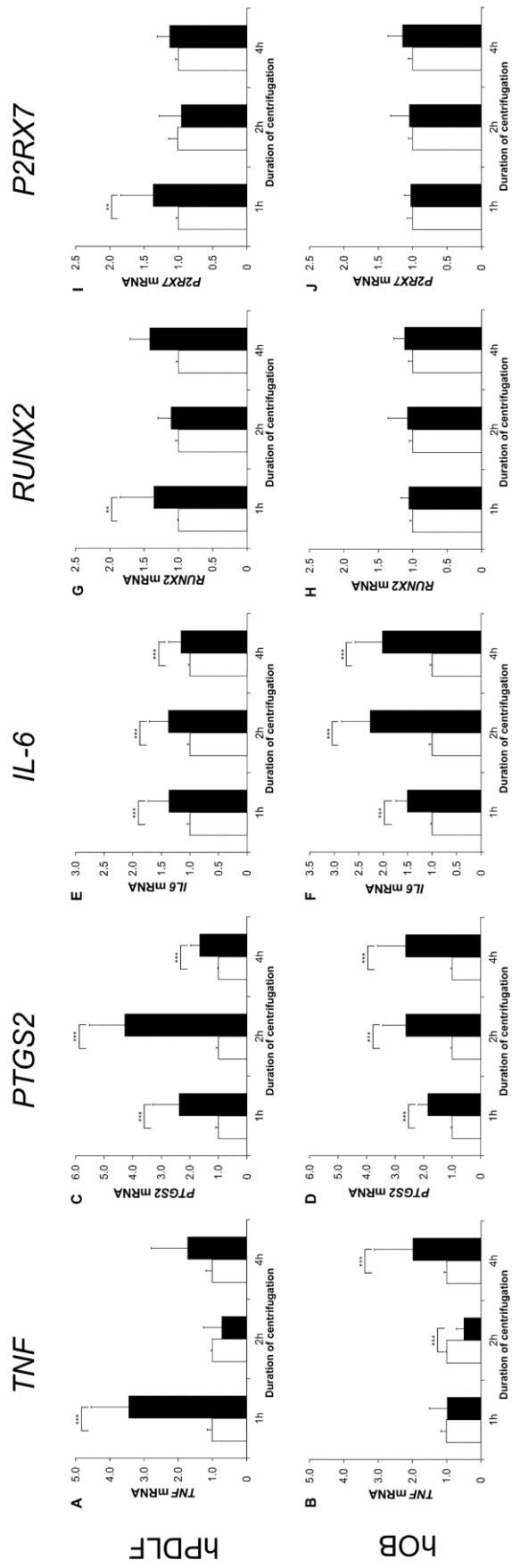


Figure 13. Gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* in mono-cultured hPDLF and hOB after 1, 2 and 4 h of CF at 73.7 g/cm². The $\Delta\Delta C_q$ method was applied and B2M was used as the reference gene. Expression level of control was defined as 1. Values were presented as mean \pm SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). \square : Control \blacksquare : CF.

5.2.3 Gene expression in co-cultured cells after lower mechanical force application

Lower (47.4 g/cm^2) CF was applied on co-cultured hPDLF and hOB and gene expression was analyzed using quantitative real-time PCR. In hPDLF, no significant gene expression regulation was detected for *TNF* (Figure 14A). *PTGS2* gene expression was upregulated 1.89-fold ($p = 0.005$) after 1 h of CF. However, it decreased significantly ($p < 0.001$) after 4 h of CF (Figure 14C). *IL6* gene expression showed significant decrease after 4 h of CF ($p = 0.001$), but no significant difference was found after 1 and 2 h of CF (Figure 14E). *RUNX2* gene expression was downregulated after 4 h of CF ($p = 0.001$) (Figure 14G). *P2RX7* gene expression was upregulated after 1 ($p < 0.001$) and 4 h of CF ($p = 0.004$) (Figure 14I).

In hOB, *TNF* gene expression slightly increased 1.74-fold ($p = 0.002$) after 2 h of CF (Figure 14B). *PTGS2* gene expression significantly increased after 1 h of CF (1.41-fold; $p < 0.001$) and 2 h of CF (2.87-fold; $p < 0.001$) but reduced to control level after 4 h of CF (Figure 14D). *IL6* gene expression was upregulated 2.91-fold ($p = 0.001$) after 2 h and 1.39-fold ($p = 0.005$) after 4 h of CF (Figure 14F). Both *RUNX2* and *P2RX7* gene expression showed significant decrease after 2 and 4 h of CF (Figures 14H, 14J).

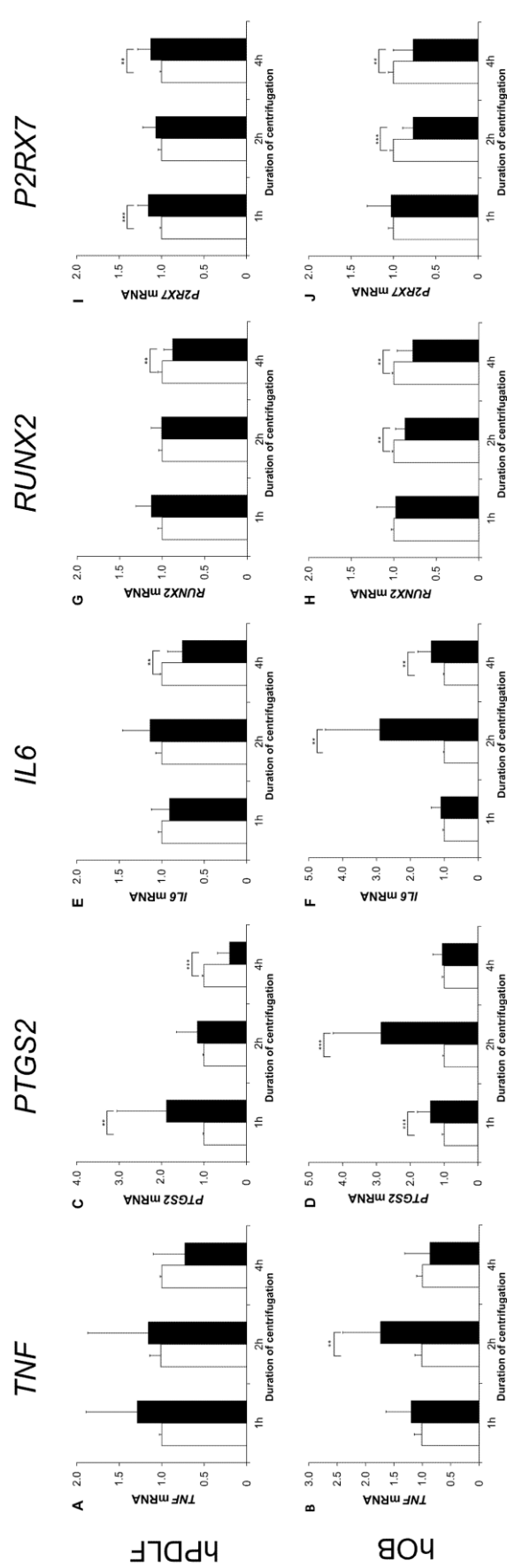


Figure 14. Gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* in co-cultured hPDLF and hOB after 1, 2 and 4 h of CF at 47.4 g/cm². The $\Delta\Delta C_q$ method was applied and B2M was used as the reference gene. Expression level of control was defined as 1. Values were presented as mean \pm SD (*p < 0.05, **p < 0.01, ***p < 0.001). □: Control; ■: CF.

5.2.4 Effects of compressive force on PGE₂, IL6 and TNF production after mechanical force application

TNF, PGE₂ and IL6 concentrations in mono- and co-cultured supernatants were measured after CF application of 1, 2 and 4 h using analytic specific ELISA. Additionally, the ratio between PGE₂ concentration after centrifugation and the corresponding control was calculated.

TNF production in the cell culture supernatant was determined using a TNF specific ELISA. In both setups (mono- and co-culture), TNF concentration was below the detection limit of the assay applied at any points after all force durations.

In mono-culture hPDLF, PGE₂ concentration was upregulated after 1, 2 and 4 h of CF application in comparison to the corresponding controls (Figure 15A). After 1 h ($p = 0.016$) and 4 h ($p = 0.004$) of CF application, PGE₂ increased significantly (Figure 15A). In hOB, the PGE₂ production increased after 1 ($p = 0.038$) and 2 h ($p = 0.038$) of CF application, but decreased after 4 h of CF (Figure 15B). Additionally, IL6 production in hPDLF was significantly higher than controls after 2 h ($p = 0.002$) and 4 h ($p = 0.041$) of CF application (Figure 15C), but no significant difference was found in hOB after 1, 2 and 4 h of CF application (Figure 15D).

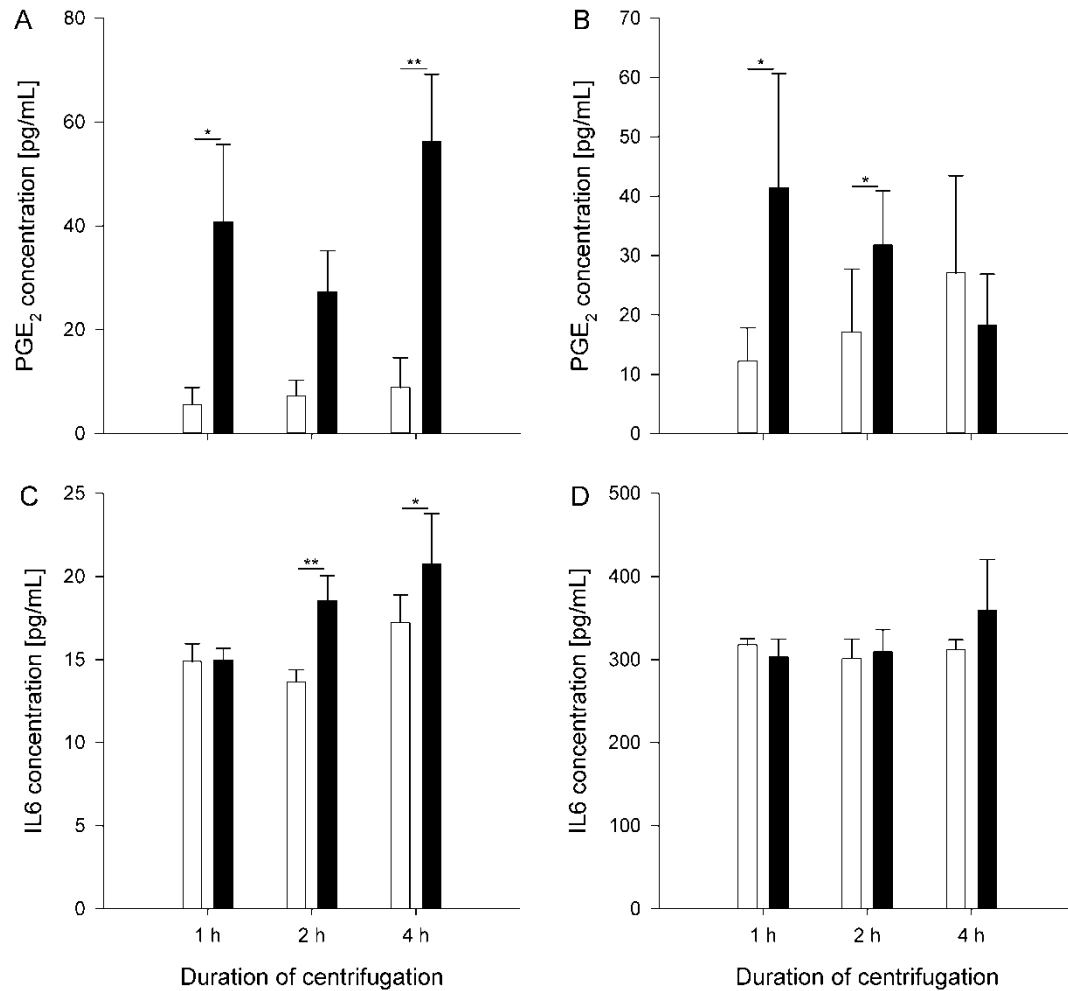


Figure 15. PGE₂ (A, B) and IL6 (C, D) concentrations in mono-cultured supernatants of hPDLF (A, C) and (B, D) hOB after 1, 2 and 4 h of CF at 47.4 g/cm² were measured using ELISA. Control groups were not subjected to CF. Values were presented as mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001). □: Control; ■: CF.

In co-culture, the production of PGE₂ was significantly lower than controls when hPDLF were exposed to 1 (p = 0.002), 2 and 4 h (p = 0.002) of CF (Figure 16A). In hOB, the PGE₂ significantly increased after 2 h (p = 0.038) of CF but was smaller after 1 h and 4 h of CF (Figure 16B). Concerning IL6 production, decrease was found in hPDLF after 1 h (p = 0.026) and 2 h of CF application (Figure 16C). However, in hOB, IL6 production showed a significant decrease after 1 h (p = 0.041) of CF, but increase was observed after 2 (p = 0.015) and 4 h of CF (Figure 16D).

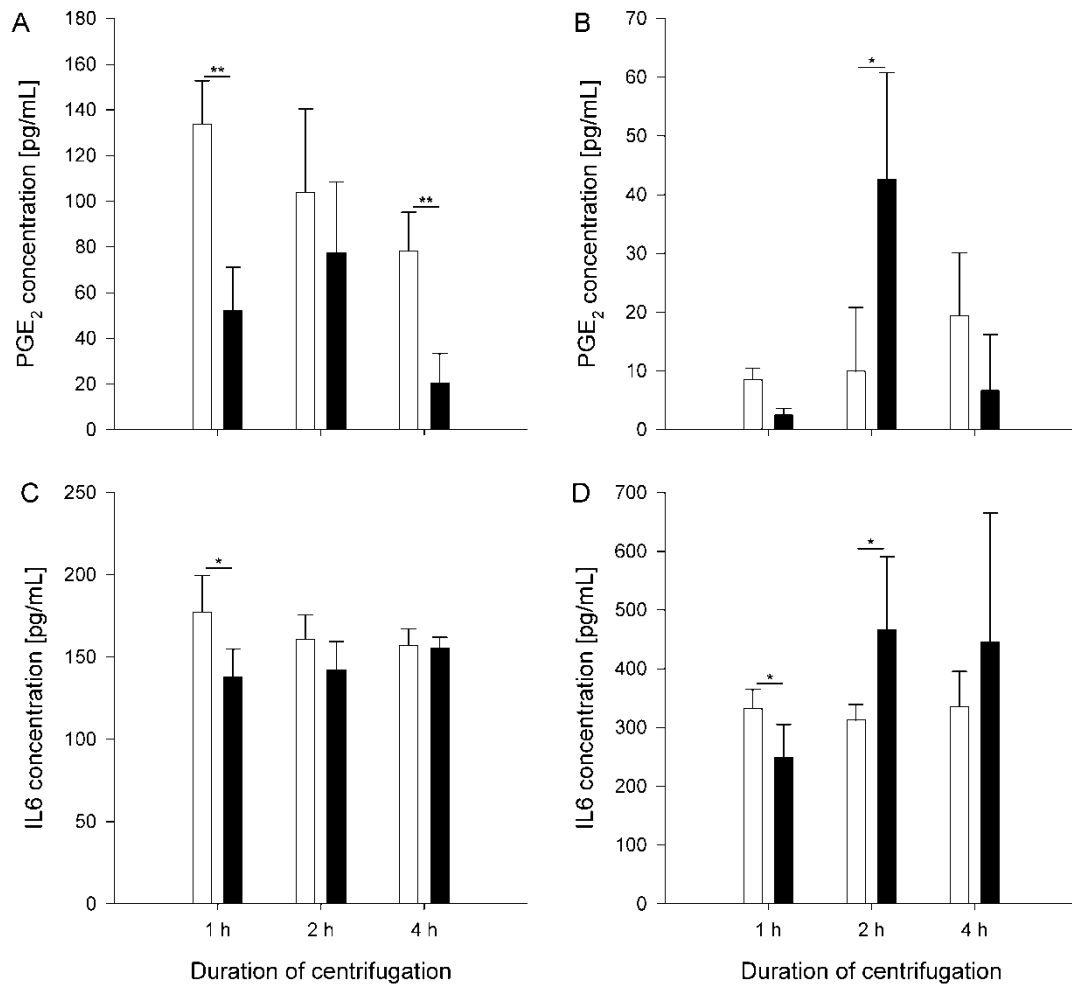


Figure 16. PGE₂ (A, B) and IL6 (C, D) concentration in co-cultured supernatants of hPDLF (A, C) and hOB (B, D) after 1, 2 and 4 h of CF at 47.4 g/cm² was measured using ELISA. Control groups were not subjected to CF. Values were presented as mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001). □: Control; ■: CF.

5.2.5 Comparison of the force induced changes in gene and protein expression according to culture conditions

Gene expression changes induced by CF showed considerable dependency on the culturing conditions. With exception of the *TNF* gene expression at 1 h the co-culturing of both tested cell types significantly attenuated the CF induced augmentation of *TNF*, *PTGS2* and *IL6* gene expression as well as the PGE₂ and IL6 protein expression in hPDLF in comparison to the separate culturing method (Table 2). A comparable reduction of the force induced changes in gene and protein expression was also found in hOB, but not for the samples that have been exposed to

CF for 2 h and for the protein expression of IL6 at 4 h. Likewise, except the *P2RX7* gene expression of hPDLF at 1 h, *RUNX2* and *P2RX7* showed subdued gene expression in both cell types when they were co-cultured in comparison to that in mono-culture.

Table 2. Comparison of co-culture vs. mono-culture gene expression and ELISA results for hPDLF and hOB. Given are the ratios of CF/control and co-/mono-culture, P values from the Mann-Whitney U test and significance levels (*p < 0.05, **p < 0.01, ***p < 0.001). ↑ = increase; ↓ = decrease.

Method	Gene, gene product, metabolite	Cell type	Time	Ratio CF/control		Comparison (co-/mono-culture)	P values	Significance
				Mono-culture	Co-culture			
qPCR	<i>TNF</i>	hPDLF	1 h	1.19	1.29	↑	0.887	
			2 h	1.74	1.15	↓	0.198	
			4 h	1.19	0.73	↓	0.089	
		hOB	1 h	4.00	1.19	↓	<0.001	***
			2 h	1.14	1.72	↑	0.014	**
			4 h	1.03	0.87	↓	0.078	
	<i>PTGS2</i>	hPDLF	1 h	2.23	1.89	↓	0.219	
			2 h	4.86	1.16	↓	<0.001	***
			4 h	1.37	0.40	↓	<0.001	***
		hOB	1 h	2.45	1.41	↓	<0.001	***
			2 h	2.56	2.87	↑	0.799	
			4 h	4.37	1.06	↓	<0.001	***
	<i>IL6</i>	hPDLF	1 h	1.33	0.91	↓	<0.001	***
			2 h	1.40	1.14	↓	0.033	*
			4 h	1.13	0.76	↓	0.024	*
		hOB	1 h	1.56	1.11	↓	0.003	**
			2 h	1.47	2.91	↑	0.114	
			4 h	5.15	1.39	↓	<0.001	***
	<i>RUNX2</i>	hPDLF	1 h	1.29	1.13	↓	0.114	
			2 h	1.35	1.01	↓	0.005	**
			4 h	1.76	0.88	↓	0.005	**
		hOB	1 h	1.50	0.98	↓	<0.001	***
			2 h	1.13	0.87	↓	<0.001	***
			4 h	0.95	0.78	↓	0.020	*
<i>P2RX7</i>	hPDLF	1 h	1.04	1.16	↑	0.843		
		2 h	1.30	1.07	↓	0.008	**	
		4 h	1.26	1.13	↓	0.178		
	hOB	1 h	1.24	1.03	↓	0.089		
		2 h	1.00	0.77	↓	0.001	**	
		4 h	0.80	0.77	↓	0.443		
ELISA	<i>PGE₂</i>	hPDLF	1 h	7.29	0.39	↓	0.010	**
			2 h	3.79	0.74	↓	0.002	**
			4 h	6.33	0.26	↓	0.002	**
		hOB	1 h	3.39	0.29	↓	0.024	*
			2 h	1.85	4.29	↑	0.009	**
			4 h	0.67	0.34	↓	0.381	
	<i>IL6</i>	hPDLF	1 h	1.01	0.78	↓	0.010	**
			2 h	1.36	0.88	↓	0.002	**
			4 h	1.20	0.99	↓	0.015	*
		hOB	1 h	0.95	0.75	↓	0.024	*
			2 h	1.03	1.49	↑	0.015	*
			4 h	1.15	1.33	↑	0.714	

5.3 “Pulse chase sampling”

5.3.1 Lower compressive force on mono-cultured cells

After mono-cultured cells were subjected to CF at 47.4 g/cm^2 , the total RNA was isolated at 2, 4, 8 and 16 min. Gene expressions of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* were measured using quantitative real-time PCR.

TNF gene expression in hPDLF increased at 2 min after 1 h ($p = 0.002$) of CF, decreased at 4, 8, 16 min. After 2 h of CF, increase was observed at 2 ($p = 0.002$) and 8 min. After 4 h of CF, *TNF* gene expression was downregulated at 2 min ($p = 0.002$), and upregulated at 4 ($p = 0.002$), 8 ($p = 0.004$), and 16 min ($p = 0.002$) (Figure 17A).

PTGS2 gene expression in hPDLF was increased at different chase points after 1 and 2 h of CF. After 4 h of CF, slight increase of *PTGS2* gene expression only observed at 4 and 8 min in hPDLF (Figure 17C).

The increase of *IL6* gene expression in hPDLF was observed at different chase points except 16 min after 1 h of CF and 2 min after 4 h of CF (Figure 17E).

RUNX2 gene expression in hPDLF after 1 and 2 h of CF increased significantly at 2 min ($p = 0.002$) and diminished at the following chase points. However, after the application of 4 h of CF, it dramatically upregulated at 4 min ($p = 0.002$) and slipped slightly at 8 min ($p = 0.002$) (Figure 17G).

Application of CF increased the *P2RX7* gene expression at 2 min after 1 ($p = 0.004$) and 2 h ($p = 0.002$) of CF in hPDLF. An upregulation was observed at 4 min ($p = 0.015$) after 4 h of CF but it rapidly dropped back to control level at 8 min ($p = 0.026$) and 16 min (Figure 17I).

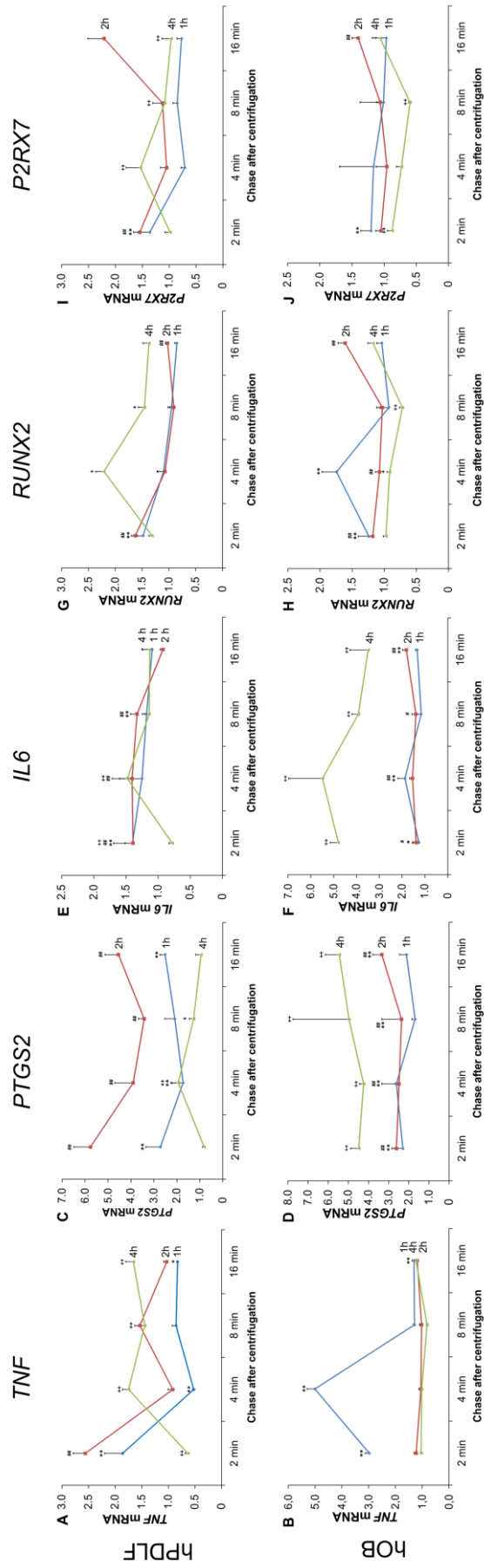


Figure 17. Real-time PCR results of the gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* in mono-cultured hPDLF and hOB at defined chase points (2, 4, 8 and 16 min) after the application of CF at 47.4 g/cm² for 1, 2 and 4 h. Values are presented as mean ± SD. Statistical significant differences between experimental condition and negative control are shown for 1 h (*), 2 h (#) and 4 h of centrifugation (+). * , #, +: p < 0.05; ** , ##, ++: p < 0.01; *** , ###, +++: p < 0.001.

In hOB, *TNF* gene expression was no change at different chase points after 2 and 4 h of CF. However, an upregulation was observed at 2 min ($p = 0.002$) and this variation even higher at 4 min ($p = 0.002$) after 1 h of CF. But it dropped back control level at 8 and 16 min ($p = 0.002$) (Figure 17B). Upregulation of *PTGS2* and *IL6* gene expression showed at all chases points (2, 4, 8 and 16 min) after 1, 2 and 4 h of CF duration. However, the ratio of upregulations of both genes after 4 h was higher than 1 and 2 h (Figures 17D, 17F). *RUNX2* gene expression increased significantly at 2 ($p = 0.002$) and 4 min ($p = 0.009$) after 1 h of CF. Besides, a significant increase at 16 min ($p = 0.002$) after 2 h of CF and a mild decrease at 8 min ($p = 0.002$) after 4 h of CF emerged (Figure 17H). The application of CF upregulated *P2RX7* at 2 ($p = 0.009$) and 4 min after 1 h of CF and at 16 min ($p = 0.002$) after 2 h of CF. But downregulation was detected at 4 and 8 min ($p = 0.002$) after 4 h of CF (Figure 17J).

5.3.2 Higher compressive force on mono-cultured cells

Total RNA of 2, 4, 8 and 16 min was isolated after 73.7 g/cm^2 loaded on mono-cultured cells. Gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* was examined using quantitative real-time PCR.

In hPDLF, the *TNF* gene expression was dramatically increased at 2 ($p = 0.002$) and 4 min ($p = 0.002$) and decreased at 8 min ($p = 0.002$) after 1 h of CF. After 2 h of CF, the gene expression was decreased at 2 min ($p = 0.002$) and increased at 4, 8 ($p = 0.002$) and 16 min. And after 4 h of CF, *TNF* gene expression was increased at 2 and 4 min but decrease at 8 ($p = 0.002$) and 16 min ($p = 0.026$) (Figure 18A).

Significant increase of *PTGS2* gene expression in hPDLF was observed at all chase points independent of the CF durations (Figure 18C).

For *IL6* gene expression in hPDLF, the upregulation emerged at different chase points after 1, 2 and 4 h of CF durations excepted 8 min after 4 h of CF (Figure 18E).

RUNX2 gene expression in hPDLF showed upregulation at different chase points excepted 8 min after 1 h and 4 min after 2 h of CF (Figure 18G).

Concerning *P2RX7* hPDLF, the application of CF only induced the gene expression significantly at 4 min ($p = 0.002$) after 1 h of CF (Figure 18I).

In hOB, significant decrease at 2 ($p = 0.002$) and 4 min ($p = 0.002$) after 2 h and increase at 4 ($p = 0.002$) and 8 min ($p = 0.002$) after 4 h was observed in *TNF* gene expression (Figure 18B).

PTGS2 and *IL6* gene expression presented upregulated at every chase point after 1, 2 and 4 h of CF durations in hOB (Figure 18D, 18F).

A slight increase was observed at 8 ($p = 0.002$) and 16 min ($p = 0.026$) after 2 h of CF as well as at 2 min ($p = 0.002$) after 4 h of CF for *RUNX2* gene expression in hOB. No significant *RUNX2* gene expression was detected at all the other chase points (Figure 18H).

P2RX7 gene expression was significantly increased at 16 min ($p = 0.002$) after 2 h and at 2 min ($p = 0.009$) after 4 h of CF in hOB (Figure 18J).

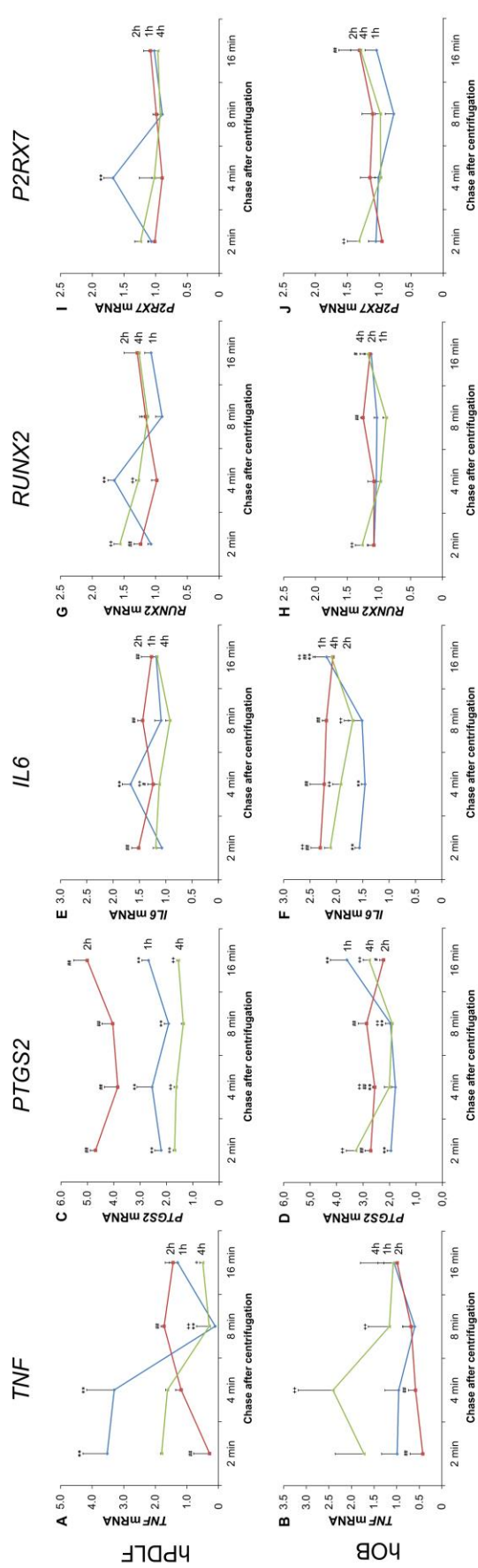


Figure 18. Real-time PCR results of the gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* in mono-cultured hPDLF and hOB at 2, 4, 8 and 16 min after the application of CF at 73.7 g/cm² (centrifuge at 400xg) for 1, 2 and 4 h. Values are presented as mean ± SD. Statistical significant differences between experimental condition and negative control are shown for 1 h (*), 2 h (#) and 4 h of centrifugation (+). *, #, +: p < 0.05; **, ##, ++: p < 0.01; ***, ###, +++: p < 0.001.

5.3.3 Lower compressive force on co-cultured cells

The pulse chase protocol was also applied to cells in co-culture submitted to a compressive force of 47.4 g/cm^2 . Total RNA was isolated at 2, 4, 8 and 16 min after CF loaded. And gene expressions of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* were measured using quantitative real-time PCR.

In hPDLF, *TNF* gene expression increased at 2 and 4 min and decrease at 8 and 16 min ($p = 0.015$) after the 1 h of CF. It was increased at 2 min and decreased at 4, 8 and 16 min ($p = 0.002$) after 2 h of CF. After 4 h, *TNF* gene expression was decreased at 2 min ($p = 0.002$) and increased at 8 and 16 min (Figure 19A).

PTGS2 gene expression was upregulated at different chase points after 1 h of CF. At 2 ($p = 0.002$), 4 and 16 min, *PTGS2* gene expression was decreased after 4 h of CF. And it showed significant increase at 2 min ($p = 0.002$) and decrease at 4 ($p < 0.002$) and 16 min ($p < 0.002$) after 2 h of CF in hPDLF (Figure 19C).

IL6 gene expression increased at 2 ($p = 0.004$) and 8 min after 2 h of CF and decreased at four chase points after 4 h and at 4 min after 1 h of CF in hPDLF (Figure 19E).

RUNX2 gene expression in hPDLF was downregulated at 8 and 16 min after 1 h of CF, at 8 min ($p = 0.002$) after 2 h of CF and at 2 min ($p = 0.002$) after 4 h of CF (Figure 19G).

Concerning *P2RX7* in hPDLF, the application of CF induced the gene expression at 2 and 4 min ($p = 0.002$) after 1 h, at 2 min ($p = 0.041$) after 2 h and at 2 and 4 min ($p = 0.015$) after 4 h of CF (Figure 19I).

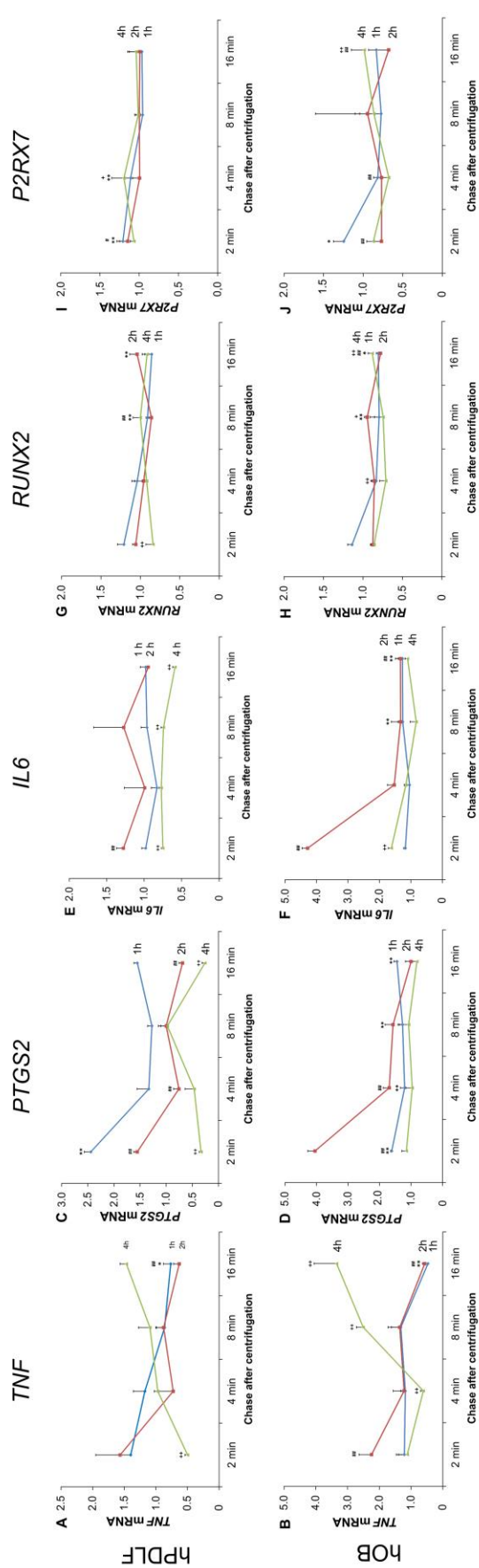


Figure 19. Real-time PCR results of the gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* in co-cultured hPDLF and hOB at 2, 4, 8 and 16 min after the application of CF at 47.4 g/cm² for 1, 2 and 4 h. Values are presented as mean ± SD. Statistical significant differences between experimental condition and negative control are shown for 1 h (*), 2 h (#) and 4 h of centrifugation (+). *, #, +: p < 0.05; **, ##, ++: p < 0.01; ***, ###, ###, +++: p < 0.001.

In hOB, *TNF* gene expression showed significantly upregulated at 2 min ($p = 0.002$) after 2 h and 8 ($p = 0.002$) and 16 min ($p = 0.002$) after 4 h of CF. But it was downregulated at 16 min after 1 ($p = 0.002$) and 2 h ($p = 0.004$) and 4 min ($p = 0.002$) after 4 h of CF (Figure 19B). *PTGS2* gene expression was increased at all the chase points after 1 h and 2, 4 and 8 min after 2 h of CF (Figure 19D). The *IL6* gene expression was upregulated at different chase points after 1, 2 and 4 h of CF durations except the 2 min after 1 h and 8 min after 4 h of CF (Figure 19F). Concerning the gene expression of *RUNX2* and *P2RX7*, slight decrease was observed at different chase points independent of the CF durations except the 2 min after 1 h of CF (Figures 19H and 19J).

6 Discussion

The effect of mechanical force on bone remodeling during orthodontic tooth movement is yet mostly studied using mono-cultured cells *in vitro* (Jacobs et al. 2014; Koyama et al. 2008). However, *in vivo*, the periodontal tissue represents a heterogeneous population of different cell types which might be functionally closely interrelated (Li et al. 2013a). During orthodontic tooth movement, these cells are subjected to mechanical force simultaneously leading to more or less intensive intercellular communication in this complex tissue microenvironment (Krishnan and Davidovitch 2006; Morikawa et al. 2016). Therefore, co-culturing of various cell types together seems more appropriate since it reflects the real physiological conditions more closer and, in addition, enables the investigation of intercellular communication *in vitro* (Hatherell et al. 2011). In this study, indirect-contact co-culture was applied to identify any kind of communication between various periodontal cell types, i.e. human periodontal ligament fibroblasts (hPDLF) and human osteoblasts (hOB) when subjected to static compressive force (CF) application. The indirect-contact co-culture model as used herein separated the hPDLF from the hOB with a porous membrane but kept both cell types under common growth conditions allowing signaling molecules and other mediators, e.g. cytokines, growth factors and metabolites to pass the membrane unrestrictedly.

6.1 Technical aspects of the experimental design

6.1.1 Force application

Cellular and molecular responses occurred under orthodontic forces during orthodontic therapy result in tooth movement. The bone remodeling markers and histological changes and performs in different pattern in the compression side and tension side of PDL (Otero et al. 2016). In orthodontic tooth movement, CF is an essential factor for bone resorption (Wise and King 2008). In previous *in vitro* studies, CF was applied using different methods such as hydrostatic pressure (Nakago-Matsuo

et al. 1996), direct weight application (Kanzaki et al. 2002), or even micro-gravity (Carmeliet et al. 1998). Additionally, centrifugation for *in vitro* mechanical stimulation of cells was also described (Baumert et al. 2004; Fitzgerald and Hughes-Fulford 1999; Inoue et al. 1993; Redlich et al. 1998; Theilig et al. 2001).

In this study, centrifuge was used as the device to produce CF on mono- and co-cultured primary cells (hPDLF and hOB). The force calculation was based on the formula published by Redlich et al. (2004a; 2004b). Since growth area and the radius of the centrifuge must be considered constant, force magnitudes can be varied via changing centrifugation speed or volume (i.e. weight) of cell culture medium. In this experimental setup, compressive forces of 47.4 and 73.7 g/cm² were applied by variation of the cell culture medium within each well of mono-cultured cells. These forces are in the range of orthodontic force during tooth movement (Ren et al. 2003; Wichelhaus 2017). The relevant inflammatory factors were measured and analyzed for 1, 2 and 4 h of force application. In mono-culture the induction of inflammatory factors was more pronounced with the application of 47.4 g/cm². Therefore, this CF was chosen for the co-culture study (see below).

6.1.2 Cell viability

In this study, pressure type of force was applied using a conventional laboratory centrifuge. This setup has been successfully applied in other studies (Baumert et al. 2004; Redlich et al. 1998) to impose CF on cells *in vitro*. Nevertheless, one of the most important parameters for cell growth is the culture temperature. In *in vitro* studies, most mammalian cells are cultivated at 37 °C to simulate the body environment. Cultivation at temperatures below 37 °C will first slow down cell metabolism (“Q₁₀ rule”) and over longer periods of time will most probably induce apoptosis and cell death. Only a few studies took this into account in their experimental setup (Hacopian et al. 2011; Ueda et al. 2016).

The centrifuge used in these experiments operated at ranges between +4 °C and +25 °C with active cooling. The temperature inside this centrifuge mainly depends on the mass of the rotor, its speed, and factors such as the amount and mass of the

samples to be centrifuged and the intraday room temperature in the laboratory. After longer periods of centrifugation the temperature within the centrifuge reached 30 °C, but not in a constant and predictable manner. In agreement with the manufacturer we tested several centrifuge presets using temperature data loggers to monitor and record the temperature data within the centrifuge during centrifugation. Here, the experimental temperature should fulfill two conditions: (1) a temperature closer to physiological characteristic of the cells used and (2) a stable temperature during centrifugation at predefined forces. Finally, CF was applied to cells with centrifugation using the protocol shown in Section 4.3.1.3. Within the limitations of the centrifuge, all experiments were run at 30 °C. The centrifuge was equilibrated to this temperature using an additional pre-step (50 min at 800×g) before cells were exposed to CF by centrifugation at 30 °C for 1, 2 and 4 h.

As mentioned above, decreased temperature can induce metabolic changes in the cells. If this occurs over longer periods of time, apoptosis is induced. Apoptosis, also known as “programmed cell death”, is accompanied by a series of characteristic morphological and metabolic changes in cells undergoing apoptosis (Kerr et al. 1972; McCance et al. 2010). Apoptosis is a naturally occurring process and basis of differentiation and growth (Hale et al. 1996) and influences many physiological processes, such as the immune system, embryonic development, metamorphosis and hormone dependent atrophy, and of course cell death (Cohen et al. 1992; McCance et al. 2010).

Apoptosis is divided into seven steps (Abcam 2017): 1. loss of membrane asymmetry; 2. caspase, calpain and cathepsin activation; 3. mitochondrial membrane potential and cytochrome c release; 4. nuclear condensation; 5. DNA fragmentation; 6. Sub G1 population increase; 7. Cell membrane blabbing. These steps happen in parallel and many of them overlap. *“Loss of membrane asymmetry or initiation of caspase cascade are biochemical features of apoptosis which do not necessarily lead to cell death. However, other downstream features such as decrease of the mitochondrial membrane potential and concomitant release of cytochrome C into the cytosol, are generally considered points of no return, after which it is very unlikely the*

cell will survive.” (Abcam 2017; p. 7) In this study, loss of membrane asymmetry, change of the mitochondrial membrane potential and DNA fragmentation were determined by annexin V-FITC, TMRE and Hoechst dye staining, respectively.

The influence of temperature on cell viability of hPDLF and hOB were tested within the experimental conditions applied (at 30 °C, up to 4 h). In comparison to the negative control (at 37 °C, up to 4 h), no apoptosis signs were observed after 4 h incubation at 30 °C. The cells maintained their physiological characteristics during the experimental conditions in this study. Hence, an experimental temperature at 30 °C is feasible for *in vitro* experiments up to 4 h. This observation is in correspondence with previous studies on PDLF viability after traumatic avulsion of permanent teeth (Souza et al. 2010).

6.1.3 *In vitro* co-culture

Co-culture is an appropriate design reflecting a situation closer to physiology and thus enabling the investigation of intra- and intercellular communication *in vitro* (Hatherell et al. 2011; Morikawa et al. 2016). Two types of co-culture systems have been reported: direct and indirect contact co-culture systems. Both are applied to co-incubate two types of cells in the same optimal cell culture environment, which can create a native functional condition to maintain cell features *in vitro*. In direct contact system two types of cells get into contact with each other thus allowing to establish direct cell-cell contacts and thus enabling intercellular communication (Jones et al. 2009). However, this may cause massive cell death of one cell type if the other one is growing faster and thus outgrows the other. From the experimental point of view, direct co-culture imposes technical challenges to isolate and purify a specific cell type from a mixed co-culture cell population. Therefore, indirect contact co-culture models were established to overcome this limitation. Both cell types are grown separately from each other but share the same cell culture environment (Figure 3). In this study, hPDLF and hOB are grown using the indirect co-culture method. Cell culture inserts with a membrane containing pores small enough, that cells cannot migrate through, were combined with 12-well plates to the reconstruct the co-culture system. HPDLF

and hOB share the same nourishment from two different areas: the insert itself and the wells. Intercellular communication is taking place via releasing cytokines and growth factors into the chamber and through the porous membrane. Both cell types can be analyzed and assessed separately after mechanical stimulation. In this setup, equivalent CF were loaded on two different types of cells simultaneously. According to the calculation in Redlich et al. (1998), the CF loading on cells was 47.4 g/cm² in the well and 49.1 g/cm² on cell culture insert respectively:

$$P = \frac{m \times r \times rpm^2 \times \pi^2}{A \times 9.8 \times 900}$$

Force (P):	47.4 g/cm²	49.1 g/cm²
Mass of medium (m):	0.9 g (~900 μL)	0.28 g (~280 μL)
Radius of centrifuge rotor (r):	0.160 m	0.1585 m
Speed of centrifuge (rpm):	970 rpm	970 rpm
Growth area of cells (A):	3.8 cm ²	1.131 cm ²

6.1.4 Sampling procedure

Two different sampling procedures are applied in studies related to CF application: (1) samples are drawn directly after force application (“direct sampling”) or (2) “pulse chase sampling”. With “pulse chase sampling”, force (i.e. the “pulse”) is applied to the specimen for a defined period of time. Then, samples are drawn at defined time intervals after force application stopped.

“Direct sampling” after force application is easy to handle and widely used in many studies (e.g. Morikawa et al. 2016; Redlich et al. 2004a). Since duration of sampling is not stated in any publication, results are not comparable between studies. “Pulse chase sampling”, on the other hand, enables to study the effect of a stress application over a longer period of time (Baumert et al. 2004). Gene expression and translation takes time and this time differs between different genes. Some genes are rapidly transcribed after stimulation, so called “primary” or “early” response genes, such as *EGR1*, *ATF3*, *IL1B*. Some genes are induced more slowly and are called

“secondary” or “late” response gene, such as *IL6* and *TNF* (Sandoval et al. 2016). This can be exemplified by two different studies, both applying “pulse-chase” sampling. Matsunaga et al. (2016) applied 12 h constant pressure on human cementoblasts using the weight approach. At 0, 7, 14 and 21 days after force release, they collected samples and analyzed gene expression of *RUNX2*, *WNT5A*, *ALP*, and *SPONI*. Baumert et al. (2004), on the other hand, applied centrifugation force on human osteoblasts for 45 and 90 min. Sampling took place at 2, 4, 8, 16 and 32 min after centrifugation stopped and *RUNX2* expression was analyzed in these samples.

In this study, samples were collected at 2, 4, 8 and 16 min after 1, 2 and 4 h of centrifugation/incubation. The results showed that gene expression always varies at different time points after mechanical force in both mono- and co-culture setups. Namely, the changes of gene expression and protein synthesis sustain even after CF application. Hence, the pulse chase multi-sampling would be necessary in future studies to monitor a dynamic process of gene expression and protein synthesis after mechanical stimulation.

6.2 Expression of genes and metabolites

Orthodontic tooth movement is a process of bone remodeling under mechanical force stimulation. Periodontal tissue cells surrounding tooth release some inflammatory factors which affect formation and absorption of bone via regulating gene expression and protein synthesis during this process. In this study, mechanical forces were applied on mono- (47.4 and 73.7 g/cm²) and co-cultured (47.4 g/cm²) hPDLF and hOB. Gene expression of several related inflammatory factors (*TNF*, *IL6*, *PTGS2*), the transcription factor *RUNX2* and the purinergic receptor *P2RX7*, were analyzed using quantitative real-time PCR for the different experimental conditions. After evaluation of different reference genes for qRT-PCR, *B2M* was selected as the reference gene. Protein synthesis (*IL6*, *TNF*) or metabolite production (*PGE₂*) were determined using ELISA.

6.2.1 TNF, IL6, PTGS2 and PGE₂

Cytokines regulate the inflammatory reaction after orthodontic force application on periodontal tissue cells (Li et al. 2013b). In this study, we found that the inflammatory cytokines TNF, PTGS2 and IL6 were regulated after CF application in a duration and culture type depending manner under the CF of 47.4 g/cm². Gene expression of *TNF*, *PTGS2* and *IL6* after all three durations (1, 2 and 4 h) of CF is upregulated in mono-cultured hPDLF and hOB, as well as the production of PGE₂ and IL6. With one exception (2 h CF in hOB), gene expression downregulation and decreased ratios of CF vs. control were found for *TNF*, *PTGS2* and *IL6* in co-cultured hPDLF and hOB compared to mono-culture. This correlates with the results of PGE₂ and IL6 ELISA measurements. In light of these findings, CF did increase the gene expression of *TNF*, *PTGS2* and *IL6* after 1, 2 and 4 h of CF in mono-culture setup and most of durations in co-culture setup in both cell types. Similar findings were reported in other studies (Kanzaki et al. 2002; Koyama et al. 2008; Mayahara et al. 2007; Römer et al. 2013). In contrast, after 2 h of CF application in hOB co-cultured with hPDLF, increased ratios of CF vs. control were found in *TNF*, *PTGS2* and *IL6* gene expression, compared to the mono-culture situation (Table 2). These findings are supported by ELISA measurements of PGE₂ and IL6. Previous studies applying co-culture showed that this cultivation technique promotes the expression of inflammatory cytokines after mechanical force (Morikawa et al. 2016). Therefore, intercellular communication might facilitate the gene expression of *TNF*, *PTGS2* and *IL6* in hOB co-cultured with hPDLF.

The aforementioned inflammatory cytokines are part of the TNF signaling pathway (KEGG pathway #ko04668; Manyam et al. 2015) regulating bone remodeling. More specifically, TNF is as a pro-inflammatory cytokine. It inhibits osteoblastic bone formation, enhances osteoclastic bone resorption and induces bone loss accordingly (Koyama et al. 2008; Kuno et al. 1994). *PTGS2* is an enzyme involved in periodontal inflammatory responses (Römer et al. 2013) and its gene expression is upregulated after force application (Kanzaki et al. 2002). *PTGS2* plays a central role in PGE₂ biosynthesis and is therefore relevant to the formation of PGE₂ during inflammatory

reaction (Kanzaki et al. 2002). In a previous study, the authors suggested that PGE₂ production in response to mechanical force can be contributed to the upregulation of *PTGS2* gene expression after force application (Shimizu et al. 1998). Additionally, IL6 is a critical regulatory factor affecting the development of skeleton (De Benedetti et al. 2006). Lack of IL6 can lower bone mass (Baker et al. 1999) via reducing numbers of osteoblasts (Bakker et al. 2014; Yang et al. 2007). Exogenous IL6 can enhance osteogenic differentiation of osteoblasts by affecting signaling molecule production (Bakker et al. 2014). Additionally, appropriate levels of mechanical force induce the secretion of IL6 (Jacobs et al. 2014; Koyama et al. 2008) which reduces bone loss (Baker et al. 1999).

Taken together, the reported *in vitro* findings support the establishment of bone resorption at the compression side during orthodontic tooth movement (Wise and King 2008). However, due to the considerable differences between mono- and co-culture results, intercellular communication (Kim et al. 2006; Kook et al. 2009; Mayahara et al. 2012) might exist between co-cultured hPDLF and hOB, leading to a downregulation of *TNF*, *PTGS2* and *IL6* gene expression after CF application. This downregulation in co-culture setup might, to some extent, influence the speed of bone resorption during orthodontic tooth movement.

Concerning the “pulse chase” design applied gene expression of *TNF*, *PTGS2* and *IL6* in both hPDLF and hOB varies from chase to chase after 1, 2 and 4 h of CF durations in mono- and co-cultured setups. Namely, the application of CF could induce gene regulation of *TNF*, *PTGS2* and *IL6*, and this was still detectable up to 16 min. These findings show that “pulse chase” sampling is a feasible way to explore delayed reactions over time in cells after experimental stimulations.

6.2.2 Runt-related transcription factor 2 (*RUNX2*)

RUNX2, one of the major genes of bone growth equilibrium, is essential for the differentiation of osteoblasts from uniform progenitor cells. Loss of *RUNX2* hinders the differentiation of osteoblast (Komori et al. 1997). *RUNX2*, thereby, is considered as a main gene for bone development and contributes a lot on signal transduction

pathways (Ducy et al. 1997). Additionally, RUNX2 influences bone matrix deposition and factors like BMP growth factor group in differentiated osteoblasts (Ducy et al. 1999). RUNX2 binding sites exist in regulatory parts of some major osteoblast-specific genes, such as osteocalcin, alkaline phosphatase, and the type-I collagen (Ducy et al. 1997; Harada et al. 1999; Kern et al. 2001).

In this study, gene expression of *RUNX2* was analyzed with quantitative real-time PCR after application of different magnitude of force (47.4 and 73.7 g/cm²) in mono- and co-cultured hPDLF and hOB. The results demonstrate nearly no variations independent of force magnitude and cell type. Only after 4 h of CF application in hPDLF, the *RUNX2* gene expression increased apparently, and this upregulation was more obvious under 47.4 g/cm² CF. Taken together, the presented results show, that mechanical force influenced the *RUNX2* gene expression, and a lighter CF was more efficient than a heavier one. It may indicate that mechanical force affects the bone remodeling in a certain extent, lower force can improve the efficiency of this process. In co-culture, *RUNX2* gene expression decreased in comparison to mono-culture setup independent of force durations or cell types. Applying the “pulse chase” design, samples were analyzed at 2, 4, 8 and 16 min after 1, 2 and 4 h of CF application on cells in mono- and co-culture. No clear pattern in relation to force magnitude in *RUNX2* gene expression was detectable, neither in mono- nor in co-culture or between the different force durations.

6.2.3 Purinergic receptor *P2RX7*

P2RX7 receptor is an adenosine triphosphate (ATP)-gated ionotropic channel and plays a crucial role in bone biology and inflammation (North 2002). After binding of extracellular ATP, a cellular stress signal that is also released with mechanical stress, P2RX7 can be activated (Brough et al. 2003; Li et al. 2005; Milner et al. 1990; Schneider et al. 2006). This process leads to accumulation of intracellular calcium and the release of fundamental inflammatory mediators, such as PGE₂, IL1A, and IL1B, which regulate bone physiology (Ferrari et al. 2006; Lister et al. 2007). Another major function of P2RX7 is to accelerate necrotic tissue metabolism. However, the role of

P2RX7 in bone remodeling during orthodontic tooth movement is still unclear (Viecilli et al. 2009).

In this study, *P2RX7* gene expression of was analyzed with quantitative real-time PCR after force application on hPDLF and hOB in mono- (47.4 and 73.7 g/cm²) and co-culture (47.4 g/cm²). In mono-culture, *P2RX7* gene expression in both cell types showed very slight upregulation after all three force durations under both magnitude of force. A higher level of *P2RX7* gene expression was found at 47.4 g/cm² CF in comparison to 73.7 g/cm² CF application. It can be concluded, that mechanical force application influences *P2RX7* gene expression and this effect is stronger under lower CF. Comparing both culture conditions, *P2RX7* gene expression either decreased or remained at the same level in co-culture if the same CF was applied. In co-culture *P2RX7* gene expression might therefore be regulated by a paracrine mechanism, e.g. by increasing extracellular ATP concentration (Brough et al. 2003; Li et al. 2005; Milner et al. 1990; Schneider et al. 2006).

Concerning pulse chase measurements, samples were analyzed at 2, 4, 8 and 16 min after CF application. *P2RX7* gene expression showed similar changes as *RUNX2* gene expression. In this case also, clear patterns related to force magnitude, force duration or culture conditions were not detectable.

6.3 Outlook

There are still some limitations in this study that can be further developed. Firstly, co-cultured two types of primary cells (hPDLF and hOB) were investigated in this study. Other types of cells, however, including osteoclasts, blood cells and bone marrow cells, also participate in bone remodeling during orthodontic tooth movement. Thereby, multi-culture setup would be considered in further studies. Secondly, in the present study, centrifuge was used for CF loading. Both temperature condition and force magnitude are related to the rolling speed of the centrifuge. It is limited to maintain the temperature at 37 °C with a demanding magnitude of force. Therefore, an innovative way could be developed to equilibrate the conditions for an optimal study setup.

7 Conclusion

Primary cells, human periodontal ligament fibroblasts (hPDLF) and osteoblasts (hOB) can survive at least for 4 h at 30 °C. Hence, culture temperature at 30 °C would be available for *in vitro* experiment within 4 h.

Mechanical force induces inflammatory reaction accompanied by sequence of cytokine regulations. These regulations influence bone remodeling which is the base of orthodontic tooth movement. Therefore, an optimal mechanical force for an effective tooth movement without side effects is desirable.

Our data indicate CF can induce the gene expression of *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* and protein synthesis of PGE₂ and IL6 in hPDLF and hOB. Communication might exist between co-cultured hPDLF and hOB subjected to CF to some extent, leading to a dominant inhibition effect on *TNF*, *PTGS2*, *IL6*, *RUNX2* and *P2RX7* gene expression and PGE₂ and IL6 protein synthesis compared to that in mono-cultured setup.

Cell reactions could be observed at least 16 min after stimulations. Namely, gene expression and protein synthesis keep varying for a period of time even after the termination of stimulation. Accordingly pulse chase setup is a feasible way to investigate the delayed reactions. Moreover, a fixed time point for collecting samples after mechanical stimulation is essential to make results comparable between different studies.

8 Summary

This study established an *in vitro* model to investigate the intra- and intercellular communication in co-cultured human periodontal ligament fibroblasts (hPDLF) and osteoblasts (hOB) and the changes in gene expression for selected genes after mechanical force loading. Identical procedures were applied to both cell types in mono-culture for comparison purposes.

Mono- and co-cultured hPDLF and hOB were subjected to CF (47.4 or 73.7 g/cm²) for 1, 2 and 4 h at 30 °C respectively. Control cells received same treatment in parallel without force application. After each centrifugation/incubation, samples (i.e. cell culture supernatants, cell lysates) were collected at 2, 4, 8, and 16 min. From each of the cell lysates, total RNA was isolated and reverse transcribed to cDNA. Gene expression of tumor necrosis factor alpha (*TNF*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), interleukin 6 (*IL6*), runt-related transcription factor 2 (*RUNX2*) and purinergic receptor *P2RX7* were determined using quantitative real-time polymerase chain reaction. Productions of TNF, IL6 and Prostaglandin E₂ (PGE₂) were determined using enzyme-linked immunosorbent assay (ELISA).

The results show, that (1) an *in vitro* model applicable to both in mono- and co-culture setups was successfully established. Within the experimental conditions applied, no significant induction of apoptosis within 4 h at 30 °C in comparison to 37 °C was detected. (2) Mechanical force induces gene expression in several inflammatory reaction-related cytokines and other genes of interest, which may influence orthodontic tooth movement. (3) Intercellular communication (paracrine regulation) between co-cultured hPDLF and hOB subjected to mechanical stimulation may exist. (4) Gene expression and protein synthesis of those genes and their gene products/metabolites analyzed varied even after termination of mechanical stimulation.

In future *in vitro* studies, the experimental temperature should be carefully considered and monitored. The developed and established indirect co-culture model is an innovative way to simulate a situation close to the physiological condition. “Pulse chase” sampling should be applied to investigate more precisely the dynamics in gene expression and protein synthesis after mechanical stimulation.

9 Zusammenfassung

In der vorgelegten Studie wurde ein *in vitro* Modell zur Untersuchung der intra- und interzellulären Kommunikation in ko-kultivierten humanen parodontalen Ligamentzellen (hPDLF) und Osteoblasten (hOB) etabliert und die Genexpression ausgewählter Gene nach Kraftapplikation untersucht. Zur Kontrolle wurden beide Zelltypen in Mono-Kultur denselben Kräften unterworfen. Die anschließende Analyse erfolgte analog.

Mono- und ko-kultivierte hPDLF und hOB wurden zentrifugalem Druck (47,4 bzw. 73,7 g/cm²) für 1, 2 und 4 Stunden bei 30 °C ausgesetzt. Als Kontrolle dienen Zellen, die den gleichen Kultivierungs- und Temperaturbedingungen unterworfen wurden, aber keinem Druck ausgesetzt waren. Vor der eigentlichen Zentrifugation und 2, 4, 8 und 16 Minuten nach jeder Zentrifugation bzw. Inkubation wurden Proben (Zelllysat, Zellkulturüberstand) genommen. Aus den Zelllysaten wurde die Gesamt-RNA isoliert und in cDNA umgeschrieben. Die Genexpression von Tumornekrosefaktor- α (*TNF*), Prostaglandinsynthase-2 (*PTGS2*), Interleukin 6 (*IL6*), Runt-related Transkriptionsfaktor 2 (*RUNX2*) und P2X-Purinorezeptor 7 (*P2RX7*) wurden mittels quantitativer Realtime-PCR bestimmt. Die Produktion von IL6, TNF und Prostaglandin E₂ (PGE₂) wurden mittel ELISA („*Enzyme-Linked ImmunoSorbent Assay*“) ermittelt.

Die Ergebnisse zeigen, dass (1) ein *in vitro* Modell erfolgreich entwickelt wurde, das sowohl für Zellen in Mono- als auch Ko-Kultur angewandt werden kann. Innerhalb der experimentellen Parameter wurde keine Apoptose-Induktion innerhalb von 4 Stunden bei 30 °C im Vergleich zu 37 °C beobachtet. (2) Mechanische Druckeinwirkung induziert die Genexpression mehrere an der Entzündungsreaktion beteiligter Zytokine und andere Gene von Interesse. Dies könnte die kieferorthopädische Zahnbewegung beeinflussen. (3) Die Ergebnisse deuten auf eine interzelluläre Kommunikation (parakrine Regulation) zwischen ko-kultivierten Zellen unterschiedlichen Typs hin, die beide einer mechanischen Stimulation ausgesetzt waren. (4) Genexpression und Proteinsynthese der untersuchten Gene bzw. Metabolite variieren nach Abschluss der mechanischen Stimulation.

In zukünftigen Forschungsprojekten sollte die Temperatur ein bedeutender Faktor bei der Durchführung von *in vitro* Experimenten sein. Das indirekte Ko-Kulturmodell ist ein innovativer und praktischer Weg, um die *in vivo* Situation zu simulieren. Probenahme nach dem „*pulse chase*“-Prozedere ermöglicht präzisere zeitliche Aussagen bezüglich der Dynamik von Genexpression und Proteinsynthese nach mechanischer Stimulation.

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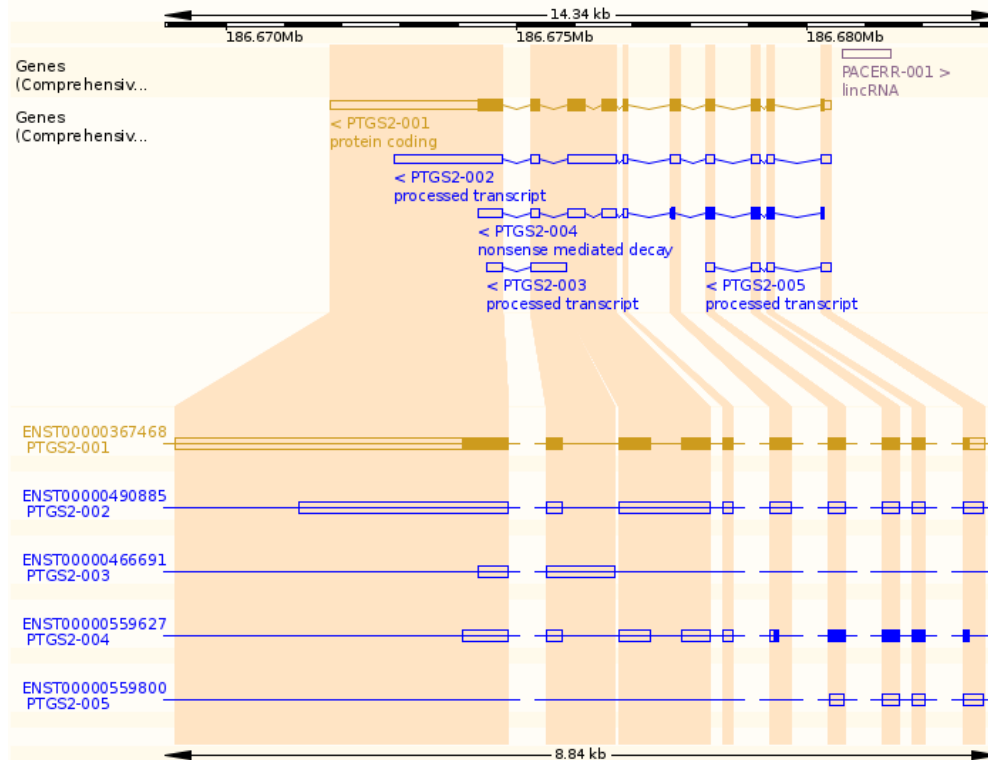
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11 Appendix - Example of bioinformatics primer testing

1. Genomic structure of the human *PTGS2* gene:

Gene-Id: 5743
 mRNA reference sequence NM_000963.3
 Genomic reference sequence NG_028206.2
 Ensemble Id: ENSG00000073756

Splice variants (Ensembl):



2. Potential primers

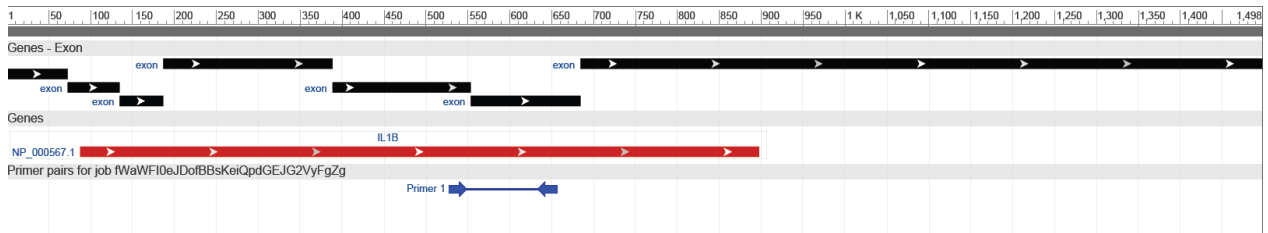
Primer no	Forward	Reverse	Product size
1	GGAACACAACAGAGTCTGAG	AAGGGGATGGCCAGTGTATAGA	
2	AGAACTGGTACATCAGCAAG	GAGTTTACAGGAAGCAGACA	
3	AAGCCTTCTCTAACCTCTCC	GCCCTCGCTTATGATCTGTC	234 bp (514-747)

Reference sequence used is GenBank accession number NM_00096.3

- Testing primer specificity with “electronic PCR” (“ePCR”) and Primer-BLAST. Since the “ePCR” service is not provided anymore, only Primer-BLAST results are shown.

For Primers #1 and #2, Primer-BLAST output generated was: “*Specified left primer cannot be found in template...make sure this primer is on the plus strand of your template.*”

For Primer #3 the following output was generated showing the primer binding site with respect to the gene’s exon structure:



Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self-comple-mentarity	Self 3' comple-mentarity
Forw.	AAGCCTTCTCTAACCTCTCC	Plus	20	514	533	55.95	50.00	5.00	0.00
Rev.	GCCCTCGCTTATGATCTGTC	Minus	20	747	728	58.21	55.00	4.00	1.00
Product length			234						

Products on intended target

>NM_000963.3 Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA
product length = 234

Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
Template 514 533

Reverse primer 1 GCCCTCGCTTATGATCTGTC 20
Template 747 728

Products on potentially unintended templates

>XM_011520202.1 PREDICTED: Homo sapiens solute carrier family 35 (GDP-fucose transporter), member C1 (SLC35C1), transcript variant X1, mRNA
product length = 231

Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
Template 915 ..C.....T.....G 934

Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
Template 1145 ...G....C.A.C..... 1126

>NM_001145266.1 Homo sapiens solute carrier family 35 (GDP-fucose transporter), member C1 (SLC35C1), transcript variant 3, mRNA

product length = 231
 Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
 Template 1661 ..C.....T.....G 1680

Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
 Template 1891 ...G....C.A.C..... 1872

>NM_001145265.1 Homo sapiens solute carrier family 35 (GDP-fucose transporter), member C1 (SLC35C1), transcript variant 2, mRNA
 product length = 231

Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
 Template 1758 ..C.....T.....G 1777

Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
 Template 1988 ...G....C.A.C..... 1969

>NM_018389.4 Homo sapiens solute carrier family 35 (GDP-fucose transporter), member C1 (SLC35C1), transcript variant 1, mRNA
 product length = 231

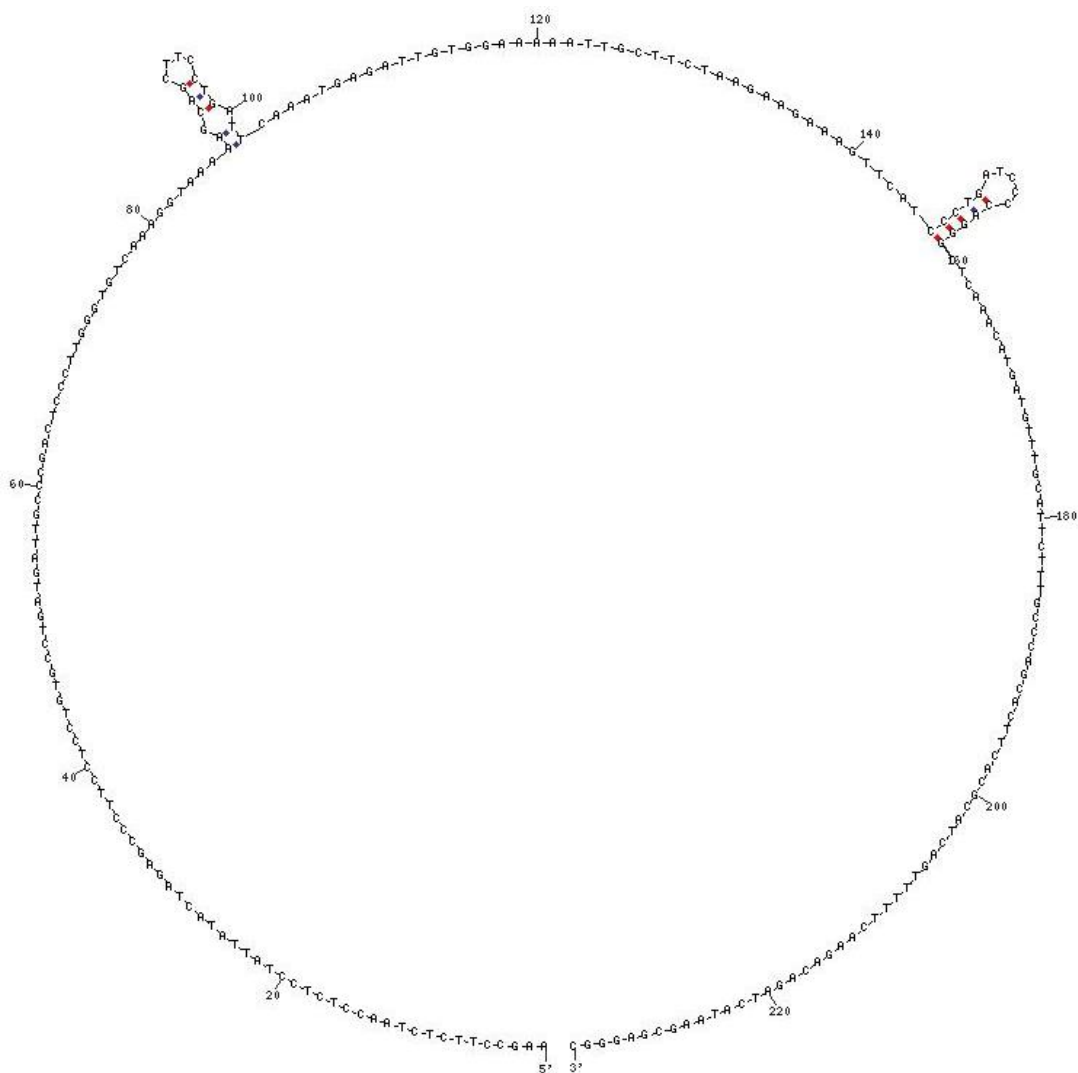
Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
 Template 2032 ..C.....T.....G 2051

Forward primer 1 AAGCCTTCTCTAACCTCTCC 20
 Template 2262 ...G....C.A.C..... 2243

4. Energy considerations on the PCR product using UNAFold service at IDT. For Primers #1 and #2 the following error message was generated: “PRIMERQUEST ERROR: Specified forward primer not in sequence; Specified reverse primer not in sequence”.

With Primer #3 the following amplification product from mRNA/cDNA is produced. The primers are shown here with underlines:

AAGCCTTCTCTAACCTCTCCTATTATACTAGAGCCCTTCCTCCTGTGCCTGATGATTGCCCG
 ACTCCCTTGGGTGTCAAAGGTAAAAAGCAGCTTCCTGATTCAAATGAGATTGTGGAAAAATT
 GCTTCTAAGAAGAAAGTTCATCCCTGATCCCCAGGGCTCAAACATGATGTTTGCATTCTTTG
 CCCAGCACTTCACGCATCAGTTTTTTCAAGACAGATCATAAGCGAGGGC



The following energy considerations were generated:

Structure Name	ΔG (kcal mol ⁻¹)	T _M (°C)	ΔH (kcal mol ⁻¹)	ΔS (cal K ⁻¹ mol ⁻¹)
1	-1.06	69	-40.5	-118.38
2	-0.65	62.6	-83.7	-249.3
3	-0.27	61.4	-61.6	-184.1

5. “Beacon Designer Free Edition” was additionally used for energy considerations.

For Primer #3 the following output was generated:

Name: PTGS2_P3

Assay Type: SYBR[®] Green

Nucleic Acid Concentration (nM)	0.25	Monovalent Concentration (mM)	50
Free Mg⁺⁺ Concentration (mM)	3	Total Na⁺ Concentration (mM)	269.09

Sense Primer: AAGCCTTCTCTAACCTCTCC

Length (bp)	Tm (°C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self-Dimer (ΔG)	Hairpin (ΔG)
20	52.87	50	2	-2.9	-0.5	0.0

Anti-sense Primer: GCCCTCGCTTATGATCTGTC

Length (bp)	Tm (°C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
20	55.23	55	2	-2.9	-2.0	0.0

Secondary Structures for Sense Primer

Dimer:-

```

          5' AAGCCTTCTCTAACCTCTCC 3'
              ||| |||
3' CCTCTCCAATCTCTTCCGAA 5'

```

-0.5

Hairpin:- Not Found

Secondary Structures for Anti-Sense Primer

Dimer:-

```

5' GCCCTCGCTTATGATCTGTC 3'
          ||||
          3' CTGTCTAGTATTCGCTCCCG 5'

```

-2.0

Hairpin:- Not Found

Cross Dimer

Cross Dimer between Sense Primer and Anti-sense Primer:-

```

          5' AAGCCTTCTCTAACCTCTCC 3'
              |||| |
3' CTGTCTAGTATTCGCTCCCG 5'

```

-2.9

```

5' AAGCCTTCTCTAACCTCTCC 3'
  |   ||| |
3' CTGTCTAGTATTCGCTCCCG 5'

```

0.3

6. Final check with Primer3plus for Primer #3

Left Primer 1:	AAGCCTTCTCTAACCTCTCC								
<u>Start:</u>	<u>Length:</u>	<u>Tm:</u>	<u>GC:</u>	<u>Any:</u>	<u>End:</u>	<u>TB:</u>	<u>HP:</u>	<u>3'</u> <u>Stab:</u>	<u>Penalty:</u>
514	20 bp	55.9 C	50.0 %	0.0	0.0	7.0	0.0	3.7	4.054
<u>Library Mispriming:</u> 13.00, reverse MLT1R MLT1-Mammalian LTR retrotransposon internal sequence - a consensus									
Problems Temperature too low; Similarity to non-template sequence too high									
Right Primer 1:	GCCCTCGCTTATGATCTGTC								
<u>Start:</u>	<u>Length:</u>	<u>Tm:</u>	<u>GC:</u>	<u>Any:</u>	<u>End:</u>	<u>TB:</u>	<u>HP:</u>	<u>3'</u> <u>Stab:</u>	<u>Penalty:</u>
747	20 bp	58.2 C	55.0 %	0.0	0.0	8.0	0.0	3.5	1.790
<u>Library Mispriming:</u> 12.00, MSTAR MStA-Mammalian LTR internal retrotransposon sequence - a consensus									

Pair:	<u>Product Size:</u> 234 bp	<u>Any:</u> 0.0	<u>End:</u> 0.0	<u>TB:</u> 15.0	<u>Penalty:</u> 5.844
<u>Library</u>	21.00, L1 Human L1 interspersed repetitive sequence - full length				
<u>Mispriming:</u>	copy				

```

1      GACCAATTGT CATACGACTT GCAGTGAGCG TCAGGAGCAC GTCCAGGAAC
[...deleted...]
451    CCAGATCACA TTTGATTGAC AGTCCACCAA CTTACAATGC TGACTATGGC
501    TACAAAAGCT GGGAGCCTT CTCTAACCTC TCCATTATATA CTAGAGCCCT
551    TCCTCCTGTG CCTGATGATT GCCCGACTCC CTTGGGTGTC AAAGGTAAAA
601    AGCAGCTTCC TGATTCAAAT GAGATTGTGG AAAAATTGCT TCTAAGAAGA
651    AAGTTCATCC CTGATCCCCA GGGCTCAAAC ATGATGTTTG CATTCTTTGC
701    CCAGCACTTC ACGCATCAGT TTTTCAAGAC AGATCATAAG CGAGGGCAG
751    CTTTCACCAA CGGGCTGGGC CATGGGGTGG ACTTAAATCA TATTTACGGT
801    GAAACTCTGG CTAGACAGCG TAAACTGCGC CTTTTCAAGG ATGGAAAAAT
[...deleted...]
4451   TTATTTTTGT ACTATTTAAA AATTGACAGA TCTTTTCTGA AGAAAAAAAA
4501   AAAAAAA

```

Statistics: Left Primer: considered 1, unacceptable product size 1, tm diff too large 1, primer in pair overlaps a primer in a better pair 1, ok 1

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Eidesstattliche Versicherung

Shi Jianwei

Name, Vorname

Ich erkläre hiermit an Eides statt,

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