Molecular Mechanisms of Cell Adhesion and Cytoskeletal Dynamics Underlying Human Mesenchymal Stem Cell Differentiation
LAURA HYVÄRI

Molecular Mechanisms of Cell Adhesion and Cytoskeletal Dynamics Underlying Human Mesenchymal Stem Cell Differentiation

ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Medicine and Health Technology of Tampere University, for public discussion in the auditorium F114 of the Arvo building, Arvo Ylpön katu 34, Tampere, on 14 June 2024, at 12 o’clock.
ABSTRACT

Stem cell differentiation during tissue development and regeneration is a complex multistep process involving temporally and spatially orchestrated regulation. Mesenchymal stem cells (MSCs) are multipotent progenitor cells of mesodermal origin that differentiate into adipocytes and osteoblasts. The differentiation capacity of these adult stem cells marks them as intriguing candidates for soft and bone tissue regeneration and engineering. However, the molecular factors and mechanisms involved in the regulation of MSC differentiation have been studied using primarily animal models and human MSC differentiation remains understudied.

In this thesis, human MSCs harvested from adipose tissue (human adipose stem cell; hASC) or bone marrow (human bone marrow stem cell; hBMSC) were studied in vitro. Adipogenic or osteogenic differentiation of hMSCs was induced with biochemical agents in culture medium. Additionally, the cells were cultured under ions extracted from bioactive glass (BaG) to stimulate osteogenic differentiation. Central and evolutionarily conserved protein kinase pathways related to cell adhesion and cytoskeletal dynamics were studied in the context of directing hMSC differentiation potential. Focal adhesion kinase (FAK) is a key regulator of cell adhesion that mediates signal through various targets, including mitogen-activated protein kinase (MAPK) pathways. Actin cytoskeleton dynamics are modulated by the Rho-associated coiled-coil kinase (ROCK) pathway. The ROCK downstream target, myocardin-related transcription factor A (MRTF-A) serves as an intriguing link between cytoskeletal dynamics and gene expression. The roles of these intracellular signaling pathways in hMSC differentiation were studied using small-molecule inhibitors of signal transduction.

The results demonstrate that cell adhesion mediated by FAK signaling acts as a molecular switch between adipogenic and osteogenic differentiation of hASCs. FAK pathway activity favored the osteogenic outcome of hASCs, but inhibition of cell adhesion by FAK inhibition resulted in enhanced adipogenesis. The activity of the FAK downstream target MAPK extracellular signal-regulated kinase (ERK) was...
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In this thesis, human MSCs harvested from adipose tissue (human adipose stem cell; hASC) or bone marrow (human bone marrow stem cell; hBMSC) were studied in vitro. Adipogenic or osteogenic differentiation of hMSCs was induced with biochemical agents in culture medium. Additionally, the cells were cultured under ions extracted from bioactive glass (BaG) to stimulate osteogenic differentiation. Central and evolutionarily conserved protein kinase pathways related to cell adhesion and cytoskeletal dynamics were studied in the context of directing hMSC differentiation potential. Focal adhesion kinase (FAK) is a key regulator of cell adhesion that mediates signaling through various targets, including mitogen-activated protein kinase (MAPK) pathways. Actin cytoskeleton dynamics are modulated by the Rho-associated coiled-coil kinase (ROCK) pathway. The ROCK downstream target, myocardin-related transcription factor A (MRTF-A) serves as an intriguing link between cytoskeletal dynamics and gene expression. The roles of these intracellular signaling pathways in hMSC differentiation were studied using small-molecule inhibitors of signal transduction.

The results demonstrate that cell adhesion mediated by FAK signaling acts as a molecular switch between adipogenic and osteogenic differentiation of hASCs. FAK pathway activity favored the osteogenic outcome of hASCs, but inhibition of cell adhesion by FAK inhibition resulted in enhanced adipogenesis. The activity of the FAK downstream target MAPK extracellular signal-regulated kinase (ERK) was
found to be significant for both the adipogenic and osteogenic differentiation of hASCs. These results indicate that FAK and ERK pathways have distinct roles in the regulation of lineage commitment.

Based on the results, the ROCK pathway and MRTF-A are relevant regulators of the hASC lineage commitment through cytoskeletal modulation. We demonstrated that actin polymerization and contractility induced by ROCK activity are required for osteogenesis. ROCK inhibition enhances the number and size of intracellular lipid droplets, thus suggesting adipogenic maturation. Active MRTF-A signaling is important for the osteogenic course of hASCs. Inhibition of nuclear translocation of MRTF-A resulted in changes in actin cytoskeleton and enhanced adipogenic fate.

The results revealed that ions dissolved from the experimental boron-containing BaG are strong inducers of osteogenesis in hASCs and hBMSCs. A novel role in the context of hMSC osteogenesis was discovered for the MAPK family member p38/heat shock protein 27 (HSP27) signaling. Activation of the p38 MAPK/HSP27 pathway occurred early and temporally during BaG-induced osteogenesis, and inhibition of HSP27 phosphorylation decreased osteogenic differentiation. The results suggest that the cytoskeletal association of phosphorylated HSP27 could function as an early regulator in the osteogenic commitment of hMSCs.

The study results reported in this thesis increase the biological knowledge and understanding of hMSC differentiation and provide a background for knowledge-based solutions in tissue engineering and for the treatment of bone and adipose tissue-related disorders.
Kantasolujen erilaistuminen kudosten kehittymisen ja uusiutumisen aikana on monitahoinen ja monivaiheinen prosessi, johon liittyvät aikaan ja paikkaan sidottu säätely. Mesenkymaaliset kantasolut ovat mesodermistä peräisin olevia monikykyisiä esisoluja, jotka pystyvät erilaistumaan rasva- ja luusoluiksi. Erilaistumiskykyäsi vuoksi näin aikuisen kantasolut ovat mielenkiintoisia kandidaatteja käyttettäväksi pehmyktukoskeen ja luukukoskeen uusiutumisessa ja kudosteknologiassa. Ihmisen mesenkymaalisten kantasolujen erilaistumista ohjaavat molekulaariset tekijät ja mekanismit tunnetaan vielä huonosti, sillä pääosa aiemmista tutkimuksista on tehty eläimille.

Based on the results, the ROCK pathway and MRTF-A are relevant regulators of the hASC lineage commitment through cytoskeletal modulation. We demonstrated that actin polymerization and contractility induced by ROCK activity are required for osteogenesis. ROCK inhibition enhances the number and size of intracellular lipid droplets, thus suggesting adipogenic maturation. Active MRTF-A signaling is important for the osteogenic course of hASCs. Inhibition of nuclear translocation of MRTF-A resulted in changes in actin cytoskeleton and enhanced adipogenic fate. The results revealed that ions dissolved from the experimental boron-containing BaG are strong inducers of osteogenesis in hASCs and hBMSCs. A novel role in the context of hMSC osteogenesis was discovered for the MAPK family member p38/heat shock protein 27 (HSP27) signaling. Activation of the p38 MAPK/HSP27 pathway occurred early and temporally during BaG-induced osteogenesis, and inhibition of HSP27 phosphorylation decreased osteogenic differentiation. The results suggest that the cytoskeletal association of phosphorylated HSP27 could function as an early regulator in the osteogenic commitment of hMSCs.

The study results reported in this thesis increase the biological knowledge and understanding of hMSC differentiation and provide a background for knowledge-based solutions in tissue engineering and for the treatment of bone and adipose tissue-related disorders.


Tulokset osoittavat, että FAK-signaalin in inhibiition välittämä soluadheesio toimii molekulaarisena kytkimenä rasvan kantasolujen erilaistumissuunnan valinnassa rasva- ja luusolujen suuntaan. FAK-signaalin aktiivisuus suosi luuerilaistumista, mutta FAK-inhibition aikaansaama heikentynyt kiinnittyminen lisäsi rasvaerilaistumista ihmisen rasvan kantasoluissa. FAK-signaaliin kytökssissä
olevan MAPK ERK-signaloinnin aktiivisuuden havaittiin olevan merkittävää sekä rasva- että luuerilaistumisen kannalta ihmisen rasvan kantasoluissa. Tulosten perusteella FAK- ja ERK-signaalireiteillä on erilliset roolit erilaistumissuunnan säätelyssä.


Väitöskirjan tutkimukset lisäisivät biologista tietoa ja ymmärrystä ihmisen mesenkymaalisten kantasolujen erilaistumisen taustalla, minkä avulla voidaan kehitää tietopohjaisia kudosteknologisia menetelmiä ja ratkaisuja rasva- ja luukudokseen liittyvien sairauksien hoitamiseen.
luukudokseen liittyvien sairauksien hoitamiseen.

kehittää tietopohjaisia kudosteknologisia menetelmiä ja ratkaisuja rasva
mesenkymaalisten kantasolujen erilaistumisen taustalla,

Väitöskirjan tutkimukset lisäisivät biologista tietoa ja ymmärrystä ihmisen
luuerilaistumista

p38 MAPK/HSP27

kantasolujen luuerilaistumisessa

ionit ovat vahvoja luuerilaistumisen indusoijia ihmisen rasvan ja luuytimen

Tulokset osoitt

aktiinitukirangassa ja

transkriptiotekijän tumakuljetuksen estäminen sai aikaan muutoksia
todettiin tärkeäksi ihmisen rasvan kantasolujen
kokoa,
luuerilaistumis
aktiivisuuden

ROCK

rasva

itäen MAPK ERK

ängöshokkiproteiini 27 (HSP27)

että luuerilaistumisen kannalta ihmisen rasvan kantasolussa.

signaloinnin ja MRTF

indikoi rasvasolujen maturaatiosta.

vatt, että kokeellisesta booria sisältävästä bioaktiivisesta lasista uutetut

roiteen aktivaation, ja HSP27

signaloinnin aktiivisuuden havaittiin olevan merkittävä sekä
-
inhibitio

rasvaerilaistumista.

aktiini

signaali

A

Bioaktiivinen lasi indusoi varhaisen ja lyhytaikaisen

kuvaillaan uudenlainen rooli

transkriptiotekijän havaittiin säätelevän

polymerisaatio

reiteillä on

lisäsi solunsisäisten lipidipisaroiden määrää ja

signaloi

solutukiran

solutukirangan muutosten avulla

viinille

MRTF

erilliset roolit

MRTF

fosforylaation

ja supistumiskyky

gan

ihmisen mesenkymaalisten

ja

- A

fosforylo

signaloinnin aktiivisuus

minkä

erilaistumissuunnan

inhibitio heikensi

avulla voidaan

p38 MAPK/
in dun

mahdollisti

HSP27

Tulosten

ihmisen

A


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<th>Description</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>minimum essential medium α</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>AM</td>
<td>adipogenic medium</td>
</tr>
<tr>
<td>AT</td>
<td>adipose tissue</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>aP2</td>
<td>adipocyte protein 2</td>
</tr>
<tr>
<td>ASC</td>
<td>adipose stem cell</td>
</tr>
<tr>
<td>BaG</td>
<td>bioactive glass</td>
</tr>
<tr>
<td>BaG BM</td>
<td>bioactive glass extract in basic medium</td>
</tr>
<tr>
<td>BaG OM</td>
<td>bioactive glass extract in osteogenic medium</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BM</td>
<td>basic medium</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BMSC</td>
<td>bone marrow stem cell</td>
</tr>
<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>C/EBP</td>
<td>enhancer-binding protein</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>core-binding factor alpha 1</td>
</tr>
<tr>
<td>d</td>
<td>day(s)</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DLX5</td>
<td>distal-less homeobox 5</td>
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<td>DMP1</td>
<td>dentin matrix protein 1</td>
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<tr>
<td>DEX</td>
<td>dexamethasone</td>
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<td>DMEM/F-12</td>
<td>Dulbecco's Modified Eagle's Medium/Ham's nutrient mixture F-12</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>ezrin-radixin-moesin</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FABP4</td>
<td>fatty acid-binding protein</td>
</tr>
<tr>
<td>FATP-1</td>
<td>fatty acid transport protein-1</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>hydroxyapatite</td>
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<tr>
<td>hASC</td>
<td>human adipose stem cell</td>
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<tr>
<td>hBMSC</td>
<td>human bone marrow stem cell</td>
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<tr>
<td>HH</td>
<td>Hedgehog</td>
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<td>HLA-DR</td>
<td>human leukocyte antigen D-related</td>
</tr>
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<td>hMSC</td>
<td>human mesenchymal stem cell</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>human serum</td>
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<td>heat shock protein 27</td>
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<td>HSC</td>
<td>hematopoietic stem cell</td>
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<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine/isobutylmethylxanthine</td>
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<td>ICC</td>
<td>immunocytochemistry</td>
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<tr>
<td>IFATS</td>
<td>International Federation of Adipose Therapeutics and Sciences</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>ISCT</td>
<td>International Society for Cellular Therapy</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LEP</td>
<td>leptin</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM domain kinase</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>MAPKAP</td>
<td>MAP kinase-activated protein</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MAPKK</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>MAPK kinase kinase</td>
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<tr>
<td>MEPE</td>
<td>matrix extracellular phosphoglycoprotein</td>
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<td>Description</td>
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</tr>
<tr>
<td>MLC</td>
<td>myosin light-chain</td>
</tr>
<tr>
<td>MLCP</td>
<td>light-chain phosphatase</td>
</tr>
<tr>
<td>MRTF-A</td>
<td>myocardin-related transcription factor A</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>OM</td>
<td>osteogenic medium</td>
</tr>
<tr>
<td>OCN</td>
<td>osteocalcin</td>
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<td>OPG</td>
<td>osteoprotegerin</td>
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<td>Oil Red O</td>
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<td>phosphatidylinositol 3-kinase</td>
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<td>p38-reactivating kinase</td>
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<td>PKA</td>
<td>protein kinase A</td>
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<td>P/S</td>
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<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor γ</td>
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<td>preadipocyte factor-1</td>
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<td>quantitative Alizarin Red staining</td>
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<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor kappa-B</td>
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<td>receptor activator of nuclear factor kappa-B ligand</td>
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<td>ROCK</td>
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<td>receptor tyrosine kinase</td>
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<td>RUNX2</td>
<td>runt-related transcription factor 2</td>
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<td>SD</td>
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<td>WAT</td>
<td>white adipose tissue</td>
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This thesis is based on the following Original Publications, referred in the text as follows.

Study I  

Study II  

Study III  
AUTHOR’S CONTRIBUTION

I–III First author. The author designed the studies in collaboration with the co-authors. The author was primarily responsible for the practical work, data collection, and analysis and drafted the manuscripts.
1 INTRODUCTION

Multipotent mesenchymal stem cells (MSCs), that possess differentiation capacity towards adipogenic, osteogenic, and chondrogenic lineages can be harvested from adult tissues of mesodermal origin (Kern et al., 2006; Pittenger et al., 1999; Varma et al., 2007). These cells have already been utilized in clinics for soft tissue and bone tissue reconstruction, although the detailed intracellular mechanisms governing MSC differentiation are still under investigation, and a large bulk of the current research is animal-based. The bone marrow was the first location where MSCs were discovered, but donor-site morbidity and low yield of bone marrow stem cells (BMSCs) have encouraged clinicians to search for other harvesting sites (Kern et al., 2006). Adipose tissue is one intriguing MSC source in which the adipose stem cells (ASCs) are embedded in the stromal vascular fraction (SVF) of the tissue (Varma et al., 2007). ASCs can be harvested in greater quantities than can BMSCs (Kern et al., 2006); however, the \textit{in vitro} differentiation potential of these cells has been debated. hBMSCs have been reported to exhibit a higher osteogenic potential than that of hASCs (C. Li et al., 2015; Mohamed-Ahmed et al., 2018; Shafiee et al., 2011), but contrasting results have been reported (Rath et al., 2016). Additionally, similar differentiation potentials of hASCs and hBMSCs have been reported (De Ugarte et al., 2003). Both BMSCs and ASCs exhibit beneficial immunomodulatory properties (Arthur & Gronthos, 2020; Patrikoski et al., 2014). Tissue engineering (TE) applications utilize biomaterials in addition to stem cells to provide structural support or to induce differentiation. Bioactive glass materials are good candidates for bone TE due to their biocompatibility and capacity to enhance the osteogenic differentiation of progenitor cells (Hench & Jones, 2015; Rahaman et al., 2011).

Protein phosphorylation is an important mediator of cellular messaging. Extracellular signals are transmitted through cell membrane receptors to activate intracellular cascades, thus regulating protein function and gene expression (Ardito et al., 2017). Studies have demonstrated that protein kinases involved in cell adhesion...
and cell cytoskeleton dynamics are also important regulators of stem cell differentiation fate (Kilian et al., 2010; McBeath et al., 2004; Sonowal et al., 2013). Integrin-mediated formation of focal adhesion complexes initiates cellular adhesion mechanisms and subsequent activation of focal adhesion kinase (FAK). FAK signaling is interconnected with various downstream targets, including mitogen-activated protein kinases (MAPK) that are important for cell proliferation and differentiation, and Rho-associated coiled-coil kinase (ROCK) that is involved in the regulation of the actin cytoskeleton (Cary & Guan, 1999; Harburger & Calderwood, 2009). Cytoskeletal remodeling regulates the activity of the transcription factor MRTF-A and the expression of actin-related genes (Olson & Nordheim, 2010). The MAPK family member p38-reactivating kinase (p38) is involved in the osteogenic differentiation of MSCs (Hoffman et al., 2017; Rodriguez-Carballo et al., 2016) and is associated with actin dynamics via the downstream target heat shock protein 27 (HSP27) (Arrigo, 2017; J. Clarke & Mearow, 2013; Hirano et al., 2004).

This thesis examined the molecular basis of human MSC differentiation fate decisions towards adipogenic and osteogenic lineages while focusing on the intracellular signaling mechanisms of cell adhesion and cytoskeletal dynamics. Differentiation was stimulated by biochemical cues, such as adipogenic or osteogenic culture supplements and bioactive ions dissolved from bioactive glass. The signaling mechanisms involved in the differentiation process were studied using small-molecule inhibitors of key signaling proteins. This thesis was written from the standpoint of a cell biologist and aimed to elucidate biological events and cell signaling mechanisms to provide a basis for the clinical translation of biological data. This thesis sheds light on the central lineage specification mechanisms that regulate human MSC differentiation.
2 LITERATURE REVIEW

2.1 Adipose tissue

Adipose tissue is a dynamic organ present in several parts of the human body. It exhibits important functions in metabolism, energy storage, and energy regulation and plays a role in heat regulation (Niemelä et al., 2007). Subcutaneous fat in the soles, palms, and tips of fingers and toes has a mechanical role by providing a barrier and padding (Berry et al., 2013). Adipose tissue is also an endocrine organ that regulates various aspects of metabolism and plays a role in regulating female reproduction via adipokine secretion (Mathew et al., 2018). Adipose tissue is formed through the generation of new adipocytes and the enlargement of existing adipocytes throughout life. It can increase in volume by 15-fold based on the nutritional environment of the individual (Berry et al., 2013). Adipose tissue is composed of precursor cells, mature adipocytes, fibroblasts, vascular endothelial cells, pericytes, smooth muscle cells, and immune cells (da Silva et al., 2020; McDonald et al., 2015; Romagnoli & Brandi, 2014) (Figure 1). Mesenchymal stem cells (MSCs) are adipocyte precursors (Berry et al., 2013). The extracellular matrix (ECM) of adipose tissue is composed of collagen, fibronectin, elastin, glycosaminoglycan, and laminin (de Sousa Neto et al., 2022).

Figure 1. Adipose tissue structure. Modified from (da Silva et al., 2020).
As presented in Figure 2, there are three distinguishable adipocyte types. The predominant type, white adipocytes, account for white adipose tissue (WAT) cells. White adipocytes are found in the subcutaneous adipose tissue (AT), thoracic and abdominal perivascular AT, and visceral AT (de Sousa Neto et al., 2022; Koenen et al., 2021). Brown adipose tissue (BAT) is predominantly present in neonates, while adults almost lack BAT (McDonald et al., 2015; J. Wu et al., 2012). The third subtype of adipocytes is a population of uncoupling protein 1 (UCP1)-positive adipocytes termed beige or brite adipocytes that develop within the white adipose tissue when exposed to cold (Pilkington et al., 2021). Additionally, pink adipocyte tissue can be distinguished in mammary glands (de Sousa Neto et al., 2022).

Figure 2. Illustration of adipocyte types. Modified from (Koenen et al., 2021).

Obesity, referring to excessive fat accumulation, has become a global epidemic and related diseases are burdening health care systems world-wide (Blüher, 2019; Fruh, 2017; Koenen et al., 2021). Excessive lipid accumulation leads to phenotypic changes and low-grade inflammation through increased levels of pro-inflammatory cytokines, ECM enlargement, and fibrosis (de Sousa Neto et al., 2022). Comorbidities linked to obesity include hypertension, insulin resistance, cardiovascular diseases, and related mortalities (Blüher, 2019; Koenen et al., 2021). The progression of certain cancers is also linked to obesity (Blüher, 2019; Fruh, 2017; Koenen et al., 2021). Adipokines secreted from adipose tissue are involved in reproductive health, mediate insulin resistance during pregnancy, and have been implicated in conditions such as polycystic ovary syndrome (Mathew et al., 2018).

2.2 Bone tissue

The skeleton in combination with other musculoskeletal tissues is the structural part of the body that allows for posture and movement. Bone serves as an important storage structure for calcium (99% of the body's calcium) and phosphate (80% of the body's phosphate) that exist primarily in the form of hydroxyapatite (HA; Ca₁₀(PO₄)₆(OH)₂) (Blair et al., 2017; Rodriguez-Carballo et al., 2016). Structurally,
bodies are composed of compact bone (also called cortical bone) lining the porous trabecular (also called cancellous) bone (Figure 3) (B. Clarke, 2008; Lopes et al., 2018). In long bones, trabecular bone is present at the ends (epiphyses), the long shaft (diaphysis) is compact bone, and the core of the shaft (medullary cavity) is filled with mostly fat-containing yellow bone marrow (B. Clarke, 2008; H. Wang et al., 2018). Active hematopoietic red bone marrow fills trabecular bone pores (Benova & Tencerova, 2020; H. Wang et al., 2018). Microscopically, the compact bone is composed of osteons (also called Haversian systems) that are cylindrical structures of a calcified matrix housing mature bone cells termed osteocytes (Figure 3) (Chang & Liu, 2021; Lopes et al., 2018). Osteocyte cell bodies (lacunae) have extensive filopodial processes (canaliculi) and this lacunocanalicular network mediates mechanical signals in the bone (Chang & Liu, 2021; B. Clarke, 2008). Blood vessels, nerves, and lymphatic vessels are located at the center of osteons in Haversian canals and in perpendicular Volkmann canals providing the cells with nutrients (Chang & Liu, 2021).

![Figure 3. Schematic presentation of long bones and cross-section of compact bone. Figure is modified from (Chang & Liu, 2021; Lopes et al., 2018).](image)

During embryonic development, the bone tissue is formed in two separate modes. Most bones are formed through a cartilage intermediate in a process termed endochondral ossification (Blair et al., 2017). Skull bones are formed directly from osteoblasts through intramembranous ossification (Salhotra et al., 2020). These craniofacial osteoblasts are derived from neural crest cells of the neural ectoderm (Salhotra et al., 2020). Bone tissue repair or the remodeling of active bone follows the same mechanisms as in bone development. After injury, a soft callus is formed...
from inflammatory cells that gradually develops into a cartilaginous intermediate, undergoes calcification, and matures into trabecular and compact bones (Houshyar et al., 2019). Most fractures heal over time; however, fractures that fail to heal have prompted the development of applications for improved bone regeneration. Bone remodeling requires the presence of bone-resorbing osteoclasts derived from the monocyte/macrophage lineage (Aeschlimann & Evans, 2004). An imbalance in osteoblast and osteoclast functions may lead to osteoporosis when bone resorption exceeds the bone-forming capacity of osteoblasts (Aeschlimann & Evans, 2004; Salhotra et al., 2020).

2.3 Mesenchymal stem cells

Stem cells are capable of self-renewal and differentiation into more specialized lineages (Choumerianou et al., 2008). Stem cells can be classified into totipotent, pluripotent, and multipotent cells based on their differentiation potential (Brignier & Gewirtz, 2010). The zygote (or fertilized egg) is a totipotent cell with the capacity to produce the entire organism, including embryonic and extraembryonic tissues (Biswas & Hutchins, 2007; Condic, 2014). Embryonic stem cells are pluripotent and can differentiate into ectodermal, mesodermal, and endodermal lineages (Brignier & Gewirtz, 2010; Condic, 2014). Multipotent stem cells that can differentiate only into specific cell types reside in fetal and adult tissues (Choumerianou et al., 2008; Pappa & Anagnou, 2009). Multipotent stem cells can be further classified based on the germ layer from which they originate (endodermal, mesodermal, or ectodermal) (Choumerianou et al., 2008). Stem cells originating from mesenchymal tissues such as the bone marrow, adipose tissue, dental pulp, dermal tissue, skeletal muscle, and umbilical cord blood are called MSCs (Mushahary et al., 2018). The role of MSCs in adult tissues is to maintain tissue integrity by regenerating tissues after trauma, cell turnover, or disease (Brignier & Gewirtz, 2010).

MSCs are characterized according to the minimal criteria provided by the International Society for Cellular Therapy (ISCT) as plastic-adherent cells with in vitro multipotent differentiation capacity towards the three main differentiation lineages of bone, adipose tissue, and cartilage based on the specific expression profile of cluster of differentiation (CD) markers (Dominici et al., 2006). The cells must express CD105, CD73 and CD90 and lack the expression of CD45, CD34, CD14 or
CD11b, CD79α or CD19 and human leukocyte antigen D-related (HLA-DR) (Dominici et al., 2006).

The MSC differentiation capacity exceeds the minimum of the three differentiation lineages determined by the ISCT as presented in Figure 4. In vitro differentiation of MSCs towards myocytes, cardiomyocytes, tenocytes, endothelial cells, hepatocytes, and pancreatic cells has been reported (Caplan & Bruder, 2001; Mushahary et al., 2018; Ullah et al., 2015). Two MSC subtypes relevant to this work are discussed in more detail in sections 2.3.1 and 2.3.2.

![Figure 4. Schematic presentation of MSC differentiation in vitro. Modified from (Caplan & Bruder, 2001; James, 2013).](image)

### 2.3.1 Bone marrow stem cells

Bone marrow is a soft heterogeneous immune and endocrine organ inside the bone cavities that is responsible for hematopoiesis, i.e. the synthesis of all cellular components of blood and blood plasma from hematopoietic stem cells (HSCs) (Benova & Tencerova, 2020). In addition to HSCs, BMSCs reside within the bone marrow (Benova & Tencerova, 2020). BMSCs are central to bone formation, as they are progenitors of bone-forming osteoblasts (Lian & Stein, 1995). The frequency of BMSCs in the bone marrow is 0.01-0.1% (Benova & Tencerova, 2020). Adipocytes that originate from these BMSCs account for the largest cell volume (70%) in bone cavities (Wang et al., 2018). In neonates the hematopoietic red bone marrow is
observed in virtually all bones, however, the adipocyte content of the bone marrow gradually increases with time and occupies the cavities (Benova & Tencerova, 2020; Wang et al., 2018). In adults red bone marrow is only present in the sternum, vertebrae, ribs, and pelvic bone (Benova & Tencerova, 2020).

MSCs from the bone marrow were first described in the seminal studies by Friedenstein et al. in mice (Friedenstein et al., 1968) and by Caplan et al. who isolated the first human BMSCs in the early 90s (Caplan, 1991). In vitro BMSC characterization follows the MSC criteria by ISCT. However, positive expression of the hematopoietic marker HLA-DR has been reported under normal culture conditions without affecting MSC identity (Grau-Vorster et al., 2019). Since their discovery, BMSCs have been studied extensively, particularly in the context of tissue engineering for bone regeneration as reviewed by Arthur and Gronthos (Arthur & Gronthos, 2020). One of the most attractive properties of hBMSCs is their immunomodulatory nature, and this makes them ideal for allogenic tissue regeneration and other clinical applications (Arthur & Gronthos, 2020). However, certain features limit the utilization of hBMSCs in research and clinical applications. The donating procedure is invasive and potentially painful, and the aspirates are small (Kern et al., 2006). Consequently, the yield of stem cells is low. The BMSC yield further decreases with increasing donor age when red bone marrow is replaced by yellow fatty bone marrow (Wang et al., 2018). Additionally, the differentiation potential of BMSCs is negatively affected by donor age (Kern et al., 2006). Importantly, a high body mass index (BMI) of a donor favors adipogenic differentiation over osteogenic and chondrogenic differentiation (Benova & Tencerova, 2020).

2.3.2 Adipose stem cells

The MSC population residing in the stromal-vascular fraction (SVF) of adipose tissue (Galateanu et al., 2012) was first identified in processed lipoaspirates (Zuk et al., 2001). SVF consists of a heterogeneous population of stromal cells, endothelial and smooth muscle cells, erythrocytes, fibroblasts, lymphocytes, monocytes, and pericytes (Romagnoli & Brandi, 2014). Despite the isolation protocol, the cultures of MSCs from adipose tissue remain heterogenous (Romagnoli & Brandi, 2014). The term adipose stem cell (ASC) was recommended by the International Federation of Adipose Therapeutics and Sciences (IFATS) (Daher et al., 2008) and has replaced the incoherent nomenclature used previously including adipose-derived stem cells.
ASCs are characterized as mesenchymal according to the ISCT criteria and surface marker expression profiles. Furthermore, ASCs can be distinguished from BMSCs by their positivity for CD36 and negativity for CD106 (Bourin et al., 2013). Moderate CD34 expression in freshly isolated ASCs has been reported in many studies, but the expression declines after passaging (Galateanu et al., 2012). The use of adipose tissue as a source of MSCs exhibits several advantages over that of BMSCs. ASCs can be harvested from lipoaspirates or excised fat samples in large quantities due to minimal donor site morbidity (Patrikoski et al., 2019; Romagnoli & Brandi, 2014). Stem cell frequency is also significantly higher in the adipose tissue (1-5%) than it is in the bone marrow (Buschmann et al., 2013; Strem & Hedrick, 2005; Varma et al., 2007). Similar to hBMSCs, hASCs possess immunosuppressive capacity (Patrikoski et al., 2014), and their differentiation potential is affected by donor characteristics such as BMI (Juntunen et al., 2021), thus making donor selection important.

2.4 Adipogenic differentiation

The details of adipogenic differentiation are presented differently based on the references, but the representations share similarities. Multipotent MSCs are progenitor cells that differentiate into mature adipocytes in a stepwise manner (Berry et al., 2013). The process of adipogenesis can be divided into two phases as presented by James 2013, including the determination phase and the terminal differentiation phase (James, 2013), or into commitment, differentiation, and terminal differentiation phases as presented by Niemelä et al. (Niemelä et al., 2007). Adipogenesis can also be described in terms of intermediate cell stages, including multipotent stem cell, preadipocyte, and adipocyte stages (Niemelä et al., 2007). The term adipoblast is also used to describe the unipotent, committed cell stage (Karahuseyinoglu et al., 2008; Niemelä et al., 2007). Figure 5 presents a simplified schematic of the stages of adipogenic differentiation and the key regulators of the differentiation.
Preadipocytes are morphologically similar to fibroblastic MSCs, and in vitro they undergo clonal expansion until they reach confluence (Gregoire et al., 1998). Cell-cell contacts encourage committed preadipocytes to undergo adipocyte conversion (Gregoire et al., 1998). Adipogenesis is accompanied by changes in cell morphology toward a round shape (McBeath et al., 2004). Adipocytes synthesize and store lipids in intracellular lipid droplets with a hydrophobic core of triglycerides shielded by a phospholipid monolayer (de la Rosa Rodriguez & Kersten, 2017; Thiam & Beller, 2017). Synthesis begins with multiple small droplets in the endoplasmic reticulum (ER), after which the droplets bud from the membrane and grow by enlargement or fusion with other lipid droplets (Thiam & Beller, 2017). Mature white adipocytes are unilocular adipocytes with large singular lipid droplets that are ideal for storage purposes (Thiam & Beller, 2017). In contrast, brown and beige adipocytes contain multiple lipid droplets and are multilocular (J. Wu et al., 2012). Adipocytes produce adipocyte-specific proteins, including perilipin family proteins, that are present in the phospholipid monolayer surrounding the lipid droplets and function to shield the droplets (Kimmel & Sztalryd, 2016; Thiam & Beller, 2017).

2.4.1 Regulation of adipogenic differentiation

The differentiation of MSCs down the adipogenic lineage is regulated through various intracellular pathways. Positive and negative regulatory mechanisms of the adipogenic phenotype targeted through transcriptional regulation have been demonstrated. Among others, hormonally induced signaling via the insulin-like growth factor-1 (IGF-1), glucocorticoid, cyclic adenosine monophosphate (cAMP), and bone morphogenetic protein (BMP) 4 pathways have been demonstrated to

![Figure 5. Schematic presentation of adipogenic differentiation of white adipocytes. Figure is based on (Ambele et al., 2020; da Silva et al., 2020; Niemelä et al., 2007).](image)
guide the adipogenic process (Ambele et al., 2020). A limitation of the previous data is that a large bulk of the results are obtained using rodent cell lines such as the mouse preadipocyte cell line 3T3-L1 or the mouse fibroblast cell line C3H10T1/2. The translation of these mechanisms into humans requires confirmation using human cells.

However, there are conserved mechanisms of adipogenic regulation in the development of adipose tissue such as the well-established master regulator of adipogenesis peroxisome proliferator-activated receptor-γ (PPARγ) (Gregoire et al., 1998; James, 2013) that is a ligand-activated nuclear receptor that binds DNA to serve as a transcriptional activator (de la Rosa Rodriguez & Kersten, 2017). During embryonic development, PPARγ marks the location of fat depots in the body, but it is also involved in terminal adipogenic differentiation (Berry et al., 2013). Another key regulator is CCAAT-enhancer-binding protein alpha (C/EBPα) (Almalki & Agrawal, 2016; Ambele et al., 2020). After initiation of adipogenic differentiation, the levels of the transcriptional regulators C/EBPβ and C/EBPδ are upregulated to activate PPARγ and C/EBPα (Almalki & Agrawal, 2016). PPARγ and C/EBPα regulate the expression of target genes that are involved in lipid storage and synthesis, including adipocyte fatty acid-binding protein (AFABP; also known as FABP4, and aP2 for adipocyte Protein 2), lipoprotein lipase (LPL), fatty acid synthase (FAS), perilipin, fatty acid transport protein-1 (FATP-1), adiponectin, and leptin (LEP) (Ambele et al., 2020; Gregoire et al., 1998).

Negative regulators of adipogenesis include Wnt family proteins that inhibit the transcription of PPARγ and maintain the undifferentiated state of preadipocytes (James, 2013; G. Lin & Hankenson, 2011; Rajashekhar et al., 2008). Other negative regulators of adipogenesis are Hedgehog (HH) and transforming growth factor beta (TGF-β) 1 and 2 that act through inhibition of PPARγ and C/EBPα expression (Ambele et al., 2020). Preadipocyte factor-1 (Pref-1) is an early negative regulator of adipogenesis and is responsible for maintaining the preadipocyte stage (da Silva et al., 2020).

### 2.4.2 In vitro induction of adipogenesis

Established cocktails of chemical agents are used to direct the adipogenic differentiation of stem cells. Common agents for the adipogenic medium for preadipocytes include dexamethasone (DEX), insulin, and 3-isobutyl-1-
methylxanthine (IBMX, also known as methylisobutylxanthine) (Galateanu et al., 2012; Koutnikova & Auwerx, 2001; Niemelä et al., 2007; Pittenger et al., 1999). DEX and IBMX upregulate C/EBP activation and PPARγ expression, and DEX inhibits Pref-1 (Galateanu et al., 2012). Insulin promotes adipogenic signaling pathways and increases the gene expression of FAS, LEP, and adiponectin (Galateanu et al., 2012). Additionally, indomethacin has been shown in an adipogenic cocktail (Pittenger et al., 1999) but has been linked to browning of the adipocytes (Overby et al., 2020). In a recent study, Herbers et al. used the PPARγ agonist rosiglitazone to preserve the white adipocyte phenotype during hASC differentiation (Herbers et al., 2022).

2.5 Osteogenic differentiation

MSC osteogenesis (Figure 6) is a stepwise process involving phases of cell proliferation, maturation, ECM synthesis, and matrix mineralization (James, 2013; Lian & Stein, 1995). Osteoprogenitor cells commit to preosteoblasts and finally differentiate into osteoblasts that are buried in the mineralized matrix as osteocytes (James, 2013; Z. Liu et al., 2023; Salhotra et al., 2020). During maturation, osteoblasts produce alkaline phosphatase (ALP), a marker of osteogenic differentiation (Golub & Boesze-Battaglia, 2007; Lian & Stein, 1995). ALP serves as a source of inorganic phosphate required for the production of calcium phosphate (CaP) minerals that accumulate in the mineralized matrix as HA crystals (Blair et al., 2017; Golub & Boesze-Battaglia, 2007). In addition to this inorganic component of bone, osteoblasts produce and secrete organic proteins, including collagen type I, osteopontin (OPN), osteocalcin (OCN), fibronectin, and bone sialoprotein (BSP) (Blair et al., 2017; Lian & Stein, 1995; Rochefort et al., 2010). Collagen type I is a fibril-forming macromolecule that forms a platform for calcium phosphate-based crystal nucleation in the ECM (Jäger & Fratzl, 2000; Oosterlaken et al., 2021). Together, the inorganic and organic components create hardness, strength, and flexibility in the bones. In vivo, osteoblasts may remain in the bone matrix as osteocytes, become bone-lining cells, or undergo controlled cell death termed apoptosis (Rochefort et al., 2010; Salhotra et al., 2020). Osteogenic marker expression in osteocytes progressively declines, and osteocyte maturation can be demonstrated using specific markers such as transmembrane glycoprotein CD44, dentin matrix protein 1 (DMP1) and matrix extracellular phosphoglycoprotein (MEPE) (Rochefort et al., 2010).
2.5.1 Regulation of osteogenic differentiation

Osteogenic differentiation of multipotent precursors into bone cells is a strictly controlled biological process. Numerous efforts have been made to elucidate the regulatory steps governing osteogenesis, and a few evolutionarily conserved signaling pathways, transcription factors and osteogenic genes have been discovered. A brief list of these central pathways is provided below.

The receptor activator of nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG) is a key regulatory pathway controlling the differentiation and activity of both osteoblasts and osteoclasts (Lv et al., 2019). OPG and RANKL compete for binding to RANK, and the binding of RANKL to RANK activates osteoclast maturation and bone resorption, whereas OPG blocks osteoclast induction (Lv et al., 2019). The HH pathway supports osteoclast maturation via RANKL activation (Lv et al., 2019). HH pathway is also involved in the early development in patterning of the limb buds and in the regulation of endochondral ossification (Lv et al., 2019; Yang et al., 2015). The Wnt/β-catenin signaling pathway is associated with osteogenic differentiation (Gao et al., 2017). In the absence of HH or canonical Wnt/β-catenin signaling, skeletal development and remodeling after injury is prevented (Houshyar et al., 2019; Salhotra et al., 2020). TGF-β/BMP pathway induces the synthesis of ECM proteins and thus promotes bone formation (Gao et al., 2017). In contrast, the Notch signaling pathway can inhibit the differentiation and promote the proliferation of osteoblasts (Gao et al., 2017). The MAPK family members extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) are involved...
in hMSC proliferation, osteogenesis, and apoptosis (Ardito et al., 2017.) MAP kinases are discussed in more detail in Section 2.6.3.

The mode of action of these regulatory pathways is to activate or inactivate target gene expression and thus regulate protein synthesis through transcription factors. Transcription factors that are activated by osteogenic pathways include SRY-box transcription factor 9 (SOX9) that marks the commitment of mesenchymal progenitors to the osteochondroprogenitor lineage (Rodriguez-Carballo et al., 2016; Salhotra et al., 2020). SOX9 is a master regulator of chondrogenesis and is involved in bone development via endochondral ossification (Salhotra et al., 2020). In MSC osteogenesis, the master regulator is runt-related transcription factor 2 (RUNX2; also known as core-binding factor alpha 1, Cbfa1) and this factor signifies the commitment of progenitor cells to preosteoblasts (Salhotra et al., 2020). Together with the osteoblast transcription factor osterix (OSX) and homeobox protein distal-less homeobox 5 (DLX5), they promote osteoblast maturation by regulating the expression of the osteoblast genes OCN, OPN, and BSP. (Rodriguez-Carballo et al., 2016; Salhotra et al., 2020).

In addition to the role of these signaling pathways in the development of the organism, they are linked to bone repair and remodeling together with signals from the cellular environment (Aeschlimann & Evans, 2004; Salhotra et al., 2020). Mechanical signals are important for activating osteogenic regulation (Stewart et al., 2020), and mechanical stimuli are linked to greater bone density in vivo (Nikander et al., 2009; Verschueren et al., 2004).

### 2.5.2 In vitro induction of osteogenesis

Osteogenic differentiation of preosteoblasts and MSCs in vitro can be stimulated with the commonly used osteogenic agents L-ascorbic acid 2-phosphate, β-Glyserophosphate and DEX (Langenbach & Handschel, 2013; Pittenger et al., 1999; Reible et al., 2017). Ascorbic acid promotes the production of collagen type I, β-Glyserophosphate serves as a phosphate source for formation of HA in the ECM, and DEX induces osteogenesis by enhancing RUNX2 gene expression (Langenbach & Handschel, 2013). Additionally, BMPs and IGF-1 have been used for osteogenic stimulation in vitro (Celil & Campbell, 2005; Reible et al., 2017; Vanhatupa et al., 2015).
Several types of biomaterials, including bioceramics, biopolymers, and hydrogels are used to support the osteogenic differentiation of precursor cells in bone TE applications (Gao et al., 2017; Ginebra et al., 2018; Hench & Jones, 2015; Ornaghi et al., 2023; Yue et al., 2020). Bioactive glasses (BaGs) are ceramic biomaterials that are discussed in more detail in Section 2.5.3.

In addition to biochemical stimulation, mechanical stimulation such as stretching, vibration, and electrical stimulation is used in vitro to induce osteogenesis by mimicking the natural environment of progenitor cells. Several recent publications have thoroughly studied the mechanical induction of osteogenesis (Halonen et al., 2020; Z. Liu et al., 2023; Pongkitwitoon et al., 2016; Tärkkönen et al., 2011; Virjula et al., 2017).

2.5.3 Bioactive glass as an inducer of osteogenesis

BaGs are amorphous surface reactive materials belonging to the class of bioceramic materials. Bioceramics share compositions similar to those of natural bone and exhibit bone-bonding and bioactive properties (Gao et al., 2017; Jones, 2013). The definitions of bioactive materials and intracellular bioactivity pathways have been discussed in a recent publication (Williams, 2022). In summary, biomaterials are reactive materials in physiological fluids that elicit desired and controllable biological effects (Williams, 2022). In addition to their bioactivity, BaGs are capable of osteostimulation, which means that they activate progenitor cells to produce bone tissue (Hench & Jones, 2015). The chemical bonding of BaG to bone was discovered in seminal studies by Hench et al. in 1969 (Hench & Jones, 2015). BaG-tissue contact and material surface reactions in body fluids leading to the formation of HA-like layer are considered major factors affecting the osteostimulatory properties of BaGs (Gerhardt & Boccaccini, 2010; Rahaman et al., 2011; Xynos et al., 2001).

BaGs have been developed for various applications, including bone fixation with porous scaffolds, and non-load-bearing site fillers with BaG particulates, granules, and even nanoscale particles (Gerhardt & Boccaccini, 2010). BaGs have also been used in combination with biodegradable polymers to fabricate composite implants with improved mechanical properties, due to their brittleness (Varila et al., 2012). In clinical settings, bone regeneration is already aided with well-established BaG compositions such as the first artificial compound 45S5 (Bioglass®) or S53P4 (BonAlive®) (Hench & Jones, 2015). The silica-based glasses 45S5 and S53P4 are
within the four-oxide system Na₂O–CaO–P₂O₅–SiO₂ (Varila et al., 2012), and release critical concentrations of soluble Na, Ca, P, and Si ions (Gerhardt & Boccaccini, 2010).

The ions released from BaGs were sufficient for osteostimulation in vitro (Gerhardt & Boccaccini, 2010; Hench, 2009; Núñez-Toldrà et al., 2019; Ojansivu et al., 2015; Ojansivu, Mishra, et al., 2018; Sun et al., 2007; Vuornos et al., 2020; Xynos et al., 2001). Si plays an important role in the early stages of osteogenesis by activating bone-related gene expression, collagen and proteoglycan synthesis, and promoting bone calcification (Gao et al., 2017). Ca is released from bone tissue during bone resorption by natural osteoclast activity (Lee et al., 2018; Nakamura et al., 2010), and studies have demonstrated that Ca induces MSC proliferation (Lee et al., 2018) and the osteogenic differentiation of MSCs and osteoblasts (Nakamura et al., 2010; Viti et al., 2016).

Novel glass compositions have been developed in search of improved biological responses. The incorporation of active elements into the silicate network has proven advantageous. Ag, Cu, and Te possess antimicrobial/anti-inflammatory properties (Miola et al., 2021; Palza et al., 2013), Cu, Zn, Mn, and Sr promote osteogenesis (Y. Lin et al., 2016; Miola et al., 2014; Naruphontjirakul et al., 2019; Westhauser et al., 2021), and Cu induces osteogenesis and angiogenesis (X. Wang et al., 2019). The addition of B exhibited promising results for the promotion of osteogenic and angiogenic differentiation (Decker et al., 2022; Ojansivu, Mishra, et al., 2018; C. Wu et al., 2011; Ying et al., 2011).

**Cell signaling mechanisms of BaGs in the context of osteogenesis**

To date, the molecular mechanisms underlying the intracellular effects of BaG remain largely unknown. Current literature suggests that silicate BaGs up-regulate central pathways involved in bone development and homeostasis (discussed in Section 2.5.1). There is evidence that the HH pathways are involved in mediating the biological responses of BaGs (Williams, 2022). Additionally, Ca ions released from BaGs have been proposed to activate Ca channel transporters and downstream signaling pathways, including the MAPK pathways, cAMP /protein kinase A (PKA), and phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) pathways to activate osteogenic differentiation (Williams, 2022). In hASCs, BaG-cell interactions activate osteogenesis through FAK, ERK, and JNK signaling (Ojansivu, Wang, et
Si and Ca ions released from BaGs may modulate the cell cycle to drive cell proliferation and subsequent osteogenic differentiation (Gerhardt & Boccaccini, 2010), but they also enhance osteogenic differentiation via the TGF-β pathway (J. Li et al., 2011). The molecular mechanisms underlying the osteogenesis-inducing function of Ba ions are not fully known, but the involvement of Wnt/β-catenin signaling pathway has been observed in hBMSCs (Yin et al., 2018).

### 2.6 Intracellular mechanisms of cell adhesion and the cytoskeleton in regulation of MSC adipogenesis and osteogenesis

Cell adhesion reflects the ability of a cell to attach to its surroundings. The majority of human cells, including MSCs, adhere to other cells or the ECM in vitro or to the culture platform in vivo, and are dependent on their anchorage. Inadequate adhesion of adherent cells acts as a death signal, ultimately leading to anoikis, a special type of programmed cell death (Frisch & Screaton, 2001). An exception is the hematopoietic cell lines that grow in suspension.

The cytoskeleton is the structural backbone of cells and is composed of microtubules, microfilaments, and intermediate filaments that are responsible for the different functions of the cytoskeleton. Microtubules are involved in cellular trafficking and play vital role in cell division by generating the mitotic spindle during cell division. Microfilaments, also termed actin filaments, are composed of monomeric G-actin subunits that polymerize to form filamentous F-actin. Actin filaments allow cells to move and adopt various shapes. Together with myosin, F-actin forms contractile bundles known as stress fibers. Intermediate filaments differ depending on the cell type, and in MSCs these filaments are primarily vimentin (Saidova & Vorobjev, 2019). The nucleus has its own dynamic nucleoskeleton composed of various structural elements, including nuclear lamins and nuclear actin (Sidorenko & Vartiainen, 2019).

The widely recognized molecular mechanisms regulating cell adhesion and cytoskeletal organization include proteins of focal adhesion complexes and actin-binding proteins that initiate the key signaling activation of the FAK, Rho/ROCK, and MAPK pathways (Ambriz et al., 2018; Kilian et al., 2010). The role of these pathways in the lineage commitment of human MSCs was investigated in this thesis, and the pathways are discussed in more detail in Sections 2.6.2. – 2.6.5. The general
mechanism of signal transduction in these pathways is briefly discussed in Section 2.6.1.

### 2.6.1 Protein kinase pathways

Protein kinase pathways, in which the message is mediated by the phosphorylation (i.e. the addition of an inorganic phosphate group) of intracellular target proteins regulate virtually all biological processes. These pathways or networks/cascades involve protein kinases, phosphatases, and phospho-binding proteins. Protein kinases are enzymes responsible for the phosphorylation of tyrosine or serine/threonine residues in the amino acid side chains of proteins. Phosphorylation activates the signaling cascade and mediates messaging through several downstream proteins. In contrast, protein phosphatases are enzymes that dephosphorylate (i.e. remove the phosphate group) proteins leading to inactivation of signaling. Protein kinase pathways are activated by several extracellular signals, thus providing an opportunity for cells to communicate with their environment. The goal of pathway activation is to regulate cellular functions at the gene expression level via the activation or inactivation of specific transcription factors that regulate protein synthesis and thereby alter cell behavior through the transcriptome. (Ardito et al., 2017).

### 2.6.2 Integrin/focal adhesion kinase signaling

Integrins are transmembrane receptors that function as links between the extracellular and intracellular spaces, and in signal transduction through the membrane. In humans, integrins consist of different combinations of eighteen α and eight β subunits forming twenty-four known αβ-heterodimers. Extracellular ligand binding (including elastic fibers, collagen family members, proteoglycans, glycosaminoglycans, and adhesive glycoproteins) to the integrin receptor induces its conformational change and the clustering of the cytoplasmic β tails. Integrin activation drives the recruitment of proteins to form intracellular adhesion complexes known as focal adhesions. Integrins lack enzymatic activity and are dependent on focal adhesion proteins, including FAK and Src kinases for signal transduction. (Cary & Guan, 1999; Harburger & Calderwood, 2009; Y. Huang et al., 2023; S. Liu et al., 2000) A schematic representation of the integrin/FAK signaling pathway is presented in Figure 7.
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Figure 7. Graphical representation of Integrin/FAK signaling. Modified from (Y. Huang et al., 2023).

FAK is a non-receptor tyrosine kinase involved in essential cell functions such as cell proliferation, survival, motility, invasion, and angiogenesis (Cary & Guan, 1999; Parsons, 2003; Tilghman & Parsons, 2008). FAK is important for anchorage-dependent cell growth, and its impairment can induce apoptosis and anoikis (Schaller, 2001). In addition to integrins, FAK is activated through growth factor receptors, G protein-coupled receptors (GPCRs), and receptor tyrosine kinases (RTKs) (Y. Huang et al., 2023). FAK overexpression is linked to tumor progression, enhanced invasiveness, and aggressiveness of metastatic tumors and has thus been targeted in the development of cancer treatments (Golubovskaya & Cance, 2007; Stokes et al., 2011; Tilghman & Parsons, 2008). The role of FAK in mediating messages from the cellular environment makes it a mechanosensitive target and key player in conversion of mechanical cues into biochemical signals referred to as mechanotransduction (Hytönen & Wehrle-Haller, 2016; Stewart et al., 2020). FAK activation results in increased FAK kinase activity and the autophosphorylation of tyrosin-397 (Y397). Y397 phosphorylation is critical for providing a highly specific binding site for Src family kinases (Y. Huang et al., 2023) and other downstream signaling pathways including Rho/ROCK and MAPK pathways (Ambriz et al., 2018; Kilian et al., 2010; Webb et al., 2004).
Cell adhesion and FAK signaling play central roles in MSC differentiation (Y. Huang et al., 2023). Osteogenesis in MSCs is more prevalent with spread actin cytoskeleton and a greater number of focal adhesions (Mathieu & Loboa, 2012). Actin polymerization can enhance FAK signaling during the osteogenic differentiation of human stromal stem cell (L. Chen et al., 2015). Additionally, mechanical stimuli guide osteogenesis through the FAK-dependent activation of RUNX2 and OSX (Stewart et al., 2020). In contrast, adipogenesis and chondrogenesis are encouraged by preventing focal adhesion attachment (Mathieu & Loboa, 2012). Adipogenesis has been demonstrated to be accompanied by the cleavage of FAK in rodent 3T3-L1 cells and primary rat preadipocytes as an essential step of the differentiation commitment, ultimately allowing for the morphological changes required for differentiation (J.-J. Li & Xie, 2007).

2.6.3 MAP kinases

The MAPK family members ERK, p38, and JNK are highly conserved serine/threonine kinases. MAPK family members share similar phases from the activating stimulus to the activation of intracellular signaling cascades and the regulation of gene expression. MAPKs are activated by phosphorylation by upstream kinases that are generically referred to as MAPK kinases (MAPKKs), and they are activated by MAPKK kinases (MAPKKKs) (Ardito et al., 2017; Lavoie et al., 2020). The MAP kinases ERK and p38 are within the scope of this thesis and are discussed below. For more details regarding JNK, previously published reviews are available (Semba et al., 2020; Weston & Davis, 2007).

MAPK ERK signaling

The MAPK ERK subfamily (Figure 8) consists of two kinases, ERK1 and ERK2. Their upstream activation is dependent on the dual-specificity kinases MEK1 and MEK2 that are capable of phosphorylating both the serine/threonine and tyrosine residues of ERK1/ERK2 (Roberts & Der, 2007). MEK1/2 are in turn activated by the small GTPase Ras and its downstream target Raf kinase (McKay & Morrison, 2007; Roskoski Jr, 2010; Schindeler & Little, 2006). Ras is primarily activated by growth factors that serve as extracellular ligands of receptor tyrosine kinases of the cell membrane (e.g. epidermal growth factor receptor; EGFR). Additionally, integrin/FAK signaling can activate the Ras/MEK/ERK axis, thus indicating the
interconnectivity of these pathways. (Gao et al., 2017; Hong et al., 2010; Webb et al., 2004.)

ERK plays a key role in the regulation of many cellular processes such as proliferation, differentiation, and apoptosis (Lavoie et al., 2020). Abnormalities in the MAP kinase cascades are closely linked to tumor progression (Roberts & Der, 2007; Takacs-Vellai, 2014). Thus, the MEK/ERK pathway has been intensively studied in the context of cancer and targeted cancer therapies, as reviewed by Caunt et al. (Caunt et al., 2015). The role of ERK in the context of differentiation has been a topic of research over the past decades. ERK is considered as a regulator of osteogenesis of progenitor cells, and it has been demonstrated to activate RUNX2 in vitro (Greenblatt et al., 2013; Shih et al., 2011; Xiao et al., 2000). The regulation of adipogenic differentiation by ERK remains inconclusive, and both positive and negative roles have been proposed in various experimental designs and cell types.
ERK1 isoform knockout in mice reduced Pref-1 expression and impaired adipocyte differentiation (Bost et al., 2005). Additionally, in the mouse preadipocyte cell line 3T3-L1, the inhibition of ERK signaling reduced adipogenic markers (Prusty et al., 2002). Studies examining human ASCs and BMSCs have indicated that ERK modulates differentiation fate between osteogenesis and adipogenesis in such a manner that active ERK favors osteogenic differentiation (Jaiswal et al., 2000; Q. Liu et al., 2009). However, conflicting results suggest that ERK is a positive regulator of hMSC adipogenesis, as ERK inhibition with MEK inhibitor U0126 attenuated gene expression of PPARγ and the formation of lipid droplets under adipogenic differentiation medium (Xu et al., 2014).

The p38 MAPK/HSP27 axis

The MAPK family p38 kinases are proline-directed serine/threonine kinases that are involved in various cellular functions, including response to environmental and intracellular stresses and cell differentiation. The p38 MAPK family consists of p38α, p38γ, and p38δ, and of these, p38α is the best characterized. (Canovas & Nebreda, 2021.) Chemical and mechanical stimuli, cell adhesion, and inflammatory signals activate p38 MAPK signaling (Rodriguez-Carballo et al., 2016). p38 kinases are activated by dual phosphorylation by MAPKK (MKK3/MKK6). Additionally, p38 can be activated through non-canonical pathways (Canovas & Nebreda, 2021). p38 MAPK phosphorylates downstream targets such as MAP kinase-activated protein (MAPKAP) kinase-2 and -3 (MK2 and MK3) (Soni et al., 2019).

HSP27, also called HSPB1 (HSP25 in mice), is one of the eleven small heat shock proteins (sHSPs) identified in humans. HSP27 is present in several tissues, and its expression is induced by heat shock. (Mounier & Arrigo, 2002; Wettstein et al., 2012.) The characteristic functions of HSP27 include oligomerization and phosphorylation that are thought to guide its activity (Mounier & Arrigo, 2002). Large oligomers ranging from 150 to 1000 kDa and consisting of up to 40 monomers can be observed in the cytosol (Mounier & Arrigo, 2002). Various pathways and kinases regulate HSP27, including the p38 MAPK/MK2, MK3, P13K/AKT, and ERK1/2 pathways (Kostenko & Moens, 2009; Mounier & Arrigo, 2002). Human HSP27 contains three serine phosphorylation sites (Ser15, Ser78, and Ser82) (Gu et al., 2023). Phosphorylation of sHSP drives the formation of small oligomers or dimers that generally activate sHSP function (Gu et al., 2023; Wettstein et al., 2012). The disaggregated form of sHSPs promotes chaperone and anti-apoptotic activities.
The disaggregated form of sHSPs promotes chaperone dimers (Wettstein et al., 2023). HSP27 can be phosphorylated by ERK1/2 and p38γ. Large oligomers ranging from 150 to 1000 expression is induced by heat shock proteins (HSPs). Phosphorylation of HSP27, also called HSPB1 (Canovas & Nebreda, 2021), that active ERK favors osteogenic differentiation. The MAPK family, including p38α, p38β, p38δ, and p38γ, are involved in cell differentiation, apoptosis, migration (Hedges et al., 1999; Hoffman et al., 2017; Kainuma et al., 2017), and the activation of HSP27 signaling has been observed in bone development in human craniofacial tissue and in rat tibiae.

Various pathways and signaling involve in the osteogenic outcome, and the activation of HSP27 signaling has been studied in the context of cell migration (Hedges et al., 1999; Hoffman et al., 2017; Kainuma et al., 2017), but its role in osteogenic differentiation remains unclear. Certain studies suggest its involvement in the osteogenic outcome, and the activation of HSP27 signaling has been observed in bone development in human craniofacial tissue and in rat tibiae.

**Figure 9.** Graphical representation of p38 MAPK/HSP27 signaling axis (Gerthoffer & Gunst, 2001; Hoffman et al., 2017).

Chaperone activity depends on the ability of HSP27 to interact with the actin cytoskeleton. HSP27 can cap the plus ends of actin filaments, thereby preventing the fixation of new actin monomers (Hirano et al., 2004). The reinforcement of actin fibers and the prevention of their aggregation by HSP27 contributes to cell-survival after heat shock or oxidative stress (Wettstein et al., 2012). Under mechanical force, HSP27 reinforces the actin cytoskeleton via p38 MAPK-mediated signaling (Hoffman et al., 2017). To date, HSP27 has been studied in the context of cell migration (Hedges et al., 1999; Hoffman et al., 2017; Kainuma et al., 2017), but its role in osteogenic differentiation remains unclear. Certain studies suggest its involvement in the osteogenic outcome, and the activation of HSP27 signaling has been observed in bone development in human craniofacial tissue and in rat tibiae.
(Leonardi et al., 2004; Tiffee et al., 2000). Its expression was also linked to osteogenic differentiation of electrically stimulated hMSCs (Hronik-Tupaj et al., 2011).

2.6.4 RhoA/ROCK pathway

The RhoA/ROCK pathway (Figure 10) is a major regulator of cell dynamics (Riento & Ridley, 2003; Yim & Sheetz, 2012). RhoA is a small GTPase that is activated by various pathways and extracellular signals such as mechanical stimuli through integrin receptors and focal adhesion complexes (Arnsdorf et al., 2009; Shih et al., 2011; Steward & Kelly, 2015; Tilghman & Parsons, 2008). Active GTP-bound RhoA binds to ROCK isoforms I and II that are multifunctional serine/threonine kinases (Riento & Ridley, 2003). ROCK substrates include myosin light-chain (MLC) phosphatase (MLCP), LIM domain kinases (LIMK), adducin, and ezrin-radixin-moesin (ERM) (Riento & Ridley, 2003). MLC plays an important role in driving the contractility of the actin and myosin cytoskeleton. ROCK regulates MLC by inhibiting MLCP, ultimately leading to increased levels of phosphorylated MLC and cell contractility. ROCK stabilizes actin stress fibers by phosphorylating LIMK that then phosphorylates coflin inactivating its ability to depolymerize actin. Adducin stabilizes actin by binding to filamentous F-actin. (Amano et al., 2010).
The reorganization of actin cytoskeleton plays an important role in osteogenic differentiation (C. Huang et al., 2015; Sonowal et al., 2013). Spread morphology with rigid stress fibers (McBeath et al., 2004; Kilian et al., 2010) and increased actomyosin tension (Ambriz et al., 2018; Clark et al., 2007; Sonam et al., 2016) are characteristics of osteocommitted hMSCs (Titushkin and Cho, 2007; Yourek, Hussain and Mao, 2007). F-actin stabilization enhances osteoblast differentiation (L. Chen et al., 2015). While ROCK activity is linked to osteogenic fate, the inactivation of ROCK signaling has been observed to instead support the adipogenic differentiation of hMSCs (McBeath et al., 2004, Kilian et al., 2010). Similarly, inhibition of LIMK, a ROCK phosphorylation target, enhanced adipogenesis in hMSCs (L. Chen et al., 2018). RhoA/ROCK-mediated actin dynamics have been implicated in beige adipogenesis as well as in white adipogenesis (Wei & Shi, 2022).

**Figure 10.** Graphical representation of RhoA/ROCK/MRTF-A signaling axis (Olson & Nordheim, 2010; Riento & Ridley, 2003).
2.6.5 MRTF-A signaling

The ROCK downstream effector myocardin-related transcription factor A (MRTF-A) (Figure 10), also known as megakaryocyte acute leukemia protein (MAL) or megakaryoblastic leukemia (MKL1), belongs to the myocardin family (Miralles et al., 2003; Olson & Nordheim, 2010). Myocardin family proteins A and B are present in various embryonic and adult tissues (Wang et al., 2002). MRTF-A activity is controlled by the balance between monomeric G-actin and polymerized F-actin. At a low actin polymerization state, MRTF-A is inactive, as it is bound and forms a stable reversible complex with G-actin through N-terminal RPEL repeats. Actin filament assembly stimulated by Rho/ROCK activity, leads to a decrease in actin monomers and the release of MRTF-A from the repressive complex. The released MRTF-A translocates into the nucleus and functions as a transcription coactivator for the serum response factor (SRF). (Olson & Nordheim, 2010.) Additionally, MRTF-A activity is modulated by nuclear actin. Nuclear actin monomers form inhibitory complexes with MRTF-A as in the cytoplasm, and nuclear actin can regulate the subcellular localization of MRTF-A by confining it to the nucleus (Sidorenko & Vartiainen, 2019).

MRTF-A/SRF signaling activates the transcription of muscle-specific, contractile, and cytoskeletal genes. These genes include alpha-, beta-, and gamma actins, integrin β1, vinculin, coflin 1, talin 1, myosin heavy chains, and myosin light chain 9 (Miralles et al., 2003; Olson & Nordheim, 2010; D. Z. Wang et al., 2002). Depletion of MRTF-A/SRF activity reduces cell adhesion, spreading, invasion, and motility in human and mouse tumor cell lines (Medjkane et al., 2009). Over the past decade, a novel role for MRTF-A on adipogenesis has emerged. Nobusue and coworkers demonstrated in mouse preadipocyte cells that G-actin controlled the expression of PPARγ by preventing nuclear translocation of MRTF-A (Nobusue et al., 2014). Furthermore, MRTF-A has been linked to the browning of white adipose tissue in mice in vivo (McDonald et al., 2015) and in vitro (Rosenwald et al., 2017). The role of MRTF-A in osteogenesis has been studied less, but there is some evidence linking MRTF-A/SRF activity to osteogenic differentiation potential. SRF, whose activity is modulated by the coactivator MRTF-A, has been demonstrated to regulate RUNX2 and thus bone formation (J. Chen et al., 2012). Bian et al. demonstrated the regulatory effects of MRTF-A on the osteogenic differentiation potential of mouse BMSCs (Bian et al., 2016).
3 AIM OF THE STUDY

The aim of the study was to characterize the roles of intracellular signaling pathways related to cell adhesion and cytoskeletal regulation in human MSC differentiation. Studies I and II focused on the differentiation fate decision between adipogenesis and osteogenesis, while Study III focused on the intracellular responses elicited by BaG in hMSC osteogenesis. The detailed aims and hypotheses of this study are as follows:

I To evaluate the role of cell adhesion-related FAK and ERK pathways, and cytoskeleton-related ROCK signaling in the fate decision of hASCs towards adipocytes and osteoblasts. We hypothesized that these mechanisms, previously studied primarily in animal models, are also significant for hASC lineage commitment.

II To determine if the cytoskeletal dynamics mediated by the MRTF-A transcription factor regulate the adipogenic and osteogenic potential of hASCs. Based on the results of Study I indicating a role for ROCK in the regulation of hASC differentiation, we hypothesized that the ROCK downstream target MRTF-A would also play a role in the lineage determination of hASCs.

III To analyze if the osteogenic differentiation of hASCs and hBMSCs induced by the ionic dissolution of bioactive glass (3-06) was regulated through the p38 MAPK/HSP27 pathway. We hypothesized that HSP27 is involved in the osteogenic differentiation of hMSCs through its ability to modulate cytoskeletal dynamics.
4 MATERIALS AND METHODS

4.1 Human mesenchymal stem cells

The stem cells used in Studies I–III were donated for research purposes with written informed consent from the donors. The studies were conducted in accordance with the ethical guidelines of the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (supportive statement R15161 for hASCs and R15174 for hBMSCs).

4.1.1 Human adipose stem cell lines

ASCs used in Studies I–III were isolated from subcutaneous adipose tissue samples received from Tampere University Hospital Department of Plastic and Reconstructive Surgery using a mechanical and enzymatic protocol as described in Original Publications I–III. All female donors were used in the studies: six donors (mean age 44 ± 11 years) in Study I, six donors (mean age 51 ± 8 years) in Study II, and three donors (mean age 59 ± 6 years) in Study III. hASCs were cultured in basic medium (BM) containing 5% human serum (HS) and 1% antibiotics (100 U/ml penicillin; 100 µg/ml streptomycin; P/S) in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) or in Minimum Essential Medium α (MEM α). 2 mM L-alanyl-L-glutamine dipeptide (GlutaMAX) was added when using DMEM/F12. The experiments with hASCs were performed in passages 2–5.

4.1.2 Human bone marrow stem cell lines

BMSCs used in Study III were isolated from bone marrow aspirates received from Tampere University Hospital Department of Orthopedics and Traumatology using a Ficoll gradient as described previously by X. Wang et al. (X. Wang et al., 2019). The donors were three females with a mean age of 86 (±5 years). hBMSCs were cultured and expanded in BM supplemented with 5 ng/ml human fibroblast growth factor (hFGF-2). The experiments were performed at passages 3–5.

4.2 Experimental setup

4.2.1 Plating densities and culture platforms

hASCs and hBMSCs in Studies I–III were seeded on culture plates 24 h prior starting the experiments in BM, which was also used as a maintenance medium.
cultured and expanded in BM supplemented with 5 ng/ml human fibroblast growth factor (hFGF-2). The experiments were performed at passages 3–5.

4.1.3 MSC characterization

The surface marker expressions of the isolated hASCs (Studies I–III) and hBMSCs (Study III) in passage one were analyzed by our technicians prior to experiments using flow cytometric analysis and fluorescence-activated cell sorting (FACS), as described in the Original Publications I–III. The mean expressions of the surface markers of the cell lines used in the individual studies are given in Table 1.

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>Surface marker expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Study I hASC, N=6</td>
</tr>
<tr>
<td>CD11a Lymphocyte (Leukocyte) function-associated antigen 1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>CD14 Lipopolysaccharide binding protein</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>CD19 B lymphocyte-lineage differentiation antigen</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>CD34 Hematopoietic progenitor cell antigen 1</td>
<td>12.6 ± 9.7</td>
</tr>
<tr>
<td>CD45 Leucocyte common antigen</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>CD73 Ecto-5'-nucleotidase</td>
<td>91.6 ± 8.6</td>
</tr>
<tr>
<td>CD90 T cell surface glycoprotein Thy-1</td>
<td>96.4 ± 4.9</td>
</tr>
<tr>
<td>CD105 Endoglin</td>
<td>98.4 ± 1.0</td>
</tr>
<tr>
<td>HLA-DR Human leukocyte antigen - antigen D related</td>
<td>0.9 ± 0.6</td>
</tr>
</tbody>
</table>

Standard deviation is indicated with ±.

4.2 Experimental setup

4.2.1 Plating densities and culture platforms

The hASCs and hBMSCs in Studies I–III were seeded on culture plates 24 h prior starting the experiments in BM, which was also used as a maintenance medium and
served as an undifferentiated control. The differentiation cultures in Studies I and II were conducted on 6- or 24-well format CellBIND polystyrene plates (Corning), with a plating density of 260 cells/cm² for other analyses, except qRT-PCR in which the plating density was 3160 cells/cm². In Study III, the culture platform was 6- or 24-well format polystyrene culture-treated plate (Nunc, Thermo Fisher Scientific) with a plating density of 1000 cells/cm². For ICC staining and fluorescence imaging in Studies II and III, hASCs and hBMSCs (1000 cells/cm²) were cultured in IBIDI µ-slides (IBIDI). Western blot analyses were conducted at higher plating densities in all studies because of the methodological requirement of sufficient protein amount for the analysis. The following plating densities were used for Western blot: 3160 cells/cm² (CellBIND 6-well plate) in Study I, 5000 cells/cm² (CellBIND 6-well plate) in Study II, and 5260 cells/cm² (Nunc 6-well plate) in Study III.

Table 2. Culture media compositions in Studies I–III.

<table>
<thead>
<tr>
<th>Medium name</th>
<th>Composition</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic medium (BM)</td>
<td>DMEM/F12, 5% HS, 1% P/S, 1% L-Glutamine (GlutaMAX)</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td>MEM α, 5% HS, 1% P/S</td>
<td>II, III</td>
</tr>
<tr>
<td>Bioactive glass BM (BaG BM)</td>
<td>MEM α, 5% HS, 1% P/S, ionic dissolution products of 3-06 BaG</td>
<td>III</td>
</tr>
<tr>
<td>Starvation BM</td>
<td>DMEM/F12, 1% HS, 1% P/S, 1% L-Glutamine (GlutaMAX)</td>
<td>I</td>
</tr>
<tr>
<td>Adipogenic medium (AM)</td>
<td>DMEM/F12, 5% HS, 1% P/S, 1% L-Glutamine (GlutaMAX), 1 µM Dexamethasone, 17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>µM Pantothenate, 33 µM Biotin and 100 nM Insulin. 0.25 mM 3-isobutyl-1-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>methylxanthine (IBMX) was added to medium once at the beginning of</td>
<td></td>
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<tr>
<td></td>
<td>differentiation.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MEM α, 5% HS, 1% P/S, 1 µM Dexamethasone, 17 µM Pantothenate, 33 µM Biotin</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>and 100 nM Insulin. 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) was added</td>
<td></td>
</tr>
<tr>
<td></td>
<td>to medium once at the beginning of differentiation.</td>
<td></td>
</tr>
<tr>
<td>Starvation AM</td>
<td>DMEM/F12, 1% HS, 1% P/S, 1% L-Glutamine (GlutaMAX), 1 µM Dexamethasone, 17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>µM Pantothenate, 33 µM Biotin and 100 nM Insulin. 0.25 mM 3-isobutyl-1-</td>
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<tr>
<td></td>
<td>methylxanthine (IBMX) was added to medium once at the beginning of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>differentiation.</td>
<td></td>
</tr>
<tr>
<td>Osteogenic medium (OM)</td>
<td>DMEM/F12, 5% HS, 1% P/S, 1% L-Glutamine (GlutaMAX), 20 µM L-ascorbic acid 2-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>phosphate, 10mM β-Glycerophosphate, 5 nM Dexamethasone</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td>MEM α, 5% HS, 1% P/S, 250 µM L-ascorbic acid 2-phosphate, 10mM β-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycerophosphate, 5 nM Dexamethasone</td>
<td>II, III</td>
</tr>
<tr>
<td>Starvation OM</td>
<td>MEM α, 5% HS, 1% P/S, 1% L-Glutamine (GlutaMAX), 20 µM L-ascorbic acid 2-</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>phosphate, 10mM β-Glycerophosphate, 5 nM Dexamethasone</td>
<td></td>
</tr>
<tr>
<td>Bioactive glass OM (BaG OM)</td>
<td>MEM α, 5% HS, 1% P/S, 250 µM L-ascorbic acid 2-phosphate, 10mM β-</td>
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<tr>
<td></td>
<td>Glycerophosphate, 5 nM Dexamethasone, ionic dissolution products of 3-06</td>
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<td></td>
<td>BaG</td>
<td>III</td>
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</tbody>
</table>
4.2.2 Cell culture media

In Studies I and II, hASCs were differentiated towards osteogenic and adipogenic lineages using osteogenic and adipogenic culture supplements listed in Table 2. In Study III, osteogenesis was stimulated with BaG ionic dissolution medium in combination or without osteogenic medium (OM). In Study I, starvation media of BM, OM, and AM containing 1% human serum were used to minimize the hormonal effect of HS in Western blot analysis of protein expression.

4.2.3 Pharmacological inhibitors

Pharmacological small-molecule inhibitors were used to study the relevance of FAK (Study I), ERK (Study I), ROCK (Study I), MRTF-A (Study II), and p38 MAPK/HSP27 (Study III) signaling in the regulation of hMSC lineage specification. Detailed information on the pharmacological inhibitors is given in Table 3. The inhibitor concentrations are described in Original Publications I–III.

Table 3. Pharmacological inhibitors used in Studies I–III.

<table>
<thead>
<tr>
<th>Target</th>
<th>Inhibitor</th>
<th>Mode of function</th>
<th>Manufacturer</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAK</td>
<td>PF-562271</td>
<td>ATP-competitive reversible inhibitor of FAK phosphorylation (Y. Wu et al., 2021)</td>
<td>Selleck Chemicals, Houston, Texas, USA</td>
<td>I</td>
</tr>
<tr>
<td>MEK-ERK</td>
<td>PD98059</td>
<td>Inhibits selectively MEK1 activation (Dudley et al., 1995)</td>
<td>Calbiochem, Millipore, Burlington, MA, USA</td>
<td>I</td>
</tr>
<tr>
<td>MRTF-A</td>
<td>CCG-1423</td>
<td>CCG-1423 binds to MRTF-A NLS, preventing its nuclear import (Evelyn et al., 2007; Hayashi et al., 2014)</td>
<td>Selleck Chemicals</td>
<td>II</td>
</tr>
<tr>
<td>MRTF-A</td>
<td>CCG-100602</td>
<td>CCG-1423 analog, molecular target unidentified (Evelyn et al., 2010)</td>
<td>Cayman Chemical Company; Ann Arbor, MI, USA</td>
<td>II</td>
</tr>
<tr>
<td>p38/HSP27</td>
<td>SB202190</td>
<td>Inhibitor of p38 phosphorylation (Wilson et al., 1997)</td>
<td>Calbiochem, Merck</td>
<td>III</td>
</tr>
<tr>
<td>ROCK</td>
<td>Y-27632</td>
<td>ATP-competitive inhibitor of the kinase activity of ROCK-I and ROCK-II (Ishizaki et al., 2000)</td>
<td>Selleck Chemicals</td>
<td>I</td>
</tr>
</tbody>
</table>
4.2.4 BaG extract medium

BaG granules used in Study III were manufactured using the melt-quenching method by our collaborators at Åbo Akademi. The manufacture and characterization were described in detail previously (Ojansivu et al., 2015). The glass composition of 3-06 BaG was [wt-%]: Na2O, 24.6; CaO, 21.6; P2O5, 2.5; B2O3, 1.6; SiO2, 50.0. BaG granules (500–1000 µm) were used in the preparation of BaG extract medium according to the protocol created by our group (Ojansivu et al., 2015) as described in Original Publication III. The ion concentrations of the 3-06 BaG extract medium before the addition of HS were determined previously as [mg/kg]: Ca, 131; K, 172; Mg, 16; Si, 56; Na, 3750; B, 2.6; P was below the limit of quantification (Ojansivu et al., 2015).

4.3 Cell viability and proliferation analyses

Cell viability was studied with live/dead assay and cell proliferation was assessed by the CyQUANT method of measuring the total DNA amount of the samples, as described in Original Publications I–III. The time points at which the analyses were performed are listed in Table 4.

Table 4. Cell proliferation and viability analyses in Studies I–III.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Detection technique</th>
<th>Time points</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell proliferation</td>
<td>CyQUANT Assay</td>
<td>7 d, 14 d</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 d, 9 d, 11 d</td>
<td>III</td>
</tr>
<tr>
<td>Cell Viability</td>
<td>Live/dead Assay</td>
<td>7 d</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 d (hBMSC)</td>
<td>III</td>
</tr>
</tbody>
</table>

4.4 Differentiation analyses

The adipogenic differentiation of hASCs and osteogenic differentiation of hASCs and hBMSCs were analyzed in the studies I–III using various markers of differentiation, listed in Table 5.
Table 5. Differentiation analyses in Studies I–III.

<table>
<thead>
<tr>
<th>Differentiation lineage</th>
<th>Marker</th>
<th>Detection technique</th>
<th>Time points</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipogenic</td>
<td>Gene expression of aP2</td>
<td>qRT-PCR</td>
<td>7 d, 14 d</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td>Gene expression of LEP</td>
<td>qRT-PCR</td>
<td>7 d, 14 d</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td>Lipid droplet formation</td>
<td>ORO staining</td>
<td>21 d</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td>Protein expression of perilipin 1</td>
<td>ICC</td>
<td>21 d</td>
<td>II</td>
</tr>
<tr>
<td>Osteogenic</td>
<td>Gene expression of RUNX2A</td>
<td>qRT-PCR</td>
<td>7 d</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td>ALP activity</td>
<td>qALP assay</td>
<td>7 d, 14 d</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 d, 9 d, 11 d</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Mineralized matrix formation</td>
<td>qAR staining</td>
<td>14 d, 21 d</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21 d</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9 d, 11 d</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Protein expression of collagen type I</td>
<td>ICC</td>
<td>14 d</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 d</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Protein expression of OPN</td>
<td>ICC</td>
<td>21 d</td>
<td>II</td>
</tr>
</tbody>
</table>

4.4.1 Quantitative real-time polymerase chain reaction (qRT-PCR)

Genetic markers of osteogenesis (RUNX2A) and adipogenesis (aP2, LEP) were analyzed with qRT-PCR in Studies I and II. The method allowed the study of relative gene expression profiles at the desired time point of differentiation. The primer sequences of the genes are presented in Original Publications I and II.

4.4.2 Quantitative alkaline phosphatase (qALP) analysis

Alkaline phosphatase (ALP) activity, which is considered as early marker of osteogenic differentiation was studied with qALP assay in Studies I–III, as described in the publications.

4.4.3 Qualitative and quantitative Oil Red O (ORO) staining

Adipogenic differentiation was assessed in Studies I and II using Oil red O (ORO) lipid droplet staining, fluorescence imaging, and image-based quantification, as described in the publications. A custom quantification pipeline designed for CellProfiler was developed in collaboration with an expert in computational analysis.
to quantify lipid formation. The pipeline is published in the Supplementary Materials of Original Publication I.

4.4.4 Qualitative and quantitative Alizarin Red (qAR) staining

In Studies I–III, qAR staining of calcium-rich depots indicating matrix mineralization and late-stage osteogenesis was performed as described in the publications.

4.5 Immunocytochemical (ICC) staining

ICC staining was used to visualize the localization of perilipin 1 (Study II), collagen type I (Studies II and III), OPN (Study II), HSP27, and pHSP27 (Study III). Additionally, ICC staining was performed to visualize the actin cytoskeleton with phalloidin-tetramethylrhodamine B isothiocyanate (TRITC; Sigma-Aldrich) and nuclei with 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The staining protocols using primary and fluorescent-labeled secondary antibodies, fluorescence imaging, and image processing have been described in Original Publications II and III.

4.5.1 Image-based analysis of stain intensity

Image-based analysis was used to show the stain intensity of the phalloidin-TRITC-stained cytoskeleton (Studies II and III) or the intensity of HSP27, and pHSP27 (Study III). Phalloidin staining, ICC staining of HSP27 and pHSP27, and the analysis with Fiji were performed as described in Original Publications II and III.

4.5.2 Image-based analysis of actin orientation

Image-based analysis of actin orientation was performed using CytoSpectre in Study II, as described in the Original Publication.
4.6 Western blot and immunodetection

Protein expression and activation by phosphorylation (Table 6) was analyzed with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Western blot and immunodetection as described in the Original Publications I–III.

Table 6. Primary and secondary antibodies used in the Western blot analyses in Studies I–III.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-α-SMA (14968)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology; Danvers, Massachusetts, USA</td>
<td>II</td>
</tr>
<tr>
<td>anti-β-actin (sc-47778)</td>
<td>mouse</td>
<td>1:2000</td>
<td>Santa Cruz Biotechnology; Dallas, Texas, USA</td>
<td>I, II, III</td>
</tr>
<tr>
<td>anti-p-ERK1/2 (T202/Y204) (#9101)</td>
<td>rabbit</td>
<td>1:2000</td>
<td>Cell Signaling Technology</td>
<td>I</td>
</tr>
<tr>
<td>anti-ERK2 (sc-154)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology</td>
<td>I</td>
</tr>
<tr>
<td>anti-p-FAK(Y397) (#3283)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>I</td>
</tr>
<tr>
<td>anti-FAK (#3285)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>I</td>
</tr>
<tr>
<td>anti-p-HSP27(S78) #2405</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>III</td>
</tr>
<tr>
<td>anti-HSP27 (D6W5V) #95357</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>III</td>
</tr>
<tr>
<td>anti-p-MAPKAPK2 (T334) #3007</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>III</td>
</tr>
<tr>
<td>anti-MAPKAPK2 #12155</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>III</td>
</tr>
<tr>
<td>anti-MKL1/MRTF-A (14760)</td>
<td>rabbit</td>
<td>1:800</td>
<td>Cell Signaling Technology</td>
<td>II</td>
</tr>
<tr>
<td>anti-p-MLC2 (3671)</td>
<td>rabbit</td>
<td>1:500</td>
<td>Cell Signaling Technology</td>
<td>II</td>
</tr>
<tr>
<td>anti-MLC2 (3672)</td>
<td>rabbit</td>
<td>1:500</td>
<td>Cell Signaling Technology</td>
<td>II</td>
</tr>
<tr>
<td>anti-p-p38 MAPK (T180/Y182) (#4511)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>III</td>
</tr>
<tr>
<td>anti-p38α (sc-728)</td>
<td>rabbit</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology</td>
<td>III</td>
</tr>
<tr>
<td>anti-mouse IgG-HRP (sc-2005)</td>
<td>goat</td>
<td>1:2000</td>
<td>Santa Cruz Biotechnology</td>
<td>I, II, III</td>
</tr>
<tr>
<td>anti-rabbit IgG-HRP #7074</td>
<td>goat</td>
<td>1:2000</td>
<td>Cell Signaling Technology</td>
<td>I, II, III</td>
</tr>
</tbody>
</table>

4.7 Statistical analyses

Statistical analyses in Studies I–III were conducted using GraphPad prism. Non-parametric statistical analyses were done using Kruskal-Wallis one way analysis of variance and Mann-Whitney U post hoc test followed by Bonferroni correction based on the number of comparisons. The results are expressed as mean and
standard deviation (SD). The differences between samples were considered significant when the adjusted p-value was <0.05.
5 SUMMARY OF RESULTS

5.1 Cell viability and proliferation under inhibitor and BaG treatments (I, II, III)

Cell viability and proliferation under different media, inhibitor treatments, and ionic extract of 3-06 BaG were assessed. In Study I, small-molecule inhibitors PF-562271, Y-27632, and PD98059 were used to target FAK, ROCK, and MEK-ERK signaling, respectively. In Study II, MRTF-A signaling was inhibited using CCG-1423 and CCG-100602 inhibitors to compare the functionality of these first- and second-generation MRTF-A inhibitors. Cell viability was studied using live/dead assay where viable cells were labelled with green fluorescent calcein AM and necrotic cells with red fluorescent EthD-1. Cell proliferation was analyzed by CyQUANT cell proliferation assay based on the total DNA amount of the samples measured using nucleic acid bound fluorescent GR-Dye.

Figure 11 shows that FAK, ROCK, and ERK inhibitor molecules PF-562271, Y-27632, and PD98059, respectively, (Study I) dose-dependently decreased the amount of adherent viable hASCs, but there was no increase in the number of dead cells. The effects of MRTF-A inhibitors CCG-1423 and CCG-100602 (Study II) on cell viability were similar. The first-generation compound CCG-1423 reduced the number of adherent viable cells more than the second-generation inhibitor CCG-100602. The hASCs tolerated the inhibitory effects of PF-562271, Y-27632, and CCG-100602 better when cultured in OM than in BM and AM conditions.
Figure 11. Cell viability of hASCs under FAK, ERK, ROCK, and MRTF-A inhibitors in BM, OM, and AM conditions after 7 days of culture. hASCs were cultured in BM, OM, and AM supplemented with PF-562271, Y-27632, PD98059, CCG-1423, and CCG-100602. Cell viability was analyzed with live/dead assay at 7 d and the cells were imaged with a fluorescence microscope using Alexa 488 for living (green) and Alexa 546 for dead (red) cells. Representative images modified from Original Publications I and II are presented. Scale bar 1.0 mm.
BaG extract media and the p38 MAPK inhibitor SB202190 used in the Study III had no effect on the viability of hBMSCs (Figure 12). A viability assay was not performed with hASCs in Study III because earlier studies by our research group demonstrated that neither 3-06 BaG (Ojansivu et al., 2015) nor the SB202190 inhibitor (Ojansivu, Wang, et al., 2018) compromised the viability of hASCs.

**Figure 12.** Cell viability of hBMSCs under p38 MAPK inhibitor in BM, BaG BM, OM, and BaG OM conditions after 6 days of culture. hBMSCs were cultured in BM, BaG BM, OM, and BaG OM supplemented with SB202190. Cell viability was analyzed with live/dead assay at 6 d and the cells were imaged with a fluorescence microscope using Alexa 488 for living (green) and Alexa 546 for dead (red) cells. Representative images modified from Original Publication III are presented. Scale bar 1.0 mm.

FAK, ROCK, and ERK inhibitors (Study I) and MRTF-A inhibitors (Study II) were demonstrated to have a significant regulatory function in cell proliferation (Figure 13). Inhibitors dose-dependently decreased the number of cells in such a manner that higher inhibitor concentrations resulted in fewer cells. We demonstrated that the second-generation MRTF-A inhibitor CCG-100602 (Figure 13D) caused less cytotoxic effects than the first-generation analog CCG-1423 (see Original Publication II Figure 1B).
Figure 13. Cell proliferation of hASCs under FAK, ERK, ROCK, and MRTF-A inhibitors in BM, OM, and AM. hASCs were cultured in BM, OM, and AM supplemented with PF-562271, Y-27632, PD98059, and CCG-100602. Cell proliferation was analyzed with CyQUANT assay at 7 and 14 d. N=12 (independent biological replicates from four donors). Significance level 5%. Modified from Original Publications I and II.

Figure 14. Cell proliferation of hASCs and hBMSCs under BaG extract and p38 MAPK inhibitor treatment. hASCs (A) and hBMSCs (B) were cultured in BM and OM supplemented with BaG extract and SB202190. Cell proliferation was analyzed with CyQUANT assay at 7, 9, and 11 d. N=9 (independent biological replicates from three donors). Significance level 5%. Modified from Original Publication III.
The p38 MAPK inhibitor used in Study III (Figure 14) did not have an adverse effect on hMSC cell proliferation. Under OM condition p38 MAPK inhibitor even increased hASC proliferation. The effect of BaG on cell proliferation was dependent on the cell type, culture medium, and culture length. BM supplemented with BaG extract enhanced cell proliferation in both cell types. BaG extract in OM increased hASC proliferation at 7 and 9 days but decreased proliferation on day 11. BaG OM decreased hBMSC proliferation at all time points studied.

Figure 14. Cell proliferation of hASCs and hBMSCs under BaG extract and p38 MAPK inhibitor treatment. hASCs (A) and hBMSCs (B) were cultured in BM and OM supplemented with BaG extract and SB202190. Cell proliferation was analyzed with CyQUANT assay at 7, 9, and 11 d. N=9 (independent biological replicates from three donors). Significance level 5%. Modified from Original Publication III.
5.2 Involvement of FAK, ROCK, ERK, and MRTF-A signaling in hASC adipogenesis (I, II)

Adipogenic potential of hASCs was studied in Original Publications I and II with ORO staining of neutral lipids. The involvement of FAK, ROCK, ERK, and MRTF-A in hASC adipogenesis was assessed by inhibiting the pathways with well-established inhibitor molecules of these signaling pathways. The ORO-stained samples were imaged using a fluorescence microscope to detect red-stained lipid droplets and DAPI-counterstained nuclei. Representative ORO-stained images of the most potent inhibitor concentrations are presented in Figure 15A. The lipid droplet morphology was unilocular-like under FAK and ROCK inhibitor treatments, as in the control AM. In contrast, the MRTF-A inhibitor induced the formation of multilocular droplets.

A custom image quantification pipeline was developed to quantify ORO staining. This method allowed us to evaluate the percentage of lipid droplets in the total image area (see Original Publication I Figure 5B and Original Publication II Figure 2B). Inhibition of ROCK and MRTF-A signaling under AM significantly increased lipid droplet formation in a concentration-dependent manner. Using a threshold value for the lipid droplet diameter we demonstrated that FAK, ROCK, and MRTF-A inhibition enhanced the portion of large lipid clusters (Figure 15B–E). Adipogenesis was also enhanced at the genetic level under FAK and ROCK inhibition, as demonstrated by qRT-PCR analysis of AP2 and LEP expressions (see Original Publication I Figure 4). However, a large concentration of the FAK inhibitor diminished both the lipid droplet amount and the total cell amount (Figure 15B). ERK inhibition reduced the lipid droplet formation induced by adipogenic culture medium (Figure 15D).
Involvement of FAK, ROCK, ERK, and MRTF-A signaling in hASC adipogenesis (I, II)

Adipogenic potential of hASCs was studied in Original Publications I and II with ORO staining of neutral lipids. The involvement of FAK, ROCK, ERK, and MRTF-A in hASC adipogenesis was assessed by inhibiting the pathways with well-established inhibitor molecules of these signaling pathways. The ORO-stained samples were imaged using a fluorescence microscope to detect red-stained lipid droplets and DAPI-counterstained nuclei. Representative ORO-stained images of the most potent inhibitor concentrations are presented in Figure 15A.

Figure 15. Inhibitor effect on hASC adipogenic differentiation analyzed by ORO staining after 21 days of differentiation. hASCs were cultured in BM and AM supplemented with PF-562271, Y-27632, PD98059, and CCG-100602. ORO staining and fluorescence imaging using Alexa 546 filter for ORO (red) and DAPI filter for nuclei (blue) was performed at 21 d. A Representative images of FAK, ROCK, ERK, and MRTF-A inhibitor-treated hASCs. Control images for separate experiments are presented. Scale bar 100 µm. B–E Quantitative data presenting the percentage of the ORO-stained lipid droplet area. N (B, C)=13–16 images (4 independent biological replicates from two donors), N(D)=19–21 images (6 independent biological replicates from three donors), N(E)=59–63 images (15 independent biological replicates from four donors). Significance level 5%. Threshold for lipid droplet cluster is 10 µm in diameter in B–D graphs and 5 µm in E graph. Modified from Original Publications I and II.
5.3 Involvement of FAK, ROCK, ERK, and MRTF-A signaling in hASC osteogenesis (I, II)

FAK, ROCK, ERK, and MRTF-A inhibition was utilized to evaluate the role of these signaling pathways in hASC differentiation potential toward bone tissue in Studies I and II. The early osteogenic differentiation potential of hASCs was assessed by analyzing ALP activity, a well-known marker for osteogenesis, and the gene expression of RUNX2A, a master-regulator of osteogenesis. Additionally, qAR staining of the mineralized matrix was used to determine late-stage osteogenesis.

Elevation of the ALP enzyme was relevant after 14 days of culture in OM (Figure 16A–D). The inhibitors significantly and dose-dependently decreased OM-induced ALP activity. Similarly, RUNX2A expression stimulated under OM was downregulated by the inhibitor function (see Original Publication I Figure 3A, and Original Publication II Figure S1C). The qAR staining gave parallel results after 21 days of culture (Figure 16E–H). Furthermore, the secretion of the ECM proteins collagen type I and osteopontin was diminished by MRTF-A inhibition (see Original Publication II Figures 3C and 4C).
Involvement of FAK, ROCK, ERK, and MRTF-A signaling in hASC osteogenesis (I, II)

FAK, ROCK, ERK, and MRTF-A inhibition was utilized to evaluate the role of these signaling pathways in hASC differentiation potential toward bone tissue in Studies I and II. The early osteogenic differentiation potential of hASCs was assessed by analyzing ALP activity, a well-known marker for osteogenesis, and the gene expression of RUNX2A, a master regulator of osteogenesis. Additionally, qAR staining of the mineralized matrix was used to determine late-stage osteogenesis.

Elevation of the ALP enzyme was relevant after 14 days of culture in OM (Figure 16A–D). The inhibitors significantly and dose-dependently decreased OM-induced ALP activity. Similarly, RUNX2A expression stimulated under OM was downregulated by the inhibitor function (see Original Publication I Figure 3A, and Original Publication II Figure S1C).

The qAR staining gave parallel results after 21 days of culture (Figure 16E–H). Furthermore, the secretion of the ECM proteins collagen type I and osteopontin was diminished by MRTF-A inhibition (see Original Publication I Figures 3C and 4C).

Figure 16. Osteogenic differentiation potential of hASC under FAK, ROCK, ERK, and MRTF-A inhibition. hASCs were cultured in BM and OM supplemented with PF-562271, Y-27632, PD98059, and CCG-100602. A–D The enzymatic activity of ALP at 14 days of differentiation. E–H qAR staining for mineralization at 21 days of differentiation. N (A–D)=9–12 independent biological replicates from 3–4 donors. N(E–G)=15–18 independent biological replicates from 5–6 donors. N(H)=9 independent biological replicates from three donors. Significance level 5%. Modified from Original Publications I and II.
5.4 Involvement of the p38 MAPK/HSP27 signaling in BaG-induced osteogenesis of hASCs and hBMSCs (III)

To evaluate the involvement of the p38 MAPK/HSP27 axis in BaG-induced osteogenesis of hASCs and hBMSCs, protein levels of p38, MK2, HSP27 and their activation by phosphorylation were studied by Western blotting and immunodetection in Study III. Temporal enhancement in the protein levels of phosphorylated p38 MAPK, pMK2, and pHSP27 was observed as a response to culture in OM (Figure 17). On the contrary, BaG OM induced a rapid peak in protein and corresponding phospho-protein levels followed by silencing of the signaling.

![Figure 17](#) Protein activation of p38 MAPK/MK2/HSP27 signaling axis in hASCs and hBMSCs under OM and BaG OM. Modified from Original Publication III.

The early osteogenic marker ALP was studied in Study III to show the effect of BaG supplementation and p38 MAPK inhibition on the hASC and hBMSC osteogenic potential. ALP activity was elevated in OM conditions compared with BM, as expected (Figure 18). In hASCs (Figure 18A), the ALP activity induced by BaG OM was enhanced with time. In hBMSCs (Figure 18B), however, BaG OM-induced ALP activity peaked on day 9 and declined thereafter. When cultured in OM alone, ALP activity increased as a function of time in both cell types. The p38 MAPK inhibition reduced BaG-induced ALP activity.

![Figure 18](#) Early osteogenic differentiation potential of hASC and hBMSC under BaG supplementation and p38 MAPK inhibition. hASCs (A) and hBMSCs (B) were cultured in BM, and OM supplemented with BaG extract and SB202190. The enzymatic activity of ALP was studied after 7, 9, and 11 days of differentiation. N=9 (independent biological replicates from three donors of each cell type). Significance level 5%. Modified from Original Publication III.
Involvement of the p38 MAPK/HSP27 signaling in BaG-induced osteogenesis of hASCs and hBMSCs (III)

To evaluate the involvement of the p38 MAPK/HSP27 axis in BaG-induced osteogenesis of hASCs and hBMSCs, protein levels of p38, MK2, HSP27 and their activation by phosphorylation were studied by Western blotting and immunodetection in Study III. Temporal enhancement in the protein levels of phosphorylated p38 MAPK, pMK2, and pHSP27 was observed as a response to culture in OM (Figure 17). On the contrary, BaG OM induced a rapid peak in protein and corresponding phospho-protein levels followed by silencing of the signaling.

Figure 17. Protein activation of p38 MAPK/MK2/HSP27 signaling axis in hASCs and hBMSCs under OM and BaG OM. Modified from Original Publication III.

The early osteogenic marker ALP was studied in Study III to show the effect of BaG supplementation and p38 MAPK inhibition on the hASC and hBMSC osteogenic potential. ALP activity was elevated in OM conditions compared with BM, as expected (Figure 18). In hASCs (Figure 18A), the ALP activity induced by BaG OM was enhanced with time. In hBMSCs (Figure 18B), however, BaG OM-induced ALP activity peaked on day 9 and declined thereafter. When cultured in OM alone, ALP activity increased as a function of time in both cell types. The p38 MAPK inhibition reduced BaG-induced ALP activity.

Figure 18. Early osteogenic differentiation potential of hASC and hBMSC under BaG supplementation and p38 MAPK inhibition. hASCs (A) and hBMSCs (B) were cultured in BM, and OM supplemented with BaG extract and SB202190. The enzymatic activity of ALP was studied after 7, 9, and 11 days of differentiation. N=9 (independent biological replicates from three donors of each cell type). Significance level 5%. Modified from Original Publication III.

The role of HSP27 signaling in late-stage osteogenesis was analyzed using qAR, and ICC staining of ECM protein collagen type I using p38 MAPK inhibition. Figure 19 shows that inhibition with SB202190 significantly decreased BaG OM-induced mineralized matrix accumulation. Collagen type I production in hASCs was diminished by HSP27 inhibition based on ICC staining (Figure 19C). In hBMSCs, SB202190 reduced collagen type I in OM, but in BaG OM the effect was indifferent (Figure 19D).
Figure 19. Matrix mineralization and collagen type I production of hASCs and hBMSCs under BaG supplementation and p38 MAPK inhibition. hASCs (A,C) and hBMSCs (B,D) were cultured in OM supplemented with BaG extract and SB202190. A,B The matrix mineralization was studied with qAR staining after 9 and 11 days of differentiation. hASCs: N=9 (independent biological replicates from three donors), hBMSCs: N=6 (independent biological replicates from two donors). Comparisons were made between the inhibited and uninhibited groups. Significance level 5%. C,D Collagen type I ICC staining was performed at 11 days of culture. Fluorescence imaging using Alexa 488 for collagen type I (green), Alexa 546 filter for phalloidin-stained F-actin (red) and DAPI filter for nuclei (blue) was performed. Small figures demonstrate the negligible collagen type I production in the basic medium (BM) controls. Scale bar 100 µm. Modified from Original Publication III.
5.5 Effect of MRTF-A and HSP27 inhibition on the actin cytoskeleton (II, III)

The intracellular F-actin cytoskeleton responded to treatments with MRTF-A and HSP27 inhibitors. In Study II, phalloidin-TRITC staining demonstrated that the fibroblastic and spindle-like shape of the hASCs cultured under control conditions was altered under CCG-100602 MRTF-A inhibition (Figure 20A). The normalized intensity of F-actin staining was significantly reduced by MRTF-A inhibition in AM condition (Figure 20B). Additionally, MRTF-A inhibition modulated the spindle-like morphology of the cytoskeleton by decreasing the coherence of actin orientation (see Original Publication II Figure 6D). Besides F-actin, MRTF-A inhibition was also demonstrated to reduce the protein levels of SRF-regulated actin-related proteins α-SMA and MLC, and MLC activation by phosphorylation (Figure 20C).

Figure 20. Phalloidin-stained actin cytoskeleton and intracellular protein levels of MRTF-A, β-actin, α-SMA, pMLC, and MLC as a response to MRTF-A inhibition. hASCs were cultured 7 d with MRTF-A inhibition. A Representative phalloidin- and DAPI-stained hASCs imaged with Alexa 546 filter for red-stained F-actin and blue DAPI filter for nuclei. Scale bar 100 µm. B Image-based analysis of mean phalloidin intensity normalized to the nuclei count. N=18–25 images (4 independent biological replicates from two donors). Significance level 5%. C Representative immunoblotted MRTF-A, β-actin, α-SMA, pMLC, and MLC at day 7. Modified from Original Publication III.
Figure 21. Phalloidin-TRITC staining and F-actin intensity of hASCs and hBMSCs. The cells were cultured 9 days in BM, BaG BM, OM, and BaG OM supplemented with SB202190 inhibitor.

A, B Representative images obtained with Alexa 546 for actin (red) and DAPI (blue) filters. Scale bar 100 µm. C, D Image-based quantitation of mean grey values of phalloidin-TRITC stained samples normalized to the nuclei count. N=15–18 images (4 independent biological replicates from two donors of both cell types). Significance level 5%. Modified from Original Publication III.
In Study III, the cell morphology of hASCs and hBMSCs was fibroblastic with thick stress fibers in the BM and OM control conditions (Figure 21A,B). The representative images and quantitated imaging data (Figure 21C,D) show that the BaG extract and p38 MAPK inhibitor treatment had parallel effects on the cytoskeleton, leading to thinner and less visible F-actin fibers and diminished F-actin intensity.

5.6 HSP27 localization and phosphorylation status during BaG-induced osteogenesis (III)

Localization and phosphorylation status of HSP27 in hMSCs cultured under BaG and SB202190 treatment was studied using ICC staining of the basal and phosphorylated HSP27 in Study III. The localization of HSP27 was cytoplasmic in both cell types, as shown in the representative Figure 22 with hBMSCs. When phosphorylated, HSP27 aligned with F-actin fibers under BM and OM conditions. However, under BaG OM the pHSP27 localization was diffuse in hASCs and hBMSCs after 9 days of culture (see Original Publication III Figure 5D, and Figure 22B, respectively). The p38 MAPK inhibition slightly increased the level of actin-associated pHSP27 under BaG OM.
Figure 22. Cellular localization of basal and phosphorylated HSP27 in hBMSCs. hBMSCs were cultured 9 days in OM and BaG OM supplemented with SB202190 inhibitor. The hBMSCs were analyzed with antibodies for HSP27 (A) and pHSP27 (B) and imaged using Alexa 488 filter for HSP27/pHSP27 (green), Alexa 546 for F-actin (red) and DAPI filter for nuclei (blue). 4x digital zoom images are presented under original images. Scale bars are 100 µm in the original images and 25 µm in the zoom images. Modified from Original Publication III.
6 DISCUSSION

6.1 Modulation of cell adhesion through FAK signaling guides hASC differentiation fate

MSCs are adherent cells, indicating that they adhere to the surrounding cells and the ECM. In vitro cell adhesion to culture plastics is one of the minimal ISCT criteria for MSCs (Dominici et al., 2006). As a fundamental characteristic of MSCs, cell adhesion is important for guiding several aspects of MSC behavior such as cell survival, self-renewal, differentiation, and motility (Geiger et al., 2009; Gumbiner, 1996; Mathieu & Loboa, 2012; Schaller, 2001; Tomakidi et al., 2014; Y.-K. Wang & Chen, 2013). The cell adhesion mechanism is dependent upon the activation of transmembrane receptors of the integrin family and subsequent recruitment of intracellular proteins such as FAK, vinculin, paxillin, talin and α-actinin to form focal adhesion complexes (Cary & Guan, 1999; Webb et al., 2004).

Study I examined the role of FAK in guiding the lineage specification of hASCs towards adipogenic and osteogenic lineages using the FAK inhibitor PF-562271 that, to our knowledge, has not been previously studied in the context of hASCs. PF-562271 (also known as VS-6062 or PF00562271) is an ATP-competitive inhibitor of FAK that was the first specific FAK inhibitor used in clinical trials as an antitumor agent (Wu et al., 2021). In Study I, the impairment of FAK phosphorylation resulted in concentration-dependent effect on hASC proliferation and differentiation. The inhibitor was supplemented with every medium change and caused a cumulative effect that resulted in a noticeable decrease in the number of adherent cells in the culture. Disruption of the adhesion mechanism thus prevented cell propagation. This is in agreement with previous studies demonstrating that without adequate attachment, MSCs are programmed into self-destruction through anoikis (Frisch & Screaton, 2001; Gilmore, 2005).

Studies on hBMSCs have demonstrated the importance of cell adhesion and activation of FAK signaling in osteogenic differentiation (Salasznyk et al., 2007; Shih et al., 2011; Xu et al., 2014). Based on our results from Study I, FAK signaling has a
parallel role in osteogenesis of hASCs. The osteogenic differentiation potential of hASCs analyzed by the expression of RUNX2/A, protein activity of ALP, and mineralized matrix formation was hindered by FAK inhibition. The induction of osteogenesis requires adequate cell density (Abo-Aziza & A.A., 2017), and our results suggest that a lack of adhesion hinders the proliferative capacity of hASCs and the subsequent initiation of osteogenesis.

To analyze the adipogenic differentiation potential we created and optimized an image quantification pipeline to quantify the results of the benchmark ORO staining. The quantification method allowed us to analyze the total lipid droplet area, and the area of enlarged droplets using limits of the lipid droplet diameter. Varinli et al. revealed a systematic shift towards larger lipid droplet sizes during the growth of human preadipocytes, thus indicating a maturation process (Varinli et al., 2015). Our results revealed that lower concentration of FAK inhibitor moderately increased the total lipid area, lipid area normalized to the cell number, and number of large lipid droplets. Enhanced formation of lipid droplets has also been previously observed in human BMSCs treated with the FAK inhibitor PF-573228 (Xu et al., 2014). The effect of FAK inhibition on adipogenesis was evident also in the gene expression level. In Study I, we observed increased AP2 and LEP, and others have reported increased PPARγ expression (Xu et al., 2014). These results suggested that adipogenesis was promoted when the adhesion mechanism was weakened. Indeed, calpain-mediated cleavage of FAK was shown essential for rodent preadipocyte differentiation (J.-J. Li & Xie, 2007). However, the Study I results also indicated that the effect of FAK inhibitor on adipogenesis was concentration-dependent, as a large inhibitor concentration that led to reduced proliferation also hindered the adipogenic course. Similarly, in vivo, FAK knockout mice exhibited decreased adipocyte survival (Luk et al., 2017). These results highlight the importance of controlled regulation of FAK signaling during cellular events such as adipogenesis.
6.2 Activity of ERK signaling is required for adipogenic and osteogenic courses of hASCs

One of the signaling mechanisms activated by cellular adhesion is MAPK ERK signaling that controls diverse cellular fates, including cell survival, proliferation, and differentiation, through transcriptional regulation (Hong et al., 2010; Roberts & Der, 2007; Webb et al., 2004; Zhang & Liu, 2002). ERK is a mechanosensing protein that functions downstream of the FAK and ROCK signaling pathways (Knapik et al., 2013; Q. Liu et al., 2009; Xu et al., 2014). The interconnection of the pathways was also observed in Study I, as the inhibition of FAK and ROCK phosphorylation also affected the protein level of phospho-ERK (see Original Publication I).

Multiple inhibitor molecules have been designed to block the signaling activity of the ERK pathway, including the first inhibitor PD98059 that was discovered by Dudley and coworkers in 1995 (Dudley et al., 1995). The importance of ERK activity in the osteogenic course has been demonstrated using ERK inhibitors. Reduced ALP activity and calcium deposition in response to ERK inhibition with PD98059 was demonstrated by Jaiswal et al. in hBMSCs (Jaiswal et al., 2000), and similar findings were obtained in hASCs (H. Gu et al., 2011; Q. Liu et al., 2009). Supporting results with hBMSCs were obtained using the ERK1/2 inhibitor FR180204 (Kilian et al., 2010) and MEK inhibitor U0126 (Shih et al., 2011). The results of Study I were in agreement with the literature, demonstrating that ERK inhibition with PD98059 dose-dependently diminished ALP activity and mineral accumulation in hASCs and indicating that ERK activity is a requisite for the osteogenic outcome.

Although the role of ERK in adipogenesis has been studied over the past few decades, there is no consensus regarding its molecular details, and the findings remain contradictory. Previous studies in mice have revealed that ERK activity is required for adipogenesis (Bost et al., 2005; Prusty et al., 2002; Tang et al., 2005). In vitro, the activation of ERK signaling was required for the expression of PPARγ, C/EBP, and other adipogenic genes in mouse preadipocytes (Prusty et al., 2002). Similarly, ERK activity has been demonstrated to drive clonal expansion of 3T3-L1 cells and their adipogenesis by associating with C/EBP (Tang et al., 2005). In vivo, ERK1 knockout mice exhibited reduced adiposity (Bost et al., 2005) and our results support these findings. In Study I, we observed a reduced adipogenic outcome under ERK inhibition and concluded that the activity of ERK signaling was significant for adipogenesis of hASCs. ERK inhibition reduced lipid droplet accumulation and
maturation, even when cells were cultured in an adipogenic medium. The crucial positive role of ERK in hMSCs has also been demonstrated by Xu et al. (Xu et al., 2014). Conversely, opposing conclusions exist in human MSCs, stating that ERK inhibition induces adipogenesis, thus making ERK a negative regulator of adipogenesis and a molecular switch between adipogenesis and osteogenesis (Jaiswal et al., 2000; Kilian et al., 2010; Q. Liu et al., 2009). Bost and coworkers postulated that ERK would be temporally activated to allow initiation of the adipogenic differentiation and then inactivated to proceed with adipocyte maturation (Bost et al., 2005), as ERK activity suppresses the transcriptional activity of its substrate PPARγ (Burns & Vanden Heuvel, 2007). However, there is still a need for further studies examining this topic, particularly in human cells.

6.3 Cytoskeletal regulation via ROCK and MRTF-A signaling determines the lineage specification of hASCs

Terminally differentiated cells of mesenchymal origin are morphologically distinct from each other, and this can be readily explained by their unique cellular functions. For example, adipogenesis is characterized by cell rounding that allows for the formation of unilocular lipid droplets to maximize energy storage in the fat tissue (de la Rosa Rodriguez & Kersten, 2017; Niemelä et al., 2007). In contrast, mature osteoblasts are cuboid and spread cells that are responsible for mineral formation and bone remodeling (Rodriguez et al., 2004; Salasznyk et al., 2007; Yourek et al., 2007). Indeed, osteogenic and adipogenic differentiation courses have been demonstrated to be mutually exclusive (James, 2013), and morphological changes have been discovered as cues that guide hMSC differentiation (Kilian et al., 2010; McBeath et al., 2004; Vasilevich et al., 2020). Studies I and II analyzed the role of cytoskeletal dynamics in the differentiation fate of hASCs while focusing on ROCK and MRTF-A signaling.

The reorganization of the actin cytoskeleton plays an important role in osteogenic differentiation (C. Huang et al., 2015; Sonowal et al., 2013). A spread morphology with rigid stress fibers (McBeath et al., 2004; Kilian et al., 2010) and increased actomyosin tension are characteristics of osteocommitted hMSCs (Ambriz et al., 2018; Clark et al., 2007; Sonam et al., 2016). Consistent with the literature, in Study I we demonstrated the importance of the key signaling pathway regulating the actin cytoskeleton, the ROCK pathway, in the osteogenic fate of hASCs. Based on our
results, inhibition of ROCK phosphorylation by Y-27632 altered hASC morphology and suppressed the phosphorylation of MLC2. MLC2 is a major player in actomyosin contraction and stress fiber assembly (Amano et al., 2010). Thus, ROCK inhibition affects cell contractility. Studies examining hBMSCs cultured with Y-27632 have demonstrated a reduced osteogenic potential (Kilian et al., 2010; Shih et al., 2011). In study I, ROCK inhibition resulted in a decrease in RUNX2A expression and halted the activity of ALP and mineral formation. These studies denote the importance of ROCK activity on the osteogenic course of hMSCs through its function on the cytoskeleton.

Actin polymerization further regulates the balance between monomeric G-actin and filamentous F-actin, thereby modulating the activity of its downstream target MRTF-A. Existing evidence of MRTF-A involvement in the osteogenic differentiation of precursor cells is limited. We hypothesized that MRTF-A could play a role parallel to that of ROCK in hASC differentiation. Our results indicate that MRTF-A is an important regulator of hASC lineage commitment through its function as a transcriptional coactivator of cytoskeletal SRF-dependent genes. Inhibition of MRTF-A nuclear localization using CCG-1423 (Evelyn et al., 2007; Hayashi et al., 2014) or its analog CCG-100602 (Evelyn et al., 2010; Watanabe et al., 2015) decreased the osteogenic potential of hASC as determined by ALP activity, secretion of ECM proteins collagen I and OPN, and matrix mineralization. Furthermore, MRTF-A inhibition diminished protein expression of α-SMA, whose activity is associated with hBMSC osteogenesis (Talele et al., 2015). Supporting our results, in murine calvarial cells, SRF knock-out decreased RUNX2 protein expression (J. Chen et al., 2012). Bian et al. reported that MRTF-A knockout mice exhibited inferior bone development compared to that of wild-type mice, and the osteogenesis of BMSCs isolated from knockout mice was reduced (Bian et al., 2016). These results indicate that ROCK and MRTF-A are important regulators of osteogenic fate.

Osteogenic and adipogenic courses are mutually exclusive, and ROCK is one of the pathways that can regulate the switch between lineage commitment (James, 2013; Kilian et al., 2010). In Study I, ROCK inhibition led to enhanced gene expression of AP2 and LEP and significant induction of lipid droplet formation and enlargement, thus suggesting the progression of adipogenic differentiation. Others have also reported that the modulation of actin polymerization through ROCK is a key regulatory step in adipogenesis (L. Chen et al., 2018; Sordella et al., 2003). The ratio of G-actin to F-actin increases during adipogenic differentiation (L. Chen et al.,
2018). This could cause MRTF-A to bind to actin monomers, thereby preventing its activity, as MRTF-A is tightly regulated by the G- to F-actin ratio in the cytoplasm and in the nucleus (Sidorenko & Vartiainen, 2019). Based on Study II, MRTF-A inhibition with either a first- or second-generation inhibitor enhanced the amount and size of lipid droplets. The repressive role of active MRTF-A on adipogenesis has been previously demonstrated in mice. Rosenwald et al. demonstrated that MRTF-A knockdown in an immortalized preadipocyte mouse cell line increased PPARγ target gene expression (Rosenwald et al., 2017). In Study II, MRTF-A inhibition reduced the alignment of F-actin fibers and inhibited MLC phosphorylation, suggesting the cytoskeletal regulation of adipogenic outcome. Interestingly, MRTF-A inhibition reduced LEP gene expression, and the inhibitor treatment promoted the formation of multilocular lipid droplets, thus suggesting a beige-like cell phenotype. This finding is corroborated by earlier studies in which MRTF-A was demonstrated to regulate beige adipogenesis in mice (McDonald et al., 2015; Rosenwald et al., 2017). These results indicate that ROCK and MRTF-A independently control the balance between osteogenic and adipogenic differentiation lineages and that the regulation of actin dynamics is relevant in the differentiation fate decision of hASCs.

6.4 BaG ionic extract induces rapid osteogenic differentiation of hMSCs

BaGs that are amorphous glass materials belonging to the biomaterial class of bioceramics, have proven to be advantageous for bone regeneration due to their capability to trigger new bone cell formation (Hench & Jones, 2015; Jones, 2013). Interestingly, BaG ions alone have proven sufficient to induce the osteogenic differentiation of precursor cells without cell-biomaterial contact (Gong et al., 2014; Núñez-Toldrà et al., 2019; Ojansivu et al., 2015; Ojansivu, Mishra, et al., 2018; Sun et al., 2007; Vuornos et al., 2019; Xynos et al., 2001), but the intracellular mechanisms underlying this induction are yet to be identified. In Study III, S53P4-based experimental 3-06 BaG with a minor difference in chemical composition due to the addition of boron (Zhao et al., 2008) was utilized for osteogenic induction. The addition of boron was rationalized by the developers to speed up the dissolution rate of the material and improve the osteogenic effect (Zhao et al., 2008). To date, only a few studies exist with this experimental BaG. The 3-06 BaG failed to adhere to soft tissue in a rat model but exhibited good biocompatibility with bone tissue (Zhao
et al., 2008). The ionic extract of the 3-06 BaG was demonstrated to induce rapid osteogenesis in hASCs (Ojansivu et al., 2015).

Our results were consistent with previous studies and revealed that hASCs and hBMSCs cultured under osteogenic medium supplemented with the 3-06 extract exhibited rapid activation of osteogenic markers, including ALP activity, collagen type I production, and formation of a mineralized matrix. The ALP activity of the hBMSCs peaked earlier than that of the hASCs. Based on this, we postulated that hBMSC osteogenesis would have proceeded further. Indeed, matrix mineralization was stronger on day 9 in BaG-treated hBMSCs than it was in hASCs. The hBMSCs mineralized and secreted collagen type I into the ECM under osteogenic medium even in the absence of BaG, thus indicating that their osteogenic potential was stronger. Similarly, the superior osteogenic potential of hMSCs harvested from bone marrow compared to that of adipose tissue has been reported previously (C. Li et al., 2015; Mohamed-Ahmed et al., 2018; Shafiee et al., 2011).

In Study III, the osteogenic outcome was accompanied by a simultaneous reduction in cell proliferation, and the proliferation of hBMSCs was observed to decline earlier, thus supporting the idea that hBMSCs differentiate faster than hASCs. In a previous study, borosilicate glasses reduced hASC proliferation and enhanced osteogenic marker genes and late osteogenic differentiation (Ojansivu, Mishra, et al., 2018). The restricting effect of BaG ions from boron-containing experimental glass on hASC proliferation was also demonstrated in a study by Vuornos et al., where they hypothesized that reduced proliferation was linked to the progression of osteogenic differentiation (Vuornos et al., 2020). Sun and coworkers demonstrated that 45S5 Bioglass® affected cell proliferation by promoting the cell cycle of human osteoblasts to pass through G1 and S phase and then enter G2 rapidly to promote subsequent osteogenesis (Sun et al., 2007). Indeed, cell proliferation is downregulated once osteogenic markers are expressed and apoptosis is a natural part of the osteogenic differentiation program (Lynch et al., 1998). The rapid initiation of cell proliferation and osteogenic commitment in response to BaG ions may explain our observations.
6.5 The p38 MAPK/HSP27 axis modulates BaG-induced osteogenesis of hMSCs through cytoskeletal interaction

In Study III, the previously understudied role of the p38 MAPK/HSP27 signaling axis in hASC and hBMSC osteogenesis was studied, as it was hypothesized to regulate differentiation through the actin-stabilizing function of HSP27 (J. Clarke & Mearow, 2013; Y. Wang et al., 2015; Wettstein et al., 2012). Upregulation of HSP27 has been demonstrated in many differentiation programs (Arrigo, 2017). Therefore, we analyzed the protein activation of phospho-HSP27, basal HSP27, and upstream kinases during osteogenic differentiation and in response to the 3-06 BaG extract. We observed that hMSC p38 MSPK/HSP27 signaling was upregulated at the protein level when osteogenic differentiation was induced. In osteogenic medium supplemented with BaG extract, HSP27 activation by phosphorylation occurred early and transiently, and in OM alone the signaling increased with time. Signal activation shared a similar trend with the osteogenic response, where pathway activation occurred earlier in hBMSCs than it did in hASCs. When HSP27 phosphorylation was inhibited by upstream kinase inhibitor SB202190, osteogenesis was reduced, thus suggesting the importance of HSP27 phosphorylation in the osteogenic outcome of hMSCs. During osteogenic differentiation, osteoblast precursors change shape from spindle-shaped cells into cuboidal osteoblasts through cytoskeletal remodeling (J. Clarke & Mearow, 2013). The cytoskeleton in mature osteoblasts becomes a meshwork of thinner actin fibers (Titushkin and Cho, 2007; Yourek, Hussain and Mao, 2007). In Study III, hMSCs exhibiting significant collagen type I secretion and ECM mineralization possessed an altered morphology with thin actin fibers indicating osteogenic maturation.

Next, we analyzed the significance of active HSP27 signaling to the cytoskeleton, as HSP27 has been proposed to stabilize actin filaments and thus regulate cytoskeletal integrity and contractility (J. Clarke & Mearow, 2013; Y. Wang et al., 2015; Wettstein et al., 2012). Interestingly, HSP27 inhibition reduced the amount of cellular F-actin and MLC2 phosphorylation, thus indicating that the phosphorylation of HSP27 was important for cytoskeleton and its dynamics. Supporting our findings, HSP27 downregulation was observed to reduce β-actin of hASCs (Fan et al., 2021). The current literature remains inconclusive regarding the phosphorylation status of HSP27 in the cytoskeletal localization (J. Clarke & Mearow, 2013; Hoffman et al., 2017; Mymrikov et al., 2011; Wettstein et al., 2012). The actin polymerization-inhibiting property of HSP27 has been considered to relate to the non-
phosphorylated monomer state (Benndorf et al., 1994). Hoffman et al. reported that phosphorylation of HSP27 could not induce its cytoplasmic association without mechanical stress caused by uniaxial stretching in mouse fibroblast cells (Hoffman et al., 2017). The results of Study III strongly suggest that in hMSCs, HSP27 co-localizes with actin when phosphorylated. Supporting our findings, Hoffman et al. concluded in another study that the phosphorylation of HSP27 is a key step in its association with actin to regulate cytoskeletal remodeling, cell spreading, and migration in mouse fibroblasts (Hoffman et al., 2022). In Study III, the cytoskeletal association of pHSP27 was linked to the undifferentiated state of hMSCs. This novel finding together with our results that the HSP27 axis was activated early in osteogenic differentiation and that inhibition of HSP27 activation resulted in diminished osteogenic outcome indicates that actin bound HSP27 is linked to the early commitment step of hMSCs into the osteogenic lineage.

6.6 Research limitations and future perspectives

This thesis focuses on intracellular cell signaling linked to the cellular phenomena of cell adhesion and cytoskeletal dynamics. Studies I–III investigated FAK, ROCK, MAPK ERK, and p38 MAPK signaling proteins that are components of central conserved protein kinase pathways, and thus their role in guiding the differentiation process is intriguing. In addition, the analysis of MRTF-A and HSP27 in Studies II and III, respectively, provided novel insights into the regulatory mechanisms governing hMSC differentiation. However, multiple other signaling molecules are involved in mediating the cues of cell adhesion and the cytoskeleton and deserve to be thoroughly investigated in the future. The studies of the thesis concentrated on two main differentiation lineages of MSCs: adipogenesis and osteogenesis, but we acknowledge the importance of the chondrogenic lineage, as well as the potential other lineages and the identification of the role of these mechanisms on additional lineages would be interesting.

Pharmacological small-molecule inhibitors were utilized in Studies I–III to analyze the relevance of FAK, ROCK/MRTF-A, ERK MAPK, and p38 MAPK/HSP27 pathways in hMSC differentiation. Future studies could include more detailed investigation using overexpression of proteins of interest and silencing of signaling proteins with siRNA to confirm the importance of these pathways in hMSC differentiation. In addition, the conflicting role of ERK signaling in the adipogenic
course of hMSCs discussed in Study I needs to be further studied. In addition to the aforementioned pathways, a broader view on the regulation of differentiation fate should be taken in future studies using proteomic tools to reveal new molecular switches regulating lineage commitment. Additionally, the transcriptome during differentiation could be studied using various sequencing techniques.

In Studies I–III, fluorescence microscopy was used to detect proteins related to adipogenesis and osteogenesis, visualize the actin cytoskeleton, and demonstrate the co-localization of actin and HSP27. Imaging techniques are continuously evolving and for example super-resolution microscopy could be used when studying intracellular interactions and localizations of regulatory proteins in the future. In Study III, we demonstrated common mechanisms regulating hASCs and hBMSCs osteogenesis but showed differences in their differentiation potential. Therefore, it would be interesting to study hMSCs from various sources, such as umbilical cord, placental tissue, synovial fluid, or dental pulp to determine the significance of the cell origin on differentiation.

On a critical note, there are limitations in generalizing of the results of this thesis. Studies conducted in vitro serve as a great model of the biological environment, but consideration must be taken when extrapolating the results into biological context. Recent advances in 3D culture methods would allow the creation of an experimental design that corresponds better to the biological setting. Due to the research and methodological limitations, the number of primary cell lines and the sample size in the studies are limited, and analysis of more cell lines from different donors would have added the assurity of the results to form general conclusions. One feature of the primary cell lines used as a study material is their donor variation. Although donor variation affected the statistical significance of the quantitative results, the variation highlights the unique characteristics of the individuals from whom the cells are harvested. Donor variation should be considered in the clinical use of autologous or allogenic MSCs.
7 CONCLUSIONS

In this thesis, the intracellular signaling mechanisms related to cell adhesion and the dynamics of the cell cytoskeleton were studied in the context of directing the differentiation potential of human MSCs.

In Studies I and II, our objective was to determine if FAK, ERK, ROCK, and MRTF-A signaling is involved in the differentiation fate decision of hASCs. The hypothesis that they are regulators of lineage specification towards adipogenic and osteogenic courses was tested using pharmacological inhibitors of signaling. Cell adhesion modulated by FAK, and the cytoskeletal dynamics regulated by ROCK and MRTF-A were observed to regulate the balance between adipogenesis and osteogenesis in hASCs. FAK and ROCK phosphorylation and the active nuclear localization of MRTF-A are required for osteogenic differentiation as demonstrated by ALP activity and matrix mineralization. Conversely, the inactivation of the FAK, ROCK, and MRTF-A pathways with PF-562271, Y-27632, and CCG-100602, respectively, enhanced lipid droplet formation and enlargement, thus indicating the adipogenic fate of hASCs. The activity of adhesion-mediated MAPK ERK signaling was demonstrated to be important for both courses of hASC differentiation, as signaling inhibition with PD98059 resulted in a decrease in both osteogenic and adipogenic markers.

Study III aimed to elucidate the currently unknown role of p38 MAPK/HSP27 in BaG-stimulated osteogenic differentiation of hMSCs. The ions extracted from the experimental boron-containing 3-06 BaG were determined to induce rapid activation of the osteogenic marker ALP and mineralization. The activity of p38/HSP27 axis was shown to be involved in the osteogenesis of hASCs and hBMSCs. Inhibition of HSP27 phosphorylation by the upstream kinase inhibitor SB202190 diminished BaG-induced osteogenesis suggesting that pHSP27 is relevant for the osteogenic commitment of hMSCs. Our results indicated that phosphorylated HSP27 is involved in the early osteogenic commitment of hMSCs through its ability to interact with the actin cytoskeleton.
In conclusion, the intracellular signaling mechanisms governing cell adhesion and cytoskeletal regulation play pivotal roles in the regulation of hMSC differentiation. This thesis provides new data regarding the mechanisms underlying hMSC differentiation. In the context of adipogenic differentiation, these studies provide a basis for advancements in TE applications for soft tissue reconstruction and cosmetic treatments and for understanding the pathophysiology of obesity and the development of new targeted therapeutics. This study adds knowledge regarding the signaling mechanisms in osteogenic differentiation and the mechanisms underlying BaG-induced osteogenesis for future knowledge-based solutions in the context of bone TE and bone tissue-related disorders such as osteoporosis.
In conclusion, the intracellular signaling mechanisms governing cell adhesion and cytoskeletal regulation play pivotal roles in the regulation of hMSC differentiation. This thesis provides new data regarding the mechanisms underlying hMSC differentiation. In the context of adipogenic differentiation, these studies provide a basis for advancements in TE applications for soft tissue reconstruction and cosmetic treatments and for understanding the pathophysiology of obesity and the development of new targeted therapeutics. This study adds knowledge regarding the signaling mechanisms in osteogenic differentiation and the mechanisms underlying BaG-induced osteogenesis for future knowledge-based solutions in the context of bone TE and bone tissue-related disorders such as osteoporosis.

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Focal Adhesion Kinase and ROCK Signaling are Switch-Like Regulators of Human Adipose Stem Cell Differentiation towards Osteogenic and Adipogenic Lineages

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Focal Adhesion Kinase and ROCK Signaling Are Switch-Like Regulators of Human Adipose Stem Cell Differentiation towards Osteogenic and Adipogenic Lineages

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Adipose tissue is an attractive stem cell source for soft and bone tissue engineering applications and stem cell therapies. The adipose-derived stromal/stem cells (ASCs) have a multilineage differentiation capacity that is regulated through extracellular signals. The cellular events related to cell adhesion and cytoskeleton have been suggested as central regulators of differentiation fate decision. However, the detailed knowledge of these molecular mechanisms in human ASCs remains limited. This study examined the significance of focal adhesion kinase (FAK), Rho-Rho-associated protein kinase (Rho-ROCK), and their downstream target extracellular signal-regulated kinase 1/2 (ERK1/2) on hASCs differentiation towards osteoblasts and adipocytes. Analyses of osteogenic markers RUNX2A, alkaline phosphatase, and matrix mineralization revealed an essential role of active FAK, ROCK, and ERK1/2 signaling for the osteogenesis of hASCs. Inhibition of these kinases with specific small molecule inhibitors diminished osteogenesis, while inhibition of FAK and ROCK activity led to elevation of adipogenic marker genes AP2 and LEP and lipid accumulation implicating adipogenesis. This denotes to a switch-like function of FAK and ROCK signaling in the osteogenic and adipogenic fates of hASCs. On the contrary, inhibition of ERK1/2 kinase activity deceased adipogenic differentiation, indicating that activation of ERK signaling is required for both adipogenic and osteogenic potential.

Our findings highlight the reciprocal role of cell adhesion mechanisms and actin dynamics in regulation of hASC lineage commitment. This study enhances the knowledge of molecular mechanisms dictating hASC differentiation and thus opens possibilities for more efficient control of hASC differentiation.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that give rise to osteoblasts, adipocytes, and chondrocytes in vitro. MSCs can be harvested from multiple adult tissues, for example, bone marrow, adipose tissue, dental tissues, and umbilical cord [1]. MSCs derived from fat tissue, adipose-derived stromal/stem cells (ASCs), are increasingly used in regenerative medicine due to their desirable immunomodulatory properties and ease of harvest [1]. Regulation of MSC differentiation has been extensively studied, but the research has been mainly conducted with the bone marrow mesenchymal stem cells (BMSC) of human or rodent origin. Although the central transcription factors and signaling pathways are conserved between cell types and species, the extrapolation of these previous results to...
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1. Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that give rise to osteoblasts, adipocytes, and chondrocytes in vitro. MSCs can be harvested from multiple adult tissues, for example, bone marrow, adipose tissue, dental tissues, and umbilical cord [1]. MSCs derived from fat tissue, adipose-derived stromal/stem cells (ASCs), are increasingly used in regenerative medicine due to their desirable immunomodulatory properties and ease of harvest [1]. Regulation of MSC differentiation has been extensively studied, but the research has been mainly conducted with the bone marrow mesenchymal stem cells (BMSC) of human or rodent origin. Although the central transcription factors and signaling pathways are conserved between cell types and species, the extrapolation of these previous results to
human ASCs cannot be made without reservation. It has been discovered that MSCs of different species are not fully comparable regarding their differentiation potential [2, 3] or immunosuppressive capacity [4]. Additionally, the differentiation potential of MSCs has been shown to vary even within species depending on the harvest site [5]. Thus, there is a need for in vitro studies elucidating the molecular mechanisms regulating differentiation potential specifically in human ASCs.

Self-renewal and differentiation of mesenchymal stem cells are tightly regulated by signals from the surrounding environment. Especially signals that regulate cell adhesion and cytoskeletal arrangements have been suggested to be important regulators of MSC differentiation [6]. Cells grow and function in association with extracellular matrix (ECM) components and respond to a wide range of external signals by converting their morphology, behavior, and fate decision accordingly [7–9]. One of the most important response mechanisms is based on the function of transmembrane adhesion receptors of the integrin family and integrin-based focal adhesion (FA) complexes. FAs work in the regulation of cytoskeletal networking and cellular signaling through a central mediator, focal adhesion kinase (FAK) [10]. FAK signaling functions through autophosphorylation of tyrosine 397 that induces interaction of FAK with Src, a nonreceptor tyrosine kinase that stabilizes as a response of this interaction and further phosphorylates other tyrosines of FAK. This leads to full activity of both kinases and subsequent activation of numerous intracellular pathways [11]. In mesenchymal stem cells (MSCs), FAK signaling is interconnected with various pathways including mitogen-activated kinases (MAPKs) and Rho-family GTPases RhoA, Rac, and Cdc42 [12].

The regulation of the cell cytoskeleton and morphology is primarily controlled by the RhoA-ROCK pathway [13], which sustains the integrity of the cytoskeleton by stimulating actomyosin contractility [14, 15]. ROCK isoforms are protein serine/threonine kinases that phosphorylate substrates such as myosin light chain (MLC) phosphatase to drive the assembly of the actin cytoskeleton [13]. The RhoA-ROCK signaling is also an important regulator of stem cell commitment [7, 16–18], and the cell shape determined by RhoA function has been proposed to be a major switch between adipogenic and osteogenic differentiation of human MSCs (hMSCs) [7]. In addition, ROCK signaling is related to the substrate stiffness-driven lineage commitment of MSCs through mechanosensing of the microenvironment via interplay with integrin-FAK signaling [19].

MAPK pathway component extracellular signal-regulated kinase 1/2 (ERK1/2) is linked to vital cellular functions such as proliferation, survival, apoptosis, motility, transcription, metabolism, and differentiation [20]. ERK1/2 has been shown to be a downstream effector of FAK-mediated signaling in MSCs [18, 21]. It has also been suggested as a mechanosensing protein, regulated by the RhoA-ROCK-mediated actin dynamics in hMSCs [22–24]. ERK1/2 activity is linked to the expression of osteogenic markers in hASCs [25]. However, the role of ERK signaling in the adipogenic differentiation fate differs depending on the experimental design and the cell type studied [25–28].

In previous studies, the cellular mechanisms of adhesion and cytoskeletal arrangements have been studied in multiple cell types and varying experimental conditions and configurations. In this study, our objective was to clarify the role of these mechanisms in the differentiation fate decision of adipose tissue-derived stem cells. The current study carefully analyzed the significance of FAK, ROCK, and ERK1/2 proteins in the adipogenic and osteogenic differentiation of hASCs. The key results demonstrated the reciprocal regulation of FAK and ROCK signaling in the interface of hASC osteogenesis and adipogenesis. Our results also consistently indicated that in hASCs, ERK1/2 activity is required for the full osteogenic and adipogenic potential. As a conclusion, our results suggested that ERK1/2 activation together with cell adhesion and actin regulation by FAK-RhoA-ROCK signaling are fine-tuning regulators of hASC fate decision. This investigation enhanced the understanding of the signaling mechanisms governing stem cell commitment and gave insight for future development of in vitro models, tissue engineering constructs, and stem cell therapies.

2. Materials and Methods

2.1. Cell Isolation and Culture. The study was carried out in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (ethical approval R15161). The hASCs were isolated from adipose tissue samples of six female donors (age, 44 ± 11 years, donor information in Table S1) with a written informed consent of the donors. Isolation of the stem cells was performed as described previously [29]. The isolated hASCs were maintained and expanded in human serum containing basic culture medium (BM) (composition in Table 1) and passaged after reaching 70–80% confluence.

2.2. Flow Cytometric Analysis of Surface Marker Expression. The cells were identified as MSCs by flow cytometry (FACSArria; BD Biosciences, Erembodegem, Belgium) at passage 1 to confirm the MSC immunophenotype of the cells. Cells were single stained using monoclonal antibodies against CD3-PE, CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD54-FITC, CD73-PE, CD90-APC (BD Biosciences, Franklin Lakes, NJ, USA), CD11a-APC, CD80-PE, CD86-PE, CD105-PE (R&D Systems, Minneapolis, MN, USA), CD34-APC, and HLA-DR-PE (ImmunoTools, Friesoythe, Germany). The FACS analysis was performed on 10,000 cells per sample and positive expression was defined as fluorescence level greater than 99% of the comparable unstained cell sample.

2.3. Osteogenic and Adipogenic Differentiation Cultures. Human ASCs were seeded into CellBIND polyurethane plates (Corning Inc., Corning, NY, USA) in BM prior to the experiments. Osteogenic and adipogenic inductions were initiated on the following day by introducing the osteogenic medium (OM) and adipogenic medium (AM) to the cells (compositions in Table 1). 0.25 mM IBMX (3-isobutyl-1-
methylxanthine; Sigma-Aldrich, Saint Louis, MO, USA) was added to the adipogenic differentiation cultures upon first change of culture media. 5 nM Dexamethasone (DEX; Sigma-Aldrich) was applied to OM when used. Fresh differentiation media were applied to the cells twice a week during the experiments. As a control, the hASCs were cultured in BM condition. The experiments were conducted at passages 3–5.

2.4. Small Molecule Inhibitors. BM, OM, and AM were supplemented with small molecule inhibitors targeted to FAK, ROCK, and ERK1/2 proteins and added to the cell cultures FAK and ROCK signaling were inhibited using PF-562271 (Selleck Chemicals, Houston, Texas, USA) and Y-27632 (Selleck Chemicals), respectively. Inhibition of ERK1/2 activation was conducted with PD98059 (Calbiochem/EMD Millipore, Billerica, Massachusetts, USA) which is a specific inhibitor of ERK1/2 upstream kinase mitogen-activated protein kinase 1 (MEK1). BM, OM, and AM conditions without the inhibitors were used as controls. Fresh media supplemented with the inhibitors were applied to the cells twice a week during the experiments.

2.5. Live/Dead Staining. The viability of the hASCs seeded 260 cells/cm² in 24-well plate and cultured 7 days in BM, OM, or AM and left untreated (control) or treated with FAK inhibitor PF-562271, ROCK inhibitor Y-27632, or MEK/ERK inhibitor PD98059 was studied with LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes; Thermo Fisher Scientific). The viable cells (green fluorescence) and dead cells (red fluorescence) were imaged using an Olympus microscope (IX51, Olympus) equipped with a fluorescence unit and camera (DP30BW, Olympus) with 4x magnification.

2.6. Fluorescence Staining of the Actin Cytoskeleton. The hASCs were cultured 7 days in BM, OM, or AM and left untreated (control) or treated with 2 μM FAK inhibitor PF-562271, 15 μM ROCK inhibitor Y-27632, and 30 μM MEK/ERK inhibitor PD98059. The cells were fixed and permeabilized with 4% PFA (Sigma-Aldrich) supplemented with 0.1% Triton X-100 for 15 min at RT. Blocking was done with 1% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at +4°C. For actin staining, the cells were incubated in tetramethyl-rhodamine B isothiocyanate- (TRITC-) conjugated phalloidin (P1951; Sigma-Aldrich) for 45 min at RT followed by 4',6-diamino-2-phenylindole (DAPI, Sigma-Aldrich) staining to visualize the nuclei.

2.7. Cell Proliferation and Quantitative Analysis of Alkaline Phosphatase Activity. Cell proliferation of control and inhibitor-treated hASCs (seeded 260 cells/cm² in 24-well plate) was assessed with CyQUANT cell proliferation assay (Molecular Probes; Thermo Fisher Scientific, Waltham, MA, USA) after 7 and 14 days of culture as described previously [29, 30]. The activity of alkaline phosphatase (ALP) was analyzed from the same cell lysates as cell proliferation as described previously [29].

2.8. Alizarin Red Staining and Quantification of Mineralization. The cells (seeded 260 cells/cm² and cultured with control and inhibitor conditions) were stained with Alizarin Red (AR) after 14 and 21 days of culture for the analysis of mineralization. The staining was done as described previously [31]. Briefly, the cells were fixed with 70% ethanol, stained with 2% Alizarin Red S (pH 4.1–4.3; Sigma-Aldrich), and photographed after three washes with water and one with ethanol. Quantitative results were obtained by extracting the dye with 100 mM cetylpyridinium chloride (Sigma-Aldrich) for 3 hours and measuring the absorbances of the samples at 544 nm.

2.9. Oil Red O Staining. hASCs (seeded 260 cells/cm² in 24-well plate) were cultured in BM, OM, and AM supplemented with inhibitor molecules for 21 days and stained with Oil Red O (ORO) staining, which indicates lipid droplet formation, as described previously [29]. Following ORO stain, the hASCs were counterstained with DAPI (Sigma-Aldrich; dilution 1:2000) for 5 minutes before the last washing steps. Fluorescence microscope images were taken with a

<table>
<thead>
<tr>
<th>Component</th>
<th>BM</th>
<th>OM</th>
<th>AM</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Medium/Ham’s Nutrient Mixture F-12 (DMEM/F-12)</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>Thermo Fisher Scientific, Waltham, MA, USA</td>
</tr>
<tr>
<td>Glutamax</td>
<td>—</td>
<td>—</td>
<td>100 nM</td>
<td>PAA Laboratories GmbH, Pasching, Austria</td>
</tr>
<tr>
<td>Insulin</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Lonza, Basel, Switzerland</td>
</tr>
<tr>
<td>Human serum (HS)</td>
<td>5%</td>
<td>5%</td>
<td>5%</td>
<td>Sigma-Aldrich, Saint Louis, MO, USA</td>
</tr>
<tr>
<td>Penicillin/streptomycin</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>L-Ascorbic acid 2-phosphate</td>
<td>—</td>
<td>200 μM</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>—</td>
<td>10 nM</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (DEX)</td>
<td>—</td>
<td>5 nM</td>
<td>1 μM</td>
<td></td>
</tr>
<tr>
<td>Pantothenate</td>
<td>—</td>
<td>—</td>
<td>17 μM</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>—</td>
<td>—</td>
<td>33 μM</td>
<td></td>
</tr>
<tr>
<td>3-Isobutyl-1-methoxyxanthine (IBMX)</td>
<td>—</td>
<td>—</td>
<td>0.25 M</td>
<td></td>
</tr>
</tbody>
</table>
Olympus microscope (IX51, Olympus, Tokyo, Japan) equipped with a fluorescence unit and a camera (DP30BW, Olympus).

2.10. Quantification of Lipid Formation. Lipid formation was quantified based on image analysis of samples stained with ORO and DAPI. Image quantification was performed with a custom analysis pipeline designed for CellProfiler (version 2.1.1, 64-bit Windows; http://www.cellprofiler.org [32]). Lipid maturation was assessed by applying a 10 μm diameter threshold for lipid droplet clusters. See Supplemental Materials for a detailed description of the analysis pipeline.

2.11. qRT-PCR. The quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was performed after 7 and 14 days of culture (hASCs seeded 3160 cells/cm² in 6-well plate) as described previously [33]. The expressions of human runt-related transcription factor 2a (RUNX2A), human adipocyte fatty acid-binding protein (FABP4 or AP2), and human leptin (LEP) were normalized with the expression of human acidic ribosomal phosphoprotein p0 (RPLP0). Gene sequences and accession numbers are presented in Table 2.

2.12. Western Blotting and Immunodetection. Human ASCs (seeded 3160 cells/cm² in 6-well plate) were starved for 24 hours in BM, OM, or AM containing 1% human serum before the 7d inhibitor-supplemented culture, which was also conducted in starvation media. Samples lysed with 2X before the 7d inhibitor-supplemented culture, which was also conducted in the BM, OM, or AM containing 1% human serum. Western Blotting and Immunodetection was conducted using GraphPad Prism 5 (La Jolla, CA, USA). Statistical differences between the inhibitor-treated samples and the respective controls were tested using the nonparametric Mann–Whitney test followed by Bonferroni post hoc test. Statistical differences with p < 0.05 were considered significant. Detailed information of the biological and technical replicates used in statistical analysis is given in Table S2.

3. Results

3.1. Characterization of hASCs. Surface marker expression of hASCs was analyzed by flow cytometry. The hASCs were characterized as MSCs due to positive expression of CD73, CD90, and CD105; lack of CD3, CD11, CD14, CD19, CD45, CD80, CD86, and HLA-DR expression; and moderate expression of CD34 and CD54 (Table S3).

3.2. Inhibition of FAK, ROCK, and ERK1/2 Activity Reduces Proliferation of hASCs. Cell proliferation capacity was evaluated in BM, OM, and AM with gradient concentrations of FAK, ROCK, and ERK inhibitors PF-562271, Y-27632, and PD98059, respectively. CyQUANT assay indicated that

Table 2: The primer sequences and accession numbers for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5′-Sequence-3′</th>
<th>Product size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP2</td>
<td>Forward GGTGGTGGAATGCGTCATG Reverse CAACGTCCCTGTCGGTTATGC</td>
<td>71</td>
<td>NM_001442</td>
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<tr>
<td>LEP</td>
<td>Forward ACAATTGTACCAGGATCATGTG Reverse TCCAAACCGGTACCTTTGTGTT</td>
<td>73</td>
<td>NM_000230</td>
</tr>
<tr>
<td>RPLP0</td>
<td>Forward AATCTCCAGGGCCACCATT Reverse CGCTGGCCTCACCACCTTTGT</td>
<td>70</td>
<td>NM_001002</td>
</tr>
<tr>
<td>RUNX2A</td>
<td>Forward CTTTATCGACCGGTGACCCACCAAC Reverse TCCTCCTGGAAGAAGTTGGCA</td>
<td>62</td>
<td>NM_001024630.3</td>
</tr>
</tbody>
</table>

bp: base pair.

Table 3: Primary and secondary antibodies used in Western blot analysis.

<table>
<thead>
<tr>
<th>Antibody type</th>
<th>Antibody</th>
<th>Host species</th>
<th>Dilution</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>Anti-β-actin¹</td>
<td>Mouse</td>
<td>1:2000</td>
<td>RT, 2 h</td>
</tr>
<tr>
<td>Primary</td>
<td>Anti-FAK²</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>+4°C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>Anti-p-FAK²</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>+4°C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>Anti-ERK2¹</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>RT, 2 h</td>
</tr>
<tr>
<td>Primary</td>
<td>Anti-p-ERK1/2²</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>+4°C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>Anti-MLC2¹</td>
<td>Rabbit</td>
<td>1:800</td>
<td>+4°C, overnight</td>
</tr>
<tr>
<td>Secondary</td>
<td>Anti-rabbit IgG²</td>
<td>Goat</td>
<td>1:2000</td>
<td>RT, 1 h</td>
</tr>
<tr>
<td>Secondary</td>
<td>Anti-mouse IgG²</td>
<td>Goat</td>
<td>1:2000</td>
<td>RT, 1 h</td>
</tr>
</tbody>
</table>

¹Santa Cruz Biotechnology, Dallas, Texas, USA. ²Cell Signaling Technology, Danvers, Massachusetts, USA.
the inhibitors have a regulatory function on cell proliferation (Figure 1(a)). The cell numbers were reduced dose dependently in the inhibitor-treated conditions compared to the control conditions. Despite a decrease in the cell number as a response to increased inhibitor concentrations, adherent cells remained viable with a negligible amount of dead cells (Figure 1(b)), as assessed with the LIVE/DEAD method. In addition to the inhibitor function on cell number, the inhibitor treatment also affected the typical fibroblast-like morphology of hASCs. Based on the immunofluorescence staining of actin cytoskeleton (Figure 2), ROCK inhibition caused the most prominent changes to the morphology of the hASCs. Y-27632 treated cells appeared spindle-like in OM and AM media, and the cells in OM had formed a network of star-shaped cells with long extensions.

3.3. FAK, ROCK, and ERK1/2 Functions Are Essential to hASC Osteogenesis. The early osteogenic differentiation potential of hASCs cultured in BM, OM, and AM in the presence or absence of the FAK, ROCK, and ERK inhibitors was assessed by quantitative real-time reverse transcriptase polymerase chain reaction analysis of the bone associated marker gene RUNX2A (Figure 3(a)) and by quantitative activity assay of ALP (Figure 3(b)) which is an early marker of osteogenesis [35]. At 7 days of culture, RUNX2A expression was markedly upregulated in the OM condition but downregulated by the addition of all studied inhibitors. However, statistical analyses were not done due to the low sample number. The enzymatic activity of ALP was the most prominent in the OM control medium after two weeks of culture, and addition of the inhibitors reduced the enzymatic activity dose dependently. ALP activation was markedly lower in BM and AM conditions, yet a similar trend in the inhibitor effect was seen.

Deposition of calcium phosphate mineral is characteristic to the maturation of osteoblasts and hence, late osteogenic differentiation capacity was studied by Alizarin Red staining protocol after 14 and 21 days of culture. Strong staining for
mineralization of the ECM occurred in the OM control medium at 21 d, as indicated by the quantitative analysis and the corresponding red-stained samples (Figure 3(c)). Mineral accumulation was significantly weakened in the inhibitor-supplemented OM conditions. BM and AM, lacking the osteogenic agents, were not able to support matrix mineralization. Although the inhibitors caused statistically significant reduction of mineralization in BM and AM conditions, the absolute values in the control conditions were too low to have any relevance for the mineralization. 2-week culture period was too short for mineral formation in the studied conditions.

3.4. Inhibition of FAK and ROCK Enhance Adipogenic Outcome of the hASCs. Adipogenic differentiation was analyzed in terms of the expression profiles of adipogenic marker genes AP2 [36] and LEP [37] (Figure 4). As expected, the expression of adipogenic marker genes was most elevated in the hASCs cultured in AM. Based on our results, FAK inhibition increased the expression of AP2 at both time points in all culture conditions. FAK inhibition also upregulated LEP in OM condition at both time points, but in AM, LEP expression was only induced at day 14. Inhibition of the Rho-ROCK signaling using Y-27632 led to enhanced AP2 expression but had an opposite downregulating effect on LEP in AM condition. ERK inhibition, on the other hand, augmented AP2 expression at 7 d but downregulated AP2 on 14 d. Moreover, ERK inhibition also repressed LEP expression at both time points in AM condition suggesting that inhibition of ERK predominantly had a repressing effect on these adipogenic marker genes. Statistical analyses were not done due to the low sample number.

To further study adipogenic differentiation of the hASCs, accumulation of lipid droplets was analyzed after three weeks of culture with a fat-soluble diazol dye Oil Red O (ORO), Figure 5(a) [38]. We also quantified the lipid accumulation by creating and optimizing a CellProfiler pipeline to analyze ORO-stained fluorescence images. We analyzed both total lipid droplet area in the cultures (Figure 5(b)) and the area of lipid droplet clusters exceeding 10 μm diameter limit (Figure S1) to visualize the ongoing adipogenic differentiation and maturation of adipocytes which is distinguished by the increasing number of lipid droplets as well as the enlargement of the individual fat vacuoles [39]. Lipid droplet cluster areas over 10 μm in diameter were further normalized with cell nuclei number to obtain results representative of the single-cell level (Figure 5(b)).

Interestingly, FAK inhibitor treatment significantly increased the proportion of large LDs in OM condition. In AM conditions 0.5 μM FAK inhibitor treatment also elevated lipid formation. However, the quantitative results showed that 2 μM FAK inhibitor led to a reduced area of LDs in AM condition. ROCK inhibition resulted in increased adipogenesis on both culture and single-cell level in OM and AM conditions. ERK inhibition reduced the area of ORO-stained LDs in the culture, also when normalized with the cell number.

3.5. Western Blot Analysis of the Inhibitor Functionality and Cross Talk between Signal Pathways. The functionality of small molecule inhibitors was confirmed by WB analysis of hASCs cultured in starvation media (Figure 6 and Figure S2). The ratio of phosphorylated and unphosphorylated forms of these proteins was analyzed with semiquantification of the band intensities using ImageJ software [40] (Figure 6(b)). Based on the visual inspection and the semiquantified results, the level of the target protein phosphorylation was clearly decreased by the specific inhibitory molecules confirming the inhibitor functionality.

Furthermore, our results pointed out that FAK, ROCK, and ERK inhibitors affected also other studied phosphoproteins and basal protein levels indicating a prospective cross

![Figure 2: Immunofluorescence staining of actin cytoskeleton and nuclei. hASCs were treated with 2 μM FAK, 15 μM ROCK, or 30 μM ERK inhibitors, and the cytoskeleton was stained with phalloidin (red) and nuclei with DAPI (blue) at day 7. Scale bar 100 μm. BM: basic medium; OM: osteogenic medium; AM: adipogenic medium.](Image 121x464 to 391x624)
Figure 3: Osteogenic differentiation of hASCs in BM, OM, and AM culture conditions supplemented with FAK, ERK, and ROCK inhibitors. (a) The cells were cultured in BM, OM, or AM supplemented with 2 μM FAK, 40 μM ERK, or 15 μM ROCK inhibitors in addition to medium controls. RUNX2A expression was analyzed with qRT-PCR at 7 d. FAK and ROCK; N = 5 (independent experiments, 5 donors), ERK; N = 3 (independent experiments, 3 donors). (b) ALP activity was analyzed with ALP assay at 7 d and 14 d. The ALP absorbance values were normalized with corresponding CyQUANT results, and the results are presented relative to the 7 d BM sample. Significance level 5%, designated with an asterisk (*). FAK, ERK, ROCK; N = 9 (independent biological replicates from 3 donors). (c) Matrix mineralization was analyzed with AR staining after 14 d and 21 d of culture. Quantitative results of AR staining are presented as graphs and corresponding representative images of the stained wells (21 d, area 1.9 cm²) are presented below; bright red dye represents mineral. Significance level 5%. FAK, ROCK; N = 18 (independent biological replicates from 6 donors, control condition values of the graphs are the same since the experiments were conducted at the same time), ERK; N = 15 (independent biological replicates from 5 donors). BM: basic medium; OM: osteogenic medium; AM: adipogenic medium; ALP: alkaline phosphatase; AR: Alizarin Red.
talk between signaling pathways. For instance, FAK inhibition had a modest decreasing effect on p-ERK1/2 in OM condition and FAK inhibitor also reduced ROCK downstream target p-MLC2 in BM and AM conditions. ROCK inhibition had a complementary decreasing effect on FAK phosphorylation in OM conditions, and also ERK inhibition decreased p-FAK levels in OM and AM conditions.

4. Discussion

Despite the fact that hASCs are already used in clinical treatments, the knowledge of the regulatory mechanisms of hASC differentiation originates from research done with varying cell types of human and nonhuman origin. Our aim was to carefully analyze the significance of cell adhesion and cytoskeleton in hASC osteogenic and adipogenic differentiation by using small molecular inhibitors for central proteins in cell adhesion and cytoskeletal dynamics.

Previous studies have also noted the importance of FAK signaling in the osteogenic potential of hMSCs [18, 21, 41]. Our results support these findings by showing that the expression of the osteogenic marker gene RUNX2A, the enzymatic activity of ALP, and eventually mineralization were distinctly decreased as a result of FAK inhibition in OM condition. In the presence of higher amounts of inhibitor, the cells failed to deposit virtually any mineral, presumably because of the decreased cell number. Role of FAK in the adipogenic differentiation has been investigated mainly with rodent cells and with diverging experimental setups [42–44]. Li and Xie [42] reported that firm adhesion is required for osteogenesis whereas morphological change accompanied with calpain-mediated cleavage of FAK is essential for preadipocytic differentiation and final maturation of adipocytes. On the contrary, a more recent in vivo study by Luk and coworkers [44] showed that the adipocyte survival was decreased by FAK knockout. We discovered that in human ASCs, inhibition of FAK activity induced expressions of adipogenic marker genes AP2 and LEP in OM and AM conditions. Moreover, the CellProfiler analysis and normalized lipid droplet values revealed that FAK inhibition significantly induced lipid droplet maturation in OM condition, and 0.5 μM concentration had moderately inducing effect in AM as well. However, the total lipid droplet area in the culture was reduced with FAK inhibition, likely due to the decreased number of adherent cells, since the inhibition also affects the cell adhesion sites. These results together with

Figure 4: Expression of adipogenic marker genes AP2 and LEP in hASCs treated with FAK, ERK, and ROCK inhibitors. The hASCs were cultured in BM, OM, or AM supplemented with 2 μM FAK, 40 μM ERK, or 15 μM ROCK inhibitors in addition to medium controls. AP2 and LEP expressions were analyzed with qRT-PCR. The expression of AP2 and LEP are normalized with the expression of the housekeeping gene RPLP0, and the results are presented relative to the 7 d BM sample. FAK and ROCK: N = 5 (independent experiments, 5 donors), ERK: N = 3 (independent experiments, 3 donors). BM: basic medium; OM: osteogenic medium; AM: adipogenic medium.
Previous findings suggest that weakening of the adhesion is needed to guide the differentiation towards adipogenic lineage, whereas too robust disruption in the adhesion affects the survival of the cells. Our results support the role of FAK signaling as a central regulator of the differentiation fate of hASCs.

FAK signaling works in cooperation with Rho-ROCK signaling to regulate cytoskeletal dynamics and cell morphology [18, 19]. We found out that both ROCK and FAK inhibition suppresses phospho-MLC2 suggesting that the actin tension is regulated by FAK-ROCK-MLC signaling cascade. Additionally, ROCK inhibition was shown to reduce phospho-FAK levels. Presumably diminished actin tension by ROCK inhibition affects upstream FA assembly and thus levels of FAK protein activation. These findings indicate a bidirectional regulation between actin assembly and cell adhesion mechanisms in hASCs. In addition to the cooperation of FAK and ROCK signaling, Rho-ROCK pathway itself has been shown to be an important regulator of the balance between osteogenesis and adipogenesis in MSCs [7, 13–15]. In the present study, we found out that the functionality of the ROCK signaling cascade was required in the commitment of hASCs to the osteoblastic lineage since inhibition of ROCK signaling caused a dose-dependent decrease of RUNX2A expression, reduced ALP activity, and hindered the mineral formation. Our results showed that ROCK

![Figure 5](image_url)

**Figure 5:** ORO staining of hASCs and quantification of lipid accumulation from ORO-stained fluorescence images. (a) Representative ORO- and DAPI-stained fluorescence images of FAK, ERK, and ROCK inhibitor-treated hASCs at 21 d. Human ASCs were stained with ORO for intracellular lipid accumulation followed by nuclei staining with DAPI. Fluorescence images were taken with Alexa546 for ORO (red) and DAPI (blue) filters. Scale bars 100 μm. (b) ORO-stained samples of FAK, ERK, and ROCK inhibitor-treated hASCs were imaged with fluorescence microscope using Alexa546 and DAPI filters and analyzed with a custom analysis pipeline designed for CellProfiler. Quantitative ORO graph presents the area of all stained LDs as percentages of the total image area. Normalized ORO graph describes LD formation on the single cell level: the area of LD clusters over 10 μm in diameter is normalized with the corresponding nuclei count. Significance level 5%, designated with an asterisk (*). FAK, ROCK: N = 13 – 16 (images from 2 donors), ERK: N = 19 – 21 (images from 3 donors). BM: basic medium; OM: osteogenic medium; AM: adipogenic medium; ORO: Oil Red O; LD: lipid droplet.
ERK has been suggested to be a mechanosensing protein downstream FAK-Rho-ROCK signaling axis guiding the differentiation fate of hMSCs [23, 24]. In this study, we saw that the inhibition of FAK and ROCK phosphorylation affected phospho-ERK levels suggesting that ERK is regulated in cooperation by the cell adhesion mechanisms and the contractility of the actin cytoskeleton. Interestingly, ERK inhibition also slightly reduced phospho-FAK levels in OM and AM conditions with a currently unknown mechanism. ERK1/2 activity has been linked to the expression of osteogenic markers in hASCs [25] and previous studies have noted that ERK inhibition obstructs osteogenesis in hMSCs [25, 28, 47]. The present study also showed that inhibition of ERK1/2 activation efficiently and dose dependently inhibited the ALP activity and mineral deposition and downregulated Runx2A expression in OM condition. Additionally, ERK1/2 has been suggested to have a regulatory role on MSC adipogenesis, though it has remained contradictory whether the role is activatory or inhibitory [25–28]. ERK1/2 pathway has been suggested to work as a molecular switch between adipogenic and osteogenic differentiation of BMSCs when cultured with osteogenic supplements [28].

![Image](image_url)

**Figure 6**: Intracellular protein activation at day 7 as a response to FAK, ROCK, and ERK inhibition. hASCs were cultured 7 days in BM, OM, and AM media containing 1% human serum supplemented with 15 μM ROCK inhibitor, 2 μM FAK inhibitor, or 30 μM ERK inhibitor. (a) Representative WB results of immunoblotted p-FAK, FAK, β-actin, p-ERK(1/2), ERK 2, p-MLC2, and MLC2. (b) Semiquantified WB results representing the ratio of phosphorylated and basal form of FAK, ERK, and MLC2 proteins. BM: basic medium; OM: osteogenic medium; AM: adipogenic medium.
On the other hand, a more recent study of Xu and coworkers [23] suggested that ERK1/2 is a positive regulator of the hBMSC adipogenesis. Our results consistently indicated that ERK1/2 activity is required for the full osteogenic but also adipogenic potential of hASCs. Inhibition of ERK activity reduced the expression of adipogenic marker genes and lipid accumulation. To our knowledge, this is the first study where ERK inhibition is shown to diminish hASC adipogenesis in both osteogenic and adipogenic culture conditions. Although the proteins investigated in this study are interconnected, the role of ERK in the hASC differentiation was not parallel with the switch-like regulation of FAK and ROCK pathways. However, the mechanism of ERK signaling in adipogenesis needs to be further studied.

5. Conclusions

This study set out to determine the significance of cell adhesion and cytoskeletal modifications regulated by FAK and ROCK signaling and their downstream target EKR1/2 for adipogenic and osteogenic differentiation potential of hASCs. The results show that EKR1/2 pathway plays a crucial positive role in both osteogenic and adipogenic courses of hASC differentiation, whereas FAK and ROCK work as molecular switches since they function as positive regulators of osteogenesis but negative regulators of adipogenesis. The investigation of these signaling proteins at the molecular level also highlights the interesting interconnection of FAK, ROCK, and EKR1/2 signaling in hASCs and implicates the complex interplay between these crucial regulators of differentiation fate. This study confirms the molecular mechanisms of cell adhesion and actin tension in human ASCs and gives us tools to modify and guide the cell proliferation and differentiation in stem cell-based applications and therapies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

The authors thank Ms. Anna-Maija Honkala, Ms. Sari Kallioloski, and Tampere Imaging Facility, BioMediTech and Faculty of Medicine and Life Sciences, University of Tampere for technical assistance. This study was financially supported by the Competitive State Research Financing of the Expert Responsibility area of Tampere University Hospital, Tekes (The Finnish Funding Agency for Innovation), Academy of Finland, and Jane and Aatos Erkko Foundation. Research funding of Doctoral Programme in Biomedicine and Biotechnology, University of Tampere, and Doctoral Programme of Computing and Electrical Engineering, Tampere University of Technology, are gratefully acknowledged.

Supplementary Materials

Figure S1: quantification of lipid droplets over 10 μm in diameter from the ORO-stained fluorescence images. Related to Figure 5(b). Figure S2: intracellular protein activation at day 7 as a response to FAK, ROCK, and ERK inhibition. Related to Figure 6. Table S1: donor information. Information of donor gender, age, and adipose tissue harvest site. Related to Materials and Methods section. Table S2: statistical analysis. Information of donor number, biological replicates, and technical replicates of statistical analyses. Related to Section 2.13. Table S3: surface marker expression. Related to Section 3.1. (Supplementary Materials)

References


Supplemental Materials and Methods

Modules of Image Quantification Pipeline

ColorToGray (input: TIF RGB images, output: OilRedO_orig, DAPI_orig)

The channels of the RGB input images were split into separate grayscale images (pixel size of the images was 0.325 µm). The red channel of the input images was stored as OilRedO_orig. The blue channel of the input images was stored as DAPI_orig.

MaskImage (input: OilRedO_orig, output: OilRedO)

Some of the images contained a scale bar, which was removed by masking all images with a mask that excludes the scale bar. The masking was applied to OilRedO_orig and the resulting images were stored as OilRedO.

Smooth (input: DAPI, output: DAPI_smooth)

Smoothing with a Gaussian filter (typical artifact diameter 15 pixels) was applied to Smooth (input: DAPI, output: DAPI_smooth).

The masking was applied to

Nuclei were segmented from the smoothed DAPI channel using global thresholding based on Otsu’s method [33]. Three-class thresholding based on minimization of weighted variance was used and the middle class was assigned to foreground (i.e. the nuclei). No additional smoothing was performed. A lower bound of 0.25 was selected for the threshold based on experimentation to avoid false positives in images containing very few or no nuclei. The upper bound was kept at 1.0. Clumped nuclei were detected based on the locations of local intensity maxima and they were separated by applying the Watershed method on the intensity image [34]. No additional smoothing was performed and a minimum distance of 15 pixels between the local maxima was used in this step. The local maxima were detected using a lower-resolution image for faster computation. Holes in the identified objects were filled after both thresholding and declumping. Detected nuclei with a diameter under 15 pixels or over 100 pixels were discarded. Nuclei touching the borders of the image were not discarded. The final nuclei objects were stored as Nuclei.

CorrectIlluminationCalculate (input: OilRedO, output: OilRedO_illum)

Background fluorescence and illumination variation in the Oil Red O channel was removed using a polynomial background fit computed separately for each image. The estimated background was stored as OilRedO_illum.

CorrectIlluminationApply (input: OilRedO, illumination function: OilRedO_illum, output: OilRedO_corrected)

The estimated background was subtracted from each image and the background-corrected images was stored as OilRedO_corrected.

ApplyThreshold (input: OilRedO_corrected, output: OilRedO_binary)

Segmentation of lipid areas from the background-corrected Oil Red O images in OilRedO_corrected was performed using global two-class thresholding based on minimization of weighted variance via Otsu’s method [33]. No additional smoothing was used. A lower bound of 0.08 was selected for the threshold based on experimentation to avoid false positive lipid areas in images that contain weak or no Oil Red O staining. The upper bound was kept at 1.0. The resulting binary segmentation was stored as OilRedO_binary.

Morph (input: OilRedO_binary, output: OilRedO_binary_opening)

Exclusion of lipid areas in OilRedO_binary smaller than a given threshold was performed using a binary morphological opening. The opening was performed once with a disk-shaped structuring element, whose diameter was 30.8 pixels (that is 10 µm). The resulting binary images, which only contained lipid areas larger than the given diameter, were stored as OilRedO_binary_opening.

MeasureImageAreaOccupied (input: OilRedO_binary, OilRedO_binary_opening)

The channels of the RGB input images were split into separate grayscale images (pixel size of the images was 0.325 µm). The red channel of the input images was stored as OilRedO_corrected. The blue channel of the input images was stored as DAPI_orig.
The total number of pixels covered by lipid areas of any size \((\text{OilRedO\_binary})\) and lipid areas larger than the given threshold \((\text{OilRedO\_binary\_opening})\) was calculated.

**ExportToSpreadsheet**

Image filenames, the total number of pixels, the number of pixels covered by lipid areas of any size, the number of pixels covered by lipid areas larger than the selected threshold and the number of nuclei in all the analyzed images was exported into a spreadsheet.

**SaveImages (input: OilRedO\_binary)**

The binary images representing the segmented lipid regions were stored in 8-bit integer TIF format to allow visual confirmation of accurate segmentation.

**SUPPLEMENTAL FIGURES**

![Figure S1. Quantification of lipid droplets over 10 µm in diameter from the ORO-stained fluorescence images.](image)

Figure S1. Quantification of lipid droplets over 10 µm in diameter from the ORO-stained fluorescence images. ORO-stained samples of FAK, ROCK and ERK inhibitor-treated hASCs were imaged with fluorescence microscope using Alexa546 and DAPI filters, and analyzed with a custom analysis pipeline designed for CellProfiler (described in main text). Graphs represent the lipid droplet clusters exceeding 10 µm diameter limit as percentages of the total image area. Significance level 5 %. FAK, ROCK: N=13-16 (images from 2 donors), ERK: N=19-21 (images from 3 donors). Abbreviations: LD, lipid droplet; basic medium, BM; osteogenic medium, OM; adipogenic medium, AM.
Figure S2. Intracellular protein activation at day 7 as a response to FAK, ROCK and ERK inhibition. Results of two individual experiments (hASC lines) per each pair of phosphorylated and basal proteins. β-actin is used as a loading control. hASCs were cultured 7 days in BM, OM and AM media containing 1 % human serum supplemented with 15 µM ROCK inhibitor, 2 µM FAK inhibitor or 30 µM ERK inhibitor. Samples were analyzed by Western Blotting and immunodetection of p-FAK, FAK, β-actin, p-ERK(1/2), ERK 2, p-MLC2 and MLC2. Abbreviations: basic medium, BM; osteogenic medium, OM; adipogenic medium, AM.

SUPPLEMENTAL TABLES

Table S1. Donor information.

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<td>55</td>
<td>subcutaneous fat from abdomen</td>
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<td>HFSC 11/13</td>
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<td>subcutaneous fat from thigh/femur</td>
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<td>HFSC 1/14</td>
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Abbreviation: HFSC, human fat stem cell.
Figure S2. Intracellular protein activation at day 7 as a response to FAK, ROCK and ERK inhibition.

Results of two individual experiments (hASC lines) per each pair of phosphorylated and basal proteins. β-actin is used as a loading control.

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<td>HFSC 1/14</td>
<td>Female</td>
<td>33</td>
<td>subcutaneous fat from abdomen</td>
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Abbreviation: HFSC, human fat stem cell.

Table S2. Statistical analysis. Information of donor number, biological replicates and technical replicates of statistical analyses.

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<td>ALP</td>
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<td>ERK: 5</td>
<td>15</td>
<td>45</td>
<td>54</td>
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<tr>
<td>ORO image analysis</td>
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<td>13-16 (images)</td>
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<td>ERK: 3</td>
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<td>19-21 (images)</td>
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Table S3. Surface marker expression.

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<td>phycoerythrin (PE)</td>
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<td>allophycocyanin (APC)</td>
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<td>BD Biosciences</td>
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<td>CD19</td>
<td>B lymphocyte-lineage differentiation antigen</td>
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<td>PECy7</td>
<td>BD Biosciences</td>
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<td>APC</td>
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Hyväri L., Vanhatupa S., Halonen H., Kääriäinen M., Miettinen S.

Stem Cells International, 2020, 8853541

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Research Article

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Previous studies have demonstrated that myocardin-related transcription factor A (MRTF-A) generates a link between the dynamics of the actin cytoskeleton and gene expression with its coregulator, serum response factor (SRF). MRTF-A has also been suggested as a regulator of stem cell differentiation. However, the role of MRTF-A in human mesenchymal stem cell differentiation remains understudied. We aimed to elucidate whether MRTF-A is a potential regulator of human adipose stem cell (hASC) differentiation towards adipogenic and osteogenic lineages. To study the role of MRTF-A activity in the differentiation process, hASCs were cultured in adipogenic and osteogenic media supplemented with inhibitor molecules CCG-1423 or CCG-100602 that have been shown to block the expression of MRTF-A/SRF-activated genes. Our results of image-based quantification of Oil Red O stained lipid droplets and perilipin 1 staining denote that MRTF-A inhibition enhanced the adipogenic differentiation. On the contrary, MRTF-A inhibition led to diminished activity of an early osteogenic marker alkaline phosphatase, and export of extracellular matrix (ECM) proteins collagen type I and osteopontin. Also, quantitative Alizarin Red staining representing ECM mineralization was significantly decreased under MRTF-A inhibition.

Image-based analysis of Phalloidin staining revealed that MRTF-A inhibition reduced the F-actin formation and parallel orientation of the actin filaments. Additionally, MRTF-A inhibition affected the protein amounts of α-smooth muscle actin (α-SMA), myosin light chain (MLC), and phosphorylated MLC suggesting that MRTF-A would regulate differentiation through SRF activity. Our results strongly indicate that MRTF-A is an important regulator of the balance between osteogenesis and adipogenesis of hASCs through its role in mediating the cytoskeletal dynamics. These results provide MRTF-A as a new interesting target for guiding the stem cell differentiation in tissue engineering applications for regenerative medicine.

1. Introduction

The actin cytoskeleton of a cell is continuously modified to allow dynamic cell functions such as stem cell differentiation [1, 2]. Actin dynamics are accomplished by continuous actin turnover and treadmilling regulated by Rho GTPase RhoA-Rho-associated coiled-coil kinase (Rho-ROCK) pathway [3, 4]. RhoA-ROCK pathway has also been reported to regulate the fate decision of mesenchymal stem cells (MSCs) [1, 2, 5]. However, the role of ROCK downstream effector myocardin-related transcription factor A (MRTF-A) in the regulation of MSC differentiation remains less studied.

MRTF-A, also known as megakaryocyte acute leukemia protein (MAL) and megakaryoblastic leukemia (MKL1), belongs to the myocardin family [6, 7] and is found in numerous embryonic and adult tissues [8]. MRTF-A generates a unique link between actin dynamics and gene expression because the activity of MRTF-A is controlled by the balance between...
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Previous studies have demonstrated that myocardin-related transcription factor A (MRTF-A) generates a link between the dynamics of the actin cytoskeleton and gene expression with its coregulator, serum response factor (SRF). MRTF-A has also been suggested as a regulator of stem cell differentiation. However, the role of MRTF-A in human mesenchymal stem cell differentiation remains understudied. We aimed to elucidate whether MRTF-A is a potential regulator of human adipose stem cell (hASC) differentiation towards adipogenic and osteogenic lineages. To study the role of MRTF-A activity in the differentiation process, hASCs were cultured in adipogenic and osteogenic media supplemented with inhibitor molecules CCG-1423 or CCG-100602 that have been shown to block the expression of MRTF-A/SRF-activated genes. Our results of image-based quantification of Oil Red O stained lipid droplets and perilipin 1 staining denote that MRTF-A inhibition enhanced the adipogenic differentiation. On the contrary, MRTF-A inhibition led to diminished activity of an early osteogenic marker alkaline phosphatase, and export of extracellular matrix (ECM) proteins collagen type I and osteopontin. Also, quantitative Alizarin Red staining representing ECM mineralization was significantly decreased under MRTF-A inhibition. Image-based analysis of Phalloidin staining revealed that MRTF-A inhibition reduced the F-actin formation and parallel orientation of the actin filaments. Additionally, MRTF-A inhibition affected the protein amounts of α-smooth muscle actin (α-SMA), myosin light chain (MLC), and phosphorylated MLC suggesting that MRTF-A would regulate differentiation through SRF activity. Our results strongly indicate that MRTF-A is an important regulator of the balance between osteogenesis and adipogenesis of hASCs through its role in mediating the cytoskeletal dynamics. These results provide MRTF-A as a new interesting target for guiding the stem cell differentiation in tissue engineering applications for regenerative medicine.

1. Introduction

The actin cytoskeleton of a cell is continuously modified to allow dynamic cell functions such as stem cell differentiation [1, 2]. Actin dynamics are accomplished by continuous actin turnover and treadmilling regulated by Rho GTPase RhoA-Rho-associated coiled-coil kinase (Rho-ROCK) pathway [3, 4]. RhoA-ROCK pathway has also been reported to regulate the fate decision of mesenchymal stem cells (MSCs) [1, 2, 5]. However, the role of ROCK downstream effector myocardin-related transcription factor A (MRTF-A) in the regulation of MSC differentiation remains less studied. MRTF-A, also known as megakaryocyte acute leukemia protein (MAL) and megakaryoblastic leukemia (MKL1), belongs to the myocardin family [6, 7] and is found in numerous embryonic and adult tissues [8]. MRTF-A generates a unique link between actin dynamics and gene expression because the activity of MRTF-A is controlled by the balance between
monomeric G-actin and the polymerized filamentous F-actin. At low actin polymerization state, MRTF-A is inactive as it is reversibly bound to cytoplasmic or nuclear G-actin through N-terminal REP1 repeats. On the other hand, when actin filament assembly is stimulated by Rho-ROCK activity, MRTF-A is released from its repressive complex with G-actin [7, 9, 10]. In the nucleus, an active MRTF-A works as a transcription coactivator of serum response factor (SRF) to activate transcription of contractile and cytoskeletal genes including alpha-, beta-, and gamma actin, integrin beta1, vinculin; collin 1; talin 1; myosin heavy chains; and myosin light chain 9 [6–8, 11].

Recently, studies have linked MRTF-A to regulation of adipogenesis through its ability to respond to actin dynamics [12]. Adipogenic differentiation towards white adipose tissue (WAT) has been reported to involve cytoskeletal changes towards rounder cell morphology, which in mature adipocytes allows optimal lipid storage [13, 14]. Adipogenesis is regulated through sequential activation of transcription factors, of which peroxisome proliferator-activated receptor gamma (PPARγ) is considered the key adipocyte-specific master switch [13–16]. Interestingly, PPARγ has been found to be one of the targets through which MRTF-A regulates adipogenesis [17, 18]. Nobusue and coworkers reported that depletion of MRTF-A with RNAi method enhanced PPARγ in murine cells [12]. To support this finding, McDonald and coworkers observed that MRTF-A and SRF were down-regulated during adipogenesis of murine embryo fibroblasts, and reciprocally, cells genetically manipulated to express MRTF-A and SRF displayed hindered adipogenesis [19]. Similarly, in a study of Mikkelsen and coworkers, SRF overexpression inhibited adipogenesis, whereas RNAi-mediated knockdown of SRF enhanced adipogenesis in mouse preadipocytes [20]. In addition to regulation of WAT, MRTF-A has been reported to regulate the browning of WAT containing mitochondria and uncoupling protein 1- (UCP1-) rich cells found in brown and beige adipose tissue [17, 19].

Adipogenesis and osteogenesis have been found mutually exclusive in MSC differentiation [2, 5, 15]. Thus, we hypothesized if MRTF-A would as well reciprocally guide the differentiation fate decision. The MSC osteogenesis is controlled by a master regulator runt-related transcription factor 2 (RUNX2), which regulates the osteoblast-specific gene expression of alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN) [15, 21]. The actin cytoskeleton is also modified during osteoblast formation: angular shape, well-defined stress fibers, but also increased actin polymerization have been reported [1, 22, 23]. To date, the existing literature addressing MRTF-A in osteogenic differentiation is sparse. Bian and coworkers implicated the regulatory effect of MRTF-A on osteogenic differentiation potential in rodent MSCs [24]. In their study, MRTF-A knockout mice had inferior bone development compared with wild type, and bone marrow mesenchymal stem cells (BMSCs) isolated from the knockout mice had decreased osteogenesis in vitro [24]. Supporting these findings, also, SRF-deficient mice have been reported to have reduced bone mineral density and bone formation rate [25].

Our study aimed to investigate the role of MRTF-A coregulator in guiding the differentiation commitment of human adipose stem cells (hASCs). Human ASCs are multipotent MSCs with the capacity to give rise to mesenchymal tissues such as fat, bone, and cartilage and have immunomodulatory properties making them suitable to be used in regenerative medicine [26]. Our approach to study the role of MRTF-A in the regulation of differentiation fate was to use two molecular inhibitors described by Evelyn and coworkers targeted to MRTF-A/SRF-mediated gene transcription, a first-generation inhibitor CCG-1423 [27] and a second-generation analog CCG-100602 [28]. CCG-100602 was designed and synthesized to improve the potency and attenuate the cytotoxicity of the lead compound by molecular modifications to the chemical structure of CCG-1423 [28–30]. The molecular target of CCG-1423 has been proposed to be the N-terminal basic domain of MRTF-A which acts as a functional nuclear localization signal (NLS) [31]. In contrast, the biological activity of the related compound CCG-100602 remains unidentified. We cultured hASCs in basic culture medium (BM) and differentiated the cells using adipogenic and osteogenic culture media, AM and OM, respectively, with or without inhibitor supplementation. Adipogenesis and osteogenesis were studied using analyses of early and late markers of differentiation, immunocytochemical staining of both intracellular and extracellular matrix (ECM) proteins, and image-based analysis methods. In addition, the F-actin formation, orientation of actin filaments, and actin-related proteins were studied to evaluate the role of MRTF-A in mediating the cytoskeletal responses during differentiation. To the best of our knowledge, this is the first study elucidating the role of MRTF-A in the regulation of the balance between hASC adipogenic and osteogenic differentiation courses. Since hASCs have been increasingly used in the clinical setting, the detailed understanding of their molecular mechanisms is of great value.

2. Materials and Methods

2.1. hASC Isolation and Culture. The hASCs used in the study were isolated from adipose tissue samples of six female donors aged 40–63 (MD = 51) with their written informed consent in accordance with the Regional Ethics Committee of the Expert Responsibility area of Tampere University Hospital, Tampere, Finland (ethical approval R15161). More detailed donor information is given in Table S1. The isolation protocol has been described previously [32, 33]. Briefly, the adipose tissue was digested mechanically and enzymatically (Collagenase type I; Thermo Fisher Scientific; Waltham, MA, USA), centrifuged, and filtrated to separate the stem cells.

The isolated hASCs were cultured adhering to a Nunclon Delta surface polystyrene culture flask (Thermo Fisher Scientific). Human ASCs were expanded and cultured in basic culture medium, designated as BM, containing 5% human serum (GE Healthcare; Chicago, IL, USA, or BioWest; Nuaillé, France) and 1% antibiotics (Penicillin/Streptomycin; Lonza, Basel, Switzerland) in Minimum Essential Medium α, no nucleosides (MEM α), or Dulbecco’s Modified Eagle
2.2. Flow Cytometry Analysis of Immunophenotype. The hASCs used in the study were characterized by flow cytometry (FACSArray; BD Biosciences, Erembodegem, Belgium) at passage 1 to evaluate their immunophenotype. Human ASCs (10,000 cells/sample) were single stained with monoclonal antibodies: CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD73-PE, CD90-APC (all mentioned antibodies from BD Biosciences, Franklin Lakes, NJ, USA), CD11a-APC, CD105-PE (R&D Systems Inc., Minneapolis, MN, USA), CD34-APC, and HLA-DR-PE (Immunotools GmbH, Friesoythe, Germany). Fluorescence level greater than 99% was considered positive.

2.3. Differentiation Media and Inhibitors. Human ASCs were induced with differentiation culture media and the MRTF-A inhibitors 24 h after plating the cells for experiments. Osteogenic differentiation was accomplished with osteogenic medium (OM) consisting of BM supplemented with 200 μM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate, and 5 mM dexamethasone (DEX) (reagents from Sigma-Aldrich, Saint Louis, MO, USA). For adipogenic differentiation, hASCs were cultured in adipogenic medium (AM) containing BM supplemented with 1 μM DEX, 17 μM pantethenate, 33 μM biotin (from Sigma-Aldrich), and 100 mM insulin (Thermo Fisher Scientific). 0.25 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) was added to AM once at the beginning of differentiation. MRTF-A inhibition was carried out by supplementing BM, OM, and AM with one of two inhibitor molecules targeted to MRTF-A/SRF signaling. We used a first-generation inhibitor CCG-1423 (Selleck Chemicals; Houston, TX, USA) and a second-generation analog CCG-100602 (Cayman Chemical Company; Ann Arbor, MI, USA). BM, OM, and AM without inhibitors were used as controls. Media and inhibitors were changed twice a week during the experiments.

2.4. Cell Viability. The inhibitor effect on the viability of hASCs was analyzed with LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells (Invitrogen™; Thermo Fisher Scientific). The cells were seeded on CellBIND culture plates (Corning) in the density of 280 cells/cm² and cultured 7 days in BM, OM, or AM conditions and the media supplemented with 3 μM, 8 μM, 15 μM, or 30 μM CCG-100602 or 15 μM, 20 μM, 25 μM, or 30 μM CCG-1423. After the 7-day culture, staining was done as described previously [34], but using a 30 min incubation time for the staining solution. Briefly, the cells were washed with Dulbecco’s Phosphate-Buffered Saline (DPBS; Lonza) and stained with a working solution of 0.5 μM calcein-AM and 0.25 μM ethidium homodimer-1 (EthD-1) in DPBS for 30 min at room temperature. After staining, fresh DPBS was changed to the wells, and the viable green-stained (calcein-AM) and dead red-stained (EthD-1) cells were immediately imaged.

2.5. Proliferation and ALP Activity. The proliferation of hASCs was analyzed using CyQUANT™ assay (Invitrogen™; Thermo Fisher Scientific). The hASCs were seeded on CellBIND culture plates (Corning) in the density of 260 cells/cm² and cultured for 7 days or 14 days in BM, OM, or AM conditions supplemented with 15, 20, or 25 μM CCG-1423 inhibitor or 10 or 12 μM CCG-100602 inhibitor. BM, OM, and AM without inhibitors were used as controls. The hASCs were lysed into 0.1% Triton X-100 buffer (Sigma-Aldrich). After a freeze-thaw cycle, the proliferation of hASCs was studied with CyQUANT GR-Dye in lysis buffer, and the fluorescence was measured with a microplate reader at 480/520 nm (Wallac Victor 1420 Multilabel Counter; Perkin Elmer, Waltham, MA, USA).

The activity of ALP, an early marker of osteogenic differentiation, was studied from the same samples as proliferation, but only the samples cultured in BM and OM conditions were studied. The lysed samples were analyzed with a colorimetric assay, as described before [35]. Briefly, the samples were incubated 15 min 37°C in a working solution containing 1:1 10.8 μM phosphate substrate and 1.5 M alkaline buffer solution. After incubation, the reaction was halted using 1.0 M sodium hydroxide (all reagents from Sigma-Aldrich). Absorbances were measured at 405 nm.

2.6. Alizarin Red Staining and Quantification of Mineralization. Late osteogenic differentiation was evaluated by assessing the calcium accumulation with Alizarin Red (AR) staining. The hASCs were seeded 260 cells/cm² on CellBIND plates (Corning) and cultured 21 days in BM or OM conditions with or without CCG-1423 and CCG-100602 inhibitors. At day 21, the cells were fixed with 70% ethanol and stained with 2% Alizarin Red S solution (pH 4.1–4.3; Sigma-Aldrich) for 10 min. After staining, wells were washed with deionized water and 70% ethanol and dried. Samples were photographed with Olympus OM-D E-M5 Mark II camera with M. Zuiko 60 mm macro lens (Olympus; Tokyo, Japan). Quantification of the staining was done by eluting the dye into 100 mM cetylpyridinium chloride (Sigma-Aldrich) for 3.5 h and measuring the absorbances at 544 nm.

2.7. Oil Red O Staining. Adipogenesis was studied by assessing the accumulation of lipid droplets with Oil Red O (ORO) staining. The hASCs were seeded 260 cells/cm² on CellBIND plates (Corning) and cultured for 21 days in BM or AM conditions. The media were supplemented with 15 or 20 μM CCG-1423 or 10 or 12 μM CCG-100602 inhibitors. BM and AM without inhibitors were used as controls. At day 21, the cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich), rinsed with deionized water, and pretreated with 60% isopropanol (2-propanol, Merck, Darmstadt, Germany). The cells were stained with 0.2% ORO staining solution for 15 min, counterstained 5 min with 1:2000 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) in deionized water, and washed several times.
2.8. Immunocytochemical and Phalloidin Staining. The hASCs were seeded 1000 cells/cm² into chambered polymer coverslips (ibiTreat μ-slide 8-well chamber; Ibidi GmbH, Gräfelfing, Germany) and cultured in BM, OM, or AM supplemented with 20 μM CCG-1423 or 12 μM CCG-100602. The culture period was 7 days for Phalloidin staining, 14 days for immunocytochemical staining (ICC) of collagen type I (COL-1), and 21 days for ICC staining of osteopontin (OPN) and periulin 1 (Plin1). After culture, the hASCs were washed with PBS (Lonza), fixed 15 min with 0.2% triton X-100 in PFA (Sigma-Aldrich), and blocked with 1% Bovine serum albumin (BSA; Sigma-Aldrich) in PBS. The samples were incubated with primary antibodies, washed, and incubated with secondary antibodies supplemented with Phalloidin-TRITC. After washes, the samples were counterstained with DAPI. Detailed reagent information is given in Table 1. Negative controls are presented in Figure S2.

2.9. Fluorescence Imaging. LIVE/DEAD-, ORO-, ICC-, and Phalloidin-stained hASC samples were imaged under an inverted microscope Olympus IX51 (Olympus) equipped with fluorescence unit and camera (DP30BW). Fluorescence images were taken using Alexa 488, Alexa 546, and DAPI filters, and 4, 10, 20, or 40x objectives. The ORO-, ICC- (COL-1, OPN, and Plin1), and Phalloidin-stained samples for quantification of mean intensity were imaged keeping the exposure times constant between the samples of different culture conditions. On the contrary, the LIVE/DEAD- and Phalloidin-stained samples for image-based analysis of actin orientation were imaged by adjusting optimal exposure times for each image to ensure visibility of the cells and the actin filaments, respectively. The image processing was done with Adobe Photoshop CC (Adobe; San Jose, CA, USA) or Fiji [36].

2.10. Image-Based Quantification of Lipid Droplet Area. Lipid droplet formation was quantified based on image analysis of ORO-stained samples with a custom analysis pipeline designed for CellProfiler (version 2.0.0, 64-bit Windows) [37]. The same protocol was used as described previously [5], but 0.1 and 1.0 were set as lower and upper bounds for lipid area segmentation, and 15 pixels was set as a threshold for exclusion of lipid droplet areas smaller than 5 μm in diameter.

2.11. Image-Based Analysis of F-Actin Intensity and Orientation. In an effort to analyze the formation of actin filaments, the Phalloidin- and DAPI-stained 40x fluorescence images of hASCs were analyzed with Fiji [36]. Briefly, the mean intensity of Phalloidin in each condition was measured, and the nuclei count of corresponding images was analyzed for normalization of the intensities.

For the analysis of actin orientation, 20x fluorescence images of Phalloidin-stained hASCs were analyzed with CytoSpectre 1.2 spectral analysis tool [38]. In brief, the images were analyzed with default settings, but specifying image magnification (20x) and camera pixel size (6.45 μm). The software was used to calculate the circular variance describing the isotropy of orientation distribution in the image field. Circular variance is bounded in the interval [0, 1], where a value closer to zero signifies distribution along the same direction (anisotropy), and a value closer to one designates spread distribution (isotropy) [38, 39].

2.12. Western Blotting and Immunodetection. Western blotting was performed to detect the protein expression of inhibitor-treated hASCs. The cells were seeded 5000 cells/cm² in CellBIND 6-well plate (Corning) and cultured 7 days in BM, OM, or AM supplemented with 20 μM CCG-1423 or 12 μM CCG-100602. Samples were washed with PBS (Lonza), lysed with 2x LAEMMLI sample buffer (Bio-Rad; Hercules, CA, USA), and separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis. After separation, the proteins were transferred onto 0.2 μm polyvinylidene fluoride (PVDF) membrane using Trans-Blot Turbo Ready-to-assemble transfer Kit (Bio-Rad). The PVDF membranes were blocked with 5% nonfat milk powder in Tris-buffered saline (TBS) supplemented with 0.05% Tween 20 (Sigma-Aldrich). Membranes were incubated with primary antibodies followed by washing steps (0.5%, 0.1%, and 0.05% Tween 20 in TBS) and secondary antibody incubation (antibodies, dilutions, and incubation times are presented in Table 2). After similar washing steps, proteins of interest were detected with chemiluminescence (ECL Prime Western Blotting Detection Reagent; GE Healthcare, Little Chalfont, UK), and the membranes were imaged with Chemi Doc MP System (Bio-Rad). Semiquantitative analysis of immunoblotted protein amounts was performed with Image J [40] to show the protein levels of MRTF-A, a-SMA, pMLC, and HLA-DR normalized with β-actin representing the cell amount in different culture conditions.

2.13. Statistical Analysis. Statistical significances were analyzed separately within each culture media (BM, OM, or AM) by comparing the inhibited conditions to the control condition without the inhibitors. MRTF-A inhibitor effects on cell proliferation (CyQUANT, 4 donors), ALP activity (4 donors), quantitative Alizarin Red staining (3 donors), and image-based analysis of actin (2 donors) and ORO (4 donors) were evaluated. All quantitative results are presented as mean and standard deviation (SD). Nonparametric Kruskal-Wallis and Mann-Whitney tests were performed using GraphPad Prism version 5.02 (GraphPad Software; La Jolla, CA, USA), followed by Bonferroni post hoc test. N values represent the number of parallel samples or images analyzed. The differences with p ≤ 0.05 were considered significant.

3. Results

3.1. hASC Characterization. The cells were identified as mesenchymal based on their plastic adherence, differentiation potential towards adipogenic and osteogenic lineages, and the surface marker expression pattern conveying the criteria given by the International Society for Cellular Therapy [41]. The expression of CD73, CD90, and CD105 was positive (≥95% cells), and there was a lack of expression of CD11a, CD14, CD19, CD45, and HLA-DR (≤2% positive cells).
The expression of hematopoietic marker CD34 was the modest, but previous studies have linked this elevation to the early passage of ASCs that declines when the cells are passaged [14, 42, 43]. Detailed surface marker expressions, their standard deviations, and fluorophore information are given in Table S2.

3.2. Viability and Proliferation. We first examined the role of MRTF-A in hASC adhesion and viability by using two inhibitor molecules (CCG-1423 and CCG-100602) in BM, OM, and AM cultures. A selection of concentrations of both inhibitors was used to find optimal concentrations for cell viability. Based on the representative LIVE/DEAD-stained images of adherent hASCs in Figure 1(a), there were viable green-stained cells, but no dead cells, in all culture conditions. The number of adherent cells decreased as a response to increasing inhibitor amount. With CCG-100602, the effect was also dependent on the culture media because the OM condition supported the viability over BM and AM conditions.

Cell proliferation, analyzed with CyQUANT assay, aligned with the viability results. Figures 1(b) and 1(c) show that the inhibitors had a dose-dependent effect on the cellular nucleic acid bound fluorescent GR-Dye denoting cell number. The inhibitor effect accumulated over time, and more significant reductions in cell amounts were seen after 14 days of culture. The hASCs in AM were more sensitive to inhibition, and lower inhibitor concentrations led to significantly reduced cell amount already at day 7.

3.3. Adipogenic Differentiation. We next investigated the significance of MRTF-A activity on the adipogenic potential of hASCs using ORO staining of cytoplasmic neutral lipids after 21 days of culture in BM or AM conditions supplemented with 15 or 20 μM CCG-1423 or 10 or 12 μM CCG-100602. The representative ORO- and DAPI-stained fluorescence images are shown in Figure 2(a). The lipid droplet accumulation was also quantified by analyzing the total ORO-stained area, as well as the area of lipid droplet clusters exceeding 5 μm in diameter to demonstrate the adipogenic maturation (Figure 2(b)). BM condition contained small red-stained lipid droplets, and both inhibitors significantly increased the size of the individual droplets. Noticeable adipogenic differentiation with large matured droplets was obtained in the AM condition, but the enlarged, clustered lipid droplets were quite scarce in AM control. MRTF-A inhibition significantly enhanced both the total area of lipid droplets and the proportion of large droplets in AM condition. However, despite their abundance, the MRTF-A inhibitor-induced lipid droplets were not as clustered as in AM control but rather more separated. Interestingly, 15 μM CCG-1423 and 10 μM CCG-100602 were found more optimal concentrations in supporting the hASC maturation. Adipogenic differentiation was further assessed with ICC staining of adipogenic marker Plin1 (Figure 2(c)). The inhibitor treatment enhanced Plin1 production of the hASCs in BM condition. Plin1 staining was stronger in AM conditions, and the staining intensity was relatively the same in control AM and with inhibition. The zoom images revealed large lipid droplets covered with Plin1 in the AM supplemented with MRTF-A inhibitors. However, these structures were absent in the AM control condition. In addition, quantitative real-time PCR (qRT-PCR) analysis was done to study the effect of MRTF-A inhibition on a genetic level using one donor cell line (Table S3 and Figure S1). We found that the
The osteogenesis-related formation of secreted ECM proteins was analyzed by COL-1 staining at day 14. In BM, hASCs produced OPN dependent on culture conditions. Interestingly, MRTF-A inhibition predominantly produced and secreting OPN to the ECM unlike the other conditions, as hypothesized, and the polystyrene control for AR staining was clear (data not shown). In OM control conditions, AR staining was low in BM conditions, after which LIVE/DEAD analysis was performed. Green dye represents living cells (Alexa 488 fluorescence images of LIVE/DEAD-stained sample). In BM, hASCs were cultured 7 d in BM, OM, or AM supplemented with 15, 20, 25, or 30 μm inhibitor (b) or 10 or 12 μm with only 25 μm within the culture period. The ALP activity was elevated in OM condition. However, cells in OM without inhibitors were lyzed by ICC staining of OPN. As displayed in Figure 4(c), the hASCs produced OPN independent of the culture conditions. Interestingly, MRTF-A inhibition constantly in the studied culture conditions, and the inhibitor treatment was lower in OM conditions with MRTF-A inhibitors, noticeably stronger, and the protein was secreted into ECM to form brous structures in the control condition. COL-1 production intensity, the total protein amount of MRTF-A in the synthesis of actin-related proteins was assessed by Western blotting and immunodetection of MRTF-A inhibitors led to enhanced or una influence on viability and proliferation of hASCs. (a) Representative fluorescence images of LIVE/DEAD-stained sample. (b) Figure 1: Continued.
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MRTF-A inhibitors led to enhanced or unaffected human adipocyte fatty acid-binding protein (AP2) expression, but both inhibitors had a downregulating effect on leptin (LEP) in AM condition.

3.4. Osteogenic Differentiation. We evaluated the early osteogenic potential of MRTF-A inhibitor-treated hASCs by measuring the activity of ALP, at day 7 and 14 time points in BM or OM conditions. Figures 3(a) and 3(b) show that the ALP activity remained relatively low at day 7, and its activity rose within the culture period. The ALP activity was elevated in OM control condition at day 14, but decreased dose-dependently with MRTF-A inhibition in both BM and OM conditions. The activity was significantly reduced in OM with only 25 μM CCG-1423, whereas the next-generation inhibitor CCG-100602 reduced ALP significantly with both concentrations. Additionally, the gene expression of an early osteogenic marker gene runt-related transcription factor 2a (RUNX2A) was decreased with CCG-1423 when analyzed with qRT-PCR (Table S3 and Figure S1).

The osteogenesis-related formation of secreted ECM proteins was analyzed by COL-1 staining at day 14. In BM, although the level of staining remained modest, inhibition of MRTF-A slightly increased COL-1 synthesis and processing in the endoplasmic reticulum (ER) and Golgi network (Figure 3(c)). In OM, however, COL-1 staining was considerably stronger, and the protein was secreted into ECM to form fibrous structures in the control condition. COL-1 production was lower in OM conditions with MRTF-A inhibitors, and the protein was sequestrated to the intracellular membranes.

The ability of the hASCs to differentiate further towards osteoblasts was evaluated by AR staining of calcium deposits at day 21 for the assessment of ECM mineralization (Figures 4(a) and 4(b)). AR staining was low in BM conditions, as hypothesized, and the polystyrene control for AR staining was clear (data not shown). In OM control condition, the hASCs showed calcium accumulation as represented by the strong red staining of the samples. Inhibition of MRTF-A signaling by CCG-1423 or CCG100602 resulted in a significant dose-dependent reduction of mineral formation in the OM condition. Late osteogenesis was also analyzed by ICC staining of OPN. As displayed in Figure 4(c), the hASCs produced OPN independent of the culture conditions. Interestingly, MRTF-A inhibition confined OPN in the intracellular space.

3.5. Actin-Related Proteins and Cytoskeleton. The role of MRTF-A in the synthesis of actin-related proteins was assessed by Western blotting and immunodetection of MRTF-A, β-actin, α-SMA, pMLC, and MLC at day 7 (Figure 5 and Figure S3). Based on the band size and intensity, the total protein amount of β-actin was relatively constant in the studied culture conditions, and the inhibitor effect on the protein level of MRTF-A varied in different culture media. However, MRTF-A inhibition predominantly

![Figure 1: Effect of MRTF-A inhibition on viability and proliferation of hASCs. (a) Representative fluorescence images of LIVE/DEAD-stained hASCs. hASCs were cultured 7 d in BM, OM, or AM supplemented with 15, 20, 25, or 30 μM CCG-1423 or 3, 8, 15, or 30 μM CCG-100602 inhibitor, after which LIVE/DEAD analysis was performed. Green dye represents living cells (Alexa 488 filter), and a negligible number of dead cells are stained with red dye (Alexa 546). Scale bar 1.0 mm, same scale in every image. (b, c) Proliferation of hASCs at 7 and 14 d was analyzed with the CyQUANT method. The hASCs were cultured in BM, OM, or AM supplemented with 15, 20, or 25 μM CCG-1423 inhibitor (b) or 10 or 12 μM CCG-100602 inhibitor (c). N = 12, independent biological replicates from 4 donors. 5% significance level was used in the statistical analysis, and the comparisons were made within a culture condition by comparing the inhibitor concentrations with the untreated medium control. BM: basic medium; OM: osteogenic medium; AM: adipogenic medium.](image)
In order to study the cytoskeleton of MRTF-A inhibitor-treated hASCs, the F-actin of the cells was stained with Phalloidin-TRITC (Figure 6(a)). Treatment significantly reduced the mean Phalloidin intensity in every culture condition, and only CCG-1423 reduced the value in BM and AM conditions. The circular variance, which represents the isotropy of the actin filaments, was the most parallel-aligned in BM condition, and a representative image is shown in Figure 6(d).

The hASCs were treated with MRTF-A inhibitors in BM, OM, and AM conditions, and the F-actin of the cells was stained with Phalloidin-TRITC. The inhibitor eled to decreased mean Phalloidin intensity in every culture condition. MRTF-A inhibition led to decreased mean Phalloidin intensity in every culture condition. The circular variance, which represents the isotropy of the actin filaments, was the most parallel-aligned in BM condition, and a representative image is shown in Figure 6(d).

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The circular variance, which represents the isotropy of the actin filaments, was the most parallel-aligned in BM condition, and a representative image is shown in Figure 6(d). The ratio of G-actin and F-actin was significantly decreased in BM and AM conditions compared to OM control conditions. MRTF-A inhibition significantly increased the circular variance, meaning that the actin filaments were disordered and less parallel-aligned compared to control conditions. The circular variance was the most parallel-aligned in BM condition, and a representative image is shown in Figure 6(d).

In order to study the cytoskeleton of MRTF-A inhibitor-treated hASCs, the F-actin of the cells was stained with Phalloidin-TRITC (Figure 6(a)). Treatment significantly reduced the mean Phalloidin intensity in every culture condition. MRTF-A inhibition led to decreased mean Phalloidin intensity in every culture condition. The circular variance, which represents the isotropy of the actin filaments, was the most parallel-aligned in BM condition, and a representative image is shown in Figure 6(d).
decreased the amount of SRF-regulated proteins α-SMA, MLC, and the phosphorylated form pMLC, although the culture media supplements also played a role on protein expression. We found that both inhibitors reduced the α-SMA protein amount in BM and AM conditions, but in OM condition, only CCG-1423 reduced α-SMA. MLC and its phosphorylated form pMLC were induced in OM, and their protein levels were lower in BM and AM conditions.

In order to study the cytoskeleton of MRTF-A inhibitor-treated hASCs in BM, OM, and AM conditions, the F-actin of the cells was stained with Phalloidin-TRITC (Figure 6(a)). The hASCs were fibroblastic and spindle-like in BM and OM control conditions. MRTF-A inhibition forced the cells to adopt more spread morphology with less coherently aligned cytoskeleton. Additionally, the inhibitor treatment led to decreased mean Phalloidin intensity in every culture media (Figure 6(c)). The inhibitor effect was the most prevalent in AM condition where the mean intensity values normalized with nuclei count of corresponding images were significantly reduced (Figure 6(c)). The circular variance, i.e., the isotropy of the actin filament orientations, was the lowest in BM and OM control conditions, meaning that the actin filaments were the most parallel-aligned (Figure 6(d), representative images in Figure S4). Based on quantitation, MRTF-A inhibition significantly increased the circular variance of the actin filaments in BM condition, and a similar trend was found in OM and AM conditions. The adipogenic culture medium itself resulted in less fibroblastic morphology compared with BM and OM.

4. Discussion

The dynamic behavior of the actin cytoskeleton is central in many cellular processes, including stem cell commitment into various differentiation lineages. Importantly, the changes in cell morphology have also been proposed to guide the differentiation. [1, 2] Actin dynamics are accomplished by actin turnover through the reversible polymerization of actin monomers into filaments. The ratio of G-actin and F-actin, in turn, regulates the activity of MRTF-A, which has been suggested as a direct link between the dynamic changes of actin cytoskeleton and regulation of gene activity [7]. Our previous study demonstrated that Rho-ROCK signaling, a central regulator of actin cytoskeleton, plays a switch like role in hASC commitment towards osteogenesis or adipogenesis [5]. This prompted us to question whether ROCK down-stream target MRTF-A would also have a similar regulatory function in the lineage commitment of human ASCs.

The role of MRTF-A and SRF-mediated transcription in regulation of differentiation has been previously studied mostly with genomic methods such as gain and loss of function of the MRTF-A gene in rodents [12, 19, 24]. Our
approach using MRTF-A inhibition with two pharmacological compounds CCG-1423 [27] and its analog CCG-100602 [28, 30] let us carry on a three-week cell cultures, typical for human MSC in vitro differentiation studies, under the influence of the inhibitors. We began by optimizing the functional inhibitor concentrations without cytotoxic effects to hASCs because no prior data was available. Both inhibitors had dose-dependent decreasing effect on cell adhesion and viability, as studied with LIVE/DEAD assay. Based on the CyQUANT analysis of cell proliferation, the inhibitors

![Figure 3: Early osteogenesis of MRTF-A inhibitor-treated hASCs. The hASCs were cultured in BM or OM supplemented with CCG-1423 (a) or CCG-100602 (b) inhibitors in addition to medium controls. ALP activity was analyzed with ALP assay at 7 d and 14 d. The ALP absorbance values were normalized with corresponding CyQUANT results, and the results are presented relative to the 7 d BM sample. N = 12, independent biological replicates from 4 donors. Significance level 5%. (c) Representative images of COL-1 (Alexa 488, green)-, actin (Alexa 546, red)-, and nuclei (DAPI, blue)-stained hASCs at 14 d, after culture with 20 μM CCG-1423 or 12 μM CCG-100602. Images with 10x and 40x magnifications are provided to give an overall view and a more detailed view of intracellular localization of COL-1. Scale bars 100 μm. BM: basic medium; OM: osteogenic medium.](image-url)
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\[
\begin{align*}
\text{Relative ALP activity} & \quad \text{CCG-1423} \\
0 \mu M & \quad 15 \mu M & \quad 20 \mu M & \quad 15 \mu M & \quad 20 \mu M \quad \star \quad \star
\end{align*}
\]

\[
\begin{align*}
\text{Relative ALP activity} & \quad \text{CCG-100602} \\
10 \mu M & \quad 10 \mu M & \quad 12 \mu M & \quad 10 \mu M & \quad 12 \mu M \quad \star \quad \star \quad \star
\end{align*}
\]

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Figure 4: Late osteogenesis of MRTF-A inhibitor-treated hASCs. Matrix mineralization of hASCs was analyzed with AR staining after 21 d of culture with CCG-1423 (a) or CCG-100602 (b) inhibitors. Quantitative results of AR staining are presented as graphs, and corresponding representative images of the stained wells (area 1.9 cm\(^2\)) are displayed below the columns, bright red dye represents mineral. \( N = 9 \), independent biological replicates from 3 donors. Significance level 5%. (c) Representative OPN-stained samples of MTRF-A inhibitor-treated hASCs at 21 d. The cells were imaged with a fluorescence microscope using Alexa 488 for OPN (green), Alexa 546 for actin (red), and DAPI (blue) filters, and 20x magnification. Scale bar 100 \( \mu m \), same scale in every image. BM: basic medium; OM: osteogenic medium.
Figure 5: Intracellular protein levels of MRTF-A, β-actin, α-SMA, pMLC, and MLC as a response to MRTF-A inhibition. hASCs were cultured 7 d in BM, OM, or AM media supplemented with 20 μM CCG-1423 or 12 μM CCG-100602. (a) Representative WB results of immunoblotted MRTF-A, β-actin, α-SMA, pMLC, and MLC. Semiquantitative results of MRTF-A (b), α-SMA (c), pMLC (d), and MLC (e) normalized with β-actin. MRTF-A and α-SMA: N = 2 independent experiments, 2 donors; pMLC and MLC: N = 4 independent experiments, 2 donors. BM: basic medium; OM: osteogenic medium; AM: adipogenic medium.
decreased cell number concentration-dependently, and the cell response was cumulative over time. The inhibitor effect was also dependent on the culture medium.

Previous in vivo and in vitro studies have suggested that MRTF-A and SRF transcription factors have a negative role in regulation of adipogenesis. This means that MRTF-A activity or overexpression is linked to decreased adipogenesis, whereas knockdown or diminished MRTF-A signaling to enhanced adipogenic differentiation fate [12, 18–20, 24]. As expected, we found that inhibition of MRTF-A activity supported the adipogenesis of hASCs. Based on ORO staining of neutral lipids and the quantitative analysis of lipid droplet area, inhibitor treatment stimulated adipogenic commitment, and large lipid droplets were detected throughout the culture area. MRTF-A inhibition also supported the maturation process, characterized with the enlargement and fusion of individual droplets [16]. In AM control with unaffected MRTF-A activity, the lipid droplets formed scarce, unevenly distributed clusters.

We also studied the adipogenesis with ICC staining of Plin1, which is one of the perilipin family proteins of the

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**Figure 6:** F-actin intensity and orientation of MRTF-A inhibitor-treated hASCs. hASCs were cultured 7 d in BM, OM, or AM media supplemented with 20 μM CCG-1423 or 12 μM CCG-100602. (a) Representative images of Phalloidin- and DAPI-stained hASCs imaged with Alexa 546 for actin (red) and DAPI for nuclei (blue) filters using 40x magnification and constant exposure time. Scale bar 100 μm. Image-based analysis done with Fiji of mean Phalloidin intensity (b) and mean intensity normalized with nuclei number (c) of the samples described in (a). N = 18–25 images, 4 independent biological replicates from 2 donors. Significance level 5%. (d) Image-based analysis of actin orientation done with CytoSpectre 1.2 spectral analysis tool. Phalloidin-stained hASCs were imaged with 20x magnification and optimally adjusted exposure times from the same biological replicates as above (representative images in Figure S4). N = 16–27 images. Significance level 5%. BM: basic medium; OM: osteogenic medium; AM: adipogenic medium.
phospholipid monolayer shielding the lipid droplet hydrophobic core [13, 44, 45]. Intense staining of Plin1 was found in all AM conditions, although large droplets with Plin1 on the surface were only detected in MRTF-A inhibitor-treated hASCs. It is possible that some lipid droplets were lost during the staining protocols, because mature adipocytes lose their attachment to the culture platform [14]. In basic culture medium, MRTF-A inhibitor enhanced Plin1 production of hASCs. Furthermore, the lipid droplet size was slightly increased, indicating that the molecular intervention was enough to support adipogenesis even without adipogenic culture supplements. Our results are in coherence with previous findings that Plin1 is one of the PPARγ target genes that is upregulated by MRTF-A depletion in mouse preadipocytes [12].

Recent studies have identified the phenotype of the MRTF-A regulated adipocytes, and their identity was reported to equivalent beige (also called brite) adipocytes [17, 19]. These adipocytes have characteristics of both white and brown adipose tissue; they are multicellular- and mitochondria-rich adipocytes involved in energy dissipation and thermogenic activities [46]. McDonald and coworkers found that the circulating levels of leptin were diminished in the MRTF-A knockout mice compared with the wild type littermates [19]. There is also some evidence that the human adipokine leptin is more associated with white adipose tissue than brown phenotype [47]. Our results of gene expression show that LEP was strongly expressed in the adipogenic control condition and markedly downregulated with MRTF-A inhibition. Additionally, the organization of the maturing lipid droplets was different from the typical fat vacuole clusters forming under adipogenic supplements. Therefore, the phenotype of hASCs with MRTF-A inhibition could be somewhat beige-like. However, the molecular identity of these cells will remain to be determined.

Next, we asked if the enhanced adipogenic differentiation with MRTF-A inhibition was linked to a reciprocal reduction in the osteogenic potential of hASCs. Our goal was to carefully study whether early osteogenesis, ECM production, and matrix mineralization were regulated by MRTF-A. Unlike adipogenesis, the relation of MRTF-A and osteogenesis has been demonstrated previously only in one study to our knowledge. Bian and coworkers showed in vivo and in vitro that the bone development and expression of osteogenic markers, respectively, were negatively affected by the loss of MRTF-A function in mice [24]. SRF knockout has been also reported to decrease the activity of early osteogenic markers RUNX2 and ALP [25]. Likewise, based on our results, MRTF-A inhibition significantly reduced the ALP protein activity stimulated by osteogenic culture condition in hASCs, and RUNX2A gene expression was hindered with CCG-1423.

Production and secretion of extracellular proteins and mineralization of ECM are an important part of skeletal development, bone remodeling, and homeostasis [48]. Therefore, we stained two constituents of the organic phase of ECM: COL-1, which provides the elasticity and flexibility to bone [48] and OPN, a regulator of matrix remodeling and tissue calcification [49]. Bian and coworkers found that MRTF-A knockout mice had lower protein expression of COL-1 and OPN [24]. As presumed, we discovered that COL-1 and OPN synthesis and secretion were enhanced by hASCs in OM condition. However, our results revealed an interesting phenomenon that these proteins were sequestered in intracellular space with MRTF-A inhibition. To study the role of MRTF-A in osteogenic maturation, we examined ECM mineral accumulation with qualitative and quantitative AR analyses. Osteogenic media-induced mineralization was significantly and concentration-dependently reduced with both MRTF-A inhibitors. The decreased ECM mineralization has also been previously reported with MRTF-A or SRF knockout murine cells [24, 25]. These results together denote that the activity of MRTF-A is important in the different stages of the osteogenic commitment of hASCs.

Finally, the actin cytoskeleton and synthesis of actin-related proteins were studied to elucidate the role of MRTF-A in regulating the differentiation fate decision of hASCs by mediating the actin dynamics. Dramatic cytoskeletal changes have been reported to occur early in the differentiation process of the fibroblastic and spindle-like mesenchymal precursor cells into mesenchymal lineages to drive the formation of specialized tissues [1, 2, 14, 50]. Nobusue and coworkers proposed that adipogenesis would require disruption of actin stress fibers and subsequent formation of MRTF-A and G-actin complexes [12]. We discovered that MRTF-A inhibition significantly decreased the coherency of actin orientation of hASCs in BM condition, and a similar trend was seen in the intensity of Phalloidin staining representing F-actin. MRTF-A inhibition also reduced the synthesis of α-SMA and MLC indicating to SRF-dependent regulation. Importantly, these cytoskeletal changes were related to the observed moderate enhancement of adipogenesis in BM condition. The adipogenic culture supplements and MRTF-A inhibition caused the hASCs to adopt more spread morphology with reduced parallel alignment of the actin filaments. Furthermore, the observed significant reduction in actin polymerization but relatively unaffected cellular protein level of β-actin may suggest that MRTF-A inhibition altered the ratio between G-actin and F-actin. Similar inhibitory effect on F-actin was also demonstrated recently in human intestinal myofibroblasts with CCG-100602 [51]. During osteogenesis, the hASC morphology was relatively spindle-like with predominant parallel actin filaments traversing the entire length of the cells. MRTF-A inhibition decreased the F-actin formation, the parallel-alignment of actin filaments, and the expression of SRF-regulated MLC and pMLC in OM condition. When activated, pMLC is involved in the formation of actomyosin complex contributing to the enhanced intracellular tension, linked to osteogenic course [1, 22]. Thus, the inhibitor-mediated changes in the cytoskeletal and protein level were linked to the suppressed osteogenic outcome. These results signify that MRTF-A regulates the differentiation fate of hASCs together with the biochemical cues by coupling the actin dynamics and target gene expression.

5. Conclusions

In summary, we have provided evidence that MRTF-A transcription cofactor is an important regulator of the inverse balance between adipogenesis and osteogenesis of hASCs.
Our results showing that MRTF-A inhibitors enhance the lipid droplet formation and maturation indicate that MRTF-A is a negative regulator of adipogenesis. Reciprocally, our novel findings of reduced osteogenesis as a response to MRTF-A inhibition highlight the necessity of MRTF-A activity on the osteogenic outcome of hASCs in vitro. MRTF-A translates the cytoskeletal changes to gene transcription via SRF and provides an essential temporarily coupled regulatory signaling node in stem cell differentiation. This study adds to the knowledge on the regulation of differentiation lineage commitment in human stem cells and provides further insight into molecular targets for pharmacological intervention to guide the differentiation fate into the desired direction.

Data Availability

The data supporting the results of this study is available upon request from the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors thank Ms. Anna-Maija Honkala, Ms. Sari Kalliokoski, and M.Sc. Kimmo Kartasalo, Tampere University, for their valuable technical assistance. The authors acknowledge the Biocenter Finland and Tampere Imaging Facility for the service. This study was financially supported by the Finnish Cultural Foundation, Ester and Uuno Kokki Fund, Jane and Aatos Erkko Foundation, and the Doctoral Programme in Biomedicine and Biotechnology, Tampere University. This study was partly supported by the Competitive State Research Financing of the Expert Responsibility area of Tampere University Hospital, Business Finland, and the Academy of Finland.

Supplementary Materials

Table S1: hASC donor information. Table S2: hASC characterization by surface marker expression. Table S3: primer sequences and accession numbers for qRT-PCR. Figure S1: relative gene expression of AP2, LEP, and RUNX2A. Figure S2: negative controls of the immunocytochemical staining. Figure S3: Western blot and immunodetection of MRTF-A, β-actin, α-SMA, pMLC, and MLC of a replicate donor cell line. Figure S4: representative images of hASC for image-based analysis of actin orientation. (Supplementary Materials)

References


# Supplementary material

## Supplementary Tables

### Table S1. hASC donor information

<table>
<thead>
<tr>
<th>Donor code</th>
<th>Age</th>
<th>Gender</th>
<th>Harvest site</th>
</tr>
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<tr>
<td>HFSC 1/13</td>
<td>55</td>
<td>Female</td>
<td>subcutaneous fat from the abdomen</td>
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<td>HFSC 11/13</td>
<td>40</td>
<td>Female</td>
<td>subcutaneous fat from the femur</td>
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<tr>
<td>HFSC 4/14</td>
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<td>subcutaneous fat</td>
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<td>HFSC 8/15</td>
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<td>Female</td>
<td>subcutaneous fat from the abdomen</td>
</tr>
<tr>
<td>HFSC 9/15</td>
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<td>subcutaneous fat from the abdomen</td>
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<tr>
<td>HFSC 10/15</td>
<td>60</td>
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<td>subcutaneous fat from the abdomen</td>
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### Table S2. hASC characterization by surface marker expression

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Surface protein</th>
<th>Surface marker expression (%)</th>
<th>SD (%)</th>
<th>Fluorophore</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>CD11a</td>
<td>Integrin alpha L (Lymphocyte function-associated antigen 1)</td>
<td>0.9</td>
<td>0.6</td>
<td>allophycocyanin (APC)</td>
<td>R&amp;D Systems Inc. Minneapolis. MN. USA</td>
</tr>
<tr>
<td>CD14</td>
<td>Lipopolysaccharide receptor</td>
<td>0.8</td>
<td>0.4</td>
<td>phycoerythrin-cyanine (PECy7)</td>
<td>BD Biosciences. Franklin Lakes. NJ. USA</td>
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<tr>
<td>CD19</td>
<td>B lymphocyte-lineage differentiation antigen</td>
<td>0.6</td>
<td>0.3</td>
<td>PECy7</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD34</td>
<td>RO isoform of leucocyte common antigen</td>
<td>1.9</td>
<td>1.2</td>
<td>APC</td>
<td>Immunotools GmbH. Friesoythe. Germany</td>
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<tr>
<td>CD73</td>
<td>Ecto-5'-nucleotidase</td>
<td>94.9</td>
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<td>phycoerythrin (PE)</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD90</td>
<td>Thy-1 (T cell surface glycoproteins)</td>
<td>99.1</td>
<td>0.8</td>
<td>APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD105</td>
<td>SH-2. Endoglin</td>
<td>97.1</td>
<td>5.1</td>
<td>PE</td>
<td>R&amp;D Systems Inc.</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Major histocompatibility class II antigen (MHC-II)</td>
<td>0.7</td>
<td>0.5</td>
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</tr>
</tbody>
</table>

Abbreviation: bp, base pair.
<table>
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<tr>
<th>Gene</th>
<th>5’-Sequence-3’</th>
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<td>62</td>
<td>NM_001024630.3</td>
</tr>
</tbody>
</table>

Abbreviation: bp, base pair.
Supplementary Figures

Figure S1. Relative gene expression of AP2, LEP, and RUNX2A. The quantitative real-time reverse transcriptase polymerase chain reaction analysis (qRT-PCR) was performed with one donor cell line after 7 and 14 days of culture in BM, OM or AM supplemented with 25 µM CCG-1423 or 12 µM CCG-100602 inhibitors. The hASC were seeded 3160 cells/cm² in the CellBIND 6-well plate (Corning). The analysis was performed as described previously (Kyllönen et al., 2013). Briefly, the total RNA was purified from hASC samples, reverse transcribed into cDNA and the qRT-PCR reactions were conducted with AbiPrism 7000 Sequence detection system (reagents from Thermo Fisher Scientific). The relative expressions of adipogenic marker genes (a) human adipocyte fatty acid binding protein (AP2, also called as FABP4), (b) human leptin (LEP) and osteogenic marker gene (c) human runt-related transcription factor 2a (RUNX2A) at 7d were measured and normalized with the expression of human acidic ribosomal phosphoprotein P0 (RPLP0). N=2, independent biological replicates from 1 donor. Gene sequences and accession numbers are presented in Table S3. Abbreviations: basic medium, BM; osteogenic medium, OM; adipogenic medium, AM.

Figure S2. Negative controls of the immunocytochemical staining. The negative control samples of hASC were cultured and treated as the actual samples with the exception that the cells were not treated with primary antibodies. Alexa 488 conjugated secondary antibodies were applied and incubated to confirm that the fluorescence signal is not unspecific, but specifically and selectively binds to the primary antibody and allows detection of the protein of interest. There was an insignificant level of green staining in the secondary antibody controls. Scale bar 100 µm. Abbreviations: basic medium, BM; osteogenic medium, OM; adipogenic medium, AM.
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Figure S4. Representative images of hASC for image-based analysis of actin orientation. Actin orientation was analyzed from the images of Phalloidin stained hASC taken with an inverted microscope Olympus IX51, using Alexa 546 filter (red), 20 X magnification and optimally adjusted exposure times for each image to ensure visibility of the cytoskeleton. DAPI stained (blue) nuclei are presented here to indicate the cell number. Scale bar 100 µm.
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Laura Hyväri1,2, Sari Vanhatupa1,2, Miina Ojansivu1,2, Minna Kelloniemi3, Toni-Karri Pakarinen4, Leena Hupa5 and Susanna Miettinen1,2,*

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*Correspondence: susanna.miettinen@tuni.fi; Tel.: +358-40-1901789

Abstract: Bioactive glass (BaG) materials are increasingly used in clinics, but their regulatory mechanisms on osteogenic differentiation remain understudied. In this study, we elucidated the currently unknown role of the p38 MAPK downstream target heat shock protein 27 (HSP27), in the osteogenic commitment of human mesenchymal stem cells (hMSCs), derived from adipose tissue (hASCs) and bone marrow (hBMSCs). Osteogenesis was induced with ionic extract of an experimental BaG in osteogenic medium (OM). Our results showed that BaG OM induced fast osteogenesis of hASCs and hBMSCs, demonstrated by enhanced alkaline phosphatase (ALP) activity, production of extracellular matrix protein collagen type I, and matrix mineralization. BaG OM stimulated early and transient activation of p38/HSP27 signaling by phosphorylation in hMSCs. Inhibition of HSP27 phosphorylation with SB202190 reduced the ALP activity, mineralization, and collagen type I production induced by BaG OM. Furthermore, the reduced pHSP27 protein by SB202190 corresponded to a reduced F-actin intensity of hMSCs. The phosphorylation of HSP27 allowed its co-localization with the cytoskeleton. In terminally differentiated cells, however, pHSP27 was found diffusely in the cytoplasm. This study provides the first evidence that HSP27 is involved in hMSC osteogenesis induced with the ionic dissolution products of BaG. Our results indicate that HSP27 phosphorylation plays a role in the osteogenic commitment of hMSCs, possibly through the interaction with the cytoskeleton.

Keywords: mesenchymal stem cells; osteogenesis; bioactive glass; p38/HSP27 signaling; phosphorylation

1. Introduction

Mesenchymal stem cells (MSCs) are the multipotent precursor cells playing a central role in bone development and regeneration [1–3], making them intriguing candidates for bone tissue engineering (TE) applications. MSCs can be obtained from adult tissues, including bone marrow and the stromal vascular fraction (SVF) of adipose tissue [4,5]. Additionally, bioactive glass (BaG) materials have been extensively studied during the past decades in the context of bone tissue engineering (TE), because of their ability to stimulate osteogenesis and bone growth [6,7]. In a clinical setting, bone regeneration is already aided by well-established silicate BaGs such as 45S5 (Bioglass ®), or in non-load-bearing sites BaG particulates, such as S53P4 (BonAlive ®) granules [8]. Novel glass compositions have been developed in search of improved biological responses, and the incorporation of metal ions such as Cu, Co, Mn, Sr, Mg, Zn, Li, and B to the BaGs has been proven advantageous for osteogenic induction [9–11]. The ionic products...
Heat Shock Protein 27 Is Involved in the Bioactive Glass Induced Osteogenic Response of Human Mesenchymal Stem Cells

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3 Department of Plastic and Reconstructive Surgery, Tampere University Hospital, Elämänaukio, Kuntokatu 2, 33320 Tampere, Finland
4 Regea Cell and Tissue Center, Tampere University, Arvo Ylpön katu 34, 33520 Tampere, Finland
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Abstract: Bioactive glass (BaG) materials are increasingly used in clinics, but their regulatory mechanisms on osteogenic differentiation remain understudied. In this study, we elucidated the currently unknown role of the p38 MAPK downstream target heat shock protein 27 (HSP27), in the osteogenic commitment of human mesenchymal stem cells (hMSCs), derived from adipose tissue (hASCs) and bone marrow (hBMSCs). Osteogenesis was induced with ionic extract of an experimental BaG in osteogenic medium (OM). Our results showed that BaG OM induced fast osteogenesis of hASCs and hBMSCs, demonstrated by enhanced alkaline phosphatase (ALP) activity, production of extracellular matrix protein collagen type I, and matrix mineralization. BaG OM stimulated early and transient activation of p38/HSP27 signaling by phosphorylation in hMSCs. Inhibition of HSP27 phosphorylation with SB202190 reduced the ALP activity, mineralization, and collagen type I production induced by BaG OM. Furthermore, the reduced pHSP27 protein by SB202190 corresponded to a reduced F-actin intensity of hMSCs. The phosphorylation of HSP27 allowed its co-localization with the cytoskeleton. In terminally differentiated cells, however, pHSP27 was found diffusely in the cytoplasm. This study provides the first evidence that HSP27 is involved in hMSC osteogenesis induced with the ionic dissolution products of BaG. Our results indicate that HSP27 phosphorylation plays a role in the osteogenic commitment of hMSCs, possibly through the interaction with the cytoskeleton.

Keywords: mesenchymal stem cells; osteogenesis; bioactive glass; p38/HSP27 signaling; phosphorylation

1. Introduction

Mesenchymal stem cells (MSCs) are the multipotent precursor cells playing a central role in bone development and regeneration [1–3], making them intriguing candidates for bone tissue engineering (TE) applications. MSCs can be obtained from adult tissues, including bone marrow and the stromal vascular fraction (SVF) of adipose tissue [4,5]. Additionally, bioactive glass (BaG) materials have been extensively studied during the past decades in the context of bone tissue engineering (TE), because of their ability to stimulate osteogenesis and bone growth [6,7]. In a clinical setting, bone regeneration is already aided by well-established silicate BaGs such as 45S5 (Bioglass®), or in non-load-bearing sites BaG particulates, such as S53P4 (BonAlive®) granules [8]. Novel glass compositions have been developed in search of improved biological responses, and the incorporation of metal ions such as Cu, Co, Mn, Sr, Mg, Zn, Li, and B to the BaGs has been proven advantageous for osteogenic induction in vitro [9–11]. The ionic products...
released from the BaGs have been found to promote proliferation [12] and the osteogenic differentiation of stem cells [13–16]. However, the molecular details of how BaGs modulate cell differentiation remain understudied.

Mitogen activated protein kinase (MAPK) family member p38 is involved in the skeletal development and bone homeostasis [17–19]. Due to the versatile involvement of p38 MAPK in many cellular processes besides cell differentiation, the details of these signaling events have been extensively studied, as reviewed by Canovas and Nebreda [20]. The pathway is activated by environmental stresses, cytokines, and mechanical stimuli [20], and there are reports showing that p38 MAPK signaling is activated by BaGs [21,22].

In vitro, osteogenesis is accompanied by the physical change of the cell cytoskeleton [19,23–25], and the cellular mechanisms regulating the cytoskeletal dynamics are significant in regulating the osteogenic lineage commitment of hMSCs [19,24,26–28]. The p38 MAPK downstream target heat shock protein 27 (HSP27), also known as heat shock protein B1 (HSPB1), is a chaperone suggested to be involved in the control of actin dynamics [17,29–31]. Therefore, we hypothesized that HSP27 would be involved in the BaG induced osteogenic fate of human mesenchymal stem cells (hMSCs).

Transient upregulation of HSP27 (or its murine analogue HSP25) has been reported in the in vitro differentiation programs of many cell types, as summarized previously [31]. Importantly, there is some prior evidence that HSP27 is involved in osteogenesis. Shakoori and co-workers showed elevated HSP27 gene expression during osteogenesis of rat osteoblasts [32], and the expression was also linked to osteogenesis of electrically stimulated human MSCs [33]. Additionally, immunohistochemical analyses have shown spatial activation of HSP27 during bone development in human fetal craniofacial tissues [34], and in rat tibiae [35].

Phosphorylation and oligomerization are important characteristics of heat shock proteins and have been proposed to guide their functions [30,36]. Unphosphorylated HSP27 exists mainly in the cytosol as large oligomers. The p38 MAPK substrate MAPK-activated protein (MAPKAP) kinase 2 (MK2) reversibly phosphorylates HSP27 on mainly Ser-78 and/or Ser-82 residues, changing its conformation into dimers. According to previous studies, this conformation promotes the nuclear and actin-related localization of HSP27. [17,36,37] We aimed to uncover the phosphorylation status of HSP27 during osteogenesis of hMSCs and reveal whether HSP27 could be involved in the osteogenic differentiation fate through its ability to interact with the cytoskeleton.

In this work, we studied the involvement of p38/HSP27 signaling in hMSC BaG induced osteogenesis. To achieve this, we cultured human adipose stem cells (hASCs) and human bone marrow stem cells (hBMSCs) harvested from adult tissues in basic medium (BM), osteogenic medium (OM), and basic medium with ionic dissolution products (Ca, K, Mg, Si, Na, and B) released from an experimental silica-based glass 3-06 [14] (BaG BM), or with the ionic dissolution of BaG in OM (BaG OM). The ion concentrations of the ionic dissolution were determined previously as [mg/kg]: Ca, 131; K, 172; Mg, 16; Si, 56; Na, 3750; and B, 2.6; P was below the limits of quantification [14]. The utilization of BaG extract allowed us to examine the effect of the bioactive ions without the cell-biomaterial contact and subsequent activation of cell adhesion-related intracellular cascades.

We analyzed the osteogenic potential of the cells with alkaline phosphatase activity, using the Alizarin Red mineralization assay and immunocytochemical (ICC) staining of collagen type I. We studied the intracellular protein activation of p38 MAPK, MK2, and HSP27 using Western Blotting of phosphorylated and basal proteins. The relevance of HSP27 phosphorylation on the osteogenic outcome was studied with the p38 MAPK inhibitor SB202190. Lastly, the intracellular localization of HSP27, pHSP27(S78), and Phalloidin-stained F-actin were studied under p38 MAPK inhibition. This study provides new knowledge on the role of p38 MAPK signaling in hMSC osteogenesis through a downstream target HSP27, and its ability to interact with the actin cytoskeleton.
2. Materials and Methods

2.1. Isolation and Expansion of hASCs and hBMSCs

The hASCs were isolated from donated subcutaneous abdominal adipose tissue samples collected at the Tampere University Hospital Department of Plastic and Reconstructive Surgery, from three female donors aged 53, 60, and 63. Isolation of the hASCs was performed as described previously by Lindroos and co-workers [38]. Briefly, the adipose tissue was digested mechanically and enzymatically (Collagenase type I; Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged and filtered to separate the stem cells. The isolated hASCs were cultured on Nunclon Delta surface polystyrene culture flasks (Thermo Fisher Scientific) in a basic culture medium (BM) consisting of 5% human serum (HS; BioWest, Nuaille, France) and 1% antibiotics (100 U/mL penicillin; 100 μg/mL streptomycin; Lonza, Basel, Switzerland), in Minimum Essential Medium α, with no nucleosides (MEM α; Thermo Fisher Scientific). The cells were cultured at 37 °C in 5% CO2, detached using TrypLE Select (Thermo Fisher Scientific), and passaged after reaching 70–80% confluence.

hBMSCs were isolated from donated bone marrow aspirates of three female donors aged 81, 87, and 91 obtained during a surgical procedure at the Tampere University Hospital Department of Orthopedics and Traumatology. hBMSC isolation from the aspirates was carried out by filtrating the samples through a cell strainer and centrifugation through a Ficoll gradient (Histopaque®-1077; Sigma-Aldrich, Saint Louis, MO, USA), as described by Wang and co-workers [10]. The isolated hBMSCs were cultured like the hASCs, with the exception that BM was supplemented with 5 ng/mL human fibroblast growth factor (hFGF-2; Miltenyi Biotec, Bergisch Gladbach, Germany).

2.2. Characterization of Immunophenotype of the Cells

The immunophenotype of hASC and hBMSC was analyzed by flow cytometry (FACSAria; BD Biosciences, Erembodegem, Belgium) at passage one. Cell samples (10,000 cells/sample) were single stained with the following monoclonal antibodies: CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD73-PE, CD90-APC (antibodies from BD Biosciences, Franklin Lakes, NJ, USA), CD11a-APC, CD105-PE (R&D Systems Inc., Minneapolis, MN, USA), CD34-APC, and HLA-DR-PE (Immunotools GmbH, Friesoythe, Germany). A fluorescence level greater than 99% was considered positive.

2.3. 3-06 Bioactive Glass Manufacturing and Extract Preparation

The bioactive glass used in this study, an experimental silica-based glass 3-06 of composition [wt%]: Na2O 24.6; CaO 21.6; P2O5 2.5; B2O3 1.3; SiO2 50.0, was manufactured using the melt-quenching method, as described previously [14]. BaG extract medium (BaG BM) was prepared according to the protocol, demonstrated in an earlier study [14]. In brief, 87.5 mg/mL 3-06 granules (500–1000 μm) were disinfected twice using 70% ethanol (Altia, Helsinki, Finland), dried, and then soaked in MEM α supplemented with 1% antibiotics (Lonza) for 24 h at +37 °C to dissolve ions from the glass. After incubation, the extract was sterile filtered, and HS (BioWest) was added to the final concentration of 5%. The BaG medium was stored at +4 °C and used within two weeks.

2.4. Cell Seeding and Differentiation Culture

The hASCs and hBMSCs were cultured separately, and the experiments were carried out at passages three to five. The plating density was 1000 cells/cm², except in Western Blot studies 5260 cells/cm². The culture platform for Western Blot samples was Corning Costar® TC-Treated 6 well plate (Corning; Corning, NY, USA), for CyQUANT®/ALP, Alizarin Red analyses Nunc™ Cell-Culture Treated polystyrene 24 well plate (Thermo Fisher Scientific), and for immunocytochemical (ICC) and Phalloidin-staining, a chambered polymer coverslip, Ibidi 8 well μ-slide was used (Ibidi GmbH, Gräfelfing, Germany). The cells were seeded into BM. The medium was supplemented with 5 ng/mL hFGF-2 (Miltenyi Biotec) when seeding hBMSCs, but hFGF-2 was excluded from the subsequent media changes.
The osteogenic differentiation was induced 24 h after cell seeding with BaG BM or osteogenic medium (OM) consisting of 5% HS (BioWest), 1% antibiotics (Lonza), 250 μM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate, and 5 mM dexamethasone (Sigma-Aldrich) in MEM α (Thermo Fisher Scientific), or with a combination of BaG extract and OM (BaG OM), prepared by supplementing BaG BM with 250 μM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate, and 5 mM dexamethasone (the supplements from Sigma-Aldrich). Control cell cultures were maintained in the BM. A total of 3 μM p38 MAPK inhibitor SB202190 (Calbiochem, Merck Millipore, Burlington, MA, USA) was used to inhibit the HSP27 phosphorylation. Fresh media with the inhibitor were changed in the cultures twice a week, during the experiments.

2.5. Western Blot and Immunodetection

hASCs and hBMSCs seeded on 6 well plates (2 donor cell lines of each cell type) were cultured in BM, BaG BM, OM, and BaG OM for 1, 3, 5, 7, 9, and 11 days to create a timeline of the differentiation process. Additionally, hASCs and hBMSCs in BM, BaG BM, OM, and BaG OM supplemented with 3 μM p38 MAPK inhibitor (SB202190; Calbiochem, Merck Millipore, Burlington, MA, USA) were cultured for 7 days.

Cell lysis and Western Blotting was performed as described previously [27]. Briefly, the samples were lysed into Laemmli Sample Buffer (2· concentrate; Bio-Rad, Hercules, CA, USA) supplemented with 5% β-mercaptoethanol (Sigma-Aldrich). The samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels using electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane (Mini Format 0.2 μm; Bio-Rad). The membranes were blocked with a 5% milk blocking solution of 5% skimmed milk powder (Valio, Lapinlahi, Finland) in 0.05% tris-buffered saline (TBS)-Tween (Tween 20; Sigma-Aldrich). Immunodetection was performed using primary antibodies and horseradish peroxidase (HRP) -conjugated secondary antibodies diluted in the blocking solution (listed in Table 1). The antibody incubations were followed by washes with 1· TBS, 0.5% TBS Tween, 0.1% TBS Tween, and 0.05% TBS Tween. The bands were detected with ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK), and ChemiDoc MP System (Bio-Rad). Semi-quantitative analysis of immunoblotted pHSP27(S78) and β-actin protein amounts was performed with Image J [39] to show the normalized pHSP27(S78) protein level.

Table 1. Primary and secondary antibodies used in immunodetection.

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Antibody</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>anti-β-actin sc-47778 †</td>
<td>mouse</td>
<td>1:2000</td>
<td>RT, 2 h</td>
</tr>
<tr>
<td>Primary</td>
<td>anti-HSP27 (D6W5V) #95357S †</td>
<td>rabbit</td>
<td>1:1000</td>
<td>+4 °C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>anti-p-HSP27(S78) #2405S †</td>
<td>rabbit</td>
<td>1:1000</td>
<td>+4 °C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>anti-MAPKAPK2 #12155T †</td>
<td>rabbit</td>
<td>1:1000</td>
<td>+4 °C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>anti-p-MAPKAP2K (T334) #3007T †</td>
<td>rabbit</td>
<td>1:1000</td>
<td>+4 °C, overnight</td>
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<tr>
<td>Primary</td>
<td>anti-p-p38 MAPK(T180/Y182) #4511S †</td>
<td>rabbit</td>
<td>1:1000</td>
<td>+4 °C, overnight</td>
</tr>
<tr>
<td>Secondary</td>
<td>anti-mouse IgG-HRP (sc-2005) †</td>
<td>goat</td>
<td>1:2000</td>
<td>RT, 1h</td>
</tr>
<tr>
<td>Secondary</td>
<td>anti-rabbit IgG-HRP #7074S †</td>
<td>goat</td>
<td>1:2000</td>
<td>RT, 1h</td>
</tr>
</tbody>
</table>

† Santa Cruz Biotechnology, Dallas, TX, USA. ‡ Cell Signaling Technology, Danvers, MA, USA.

2.6. Cell Proliferation and Quantitative Analysis of Alkaline Phosphatase Activity

Cell proliferation, i.e., total DNA amount of hASCs and hBMSCs, was determined quantitatively by CyQUANT® cell proliferation assay (Thermo Fisher Scientific) after 7, 9, and 11 days of culture. Before cell lysis, time point images were taken with a Nikon eclipse TS100 inverted phase contrast microscope (Nikon, Tokyo, Japan), with 4 X light objective. The analysis was performed on samples collected in 0.1% Triton buffer (Sigma-
Alkaline phosphatase activity denoting the early osteogenic differentiation of hMSCs was analyzed from the same cell lysates as cell proliferation, as previously described [38,40]. Briefly, the samples were incubated 15 min at 37 °C in working solution consisting of 1:1 10.8 μM phosphatase substrate (Sigma-Aldrich), and 1.5 M alkaline buffer solution (Sigma-Aldrich), after which the reaction was halted with 1.0 M sodium hydroxide (Sigma-Aldrich). The chromogenic reactions of the samples were detected by measuring the absorbance at 405 nm with a Victor 1420 microplate reader (Wallac).

2.7. Alizarin Red Staining of Matrix Mineralization

Extracellular mineral formation was studied with Alizarin Red (AR) analysis after 9 and 11 days of differentiation culture, as reported before [27]. In brief, the wells were fixed with 70% ethanol (Altia), stained with Alizarin Red S solution, (pH 4.1–4.3; Sigma-Aldrich) for 10–15 min, washed with water and then with 70% ethanol (Altia). Dry wells were photographed for macroscopic images, and AR staining was quantified by extracting the dye for 3 h into 100 mM cetylpyridinium chloride (Sigma-Aldrich). The absorbances of the extracted dye were measured at 544 nm with a Victor 1420 microplate reader (Wallac).

2.8. Immunocytochemical Staining

ICC staining of hASCs and hBMSCs (2 donor cell lines of each cell type) was conducted to analyze the protein expression of HSP27 (time point 9 d) and pHSP27(S78) (time points 3 d and 9 d), and osteogenic marker protein collagen type I, at 11 days of culture. ICC staining was performed as described previously [27]. The cell cultures were fixed for 15 min with 0.2% Triton X-100 in paraformaldehyde (PFA; Sigma-Aldrich) and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in phosphate buffered saline (PBS; Lonza). The samples were incubated with primary antibodies, washed, and incubated with secondary antibodies (detailed antibody and reagent information is given in Table 2). Phalloidin-Tetramethylrhodamine B isothiocyanate (TRITC), for actin cytoskeleton staining, was added simultaneously with the secondary antibodies. The samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for visualization of nuclei. Secondary antibody control staining was conducted as described, with the exception that the primary antibody incubation was left out.

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Antibody</th>
<th>Host Species</th>
<th>Dilution</th>
<th>Incubation</th>
</tr>
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<tr>
<td>Primary</td>
<td>anti-collagen type I (ab90395) †</td>
<td>mouse</td>
<td>1:2000</td>
<td>+4 °C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>anti-pHSP27 (S78) (ab32501) †</td>
<td>rabbit</td>
<td>1:500</td>
<td>+4 °C, overnight</td>
</tr>
<tr>
<td>Primary</td>
<td>anti-HSP27 (D6W5V) #95357S †</td>
<td>rabbit</td>
<td>1:500</td>
<td>+4 °C, overnight</td>
</tr>
<tr>
<td>Secondary</td>
<td>anti-mouse IgG Alexa fluor 488 (A11029) §</td>
<td>goat</td>
<td>1:500</td>
<td>+4 °C, 45 min</td>
</tr>
<tr>
<td>Secondary</td>
<td>anti-rabbit IgG Alexa fluor 488 (A21206) §</td>
<td>donkey</td>
<td>1:500</td>
<td>+4 °C, 45 min</td>
</tr>
<tr>
<td>-</td>
<td>Phalloidin-TRITC §</td>
<td>-</td>
<td>1:500</td>
<td>+4 °C, 45 min</td>
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<tr>
<td>-</td>
<td>DAPI §</td>
<td>-</td>
<td>1:2000</td>
<td>RT, 5 min</td>
</tr>
</tbody>
</table>

† Abcam, Cambridge, United Kingdom. † Cell Signaling Technology, Danvers, MA, USA. § Sigma-Aldrich, Saint Louis, MO, USA.

2.9. Fluorescence Imaging and Image-Based Analysis of Stain Intensity

Fluorescence images of hASC and hBMSC samples were taken with an Olympus IX51 inverted microscope (Olympus, Tokyo, Japan) equipped with a fluorescence unit and camera (DP30BW). Alexa 488 filter was used for detection of HSP27, pHSP27(S78), and collagen type I, an Alexa 546 filter for β-actin, and a DAPI filter for nuclei. The samples
were imaged with 40 X objective. Exposure time was kept constant within experiments. Image panels were processed, and adjustments of brightness and contrast were made with Adobe Photoshop CC (Adobe, San Jose, CA, USA). The 4 X digital zoom images were processed with Fiji [41]. The mean grey values representing the intensity of HSP27, pHSP27, and Phalloidin-TRITC signals in the fluorescence images, and the nuclei count of corresponding images for normalization of the intensities, were analyzed with Fiji [41].

2.10. Statistical Analysis

Statistical analyses were performed to evaluate the differences between samples in the ALP, CyQUANT, and Alizarin Red analyses, and the image-based quantitation data of HSP27, pHSP27, and F-actin. Results are expressed as mean and standard deviation (SD). Statistical analyses were conducted using the non-parametric Mann–Whitney test, followed by the Bonferroni post-hoc test with GraphPad Prism 5 (La Jolla, CA, USA). Statistical differences with \( p < 0.05 \) were considered significant. BM BaG, OM, and OM BaG conditions were compared to BM control. Comparisons of BM BaG vs OM BaG, OM vs OM BaG, and the comparisons between BM, BaG BM, OM, and BaG OM, and the corresponding SB202190-inhibited conditions were also made due to their relevance.

3. Results

3.1. Characterization of the Mesenchymal Origin of the CELLS

The cells were identified as mesenchymal based on the criteria given by the International Society for Cellular Therapy [42]. The cells were adherent to plastic culture surfaces and the surface marker expression pattern studied by flow cytometry conveyed the criteria. All hASC and hBMSC donor cell lines in this study had positive expression of CD73, CD90, and CD105, and lacked the expression of CD11, CD14, CD19, and CD45 (Table 3). The expression of CD34 was negative in hBMSCs, but moderate in hASCs. The elevated expression of CD34 in freshly isolated hASCs declines after passaging [43]. HLA-DR expression of hASCs was negative and in accordance with the criteria, but hBMSC donor cell lines were HLA-DR positive. This feature of BMSCs has been reported under normal culture conditions and does not compromise the MSC identity [44].

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Surface Protein</th>
<th>Surface Marker Expression hASC (%)</th>
<th>Surface Marker Expression hBMSC (%)</th>
<th>Fluorophore</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a</td>
<td>Integrin alpha L (Lymphocyte function-associated antigen 1)</td>
<td>1.5 ± 0.7</td>
<td>0.7 ± 0.4</td>
<td>APC</td>
<td>R&amp;D Systems Inc. Minneapolis, MN, USA</td>
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<tr>
<td>CD14</td>
<td>Lipopolysaccharide receptor</td>
<td>1.2 ± 0.9</td>
<td>5.4 ± 1.8</td>
<td>PECy7</td>
<td>BD Biosciences. Franklin Lakes, NJ, USA</td>
</tr>
<tr>
<td>CD19</td>
<td>B lymphocyte-lineage differentiation antigen</td>
<td>1.1 ± 0.9</td>
<td>4.3 ± 2.4</td>
<td>PECy7</td>
<td>BD Biosciences</td>
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<tr>
<td>CD34</td>
<td>Hematopoietic progenitor cell antigen I</td>
<td>22.2 ± 23.4</td>
<td>2.5 ± 0.5</td>
<td>APC</td>
<td>Immunotools GmbH, Friesoythe, Germany</td>
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<tr>
<td>CD45</td>
<td>RO isoform of leucocyte common antigen</td>
<td>1.8 ± 0.3</td>
<td>7.1 ± 1.4</td>
<td>APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD73</td>
<td>Ecto-5′-nucleotidase</td>
<td>91.1 ± 3.7</td>
<td>93.4 ± 3.7</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD90</td>
<td>Thy-1 (T cell surface glycoproteins)</td>
<td>98.8 ± 1.0</td>
<td>88.6 ± 6.5</td>
<td>APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD105</td>
<td>SH-2. Endoglin</td>
<td>94.7 ± 6.9</td>
<td>92.9 ± 6.1</td>
<td>PE</td>
<td>R&amp;D Systems Inc.</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Major histocompatibility class II antigen (MHC-II)</td>
<td>1.3 ± 1.1</td>
<td>85.7 ± 8.3</td>
<td>PE</td>
<td>Immunotools GmbH</td>
</tr>
</tbody>
</table>

Abbreviations: human adipose stem cell, hASC; human bone marrow stem cell, hBMSC; cluster of differentiation, CD; allophycocyanin, APC; phycoerythrin-cyanine, PECy7; phycoerythrin, PE. Standard deviation is indicated with ±.
3.2. Proliferation and Osteogenic Differentiation

The CyQUANT assay results of cell proliferation (Figure 1A,B) show, that BaG extract enhanced the total DNA amount of both hASCs and hBMSCs, when cultured in BM. In hASCs, the cell number was increased with OM, at all time points studied. Compared to the OM condition, BaG OM significantly enhanced cell proliferation of hASCs on day 7, but diminished the cell number on day 11. Proliferation was significantly induced with OM in hBMSCs, but BaG OM reduced the proliferation compared with the OM condition. Micrographs taken before the sample lysis show that the cells cultured under BaG OM for 9 and 11 days had produced a mineral layer on top of the cell layer (Figure S1).

![Figure 1](image)

**Figure 1.** Cell proliferation and osteogenic differentiation of hASCs and hBMSCs. hASCs and hBMSCs were cultured in BM, BaG BM, OM, and BaG OM. (A,B) Cell proliferation was studied with CyQUANT assay after 7, 9, and 11 days of culture. (C,D) ALP activity was studied after 7, 9, and 11 days of culture and is presented normalized, with the corresponding cell amounts. (E,F) Matrix mineralization was analyzed with Alizarin Red staining after 9 and 11 days of culture. Qualitative representative results (stained wells, area 1.9 cm²) are presented below the corresponding graphs. Bright red staining represents the mineral. (G,H) Representative images of collagen type I stained hASCs and hBMSCs at 11 d. Scale bars 100 μm. Statistical analysis: cell proliferation and ALP analyses; N = 9, independent biological replicates of three donors of each cell type. Mineralization; hASCs: N = 9 independent biological replicates of three donors; and hBMSCs: N = 6 independent biological replicates of two donors. Statistical analysis was conducted within time point, *p < 0.05. The asterisk above the error bar indicates the statistical difference compared to the BM control, other
comparisons are indicated with lines. Abbreviations: alkaline phosphatase, ALP; basic medium, BM; osteogenic medium, OM; and bioactive glass, BaG.

We evaluated the osteogenic potential of hASCs and hBMSCs by analyzing the activity of an early osteogenic marker, alkaline phosphatase (ALP), normalized with the corresponding cell amount (Figure 1C,D). The ALP activity of hASCs was time-dependently increased with OM and BaG OM conditions. OM induced a significant increase in ALP activity of hASCs on day 11, which was further enhanced by BaG OM. OM significantly increased the ALP activity of hBMSCs at all time points studied. Furthermore, BaG OM stimulated a significant increase in the ALP activity of hBMSC already on day 7, compared with OM alone. ALP activity of both cell types was low under BM and BaG BM conditions.

Mineral accumulation in the extracellular matrix (ECM), characteristic of late osteogenic differentiation, was analyzed by Alizarin Red (AR) staining of hASCs and hBMSCs, after 9 and 11 days of osteogenic differentiation (Figure 1E,F). Based on the quantitative AR staining and corresponding qualitative images shown below the columns, BaG OM significantly enhanced matrix mineralization of both hASCs and hBMSCs. OM alone induced mineralization compared with the BM control, but the level of mineral accumulation was markedly lower without BaG. Only hBMSCs demonstrated strong red AR staining in OM, on day 11. BaG without osteogenic supplements could not stimulate mineralization of the ECM during the culture period.

To further analyze the osteogenic potential of the hASCs and hBMSCs, the production of ECM protein collagen type I was analyzed with ICC staining on day 11 (Figure 1 G,H, secondary antibody controls in Figure S2a). In hASCs, OM enhanced the collagen type I production, which appeared to localize intracellularly in the perinuclear area. BaG OM markedly enhanced the amount of collagen type I in hASCs, which was found secreted into the extracellular space. In hBMSCs, extracellular fibrils of collagen type I were formed under OM condition, but there was more collagen type I present in BaG OM. The production of collagen type I was negligible under the BM and BaG BM conditions.

3.3. Activation of the p38/MK2/HSP27 Pathway

The activation of p38/MK2/HSP27 pathway during hASC and hBMSC osteogenesis was analyzed with Western Blotting. The timeline figures (Figures 2A,B and S3 presenting additional donor cell lines) show the sequential activation of p38 MAPK, MK2, and HSP27 through phosphorylation, and the corresponding unphosphorylated protein levels. The pathway constituents were detected in hASCs and hBMSCs cultured under all culture conditions, but the protein expressions were upregulated more in OM conditions. The protein levels of p-p38, p38, pMK2, MK2, and pHSP27(S78) increased with time during culture in OM, whereas in BaG OM, the expression levels peaked and declined during the culture. Unphosphorylated HSP27 and β-actin were present throughout the culture period, under all culture conditions. Semi-quantification of the pHSP27(S78) normalized with the β-actin, representing the cell amount, was performed to highlight its activation (Figure 2C,D). The normalized pHSP27(S28) peaked later in hASCs compared to hBMSCs, under BaG OM condition. In hBMSCs the normalized pHSP27(S28) increased time-dependently in OM without BaG, while a similar trend was absent in the hASCs.

3.4. Inhibition of HSP27 Phosphorylation

The relevance of pHSP27(S78) activation in BaG induced osteogenesis of hASCs and hBMSCs was further studied with p38 MAPK inhibition, under OM and BaG OM conditions. SB202190 inhibitor with a concentration of 3 μM was chosen based on the literature and prior optimization [45,46]. SB202190 was shown by Western Blotting to specifically reduce the phosphorylation, and thus activation of pHSP27(S78), without affecting the basal level of HSP27 (Figure S5a,b). CyQUANT analysis was performed to confirm that the inhibitor did not compromise cell proliferation (Figure S5c,d).
without BaG, while a similar trend was absent in the hASCs. The timeline figures (Figures 2A,B and S3 presenting AR staining and corresponding qualitative images shown below the columns, BaG OM sig-

3.4. Inhibition of HSP27 Phosphorylation
did not compromise cell proliferation (Figure S5c,d). The quantitative data showed that p38 MAPK inhibition had a reducing effect on HSP27 in hASCs, when cultured under OM and BaG OM, whereas the inhibition had no effect in hBMSCs (Figure 4B,C).

Figure 2. Timeline of p38/MK2/HSP27 activation in hASCs and hBMSCs. (A,B) hASCs and hBMSCs were cultured in BM, BaG BM, OM, and BaG OM for 1, 3, 5, 7, 9, and 11 days, and analyzed with Western Blotting and immunodetection. The figure presents cropped blots of p-p38, p38, pMK2, MK2, pHSP27(S78), HSP27, and β-actin of the representative donor cell lines. Full-length blots are presented in Figure S4. (C,D) Semi-quantification of the pHSP27(S28) bands presented normalized to β-actin in hASCs and hBMSCs. Abbreviations basic medium, BM; osteogenic medium, OM; and bioactive glass, BaG.

The p38 MAPK inhibition had a reducing trend on BaG OM -induced ALP activity (Figure 3A,B). SB202190 significantly decreased the BaG OM induced mineralization of the ECM in both cell types on day 11 (Figure 3C,D), although mineralization was not fully inhibited. Similarly, ICC staining showed that inhibition with SB202190 diminished collagen type I production of hASCs, under OM and BaG OM conditions (Figure 3E,F). In hBMSCs, the level of collagen type I staining was diminished by p38 MAPK inhibition in OM, but there was no clear difference in BaG OM.

3.5. HSP27 and pHSP27 Localization
Cellular localization of HSP27 and its phosphorylated form, cultured under BaG and SB202190 treatment, were visualized with ICC staining, and the stain intensities were quantified and normalized with the cell number. After 9 days of culture, ICC stained basal HSP27 was scarce under BM and BaG BM conditions (Figure 5a), but found abundantly in the cytosol of hASCs and hBMSCs cultured, under OM and BaG OM conditions (Figure 4A).
Figure 3. The effect of p38 MAPK inhibition with SB202190 on hASC and hBMSC osteogenesis. hASCs and hBMSCs were cultured in BM, BaG BM, OM, and BaG OM supplemented with 3 µM SB202190 inhibitor. (A,B) Early osteogenic differentiation was assessed with ALP activity. (C,D) Matrix mineralization was analyzed with Alizarin Red staining (stained wells, area 1.9 cm²). (E,F) ICC staining of collagen type I. Scale bars are 100 µm. Values for OM and BaG OM conditions without the inhibitor, and qualitative representative results of Alizarin Red staining are the same as presented in Figure 1. Statistical analysis: ALP analysis (and cell proliferation used to normalize the ALP activity data); N = 9, independent biological replicates of three donors of each cell type. Mineralization; hASCs: N = 9 independent biological replicates of three donors; hBMSCs: N = 6 independent biological replicates of two donors. Statistical analysis was conducted within the time point, and comparisons were made between OM and OM + SB202190 or BaG OM and BaG OM + SB202190. *p < 0.05. The asterisk above the error bar indicates the statistical difference compared to the control group. Abbreviations: osteogenic medium, OM; and bioactive glass, BaG.

ICC staining of pHSP27(S78) after 3 days of differentiation showed that when phosphorylated at Serin 78, HSP27 co-localized with the rigid and aligned Phalloidin-stained F-actin fibers (Figures 5A and S6b). Similarly on day 9, pHSP27(S78) associated with the actin filaments in hASCs and hBMSCs, cultured under BM, BaG BM, and OM conditions (Figures 5D and S6c). Interestingly, pHSP27(S78) was found diffusely located throughout the cytosol after 9 days of differentiation in BaG OM, in both cell types. The diffuse localization of pHSP27(S78) was also partly present in hBMSCs cultured in OM. The p38 MAPK inhibition on day 9 reversed the BaG OM related diffuse localization of pHSP27(S78) to more F-actin aligned organization (Figure 5D). The image-based quantitation of pHSP27(S78) stain intensity showed that p38 MAPK inhibition reduced the normalized amount of the phosphoprotein, especially in the later time point (Figure 5E,F).
3.6. The Cell Morphology and F-Actin Intensity

The role of HSP27 phosphorylation on the cytoskeletal organization was further analyzed with Phalloidin-TRITC staining and image-based quantitation of F-actin intensity. After 9 days of differentiation, the cell morphology of hASCs and hBMSCs (Figure 6A), was fibroblastic with thick stress fibers traversing the cells, in the BM and OM conditions. BaG extract or p38 MAPK inhibitor treatment altered the morphology leading to thinner and less visible F-actin fibers. The osteogenically committed hMSCs in BaG OM condition had markedly different appearance with disrupted cytoskeleton and some disintegrated nuclei. Quantitation of the imaging data revealed that the mean F-actin intensity, normalized with cell amount (fragmented nuclei were excluded), was significantly diminished by BaG extract addition in both cell types after 9 days of differentiation (Figure 6B,C). Similarly, p38 MAPK inhibition significantly reduced the mean F-actin intensity in all other conditions except BaG OM in hBMSCs, where the amount of F-actin was already low without the inhibition.
with lines. Abbreviations: basic medium, BM; osteogenic medium, OM; and bioactive glass, BaG.

indicates the statistical difference compared to the BM control, and other comparisons are indicated

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2 independent biological replicates of 2 donors. Representative images of hASCs and hBMSCs

and hBMSCs normalized with nuclei count. Statistical analysis: hASCs: N = 6–9 (3 d) /8–10 (9 d)

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Figure 6. Actin cytoskeleton and F-actin intensity of hASCs and hBMSCs. (A) Representative Phalloidin-TRITC stained hASCs and hBMSCs cultured 9 days in BM, BaG BM, OM, and BaG OM supplemented with 3 μM SB202190 inhibitor. The cells were imaged with a fluorescence microscope using constant exposure times with Alexa 546 for actin (red) and DAPI (blue) filters, and a 40× magnification. Scale bar is 100 μm, and is used in every image. (B,C) Image-based quantification of mean grey values of Phalloidin-TRITC stained hASCs and hBMSCs normalized with nuclei count. Statistical analysis: hASCs: N = 6–9 (3 d) / 8–10 (9 d) independent replicates based on donors; hBMSCs: N = 15–18 images, 4 independent biological replicates of 2 donors. *p < 0.05. The asterisk above the error bar indicates the statistical difference compared to the BM control, and other comparisons are indicated with lines. Abbreviations: basic medium, BM; osteogenic medium, OM; and bioactive glass, BaG.

4. Discussion

Bioactive glasses and their combination with stem cells are considered promising remedies for bone defects, although the molecular details on how BaGs induce osteogenesis remain understudied. The importance of the regulation of the actin cytoskeleton in the osteogenic commitment of MSCs has been demonstrated in several studies [19,24,26–28,47]. MAPK p38 downstream substrate, a chaperone HSP27, has been proposed as a regulator of cytoskeletal dynamics, but its role in osteogenesis is not established. We hypothesized that HSP27 driven cytoskeletal modulation would be linked to the osteogenic course of hMSCs. The efforts made in unraveling the signaling pathways involved in BaG induced osteogenesis are important for creating knowledge-based biomaterials for bone regeneration.

We differentiated hMSCs derived from bone marrow and adipose tissue to make our data representative of hMSCs in BaG extract medium (BaG BM), osteogenic culture medium (OM), or with a combination of BaG extract and osteogenic supplements (BaG OM). BaGs have been found to be strong inducers of osteogenic differentiation of hMSCs [6,8], and our research group has confirmed that solely the ions extracted from bioactive glasses can enhance osteogenesis of hASCs [13,14]. The experimental BaG used in this study was a S53P4-based BaG with the substitution of silica with boron, shown to have a fast glass dissolution rate and osteogenic response in hASCs [14], and a good bone attachment in a rat model [48]. Indeed, boron has been demonstrated as an important trace element in bone formation [9,49–52].
In accordance with our previous publications [13,14], we demonstrated here that ionic dissolution of the 3-06 BaG supplemented with osteogenic agents induced significant and rapid osteogenic responses in hMSCs harvested from adipose tissue and bone marrow, as demonstrated with enhanced ALP activity and mineralization of the ECM, already on day 9 of differentiation. The osteogenic response of the cells was accompanied with a decrease in cell proliferation, as shown previously [53,54], but no toxic effects from the ionic extract were observed. The strong mineralization of hMSCs as a response to the 3-06 BaG was in coherence with the earlier borosilicate glass studies [51,55]. The ALP activity and mineralization also elevated with time in OM without BaG, suggesting to delayed osteogenic response without the ionic stimulus.

Collagen type I is the major component of the bone ECM, accounting for 90% of the organic matrix [56], and it is linked to osteogenic maturation [57]. Here we showed that collagen type I production was enhanced by osteogenic condition, and further increased by the ionic extract of 3-06 BaG. The collagen network in the ECM provides a platform for calcium phosphate -based crystal formation, and thus mineralization of the bone matrix [58–60]. The strong collagen type I staining induced by BaG OM was in coherence with the mineralization assay, indicating late-stage osteogenesis of the hMSCs.

BaGs have been reported to activate osteogenesis even without osteogenic culture supplements [15,61]. In this study, the ionic dissolution of 3-06 bioactive glass in basic medium did not elicit osteogenesis. Supporting these findings, in our previous study, the ionic dissolution products of BaGs alone could not induce the osteogenic response of hASCs [14]. However, the culture period in this study could have been simply too short, or the ionic cocktail insufficient, for BaG induced osteogenesis without other culture supplements.

The expression of HSP27 is upregulated as a response to conditions that alter protein folding, such as heat shock [31], but also mechanical stimuli [17], and differentiation [32]. In an earlier study with hBMSC, HSP27 gene expression was upregulated with electrical stimulation starting on day 10, and the osteogenic differentiation markers ALP and collagen type I were upregulated shortly after [33]. Additionally, in hASCs, HSP27 protein activity was upregulated with osteogenic differentiation medium [62]. We analyzed the protein expression of the p38/MK2/HSP27 pathway constituents p38, MK2, and HSP27, and their phosphorylated forms, and found coherently that the signaling axis was activated with osteogenic induction. A 3-06 BaG extract induced strong, transient HSP27 phosphorylation at Ser-78, which was temporally coupled to the activation of upstream kinases p38 MAPK and MK2, by phosphorylation. The early activation of signaling seemed to be linked to the fast osteogenic response of hASCs and hBMSCs, under BaG OM. Supporting our findings, the ionic products of Bioglass 45S5 have been reported to enhance p38 MAPK and MK2 gene expression in osteoblasts [16]. The cells under OM showed early markers of osteogenesis and enhanced activation of p38/HSP27 signaling throughout the culture period, suggesting its role in the osteogenic commitment.

The role of pHSP27(S78) in directing the osteogenic course of hMSCs was further assessed by an upstream kinase inhibition. The pharmacological inhibition of HSP27 has been previously conducted with p38 MAPK inhibitors SB202190, and a structurally related SB203580 (Adezmapimod) [29,46,63]. Here, we confirmed that SB202190 specifically inhibited phosphorylation of HSP27 at Ser-78 in hBMSCs and hASCs. Under inhibitor treatment, the BaG induced ALP activity was slightly reduced, and the matrix mineralization was significantly reduced, although not fully inhibited. p38 MAPK inhibition also hampered the production of collagen type I. Our results suggest that HSP27 phosphorylation is involved in the osteogenic commitment of hMSCs, but presumably is not a determining factor of differentiation fate.

The cytoskeletal association of HSP27 has been demonstrated in earlier studies, but the role of the HSP27 phosphorylation status in the cytoskeletal localization remains contradictory [17,29,36,64]. In this study, the localization of unphosphorylated HSP27 was cytoplasmic, similar to previously described results [29,65]. HSP27 co-localized with actin only when phosphorylated, forming linear structures. Although HSP27 has been more
commonly described as an actin capping protein [30], it may also bind to the sides of actin filaments as monomers [66,67], as in our observations. Similar results were reported in mouse fibroblasts, where uniaxial cyclic stretch upregulated HSP27 phosphorylation and subsequent co-localization of pHSP27 with actin stress fibers [17]. This cytoskeletal organization of pHSP27 was shown to reinforce the actin filaments [17]. In this study, the inhibition of p38/HSP27 axis reduced the pHSP27(S78) protein level and had a parallel effect on the F-actin intensity, suggesting a stabilizing function of pHSP27 on the actin filaments.

Studies have demonstrated that actin polymerization drives the MSC differentiation towards an osteogenic fate [19,68]. We discovered that the cytoskeletal association of pHSP27(S78) was linked to the undifferentiated or to the early osteocommitted hMSCs state with prevalent actin cytoskeleton, and was not present in the cells, strongly demonstrating mineralization of the collagenous matrix. Indeed, the cytoskeletal rigidity and tension are cues of the osteogenic commitment, but the differentiation process involves dynamic remodeling of the actin cytoskeleton [19,24,28]. Our results suggest that phosphorylation of HSP27 at Ser-78 could perform as an early regulatory step in promoting the hMSCs to commit to the osteogenic course through its interaction with the actin filament cytoskeleton. However, it is possible that this regulation involves a combination of multiple signaling molecules downstream of p38 MAPK.

5. Conclusions

In this study, we investigated the currently unstudied role of the p38 MAPK substrate HSP27 in hMSC BaG induced osteogenesis. We discovered that the ionic extract of an experimental silica-based 3-06 BaG, together with osteogenic culture supplements, induced rapid osteogenesis in hMSCs of two different origins, demonstrated by enhanced ALP activity, matrix mineralization, and secretion of collagen type I. The protein expression and activation by phosphorylation of p38 MAPK, MK2 and HSP27(S78) was linked to the osteogenic outcome, and the inhibition of HSP27 phosphorylation reduced the osteogenic markers, indicating the importance of this pathway to hMSC osteogenic course. Co-localization of pHSP27(S78) with actin stress fibers was linked to the early osteogenesis of hMSCs, whereas its localization was diffuse in the differentiated hMSCs. Due to the interplay of p38 MAPK signaling with several other targets, further studies on the topic are required.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12020224/s1, Figure S1. Phase contrast micrographs of hASC and hBMSC cultures. Figure S2. Secondary antibody controls for ICC staining. Figure S3. Supplemental donor cell lines related to Figure 2: timeline of p38/MK2/HSP27 activation in hASCs and hBMSCs. Figure S4. Full-length blots related to Figures 2 and S3. Figure S5. Inhibitor effect on pHSP27(S78) and HSP27 protein activity, and cell proliferation. Figure S6. Cellular localization of basal HSP27 and pHSP27 in hASCs and hBMSCs. Figure S7. Full-length blots related to Figure S5.

Author Contributions: Conceptualization, L.H. (Laura Hyvärä), S.V. and S.M.; Methodology, Formal Analysis, and Data Curation L.H. (Laura Hyvärä) and M.O.; Resources, S.M., L.H. (Leena Hupa), T.-K.P. and M.K.; Writing—Original Draft Preparation, L.H. (Laura Hyvärä); Writing—Review & Editing, L.H. (Laura Hyvärä), S.V., M.O., L.H. (Leena Hupa), T.-K.P., M.K. and S.M.; Project Administration, S.M.; Funding Acquisition, L.H. (Laura Hyvärä) and S.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. The study was carried out in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland with the supportive statements R15161 for hASCs and R15174 for hBMSCs.

Informed Consent Statement: Written informed consent was obtained from all adipose tissue and bone marrow donors.

Data Availability Statement: The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Molecular Mechanisms of Cell Adhesion and Cytoskeletal Dynamics Underlying Human Mesenchymal Stem Cell Differentiation

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