The impact of signaling factors on intervertebral disc degeneration and regeneration

Studies on disc and mesenchymal stem cells from chronic low back pain patients

Daphne Hingert

Department of Orthopaedics

Institute of Clinical Sciences

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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daphne.hingert@gu.se
daphnebhingert@gmail.com

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With great power, comes great responsibility

- Uncle Ben (Ben Parker)

To my beloved family

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ချစ်ဖေဖေသို့

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ถึงคุณแม่ที่รักของฉัน

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मेरी प्यारी दादी को

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ABSTRACT

INTRODUCTION: Chronic low back pain (LBP) is associated with degeneration of the intervertebral discs (IVDs). Increased expressions of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and matrix metalloproteinases (MMPs) in degenerated IVDs lead to loss of proteoglycan and extracellular matrix (ECM) which affect the viability of the disc cells (DCs). Treatment approaches using growth factors, cell therapy and extracellular vesicles (EVs) derived from human mesenchymal stem cells (hMSCs) could improve current treatment models by directly influencing the IVD degeneration processes.

AIMS: To explore the effects of growth factors, hMSC derived signaling peptides and small EVs (sEVs) on degenerated DCs in terms of cell viability and ECM production and to investigate the impact of stress hormone cortisol on DCs and hMSCs in *in vitro* models.

METHODS: DC and hMSC isolation from patients' tissue, cell cultures in monolayer and 3D pellets, cell viability assay, histological staining, and immunohistochemistry were carried out. In Study I, hMSCs were encapsulated in a hydrogel and stimulated with bone morphogenetic growth factor 3 (BMP-3), or IL-1β pre-treatment followed by BMP-3 stimulation. *In situ* hybridization was used to investigate the gene expressions of COL2A1 and OCT4. In Study II, the effects of cortisol at physiological and increased levels were studied on DCs and hMSCs in the 3D pellet model. Apoptosis assays were carried out and immunohistochemistry was used to evaluate cytokine expressions. Study III was a follow-up study of Study I in a 3D pellet model investigating the effect of BMP-3 and pre-treatment on DCs, hMSCs and co-culture (DCs and hMSCs in 1:1 ratio). In Study IV, the effects of hMSC conditioned media (CM) and connective tissue growth factor (CTGF) were investigated on DC pellets. The constituents of CM were further identified using mass spectrometry analysis. In Study V, the concentration of MMP-1 was quantified by enzyme-linked immunosorbent assay in disc tissue. Furthermore, the ability of CM to mitigate the effects of MMP-1 at different concentrations was studied. In Study VI, small EVs were isolated with differential centrifugation, and further characterized using flow cytometry, nanoparticle tracking analysis, and western blot. DC pellets were then stimulated with sEVs and cell proliferation, ECM production, apoptosis, lactate dehydrogenase activity, cytokine and chemokine secretions were evaluated.

RESULTS: Pre-treatment of IL-1β followed by BMP-3 enhanced chondrogenic differentiation in hMSCs in the hydrogel model (Study I) as well as in the 3D model (Study III). BMP-3 promoted chondrogenesis in DC pellets while a stronger effect was observed in co-culture (Study III). Study II demonstrated that exposure to cortisol even at physiological concentration restricted proliferation and compromised chondrogenesis in both DCs and hMSCs. CM from hMSCs enhanced viability and ECM production in DCs and mass spectrometry analysis revealed more than 120 peptides with high relative abundance (Study IV). Study V demonstrated that CM has the ability to mitigate the effect of MMP-1 on DCs, however, the potency of CM decreased with increased concentration of MMP-1. Lastly, Study VI demonstrated that sEVs enhanced cell proliferation while suppressed apoptosis. Early and increased ECM production was also observed in the DCs with sEVs treatment.

CONCLUSION: Signaling factors from hMSCs have positive effects on DCs and can mitigate the degenerative properties of pro-inflammatory cytokines and enzymes known to be present in the degenerated IVDs. Further, pain-induced stress regulated by cortisol may be a contributing factor of IVD degeneration.

KEYWORDS: signaling peptides, MSCs, disc cells, BMP-3, IL-1beta, cortisol, co-culture, conditioned media, MMP-1, extracellular vesicles, chondrogenesis, low back pain.

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SAMMANFATTNING PÅ SVENSKA

INTRODUKTION: Kronisk smärta i ländryggen kan vara förknippad med degeneration av intervertebraldiskarna i ryggraden. Vissa ämnen såsom pro-inflammatoriska cytokiner, t.ex. interleukin-1beta (IL-1β), och matrix metalloproteinaser (MMPs) ökar i degenererade diskar. Detta leder till försämrad funktion av cellerna i disken (DCs) och förlust av ämnen runt cellerna som bidrar till diskens stötdämpande förmåga kallad extracellulär matrix. Utveckling av nya behandlingsmetoder som minskar nedbrytningen av diskvävnaden skulle kunna minska problemen för vissa patienter med svår ryggsmärta. Detta skulle kunna innefatta att tillföra lösliga faktorer eller celler som stimulera diskens befintliga celler såsom olika tillväxtfaktorer, humana mesenchymala stamceller (hMSCs) eller faktorer som stamcellerna frisätter.

SYFTE: Att undersöka effekten av både potentiellt positiva och negativa effekter av olika faktorer såsom tillväxtfaktorer, signalpeptider och extracellulära vesiklar (sEVs) från hMSCs, cytokiner och stresshormon på diskcellers från degenererade diskar avseende funktion och överlevnad i in vitro-modeller.

METODER: Diskceller och mesenchymala stam celler isolerades från patienter som opererades för kronisk ryggsmärtsproblematik och studerades i olika typer av cellodlingar (monolayer, 3D pelletodling och i odling med bärarmatris). Olika analyser avseende bland annat cellviabilitet och proteoglycanproduktion utfördes. Celler från minst 3 patienter användes i varje studie. I studie i inkapslades hMSCs i en hydrogel och stimulerades med BMP-3 (bone morphogenic protein-3), med eller utan förbehandling med IL-1β under 24 timmar. *In situ* hybridisering användes för att undersöka genuttryck av generna COL2A1 och OCT4. I studie II studerades effekten av kortisol på DCs och hMSCs i en 3D-pelletmodell vid fysiologisk och förhöjd koncentration av kortisol. Analys av celldöd genomfördes och immunohistokemi användes för att utvärdera cytokinuttryck. Studie III var en uppföljningsstudie av studie I där effekten av BMP-3 och med eller utan förbehandling med IL-1β på DCs och hMSCs separat samt i samkultur undersöktes. I studie IV undersöktes effekten av utsöndrade ämnen från hMSC, konditionerat media (CM), samt en tillväxtfaktor, CTGF (conective tissue growth factor) på DC i pelletodling. Beståndsdelarna i CM identifierades vidare med hjälp av masspektrometri-analys. I studie V mättes koncentrationen av MMP-1 i diskvävnad med ELISA. Vidare studerades möjligheten att med CM från hMSC motverka den negativa effekten av olika koncentrationer av MMP-1 på diskceller. I studie VI undersöktes effekten av från hMSC

utsöndrade sEVs på DC. sEVs isolerades med differentiell centrifugering och karakteriserades med flödescytometri och NTA (nano particle tracking analysis). DC-pellets stimulerades med sEVs och cellviabilitet, ECM-produktion, cytokin- och kemokinproduktion utvärderades.

RESULTAT: Förbehandling med IL-1β följt av BMP-3 stimulering förbättrade differentieringen av hMSC till diskcellsliknande celler (kontrocytlika celler) i både hydrogel (studie I) såväl som i 3D-modell (studie III). BMP-3 i koncentrationen 10 ng/mL främjade också DC celler positivt i 3D odling. Den starkaste effekten av BMP-3 observerades i samkultur av hMSC och DC (studie III). Studie II visade att exponering för kortisol, även vid fysiologisk koncentration, begränsade både celldelning och ECM produktion för såväl DCs och hMSCs. CM från hMSC förbättrade livskraften och ECM-produktionen från DCs och masspektrometri-analys identifierade mer än 120 peptider i CM (studie IV). I Studie V visades att CM har förmåga att motverka effekten av MMP-1 på DCs. Dock så minskade effekten av CM med ökad koncentration av MMP-1. Slutligen visades i studie VI att sEVs minskade celldöd och förbättrade viabiliteten av diskceller i odling. Stimulering av DC med sEVs från hMSCs ledde också till tidigare och ökad ECM-produktion.

SLUTSATS: Förhöjda nivåer av kortisol, vilket kan orsakas av stress och kronisk smärta, kan vara en bidragande faktor till disk degeneration. Signalmolekyler från humana mesenchymala stamceller har positiva effekter på diskceller från degenererade diskar och kan mildra de negativa effekterna av pro-inflammatoriska cytokiner och enzymer som förekommer i degenererade diskar.

ကောက်နုတ်ချက် အကျဉ်းချုပ် (Abstract in Burmese)

မိတ်ဆက်- နာတာရှည်ကျောအောက်ပိုင်းနာကျင်မှုဆိုရာတွင် ကျောရိုးအတွင်းပိုင်း အရိုးပြားများ (IVDs) ၏ယိုယွင်းမှုများပါဂင်သည်။ အရိုးပြားယိုယွင်းမှုအတွင်းရှိ တိုးမြင့်လာသည့် ခုခံအားတုန့်ပြန်ထုတ်လွှတ်မှုပမာဏများ interleukin-1beta (1L-1beta) နှင့် သတ္ထုဓာတ်အင်ဇိုင်းပမာဏများမှ တစ်ဆင့်တစ်ရှူးပရိုတီန်းနှင့်ဆဲလ်ပြင်ပဒြပ်စင်များ ECM အားဆုံးရှုံးလာစေသည်၊ ယင်းအချက်သည် အရိုးပြား ဆဲလ်များ disc cells (DCs) ၏ ရှင်သန်မှုအပေါ် ဆိုးကျိုးသက်ရောက် စေသည်။ ဤရောဂါဇေဒနာအား ဆဲလ်ကြီးထွားမှုကုထုံးနည်းသစ်များဖြင့် စမ်းသပ်ကုသလျက်ရှိသည်။ လူသားပင်မဆဲလ်များ mesenchymal stem cells (hMSCs) မှဆင်းသက်လာသည့် အပိုဆဲလ် အိတ်များမှ IVD ယိုယွင်းမှုလုပ်ငန်းစဉ်အပေါ်တွင် တိုက်ရိုက်သက်ရောက်မှုစွမ်းအင်ကို ထိန်းသိမ်းပေးနိုင်သည်။ ဤနည်းအားဖြင့် ကျွန်တော်တို့မှယနေ့မျက်မှောက်ကာလ၏ ကုသထုံးများကိုပြောင်းလဲတိုးတက်စေနိုင်သည်။

ဦးတည်ချက်များ- ကြီးထွားမှုအရင်းအမြစ်များအားလေ့လာရှာဖွေရန် ယိုယွင်းနေသည့် အရိုးပြားများအတွင်းရှိ hMSCs မှ ဆင်းသက်လာသည့် အမှတ်လက္ခဏာပြသသည့် အက်ဆစ်အစုအဝေးနှင့် small extracellular matrix (sEVs) တို့မှဆဲလ်ရှင်သန်မှုအား ထောက်ပံ့ ပေးသည့်အလျောက်၊ DCs အပါအဝင် နည်းလမ်းအသစ်များဖြင့် ဖြစ်ပေါ်စေသည့်ဇေဒနာအလျောက် ပင်မဟိုမုန်းဓာတ် cortisol သက်ရောက်မှုအပေါ်တွင် ဆန်းစစ်လေ့လာခဲ့ကြသည်။

နည်းလမ်းများ- လူနာတစ်ရှူးများထံမှ DCs နှင့် hMSCs အစအနများ၊ အလွှာတစ်လွှာချင်း (monolayer) နှုင့် 3D အပြားများအတွင်း ရှိဆဲလ်ဖွဲ့စည်းပုံများ အတွင်းရှိဆဲလ်အရေအတွက် တစ်ရှူးနှင့်ခုခံအားမှတ်တမ်းဓာတုဗေဒအစအနများကိုလည်းလေ့လာခဲ့သည်။ လေ့လာချက် (၁) တွင် hMSCs များအား PuraMatrix hydrogel အတွင်း အတောင့်ငယ်များအဖြစ်ဖွဲ့စည်းစေပြီး Bone morphogenetic protein 3 (BMP-3) (သို့) 1L-1beta ကြိုတင်ကာကွယ်ကုသမှုကို (၂၄) နာရီ လုပ်ဆောင်ပြီး ယင်းနောက်တွင် BMP-3 အားဆက်လက် လုပ်ဆောင် ခဲ့သည်။ အက်တမ်လမ်းကြောင်းပြောင်းလဲမှု (*in situ* hybridization) နည်းလမ်းဖြင့် COL2A1 and OCT 4 ဗီဇရှင်းတမ်းများ အား လေ့လာခဲ့သည်။ လေ့လာချက် (၂) တွင် cortisol ဇီဂကမ္မအတွင်းရှိ ဟိုမုန်းနှင့် တိုးမြင့်လာသည့် အဆင့်များအား 3D pellet model အသုံးပြု၍ DCs နှင့် hMSCs များအရလေ့လာခဲ့သည်။ ဆဲလ်သေဆုံးလမ်းကြောင်း များအပေါ် ဆက်လက်လေ့လာခဲ့ပြီး ခုခံအားမှတ်တမ်းဓာတုဗေဒ ပညာရပ်ဖြင့် cytokine ရှင်းတမ်းများအားလေ့လာခဲ့သည်။ လေ့လာချက် (၃) တွင် BMP-3 အပေါ်သက်ရောက်မှုအား 3D pellet model စနစ်ဖြင့် လေ့လာ ခဲ့ပြီး DCs, hMSCs နှင့် မဖွဲ့စည်းမီ DCs နှင့် hMSCs (1:1နှုန်းထား) ဖြင့်ကြိုတင်ကုသပေးခဲ့သည်။ လေ့လာချက် (၄) တွင် hMSCs လက္ခဏာပြသသည့် အရိုးပြားများအပေါ်တွင် Conditioned Media (CM) နှင့်ချိတ်ဆက်သည့်တစ်ရှူးကြီး ထွားမှုအရင်းအမြစ် Connective tissue growth factor (CTGF) တို့ အားတွေ့ရှိရသည်။ CM ဖွဲ့စည်းပုံအား ထုထည်မီတာစနစ်ဖြင့် နောက်ဆက်တွဲဖော်ပြထားသည်။ လေ့လာချက် (၅) တွင် matrix metalloproteinase 1 (MMP-1) ၏ တစ်ရှူးအဆင့်များကို အရိုးပြားများအတွင်းရှိ enzyme-linked immunosorbent assay စနစ်ဖြင့် တွက်ချက်ထားသည်။ ယင်နောက်ပိုင်း လေ့လာချက် (၆) တွင် sEVs မှ DCs အပေါ် hMSCs သက်ရောက်မှုများကို လေ့လာရာခဲ့သည်။ နောက်ဆက်တွဲလက္ခဏာ များအတွက် flow cytometry နှင့် nanoparticle tracking analysis လမ်းကြောင်းများဖြင့်လေ့လာခဲ့သည်။ DC pellet များ၏ဆဲလ်ရှင်သန်မှု၊ ECM ထုတ်လွှတ်မှု၊ apoptosis၊ lactate dehydrogenase လုပ်ငန်းစဉ်- cytokine နှင့် chemokine စွန့်ထုတ်မှုများအားတွေ့ရှိရသည်။

ရလဒ်များ- hydrogel model (လေ့လာချက် ၁) အတွင်းရှိ hMSCs IL-1beta နှင့် BMP-3 ဖွံ့ဖြိုးဆဲလ် chondrogenic ကွဲပြားမှုများ မှာ 3D model လေ့လာချက် နှင့် တူညီသည် ။ BMP 3 အတွင်းရှိ DC pellets အတွင်း chongrogenesis တိုးမြင့်လာစဉ်တွင် ပိုမိုပြင်းထန်သည့် သက်ရောက်မှု တစ်ခုကို ပူးတွဲဖွဲ့စည်းပုံအတွင်းတွေ့ရှိရသည် (လေ့လာချက် ၃) ။ လေ့လာချက် ၂ တွင် DCs နှင့် hMSCs များအတွင်းရှိ ကန့်သတ် proliferation နှင့် ထိန်းညှိထားသည့် chondrogenesis အတွင်း ဇီဝကမ္မအဆင့်ပျော်ဝင်မှတ်အတွင်းရှိတွင်ပင်cortisolထိတွေ့မှုများရှိကြောင်းပြသနိုင်ခဲ့သည်။
ယင်းအချက်သည် ရှင်သန်မှုနှင့် DCs အတွင်း ECM ထုတ်လွှတ်မှုနှင့် ထုထည်မီတာ ဆန်းစစ်မှုများ အတွင်း (လေ့လာချက် ၄) ပါ 129 peptides ပမာဏ ကို ကျော်လွန်ခဲ့သည်။ လေ့လာချက် ၅ တွင် CM မှ MMP-1 အား DCs အတွင်းတိုက်ခိုက်နိုင်ကြောင်းပြသခဲ့သည်။ မည်သို့ပင်ဆိုစေ CM ၏ စွမ်းဆောင်ရည်မှာ တိုးမြင့်လာသည့် MMP-1 ပျော်ဝင်မှတ်အလျောက် နောက်ဆုံးတွင် ကျဆင်းခဲ့ရသည်။ လေ့လာချက် ၆ အရ apoptosis အားဖိအားပေးသည့်အလျောက် sEVs ဖွံ့ဖြိုးဆဲလ်၏ ရှင်သန်နှုန်းတိုးမြင့်လာပြီး ၊ sEVs ကုထုံးဖြင့် DCs အတွင်းကုသရာတွင် ECM ထုတ်လွှတ်မှုကိုလည်း တွေ့ရှိခဲ့သည်။

ကောက်နုတ်ချက်- hMSCs အတွင်းရှိ အမှတ်အသားပြအရင်းအမြစ်များတွင် DCs အတွက် ကောင်းကျိုးများရှိစေပြီး IVDs ယိုယွင်းမှုအတွင်း တည်ရှိသည့် မရောင်ရမ်းမီအတွင်းရှိ cytokines နှင့် enzymes များ၏ စွမ်းရည်များအား ယိုယွင်းမှုကို လျော့ပါးသက်သာလာစေသည်။ ယင်းနောက်ပိုင်းနာကျင်မှုများလျော့နည်းလာပြီး cortisol မှလည်ပတ်လုပ်ဆောင်သည့်ဖိ အားမှာ IVDs ယိုယွင်းမှုနှင့်ချိတ်ဆက်သည့်အရင်း အမြစ်တစ်ရပ်ဖြစ်လာခဲ့သည်။

บทคัดย่อภาษาไทย (Abstract in Thai)

บทนำ: อาการปวดหลังส่วนล่างเรื้อรังพบว่ามีความเกี่ยวข้องกับการเสื่อมสภาพของหมอน รองกระดูก (intervertebral discs) โดยการแสดงออกที่เพิ่มขึ้นของตัวบ่งชี้การอักเสบ cytokine เช่น interleukin-1beta (IL-1beta), เอนไซม์กลุ่ม matrix metalloproteinases (MMPs)ในหมอนรองกระดูก ที่เสื่อมสภาพนำไปสู่การสูญเสีย proteoglycan และ extracellular matrix (ECM) ซึ่งส่งผลกระทบต่ออัตราการมีชีวิตของเซลล์หมอนรองกระดูก (disc cells). แนวทางการรักษานั้นมีหลายทางอาทิ เช่นการใช้สารกระตุ้นการ เจริญเติบโต (growth factors), การรักษาด้วยเซลล์ (cell therapy) และการใช้ small extracellular vesicles (sEVs) ซึ่งแยกจาก stem cell ของมนุษย์โดยแนวทางการรัก ษาเหล่านี้จะช่วยพัฒนาการฟื้นฟูหมอนรองกระดูกโดยตรง

จุดประสงค์: เพื่อสำรวจผลกระทบของสารกระตุ้นการเจริญเติบโต (growth factor), signaling peptides จาก mesenchymal stem cells ของมนุษย์ และ sEVs ต่อหมอนเรองกระดูกที่เสื่อมสภาพในเชิงของอัตราการมีชีวิตรอดของเซลล์หมอนรอง กระดูกการผลิต ECM และเพื่อที่จะตรวจสอบผลกระทบของฮอร์โมนที่ส่งผลต่อความเครียดอย่าง cortisol ต่อเซลล์หมอนรองกระดูกและ stem cells ของมนุษย์ในหลอดทดลอง

วิธีการทดลอง; เซลล์หมอนรองกระดูกและ stem cells ถูกแยกจากเนื้อเยื่อของคนไข้แล้วนำไปเลี้ยงแบบ monolayer แล้วเลี้ยงแบบสามมิติในรูปแบบของ pellet จากนั้นทำการทดสอบอัตราการมีชีวิตของเซลล์,ศึกษาจุลกายวิภาคศาสตร์ของเซลล์ (histologi cal staining), และ immunohistochemistry. ในการศึกษาที่ 1, stem cells ถูกเลี้ยงใน hydrogel และถูกกระตุ้นด้วย สารกระตุ้นการเจริญเติบโตของเซลล์ bone morphogenetic growth factor 3 (BMP-3) หรือกระตุ้นด้วย IL-1beta ก่อนตามด้วย BMP-3 ซึ่งในการทดลองนี้วิธีการ *in situ* hybridization ได้ถูกใช้เพื่อตรวจสอบการแสดงออกของยีน COL2A1 และ OCT4. ในการทดลองที่ 2 เป็นการศึกษาผลกระทบของการเปลี่ยนแปลงระดับฮอร์โมน cortisol ที่เพิ่มขึ้นต่อเซลล์หมอนรองกระดูกและ stem cells ซึ่งทำการเลี้ยงเซลล์แบบสามมิติโดยมีการศึกษาการตายของเซลล์ด้วย apoptosis assay และการศึกษาการแสดงของออกของ cytokine ด้วยเทคนิค immunohistochemis try.ในการทดลองที่ 3 ซึ่งเป็นการศึกษาเพิ่มเติมจากการศึกษาที่ 1 โดยทำการเลี้ยงเซลล์ในรูปแบบสามมิติ เพื่อตรวจสอบผล กระทบของเซลล์หมอนรองกระดูก, stem cells, เซลล์หมอนรองกระดูก ที่เลี้ยงควบคู่กับ stem cells (ในอัตราส่วน 1:1) หลังจากกระตุ้นด้วยสารกระตุ้นการเจริญเติบโต BMP-3 หรือกระตุ้นด้วย IL-1beta ก่อนตามด้วย BMP-3. การทดลองที่ 4 เป็นการศึกษาผลกระทบของเซลล์หมอนรองกระดูกที่เลี้ยงแบบสามมิติหลังจากถูกกระตุ้นด้วย conditioned media(CM) ที่ได้จาก stem cells และสารกระตุ้นการเจริญเติบโต CTGF ส่วนประกอบของ CM นั้นถูกวิเคราะห์เพิ่มเติมโดยการใช้เทคนิค mass spectrometry. ในการศึกษาที่ 5 เป็นการหาปริมาณหรือความเข้มข้นของเอนไซม์ MMP-1 ในเนื้อเยื่อหมอนรองกระดูกโดยวิธี enzyme-linked immunosorbent assay จากนั้นทำการศึกษาความสามารถของ CM ในการยับยั้งฤทธิ์ของเอนไซม์ MMP-1 ในความเข้มข้นต่างๆ. ในการศึกษาที่ 6 เป็นการตรวจสอบผลกระทบของ sEVs ต่อเซลล์หมอนรองกระดูกโดยเซลล์หมอนรองกระดูกที่ถูกเลี้ยง

แบบสามมิตินั้นจะถูกกระตุ้นด้วย sEVs แล้วจึงนำไปศึกษาอัตราการมีชีวิตของเซลล์ การผลิต ECM การตายของเซลล์แบบ apoptosis การทำงานของเอนไซม์ lactate dehydrogenase การหลั่งของ cytokine และ chemokine ซึ่ง sEVs จะถูกแยกด้วย วิธีการ differential centrifugation แล้วนำไปศึกษาคุณสมบัติ ด้วยเทคนิค flow cytometry และวิเคราะห์ด้วย nanoparticle tracking analysis.

ผลการทดลอง: พบว่าการกระตุ้น stem cells ด้วย IL-1beta ก่อนตามด้วย BMP-3 นั้นส่งผลให้เซลล์พัฒนาไปเป็นเซลล์กระดูกอ่อน (chondrogenic differentiation) ในการเลี้ยงเซลล์ใน hydrogel (การศึกษาที่ 1) และเช่นเดียวกันกับในการเลี้ยงเซลล์แบบสามมิติ (การศึกษาที่ 3) โดยสารกระตุ้นการเจริญเติบโต BMP-3 นั้นช่วยในกระบวนการสร้างกระดูกอ่อน (chondrogenesis) ในเซลล์หมอนรองกระดูก ที่เลี้ยงแบบสามมิติและออกฤทธิ์ได้ดียิ่งกว่าในการเลี้ยงเซลล์แบบควบคู่ระ หว่างเซลล์หมอนรองกระดูกและ stem cells (การศึกษาที่ 3). จากการศึกษาที่ 2 พบว่าเซลล์หมอนรองกระดูกและ stem cells ที่ถูกเลี้ยงในสภาวะที่มีฮอร์โมน cortisol แม้ในระดับปกติเทียบเท่าในร่างกายมนุษย์ทำให้การเจริญเติบโต ของเซลล์และการพัฒนาไปเป็นกระดูกอ่อนนั้นมีการชะลอตัว.ในการศึกษาที่ 4 พบว่า CM ที่ได้จาก stem cells นั้นสามารถเพิ่มอัตราการมีชีวิตและการผลิต ECM ของเซลล์หมอนรองกระดูก ซึ่งจาก การวิเคราะห์โดยเทคนิค mass spectrometry พบว่า CM ประกอบด้วยมากกว่า 120 peptides นั้นมีค่าความชุกชุมสัมพัทธ์ (relative abundance) ในระดับที่สูง จากการศึกษาที่ 5 นั้นแสดงให้เห็นว่า CM มีความสามารถในการชะลอฤทธิ์ของเอนไซม์ MMP-1 ได้ในเซลล์หมอนรองกระดูกอย่างไรก็ตามความสามารถของ CM ลดลงด้วยความเข้มข้นของเอนไซม์ MMP-1 ที่สูงขึ้น. ในท้ายที่สุดจากการศึกษาที่ 6 แสดงให้เห็นว่า sEVs นั้นสามารถช่วยเพิ่มอัตราการมีชีวิตของเซลล์และในขณะเดียวกันลดการ ตายแบบ apoptosis ของเซลล์หมอนรองกระดูกได้ในช่วงแรกนั้นยังพบว่าเซลล์หมอนรองกระดูกมีการผลิต ECM มาก ขึ้นเมื่อถูกกระตุ้นด้วย EVs.

ข้อสรุป: signaling factors ที่ได้จาก stem cells ให้ผลในเชิงบวกโดยการบรรเทาการอักเสบจาก cytokine และเอนไซม์ที่มักพบในหมอนรองกระดูกที่เสื่อมสภาพ นอกจากนั้นยังพบว่าความเครียดที่เกิดจากความเจ็บปวดโดยฮอร์โมน cortisol ยังเป็นอีกหนึ่งปัจจัยที่ก่อให้เกิดการเสื่อมสภาพของหมอนรอง กระดูก

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Hingert D, Barreto Henriksson H, Brisby H. Human Mesenchymal Stem Cells Pretreated with Interleukin-1β and Stimulated with Bone Morphogenetic Growth Factor-3 Enhance Chondrogenesis. Tissue Eng Part A. 2018 May; 24(9-10):775-785.doi: 10.1089/ten. TEA.2017.0087

II. Hingert D, Nilsson J, Barreto Henriksson H, Baranto A, Brisby H. Pathological Effects of Cortisol on Intervertebral Disc Cells and Mesenchymal Stem Cells from Lower Back Pain Patients. Cells Tissues Organs. 2019;207(1):34-45.doi: 10.1159/000500772.

III. Hingert D, Barreto Henriksson H, Baranto A, Brisby H. BMP-3 Promotes Matrix Production in Co-cultured Stem Cells and Disc Cells from Low Back Pain Patients. Tissue Eng Part A. 2020 Jan; 26(1-2):4756.doi:10.1089/ten.TEA.2019.0125

IV. Hingert D, Nawilaijaroen P, Aldridge J, Baranto A, Brisby H. Investigation of the effect of secreted factors from mesenchymal stem cells on disc cells from degenerated discs. Cells Tissues Organs. 2019; doi: 10.1159/000506350

V. Hingert D, Nawilaijaroen P, Ekström K, Baranto A, Brisby H. Human levels of MMP-1 in degenerate discs can be mitigated by signaling peptides from mesenchymal stem cells. Submitted.

VI. Hingert D, Ekström K, Aldridge J, Crescitelli R, Brisby H. Extracellular vesicles from human mesenchymal stem cells expedite chondrogenesis in 3D human degenerative disc cell cultures. Submitted.

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ABBREVIATIONS

AC	Articular cartilage
ACAN	Aggrecan
ADAMs	A disintegrin and metalloproteinases with thrombospondin motif
AF	Annulus fibrosus
BMA	Bone marrow aspirate
BMP-3	Bone morphogenetic protein-3
CCK-8	Cell counting kit 8
CM	Conditioned media
COL2A1/COLIIA1	Collagen type II
CTGF	Connective tissue growth factor
CXCR2	Chemokine receptor 2
2D	Two dimensional
3D	Three dimensional
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DCs	Disc cells
DDD	Disc degeneration disease
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EVs	Extracellular vesicles

FGF-2	Fibroblast growth factor-2
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GC	Glucocorticoids
hMSCs	Human mesenchymal stem cells
HPA	hypothalamus-pituitary-adrenal
IHC	Immunohistochemistry
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-1R	Interleukin-1 receptor
ISCT	International Society for Cellular Therapy
ISEV	International Society for Extracellular Vesicles
ISH	*In situ* hybridization
IVD	Intervertebral disc
LDH	Lactate dehydrogenase
LBP	Low back pain
MMPs	Matrix metalloproteinases
MMP-1	Matrix metalloproteinase-1
MRI	Magnetic resonance image
MS	Mass spectrometry
MSCs	Mesenchymal stem cells

NP	Nucleus pulposus
NTA	Nano-particle tracking analysis
OCT4	Octamer-binding transcription factor 4
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PI	Propidium iodine
PRE-T	Pre-treatment
RADA	Arginine (R)-Alanine (A)-Aspartic acid (D)-Alanine (A)
RGD	Arginine (R)-Glycine (G)-Aspartic acid (D)
SEM	Scanning electron microscope
sEVs	Small extracellular vesicles
Sox9	SRY-Box Transcription Factor 9
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscope
TGF-β	Transforming growth factor-beta
TIMPs	Tissue inhibitor of matrix metalloproteinases
TIMP-1	Tissue inhibitor of matrix metalloproteinase-1
TRPS	Tunable resistive pulse sensing
TUNEL	TdT- mediated dUTP Nick End Labeling
UCF	Ultra-centrifugation
VEGF	Vascular endothelial growth factor
WB	Western blot

1 INTRODUCTION

Low back pain (LBP) is the leading cause of years lived with disability. The life time prevalence of the disease is as high as 84% and its burden is increasing worldwide due to the growing aging population [1-3]. The symptoms of LBP resulted in more severe disability in comparison to other diseases. The total cost of a single patient suffering from LBP has been estimated to be 20,700 euros per annum in Sweden alone [4] and is mainly due to the loss of productivity and sick leave [5]. Therefore, there is a strong need for research in this field.

One of the main causes of LBP is believed to be the intervertebral disc (IVD) degeneration disease (DDD) [2, 6]. In DDD, a loss of proteoglycans and degradation of collagen structure occurs due to matrix degrading enzymes such as matrix metalloproteinases (MMPs). The degradation of the matrix results in reduced disc height and dehydration of the disc tissue. The presence of pro-inflammatory cytokine like interleukin 1 beta (IL-1β) in the microenvironment of the IVDs also leads to a reduction in the number of chondrocytes and the scarcity of essential nutrients, such as oxygen and glucose [2, 7, 8]. Genetics and aging also play a role in IVD degeneration [9]. Psychological distress may intensify pain symptoms [10] and trigger a stress response in the endocrine system, nervous system, and immune system [11]. Chronic pain-induced stress may thereby lead to disruption of hormonal balance, decrease resistance to infection, delay wound healing [12] and induce the production of pro-inflammatory cytokines [13]. Besides, chronic stress triggers the production of glucocorticoids (GC) such as corticosterone (cortisol). Cortisol has been demonstrated to play a vital role in inhibiting the viability and differentiation of the cells in many disease models [14-16].

Current treatments for LBP involve exercise, physiotherapy and surgical interventions, which have limited effect in some patients and do not address the underlying mechanisms of IVD degeneration. Hence, it would be beneficial to develop new therapeutic alternatives that are less invasive and could possibly induce IVD regeneration [17, 18]. Mesenchymal stem cells (MSCs)-based therapies have been suggested due to their multi-lineage differentiation and immunomodulatory abilities [19]. One novel strategy is to inject MSCs into degenerated IVDs with or without the incorporation of growth

factors to boost differentiation [20, 21]. Growth factors such as transforming growth factor-beta (TGF-beta), bone morphogenetic growth factor 3 (BMP-3) and connective tissue growth factor (CTGF) have been reported as major factors in protein interaction networks that act as the main inducer of chondrogenesis [22]. However, some reports reveal that transplanted cells may not survive for long and that the observed therapeutic effects could be due to the vast array of bioactive factors secreted by the MSCs [23]. Extracellular vesicles (EVs) are secreted by the MSCs as mediators of intercellular communication in addition to their secretion of chemokines, cytokines, or growth factors. EVs have been shown to be able to drive regenerative processes in many diseases [24-26].

In this thesis, key aspects of cell interaction between MSCs and disc cells from degenerated IVDs, as well as the impact of various signaling factors such as growth factors, cytokine, hormone and small EVs (sEVs) on IVD regeneration were investigated.

1.1 The anatomy of the human spine and the intervertebral discs

The architecture of the human vertebral column consists of bony vertebrae interconnected with fibrocartilaginous discs as shown in figure 1. The structure of the spine contains 33 vertebrae, consisting of 5 sacral, 4 coccygeal, 5 lumbar, 12 thoracics, and 7 cervical vertebrae. The human spine possesses 23 fibrocartilaginous discs that are also known as the intervertebral discs (IVDs) [27]. The IVDs account for 30% of the total height of the spinal column and are essential for activities of the muscles such as bending, flexion, torsion and mechanical loading. An IVD in the lumbar region is approximately 7-10 mm in thickness with a diameter of 4 cm [1]. The IVD is composed of two main tissues, the nucleus pulposus (NP) and the annulus fibrosus (AF). The NP is a gelatinous core structure trapped within the inferior and superior cartilage end-plates and is made up of collagen fibers, mainly collagen type II with a mixture of elastin fibers. The water and proteoglycan give a hydrogel-like feature [2, 28, 29] and promote swelling pressure in the NP while collagen provides swelling resistance. The swelling property and high water content are also necessary to compensate for compression and mechanical loading. However, water and collagen content decreases as aging progresses [30].

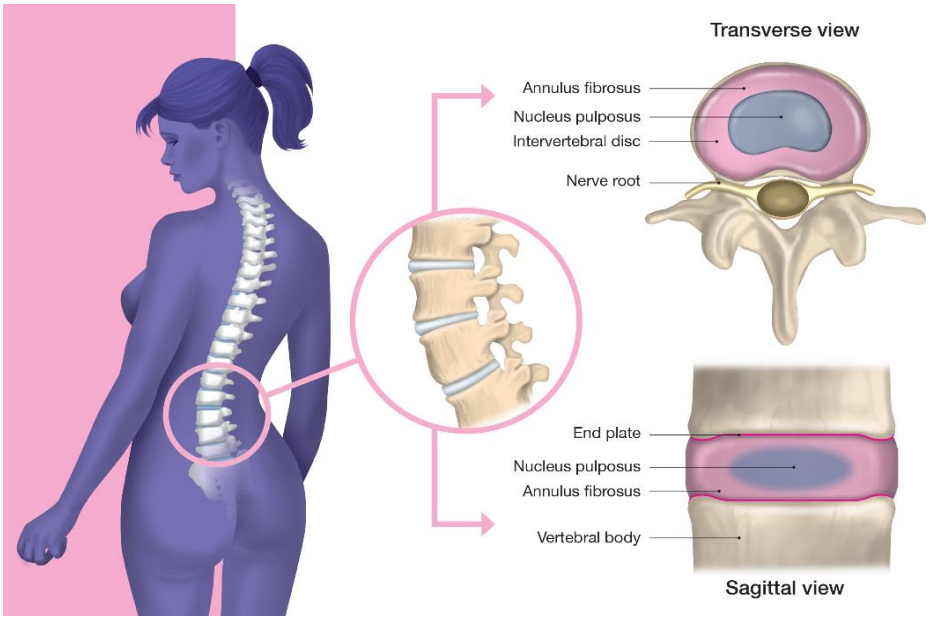

Figure 1. The anatomy of the human spine and the intervertebral discs

The AF is the ring that surrounds the NP consists of lamellae made up of organized collagen fiber, mainly collagen type I [31]. The elastin network within the lamellae assists in the readjustment of the disc to its native state following flexion or bending [32].

1.1.1 Cells within the intervertebral discs (IVDs)

The population of the cells in an adult IVD is about 4,000,000 cells/cm^3 in the NP and approximately 9,000,000 cells/cm^3 in the AF [30]. Similar to chondrocytes in articular cartilage (AC), NP cells have chondrocyte-like cells characteristics with plump morphology as shown in figure 2 and are identified by markers such as Sox-9, aggrecan (ACAN), and collagen type II (COL2A1) [33], etc. Sox-9 regulates the gene expression of COL2A1 and ACAN, which are the main ECM protein of the cartilage [34]. No specific markers for NP cells are available today as these markers can also be found in chondrocytes [35]. Hence, specific markers that can verify and distinguish the NP cells from AC cells are needed. The suggested markers used for NP cells are SOX9, COL2A1, ACAN, KRT19 and NCAM1 [35, 36].

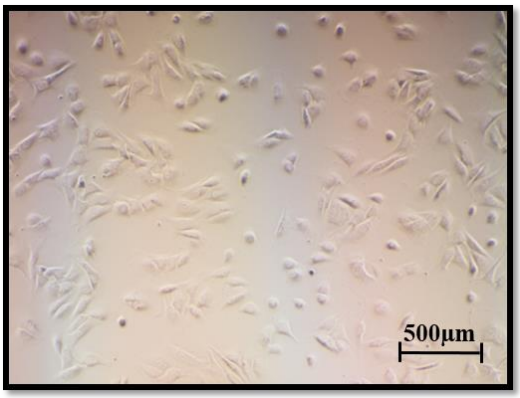

Figure 2. Image of disc cells isolated from degenerated IVD tissue in cell culture.

The cells in the AF are elongated fibroblast-like cells surrounded by collagen fibers [37]. The population of the immature cell is also found in this area of the IVD [38, 39]. These cells express progenitor and stem cell markers indicating a potential stem cell- niche in the outer area of the AF [40].

1.1.2 Extracellular matrix of the IVDs

The IVDs possess multiple types of collagen and proteoglycan within its extracellular matrix (ECM). The most prevalent types of collagen in the IVDs are collagen type I in the outermost layer of the AF and collagen type II in the NP [29]. Various proteoglycans in the IVDs are aggrecan (ACAN), glycosaminoglycan (GAG), versican, decorin, perlecan, fibromodulin, biglycan, lumican, etc. ACAN is found in both NP and AF and it is the most abundant proteoglycan [7]. The presence of ECM facilitates the interaction of the cells including cell migration and cell-cell contacts [30]. The ratio of proteoglycan to collagen in a healthy adult IVD is approximately 27:1 [41].

1.1.3 Proteinases and their inhibitors in the IVDs

Degradation of ECM is caused by proteolysis and this process is responsible for the physical and structural changes of the degenerating IVD. The matrix metalloproteinases (MMPs) are the most common native proteinases that break down the ECM within the IVDs and thereby contribute to IVD degeneration [30]. Members of the MMP family include collagenases (MMP-1/8/13), gelatinases (MMP-2/9), and stromelysin (MMP-3), which degrade collagen, versican, ACAN and even the linking proteins. A disintegrin and metalloproteinases with thrombospondin motif (ADAMs) is the close family member of MMPs, which also degrades versican and ACAN in the IVDs [30].

Matrix Metalloproteinase-1

Matrix metalloproteinase-1 belongs to the collagenase subfamily of the MMP family. It has the ability to breakdown collagen type II and is one of the main players responsible for collagen degradation during IVD degeneration [42]. Recent study reports increase levels of MMP-1 with increased severity of IVD degeneration [43].

Tissue Inhibitor of Metalloproteinases

The catabolic activities of MMPs are kept in check by tissue inhibitors of MMPs (TIMPs) in a positive feedback mechanism in the IVDs [44]. TIMPs regulate cell growth, tissue repair and ECM remodeling [45] and TIMP-1 is an important inhibitor of MMP-1 [46].

1.2 IVD degeneration

The process of IVD degeneration generally involves increased degradation of IVD's matrix by the MMPs and ADAMs, leading to changes in ECM composition within the NP and AF while threatening the viability of the cells in the IVDs [8]. This change in the biochemical composition of the discs triggers a drop in osmotic pressure [37] and swelling property of the IVDs matrix [32, 47]. The drop in hydration also decreases structural integrity and reduces the ability of the IVD to withstand physical loading [8]. Thus, degenerated discs decrease in height and bulges when subjected to physical loading [48].

Additionally this change in biochemical composition triggers the production of pro-inflammatory cytokines and MMPs. Increased production of the cytokines and MMPs accelerates the degradation rate of ECM while disrupting the secretion of tissue inhibitors of metalloproteinases (TIMPs) to maintain homeostasis [7]. The formation of cell clusters and morphological changes of the cells occurs in degenerated IVDs [49]. Furthermore, excessive necrosis, apoptosis, and autophagy are also reported [50, 51].

Ingrowth of nerves and blood vessels into the disc also occur during the process of degeneration especially in clefts and fissures in the outer regions of the IVDs. In severe cases, fissures are also detected within the NP [32]. Calcification of the endplate cartilage also occurs during IVD degeneration limiting the blood supply, which compromises the diffusion of oxygen and nutrients [7, 52]. Decreased oxygen supply triggers the production of lactic

acid [32, 52]. Accumulation of lactic acid over time decreases the pH in the environment affecting cell viability and ECM production in the IVDs [30].

Aging is one of the factors that contributes to the development of disc degeneration. In human, the level of degeneration was reported to be approximately 6% by the age of 20, 31% by 30, and 79% from the age of 60 [53, 54]. Environmental factors such as smoking cigarettes and physical loading are reported to be associated with IVD degeneration [37, 55]. Genetic inheritance also plays a role as several genes are reported to associate with DDD [37].

The severity of IVD degeneration can be classified by different scaling systems such as the Thompson or Pfirrmann grading scales, which are widely used in the clinics. The grading scheme of the Thompson scale is developed by evaluating the gross morphology of human IVD from the display of the disc tissues in histological sections [56]. The Pfirrmann grading system was on the other hand, widely used to evaluate morphologic and modic changes of IVD degeneration on magnetic resonance images (MRI). However, none of these grading schemes provide detailed information on the underlying symptom of the degeneration [57].

1.3 Current treatment for low back pain

Current treatments for IVD degeneration leading to LBP are symptomatic treatments. This includes exercise, physiotherapy, pain killers, and in patients with longstanding severe pain, disc replacement or spinal fusion surgery [58]. Surgery is recommended only if the pain is chronic and when physical therapy program over a longer period fails to alleviate the pain. Although surgery often relieves the pain, at least partly, the normal movement (segmental motion) of the spine is compromised due to the fusion of two or more spinal segments [59].

Other less invasive methods suggested are injections of protein and/or growth factors directly into the IVD with fluoroscopic guidance [60]. The rationale behind administering growth factors and proteins into the degenerated area is to stimulate anabolic responses that could reverse IVD degeneration processes [61]. Several *in vitro* and *in vivo* studies using this approach in small animals have shown satisfying results [62]. However, determining the right concentration remains a challenge as the physiological concentration is relatively low, especially in the avascular IVD. The limitations for such treatment options are also the short halftime of those proteins and risks for causing inflammation and ossification [60].

1.3.1 Cell therapy

In addition to administering proteins into the site of degeneration, cell therapy has been suggested as an approach that could potentially cease and/or reverse disc degeneration, especially in its early stage [60, 63]. The treatment strategy could involve injection/transplantation of healthy cells such as non-degenerated NP cells incorporated with growth factors into the affected region, with the ambition that these cells will repopulate the IVD and the growth factor may boost regeneration and eventually bring back homeostasis. However, obtaining healthy NP cells remains a challenge as harvesting these cells from healthy discs can induce disc degeneration [64].

MSC-based therapy

Cell therapy with the use of embryonic or adult stem cells maybe a viable approach for regeneration of the IVDs [63, 65]. However, the application of embryonic stem cells is restricted due to ethical controversies and their tendency for mal-differentiation and teratoma formation *in vivo* [66]. Mesenchymal stem cells (MSCs) - based therapy is gaining popularity in recent years due to their multipotency, immunomodulatory abilities and regenerative effects in many disease models [67-69]. MSCs could undergo chondrogenic differentiation into chondrocyte-like cells when stimulated with the right growth factor [70]. These cells, like NP cells, could produce ECM and when transplanted, has the potential to replenish the degenerated IVD [71, 72]. However, the limitations of this treatment approach are that transplanted cells may not survive for long after transplantation and could risk undergoing mal-differentiation or mutations [68, 73, 74].

1.4 Human mesenchymal stem cells (hMSCs)

Human MSCs were first discovered in the 1960s when a Russian scientist, Friedenstein, demonstrated the formation of new bone after transplanting bone marrow fragments or bone marrow suspension in diffusion chambers [75]. It was from these results where the bone marrow cell suspension was subsequently categorized into two main populations; hematopoietic stem cells and non-hematopoietic stromal cells [76, 77]. The role of non-hematopoietic stromal cells was thought to be involved merely with hematopoiesis [78]. However, differences in density independence, the capacity to adhere to plastic and to form fibroblast-like clonal growth (colony forming unit) were observed *in vitro* when compared to hematopoietic stem cells [76].

Later on, the ability of bone marrow stromal cells to differentiate into various mesenchymal lineage (multipotency) was reported. This means they are undifferentiated cells that can differentiate only within their lineage of origin i.e. endoderm, mesoderm and/or ectoderm [79]. They are found in adult human tissues and the cells that can differentiate into mesenchymal tissue (bone,

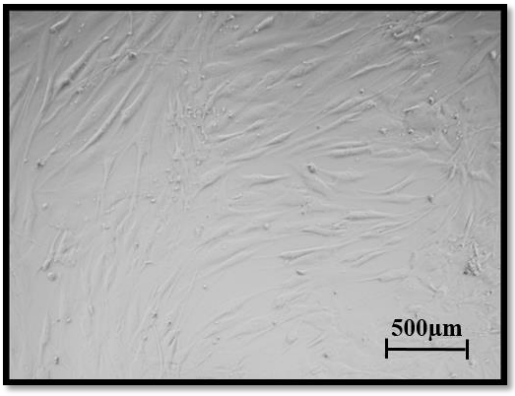

Figure 3. Image of hMSCs isolated from bone marrow aspirates of a LBP patient in cell culture.

muscle, cartilage, tendons, adipose, ligament, etc.) and are termed human mesenchymal stem cells (hMSCs) [80]. These hMSCs, as shown in figure 3, are referred to as bone marrow stromal cells when they are isolated from the bone marrow [81]. To date, hMSCs have been extensively studied [82] even though their differentiation potential is more limited compared to embryonic stem cells.

1.4.1 Sources and characterization of hMSCs

hMSCs were first discovered in bone marrow stroma, however, other tissues such as cell niches in the skin, synovial membrane, adipose tissue, dental pulp, muscle, uterine cervix, neonatal tissue, intestine, brain, umbilical cord and biological fluids (nasal mucosa, breast milk, peripheral blood, umbilical cord blood and menstrual blood) have been reported to be sources of hMSCs [83, 84]. Among these different sources, bone marrow derived hMSCs are the most studied, even though the scarcity of the tissue and the painful harvesting technique resulted in limited application [85]. Human MSCs from different sources are now used to conduct various studies, however, it was suggested that differences in the niches and tissue origin of the cells may partially reflect differences in multi-lineage differentiation ability [86-88].

The idea of standardizing the characterization of hMSCs has evolved due to the diversity and heterogeneity of hMSCs' sources. hMSCs have no single cell marker and therefore characterized by both the presence (positive) and absence (negative) of cell surface markers [81]. In addition, the International Society

for Cellular Therapy (ISCT) has proposed 3 criteria [89] for defining hMSCs as follow:

- hMSCs must exhibit the presence and absence of cell surface markers as shown in table 1.
- hMSCs must adhere to plastic surfaces.
- hMSCs must exhibit multipotency by differentiating into osteocytes, chondrocytes, and adipocytes *in vitro* and *in vivo* (mouse models).

Table 1. Cell surface markers for characterization of hMSCs

Positive Markers	***Negative Markers***
→ $CD73^+$	→ $CD45^-$
→ $CD90^+$	→ $CD34^-$
→ $CD105^+$	→ $CD14^-$ or $CD11b^-$
→ $CD166^+$	→ $CD79\alpha^-$ or $CD19^-$
→ $CD106^+$	→ $HLA\text{-}DR^-$
→ $STRO1^+$	

1.4.2 Multi-lineage differentiation of hMSCs

Various *in vitro* assays can be carried out to verify the multi-lineage differentiation ability of hMSCs [72]. Osteogenic differentiation can be induced by supplementing specific factors such as ascorbate-2-phosphate, dexamethasone, and beta glycerol phosphate into their culture system [90]. Adipogenic differentiation can be triggered by administering specific factors including indomethacin, dexamethasone, peroxis proliferator-activated receptor γ (PPARγ) insulin, and isobutylmethylxanthine in the media [72]. Their multi-lineage differentiation ability acts as a strong characteristic of stem cell, however, they do not maintain these features indefinitely. They lose their proliferative and differentiation ability with extensive sub-cultivation in *in vitro* over time [91].

1.4.3 Chondrogenic differentiation and its assessment techniques

Bone marrow derived hMSCs can be directed to differentiate into chondrocyte-like cells with specific stimulation during culture. A defined culture media is required with the addition of TGF-β, a growth factor crucial for chondrogenic differentiation. Together with these supplements, cell culture in three-

dimensional (3D) aggregates with high cell density that can induce a cell-cell interaction is important for chondrogenesis [92, 93]. hMSCs undergo chondrogenic differentiation within 14-21 days under these culture conditions [72]. After differentiation into chondrocyte-like cells, they start producing ECM, mainly proteoglycans and collagens, just like chondrocytes. To be specific, collagen type I are first produced and by day 5, collagen type II can be detected in the matrix [94]. By day 14, collagen type II and X are produced throughout the cells aggregates with collagen type I present in abundance at the outer layer of flattened cells. ACAN and linking protein can also be detected as time progresses [92, 95]. The expressions and the presence of these protein markers can be used to confirm the occurrence of chondrogenic differentiation in hMSCs.

In histological sections, chondrogenic differentiation can be assessed by Alcian blue van Gieson or toluidine blue staining of the aggregate, where sulphated glycosaminoglycans stains blue (Alcian blue) and collagen stains pink (van Gieson) as shown in figure 4. For the latter staining ECM stains purple and fibrous tissue stain blue [96, 97]. Immunohistochemistry (IHC) staining of multiple collagens can also be used to assess chondrogenic differentiation [98]. In general, chondrogenic cultures of hMSCs *in vitro* are typically harvested after 3-4 weeks depending on the objectives of the experiment.

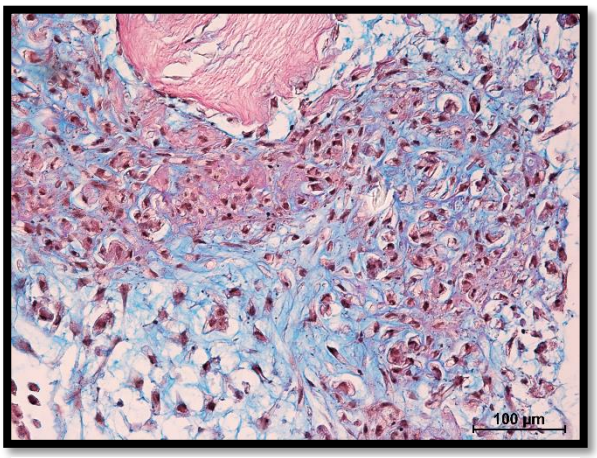

Figure 4. Histological evaluation of chondrogenic differentiation of hMSCs by Alcian blue van Gieson staining. Collagen component stains pink while proteoglycan stains blue. The black dots are the cell nuclei.

While the above-mentioned techniques are informative, they are merely qualitative and the interpretation of the result could be subjective. In order to assess a successful chondrogenic differentiation quantitatively, the ECM accumulation can be measured by the GAG content in the cell aggregates/pellets through GAG assay [94]. The number of cells can be determined by measuring DNA content by DNA assay. The amount of ECM production per cell can then be evaluated quantitatively by normalizing the amount of GAG to its DNA content [94]. Alternatively, the chondrogenic process can be evaluated by the gene expression level of Sox5, 6, 9, COL2A1 genes in the pellets using the polymerase chain reaction method [91, 99].

1.5 Secretome from hMSCs

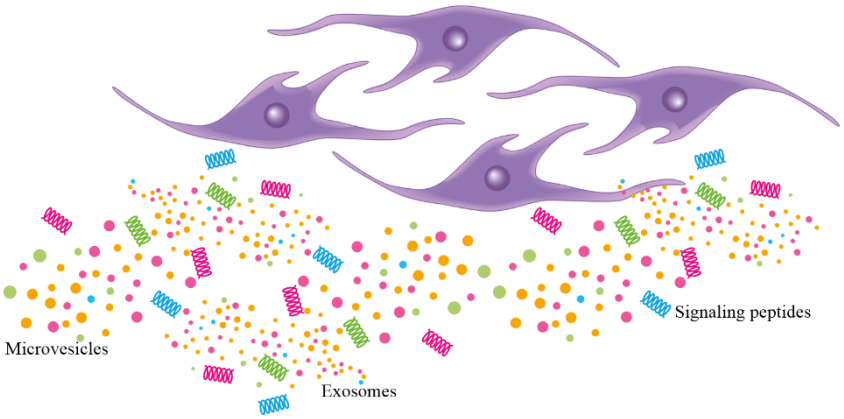

Figure 5. Illustration of hMSCs and their ability to secrete multiple bioactive factors.

Recent studies have brought attention to the paracrine signaling mechanism of the hMSCs as shown in figure 5 where they produce a vast array of bioactive factors that may have a therapeutic impact in many disease models [23, 100]. Hence, the constituent of the secretome from the hMSCs has captivated the attention of researchers for its potential usage in tissue regeneration and repair [101-104]. The secretome is defined as sets of factors/peptides such as soluble protein, growth factors, free nucleic acids, lipids, extracellular vesicles (EVs) secreted to the extracellular space [105]. hMSC conditioned media (CM) has been shown to contain secretome such as soluble components capable of independently signaling and mediating repair and regeneration [102, 106]. The secretion is tissue-specific and fluctuates in response to changing physiological states and pathological conditions of the cells [85].

1.5.1 hMSC derived extracellular vesicles

Extracellular vesicles (EVs) are the generic term for the component of cellular secretions with a lipid bilayer deprived of a functional nucleus found in biological fluids [107]. They are secreted by cells continuously or after stimulation, for instance, calcium flux, cellular stress, etc. These EVs also play a role in tissue homeostasis and intercellular communication. EVs take part in tissue repair, immunomodulation, and proliferation [108]. They are involved in transporting proteins, lipids, nucleic acids between cells [109]. EVs have the ability to not only exchange biological material between surrounding cells but also travel long distances disseminating genetic content among distal organs and regulating gene expression of the host tissues [110]. EVs also relay messages including regeneration, resistance to apoptosis, and induction of intrinsic repair cascades of injured cells. Furthermore, an inflammatory environment could impact the composition of EVs and the biological activities of immune effector cells. Different kinds of EVs are classified according to their size and biogenesis [111]. The largest EVs are called apoptotic bodies as they are secreted after cell apoptosis and are from 1 to 5µm [108]. EVs from 0.1 to 1µm are known as micro particles, ectosomes, or microvesicles. They are produced by cells during metabolic changes or stress. Microvesicles, unlike the apoptotic bodies, do not contain fragmented DNA [112]. Smaller vesicles (exosomes), 30-100nm, are secreted continuously regardless of the cellular condition [108]. However, the international society for extracellular vesicles (ISEV) has recently endorsed that operational terms of the subtypes of EVs be related either to physical characteristics, biochemical composition, or description of conditions and derivative cells [107]. EVs of sizes within >100nm <200nm are termed small EVs (sEVs) while the ones > 200nm are termed large and/or medium EVs [107]. EV markers such as CD63 and/or CD81 are also suggested to characterize the biochemical composition of the EVs.

Multiple techniques such as differential ultracentrifugation (UCF), size exclusion chromatography, density cushion are used to isolate EVs/exosomes from CM of hMSCs [113, 114]. The method of isolation influences the type, amount and purity of EVs recovered [114]. Until the turn of the year 2015, UCF is the most widely used methods involving low-speed centrifugation (300g, 500g, or 2,000g) of the CM as the first step to remove dead cells and cell debris. Medium speed centrifugation (10,000-20,000g) is further applied on the supernatant to eliminate large vesicles and lastly, sEVs/exosomes are pelleted using high-speed ultracentrifugation (100,000-120,000g) [115]. Also, the use of UCF is often-times complemented with other techniques such as density gradients, size exclusion chromatography, filtration, precipitation, and

immune-isolation [114]. In recent years, various additional techniques are emerging in order to achieve better recovery and specificity [107].

In order to ensure successful isolation of the EVs, it is recommended to perform multiple verification techniques determining the morphology, size, density, and specific proteins. Transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy or cryo-electron microscopy are used widely to visualize the EVs isolated as shown in figure 6 [114]. In addition to morphological evaluation, the size and density of the EVs can be determined using nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), or dynamic light scattering (DLS) methods. The difference in the mode of actions between these techniques is that NTA collects information from individual vesicle scattering from a laser beam overtime [116], TRPS measures changes in the ionic current generated by the transport of vesicles through a size-tunable nanopore [117], while DLS detects bulk scatter light from vesicles in Brownian motion [118]. However, NTA and TRPS are more favorable as they can quantify the concentration of vesicles for further calculation of the total number of vesicles isolated [119].

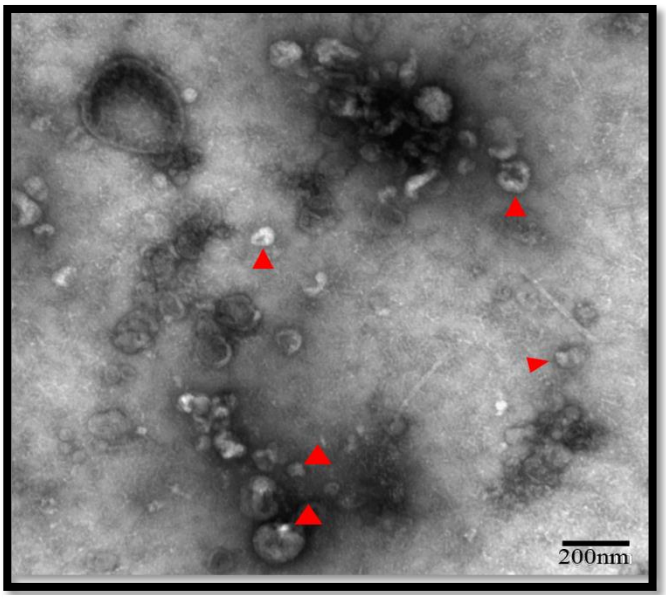

Figure 6. Image illustrating extracellular vesicles secreted from hMSCs observed under a transmission electron microscopy. Extracellular vesicles of different sizes can be seen as indicated by the red arrows. EV isolation by D. Hingert, image captured by R. Crescitelli.

Additionally, light microscopic single EV analysis method can be employed to visualize immobilized EVs inside a microfluidic chamber [114]. In recent years, the use of nano-flow cytometry has emerged as it allows multi-parameter analysis of single EV up to the size of 40nm [120, 121].

Bulk analysis of EV proteins/markers can be detected using flow cytometry and Western blotting (WB) [122]. Beads bound flow cytometry using micro-beads conjugated with antibodies such as CD9, CD63, and CD81 is used due to limited sensitivity and resolution of the conventional flow cytometry [121, 123].

1.6 Selected growth factors and cytokine that are of importance in regulating chondrogenesis

Growth factors are signaling molecules composed of polypeptides that regulate various cell processes [124]. They are secreted by the cells to mediate cell-cell communication. These growth factors can diffuse and bind to specific cell membrane receptors on neighboring cells (paracrine) or the secreting cells (autocrine) [125]. This binding triggers cellular signaling cascades regulating proliferation, differentiation, ECM production, migration, etc. Growth factors can bind to different types of receptors conveying different signals with different cell types. The cells can, however, react differently depending on the dose of the stimulating growth factor, the stage of the signaling pathways (active or inactive) and the phase of the cell cycle. [124].

1.6.1 Transforming Growth Factor β

Transforming growth factor β (TGF-β) is regarded as the main inducer of chondrogenesis in hMSCs and it is one of the main regulating factors of cartilage and bone development [126]. It is also supplemented in defined media to induce chondrogenesis in hMSCs in *in vitro* cultures [94].

1.6.2 Bone Morphogenetic Protein 3

Bone morphogenetic protein 3 (BMP-3) is a member of multivariate BMPs family belonging to the superfamily of TGF-β, which can induce cartilage and bone formation [127]. BMP-3 regulates the proliferation of cartilage cells and the formation of endochondral bone during skeleton development. It also promotes the proliferation of hMSCs and chondrocytes through the TGF-β signaling pathway [128, 129].

1.6.3 Fibroblast Growth Factor

Fibroblast growth factor (FGF-2) enhances chondrogenic potential and maintain the multi-lineage differentiation ability of hMSCs in culture. It also facilitates the proliferation of hMSCs, preserving their immature state [130, 131]. FGF-2 further enhances the effect of BMP-3 [128] and promotes collagen synthesis in tendon tissues [132].

1.6.4 Connective Tissue Growth Factor

Connective Tissue Growth Factor (CTGF) belongs to the cysteine-rich protein CCN family. It induces the development of cartilage and bone formation as well as chondrogenesis [22]. It is reported to work synergistically with TGF-β in promoting ECM production [133].

1.6.5 Interleukine-1β

IL-1 was first discovered in 1940 as a 'fever stimulating substance' secreted by immune cells [134]. IL-1 family consists of 11 members and each member modulates different innate inflammatory and immune responses. IL-1β is reported to be the most vital cytokine in provoking IVD degeneration [134]. It activates the secretion of multiple pro-inflammatory mediators (cytokines, chemokines, and MMPs) responsible for pathological activities [134]. Significantly high expression of IL-1β is reported to be found in degenerated IVD tissues and cells [135]. IL-1β is also involved in various pathological processes such as inflammatory responses, ECM degradation, oxidative stress, cellular senescence, cellular apoptosis, and angiogenesis/neo-innervation [134].

1.7 Hydrogels as a cell carrier

Hydrogels are used as a scaffold or as a substitute for ECM in experiments to mimic the micro-environment in the tissue to enable cell attachment, proliferation, differentiation, and migration [136, 137]. Hydrogels are crosslinked, water-rich 3D macromeres structure that can be obtained from nature or manufactured synthetically. Common hydrogels are calcium alginate, agarose, and collagen gel [138]. Cells are sometimes seeded on or encapsulated in a hydrogel upon transplantation into the site of injury e.g. an IVD [20]. Hydrogels are also reported to influence the direction of differentiation in hMSCs [139].

1.7.1 PuraMatrix™

PuraMatrix™ is a self-assembling peptide consists mainly of water (99%) and repeating sequence of amino acids ((1% w/v) of Arginine (R)-Alanine (A)-Aspartic acid (D)-Alanine (A)) forming RADA sequence commercially available in the market as shown in figure 7 [140]. The RADA sequence mimics the RGD sequence (Arginine (R)-Glycine (G)-Aspartic acid (D)), which serves as ligands for cell attachment [141]. The pore size is around 5-200nm [140]. It has been reported to induce chondrogenesis of bovine MSCs *in vitro* [142] and in the equine model [143, 144]. *In vivo* studies also showed survival of encapsulated hMSCs in PuraMatrix for at least 6 months after injection into the IVDs of the injured porcine disc [20].

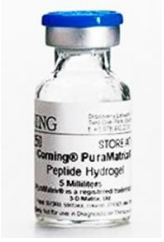

Figure 7. PuraMatrix™ hydrogel (PuraMatrix™, 2016)

1.8 Pain and Stress

Pain is the primary symptom of LBP provoking patients to seek out treatments and its attenuation commonly defines a successful treatment [145]. Nociceptive pain is primarily positive, meaningful, and necessary for survival. But it loses its positive function when it is prolonged and sensitized. Perpetual long term pain turns into a disabling and catastrophic phenomena that might lead to excessive stress and even more pain [11]. This adds on to the vicious cycle of pain and stress as increased psychological stress increases the risk of LBP [9]. It is reported in animal models that chronic pain is associated with activation of the hypothalamus-pituitary-adrenal (HPA) system where chronic pain serves as an unavoidable stressor [146].

Stress, also known as 21st century health epidemic is defined as experiences that are uncontrollable, unpredictable or a threat to one's ego [13]. Initially, stress-induced psychological changes serve an adaptive role as the body tries to achieve homeostasis despite the stressor, but an increase in allostatic load has detrimental effects on the host. These include exacerbation and development of other ailments such as hypertension, cardiovascular diseases,

obesity, LBP, and even reduced fertility and poor pregnancy outcome in females [147]. Psychological stress is first perceived by the HPA. As the first response to stressors, the hypothalamus in the brain secretes the corticotrophin-releasing hormone. This hormone triggers the pituitary gland to release adrenocorticotrophine into the bloodstream. When the hormone reaches the adrenal glands, the adrenal cortex secretes cortisol. Corticosteroids regulate many organs and brain areas through mineralocorticoid receptors and the glucocorticoid (GC) receptors [148]. As the body's adaptive response to stress (allostasis), pro and anti-inflammatory cytokines are secreted by different cells and they coordinate each other [13]. As the HPA system is receptive to both acute and chronic stress, corticoids are suggested as suitable biomarkers for stress [149]. To assess psychosocial stress, cortisol level is measured in biological samples for current systemic concentration in saliva and blood or cumulative/overall secretion over time in urine and hair [148].

1.8.1 Cortisol

Cortisol, also known as corticosterone is a glucocorticoid (steroid hormone) secreted by adrenal glands due to different stimuli as mentioned above. Glucocorticoids are widely used as biomarkers for stress [147]. In both human and non-human primates, cortisol is the most common glucocorticoid, whereas corticosterone is the main stress hormone in other vertebrates [147, 150]. In healthy physiological concentration, cortisol plays a role in mobilizing energy stores and regulating the immune system [146]. However, prolonged exposure to cortisol has been shown to compromise the differentiation ability of MSCs as well as cause apoptosis in chondrocytes [14, 16].

1.9 *In vitro* models

Over the last century, cell-based research has been performed on multiple surfaces modified to promote cell growth in two-dimensional (2D) monolayers [151]. These 2D cultures are used for various *in vitro* experiments and have proven effective as they provide a convenient techniques for treating and analyzing cells [151]. However, 2D cultures do not allow the formation of multicellular structures, which mimic the microenvironment found *in vivo* [152, 153]. They also do not permit cell-cell interaction based on mechanical and biochemical cues due to the absence of a true ECM [154]. ECM is a crucial cellular factor that is critical for the regulation of homeostasis as it influences cellular viability and outcomes of stimulation to soluble factors. Hence, biologically more relevant *in vitro* models, such as three-dimensional (3D) culture system was developed. 3D cell cultures represent a closer resemblance to the microenvironment observed in *in vivo* [155]. They also provide relevant

cell-cell and cell-ECM signaling as such signaling is important for multiple cellular processes including proliferation and differentiation [155, 156].

1.10 Gaps in knowledge associated with research questions

→ #1. What effect does cortisol have on hMSCs and DCs on a cellular level?

→ #2. What effect does pre-exposure to pro-inflammatory cytokine have on hMSCs' differentiation and chondrogenesis?

→ #3. Can growth factors be used to enhance chondrogenesis in mesenchymal stem cells and disc cells from LBP patients *in vitro*?

→ #4. Positive effects seen on disc cells in co-culture with hMSCs – What causes these effects?

→ #5. How do hMSCs exert their therapeutic effects on DCs? What do they secrete and what is the outcome of stimulating disc cells with the secretome of hMSCs?

2 AIM

The overall aim of this thesis was to identify and better understand the role of signaling factors that can ameliorate and reverse IVD degeneration in order to pave ways for treatment strategies where these signaling factors can be applied to directly influence IVD regeneration.

2.1 Specific Aims

- Investigation of the underlying effects of BMP-3, and IL-1β on hMSCs and DCs in terms of proteoglycan accumulation, cellular differentiation of hMSCs and cellular cross-talk between hMSCs and DCs in hydrogel environment and 3D pellet system.

- Investigation of the impact of cortisol on hMSCs and DCs.

- Investigation of the outcome of hMSCs secretome on DCs and hMSCs in chondrogenesis

- Investigation of the effect of hMSC derived extracellular vesicles on DCs from degenerated discs.

3 METHODS

3.1 Patients and human tissues collected

Bone marrow aspirates (BMA) from the iliac crest and disc tissues from degenerated IVDs were collected from patients undergoing spinal fusion surgery including the removal of the disc(s) as part of the surgical procedure. These patients have long-standing low back pain and were diagnosed with degenerated disc disease (table 2). The disc tissues were harvested from the center of the disc. The disc tissues used in Studies IV and V were previously graded with the Pfirrmann grading system on MRI.

Table 2. Donor information and details of samples collected as well as the use of donor tissues in different studies conducted in this thesis. All the donors were diagnosed with disc degeneration disease (DDD).

Donor	Gender	Age	Samples collected	Study
1	Female	32	BMA, disc tissue	I, II, III
2	Male	50	BMA, disc tissue	I, III
3	Male	44	BMA	I
4	Female	40	BMA, disc tissue	II
5	Female	34	BMA, disc tissue	II, III, VI
6	Male	37	BMA	II, IV
7	Male	31	BMA, disc tissue	II, V
8	Female	38	BMA, disc tissue	III, VI
9	Female	28	BMA, disc tissue	III, IV, V, VI
10	Female	50	BMA	III
11	Male	51	BMA	III
12	Male	32	Disc tissue	III, IV
13	Female	48	BMA, disc tissue	IV, V, VI
14	Female	36	Disc tissue	V
15	Male	46	Disc tissue	V
16	Male	34	Disc tissue	V
17	Female	49	Disc tissue	V
18	Female	56	Disc tissue	V
19	Female	46	Disc tissue	V
20	Female	32	Disc tissue	VI
21	Male	34	Disc tissue	VI

3.1.1 Ethical Permission

All the patients provided informed consent and all the studies were approved by the Regional Ethics Review Board at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden (Dnr: 532-04).

3.2 Isolation of hMSCs and DCs from human tissues

Bone marrow aspirates (BMAs) were transferred to cell separation Ficoll tubes and centrifuged by density gradient centrifugation (470g, 20 minutes). The mono-nuclear phase was collected and cultured (37℃ and 5% CO_2) in MSC media containing DMEM-low glucose media (Thermo Fisher Scientific, MA, USA) supplemented with penicillin/streptomycin, L-glutamine, 5ng/mL FGF (Thermo Fisher Scientific) and 10% human serum.

Disc tissues were cut into small pieces and digested with type II collagenase (Gibco Life, MA, USA) overnight. The cell suspension was then centrifuged (470g, 4℃, 5 minutes) and cultured (37℃ and 5% CO_2) in DC media containing DMEM-low glucose supplemented with penicillin/streptomycin, L-glutamine (Thermo Fisher Scientific), and 10% human serum.

For both cell types, the media was changed every 2-3 days and the cells were passaged at 90% confluency. The cells used were between passages 3-7 (Studies I-VI).

3.3 Characterization of hMSCs

The phenotypical morphology, proliferation and chondrogenic differentiation of hMSCs isolated from BMA were characterized in Studies I-IV. The morphology was assessed through light microscopy in passages 2-3. Proliferative ability was assessed through proliferation assays and chondrogenic differentiation through histological staining and immunohistochemistry as described in section 1.4.3.

The phenotypic characteristics of hMSCs were analyzed in Studies V-VI with a BD Stemflow hMSC analysis kit by flow cytometry (BD FACSVerse™ instrument, BD Biosciences, CA, USA) to confirm the positive expressions of CD73, CD90 and CD105 and negative expressions of CD19, CD11b, CD34, CD45, and HLA/DR. The data were further analyzed using FlowJo™10 (Flow Jo, LLC, Ashland, OR, USA). All the hMSCs analyzed were in passages 3-5.

3.4 *In vitro* models and chondrogenic induction

3.4.1 *In vitro* model – monolayer culture

Monolayer culture was used to expand the cells after isolation. In addition this system was also used to study the cells in Study II where both hMSCs and DCs were seeded at a density of 100,000 cells per well in 6 well-plates (Corning, NYC, USA) and cultured (37℃ and 5% CO_2) for 2 weeks. Media was replaced 2-3 times a week and passaged at 90% confluency.

3.4.2 *In vitro* model – encapsulation of cells in hydrogel

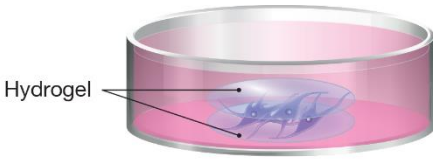

Figure 8. Illustration of hMSCs encapsulation in the hydrogel model used in Study I.

Approximately 300,000 hMSCs were re-suspended in 10% sucrose solution and seeded on top of PuraMatrix™ hydrogel [140]. Another layer of hydrogel was placed on top of the cell forming a 'sandwich-like' structure as shown in figure 8. The hydrogel solidified upon coming in contact with the sucrose solution. This encapsulation technique was carried out in a cell culture insert in a 24 well-plate (Corning) in triplicates. The media was changed every 2 days and the encapsulated cells were cultured (37℃ and 5% CO_2) for 28 days and harvested at day 7, 14 and 28 (Study I).

3.4.3 *In vitro* model – 3D pellet culture

Approximately 200,000 cells were placed in a conical tube (Corning) and centrifuged at 473g for 5 minutes and incubated to allow spheroid formation as shown in figure 9. Both hMSCs and DCs formed rounded cell pellets (spheroids) in this *in vitro* model. The pellets were cultured (37℃ and 5% CO_2) for 28 days and harvested on days 7, 14, and 28. The pellets were cultured in four replicates from each donor cells (Studies II-VI).

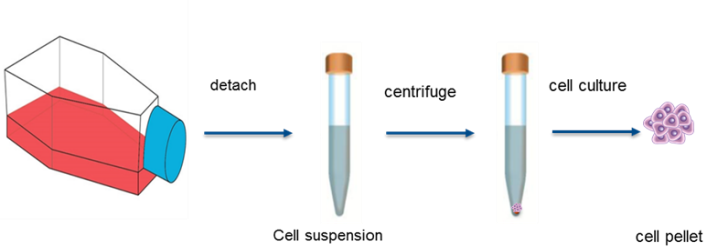

Figure 9. Procedures for cultivation of 3D pellets.

3.4.4 *In vitro* model – 3D co-culture

To study cell-cell interaction between hMSCs and DCs, 3D pellets were cultures as mentioned in 3.4.3 with hMSCs and DCs in a 1:1 ratio as shown in figure 10. The pellets were cultured for 28 days and harvested at days 7, 14, and 28. Four replicates from each donor cells were cultured (Study III).

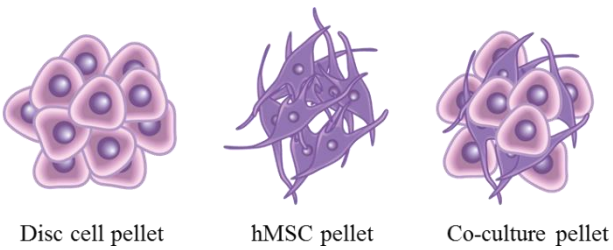

Figure 10. Illustration of DC, hMSC and co-culture pellets.

3.4.5 Chondrogenic induction

To induce chondrogenesis *in vitro* (Studies I-VI), the cells were cultured in chondrogenic media containing DMEM-high glucose (Life Technologies, CA, US), supplemented with dexamethasone (Sigma-Aldrich, MO, USA), insulin, selen, transferrin (Gibco Life), 10ng/mL TGF-β (R&D systems, UK), human serum albumin (Equitech-Bio, TX, USA), penicillin/streptomycin (Gibso Life), linoleic acid (Sigma-Aldrich), and L-ascorbic acid (R&D systems).

3.5 Conditioned media collection

Conditioned media (CM) used in Studies IV and V were media collected from cultured hMSCs from 3 donors cultured in serum containing media as depicted in figure 11. The hMSCs were seeded at a density of approximately 10,000 cells/cm^2 and CM was harvested every 48 hours. The cells were passaged at 90% confluency. The collected CM was then centrifuged at 300g for 5 minutes

to eliminate cell debris and the supernatants from all 3 donors were sterile-filtered, pooled and stored at -80°C. The hMSCs used in these studies were in passages 4-5. In study VI, CM was collected from hMSCs from 1 donor

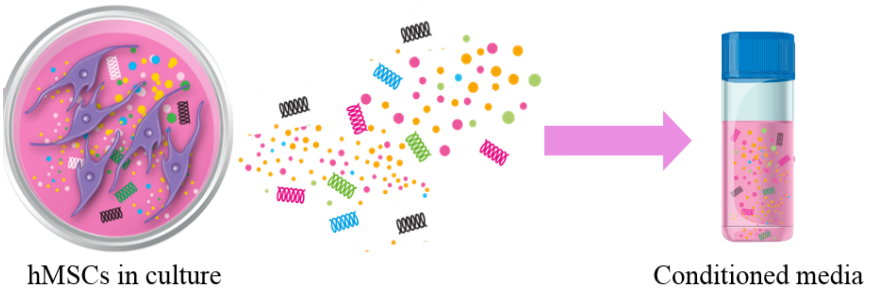

Figure 11. Graphical illustration of conditioned media collection from hMSCs culture.

cultured in serum-free growth media commercially available as NutriStem XF medium (Biological Industries, Kibbutz Beit-Haemek, Israel). The CM was collected every 2-3 days. The collected CM was then centrifuged (300g, 4℃, 5 minutes) and stored at -80℃ until use. The hMSCs were in passages 4-7.

3.6 Isolation of extracellular vesicles

Extracellular vesicles (EVs) were isolated from the serum-free CM using a series of differential centrifugation/ultra-centrifugation (UCF) technique and filtration (Study VI). The technique includes centrifugation of CM at 16,500g for 20 minutes followed by filtration through 0.2µm filters to eliminate debris and large vesicles. The filtered supernatant was then ultra-centrifuged at 120,000g for 70 minutes in a T-645.5 rotor (Sorvall wx Ultra series). The small EV (sEVs) pellets were then re-suspended in ice-cold phosphate buffer saline (PBS) and kept at -80°C. The entire procedure was performed at 4℃.

3.7 Characterization of extracellular vesicles

3.7.1 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to determine the concentration and size distribution of the EVs applying the NanoSight LM10/LM14 system (NanoSight Ltd, Malvern, UK). Six batches of sEVs were isolated and were analyzed with NTA with appropriate dilution made with PBS prior to the analysis. An automatic injection of the samples for each capture was applied

by a syringe pump and three 60 seconds videos were recorded and the analysis performed by NanoSight NTA 3.2 software. The particle concentration and size distribution were presented in a histogram depicting the average concentration per mL ± SEM and mean size ± SEM in nanometer (nm) respectively. The total number of particles in the stock solution was back-calculated based on NTA results (Study VI).

3.7.2 Flow cytometry

Small EVs (10µg protein content) were incubated with 100,000 CD63-coated magnetic beads (Thermo Fischer Scientific) overnight with gentle agitation. The beads-bound sEVs were washed and incubated with CD9-APC-Vio-770, CD63-PE-Vio-770, and CD81-PerCP-Vio700 (Miltenyi Biotec, Bergisch Gladbach, Germany) or corresponding isotype control for 40 minutes at room temperature with frequent gentle agitation. After washing the beads-bound sEVs for three times, the samples were acquired with FACSVerse (BD Bioscience) for the presence of EV markers such as tetraspanins CD9, CD63, and CD81. Data analysis was conducted with FlowJo™10 (Study VI).

3.7.3 Transmission electron microscopy

Small EVs (10µg protein content) were deposited on glow discharged 200-mesh formvar/carbon copper grids (Electron Microscopy Sciences, Hatfield Township, USA). The sEVs were fixed with 2.5% glutaraldehyde after washing twice in distilled water. The samples were then stained with 2% uranyl acetate for 90 seconds after two more washes and visualized under a transmission electron microscopy (TEM) with digitized LEO 912AB Omega electron microscope (Carl Zeiss SMT, Oberkochen, Germany) equipped with a Veleta CCD camera (Olympus-SiS, Münster, Germany) (Study VI).

3.7.4 Western blot

An equal amount (20µg) of proteins from sEVs and hMSCs were loaded and separated on precast 4-20% Mini-Protean TGX gels and transferred to PVDF membranes (Bio-Rad Laboratories, Sweden). EveryBlot blocking buffer (Bio-Rad Laboratories, Sweden) was used to block the membranes to prevent unspecific binding of the protein. Primary antibodies such as Grp94 (1:1000 dilution) (Enzo, Solna, Sweden), anti-CD63 (1:1000) (BD Biosciences), anti flotillin-1(1:1000) (Abcam), anti-CD81 (1:1000) (Abcam) and anti-Tom20 (1:200) (Santa Cruz Biotechnology, TX, USA) were added to the membranes and incubated at 4°C overnight. The membranes were then washed and incubated for 60 minutes with secondary antibodies such as anti-grp94 anti-rat IgG (1:5000) (Harlan Sera-Lab, Loughborough, UK), anti-CD63, anti-CD81,

and anti-Tom20 anti-mouse IgG (1:5000) (Harlan Sera-Lab), and anti-flottilin-1 anti-rabbit IgG (Harlan Sera-Lab). The specific proteins were then detected using a ChemiDoc Imaging System (Bio-Rad laboratories).

3.8 *In vitro* stimulation of cells with signaling factors

In order to study the effects of signaling factors on influencing hMSCs and DCs towards chondrogenesis, different signaling factors were administered to the cell media in multiple *in vitro* models and studied as follow:

Table 3. The different signaling factors and cell release factors investigated in studies I-VI.

Signaling factor	Type	Dosage in media	Recipient cells	*In vitro* model used	Study
BMP-3	Growth factor	1-10ng/mL	hMSCs, DCs	Hydrogel, 3D pellet, co-culture	I, III
IL-1β	Cytokine	1-10ng/mL	hMSCs, DCs	Hydrogel, 3D pellet, co-culture	I, III
Cortisol	Hormone	150-300ng/mL	hMSCs, DCs	Monolayer, 3D pellet	II
CTGF	Growth factor	10ng/mL	hMSCs, DCs	3D pellet	IV
CM	Soluble proteins, EVs	1:1 ratio with chondrogenic media	DCs	3D pellet	IV, V
MMP-1	Collagenase	5-100ng/mL	DCs	3D pellet	V
sEVs	Vesicles	$5x10^{10}$ vesicles/mL	DCs	3D pellet	VI

3.9 Histological investigation

Tissues and cell pellets were fixated with 4% formaldehyde immediately after harvesting and sent for paraffin embedding at HistoCenter AB, Sweden. The paraffin-embedded sections, which were between 5-10μm in thickness were then used to investigate the morphology, ECM accumulation, and expressions of multiple genes and proteins.

3.9.1 Histological staining

Alcian blue van Gieson combined staining (Studies I-VI) and Von Kossa (Study I) staining of the cell pellets and tissues were performed by HistoCenter AB, Sweden.

3.9.2 *In situ* hybridization

In situ hybridization (ISH) is a combination of molecular biological and histochemical techniques that detects gene expression in tissue sections through the hybridization of a labeled nucleotide probe, which binds to a complementary RNA or DNA sequences [157]. ISH allows the detection of individual RNAs at a high resolution with high accuracy and sensitivity [158]. ISH was used to examine the expressions of OCT4 and COL2A1 genes in tissue samples in Study I. Briefly, ISH was performed with RNAscope® Fluorescent Multiplex Assay (Advanced Cell diagnostics, Abingdon, UK) by adding the probes to the sections after de-paraffin to allow hybridization. The sections were then incubated for 2 hours at 40°C and 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Gibco Life) staining was conducted. The sections were examined under a fluorescence microscope.

3.9.3 Immunohistochemistry

Immunohistochemistry (IHC) is a method that uses an antibody to bind to a certain protein epitope (antigen) present on the tissues. The mode of action of IHC involves the binding of a primary antibody to the epitope followed by the binding of a secondary antibody [98, 159]. In order to visualize the expression, secondary antibodies are often labelled with a fluorescence molecule [159]. IHC was used to examine the expressions of SOX9, COLIIA1, ACAN, PCNA (proliferating cell nuclear antigen), KRT19, IL-1R, and CXCR2 (Studies I-VI). Briefly, paraffin-embedded tissue/cell pellet sections were deparaffinized, rehydrated followed by antigen retrieval with citrate buffer to re-activate the proteins. Primary antibodies such as anti-Sox9 (1:1000), anti-COLIIA1 (1:100), anti-ACAN (1:500), anti-PCNA (1:100), anti-KRT19 (1:100), anti-IL1 receptor 1 antibody (1: 50), and anti-CXCR2 antibody (1: 50) (R&D systems) were added to the sections. After overnight incubation at 4°C,

secondary antibodies such as donkey/goat anti-rabbit IgG Alexa Fluor 546 (against SOX9, ACAN, PCNA, KRT19, IL-1R, and CXCR2) and goat anti-rabbit IgG Alexa Fluor 546 (against COLIIA1) (R&D systems) were added. Nuclear counter-stain with DAPI was carried out and the sections were examined under a fluorescence microscope (Studies I-VI).

3.9.4 Microscopy and image analysis

Light microscopy, Nikon Eclipse 600 (Nikon Instruments Europe) was used to examine cell morphology and ECM production in histological sections stained with ABvG or Von Kossa staining (Studies I-VI). Fluorescence microscopy (Nikon Eclipse 600) was used to determine the presence and localization of gene and protein expressions in the histological sections (Studies I-VI).

Image analyses were conducted with NIS Elements software (Nikon Instrument Europe, Amsterdam, Netherland) for counting of positive cells (Studies II, VI), fluorescence intensity quantification and to determine the area of the tissues (Studies III-VI). Positive cells were presented as a percentage of the total cell count (Studies II, VI), while fluorescent intensity quantification and area of the pellets were presented as pixels/μm^2 and cells/μm^2 respectively (Studies III-VI).

3.10 Apoptotic assays

3.10.1 Annexin V assay

Annexin V FITC is a fluorescence assay (Dojindo Molecular Technologies, Munich, Germany) used to evaluate apoptosis in monolayer culture models (Study II). The presence of fluorescein isothiocyanate (FITC) labeled with Annexin V and propidium iodine (PI) dyes allow the dyes to bind to cells with raptured cell membranes due to apoptosis or cell necrosis. FITC is a green fluorescent stain that binds to the phospholipid bilayer in the cell membrane while PI is a red fluorescent nuclear and chromosome counter-stain that binds to the DNA. With this assay, early apoptotic cells are detected with green stain and necrotic cells are observed with both red and green stainings. An annexin V, FITC apoptosis detection kit was used to perform the assay on cell suspensions. The cells were then deposited on glass slides and examined with a fluorescence microscope (excitation/emission: annexin VFITC 494 nm/518nm, PI 535 nm/617 nm).

3.10.2 TdT- mediated dUTP Nick End Labeling (TUNEL) assay

TdT- mediated dUTP Nick End Labeling (TUNEL) assay is a fluorescent assay for the detection of apoptotic cells in histology sections. The mode of action of this assay involves the labeling of fragmented DNA with the polymerase terminal deoxynucleotidyl transferase (TdT). Fluorescein, a green fluorescent dye along with DAPI is commonly used for visualization of the cells with and without DNA fragmentation [160]. TUNEL assay was carried out on cell pellet histology sections to observe the occurrence of apoptosis (Studies II, VI). In short, paraffin-embedded sections were de-paraffined and TUNEL assay was carried using FragEL DNA Fragmentation Detection Kit (Merck Chemicals and Life Science AB, Solna, Sweden) according to manufacturer's instructions. A positive control was produced by adding DNase to the specimen for 20 minutes. The sections were examined under a fluorescence microscope and fluorescence intensity and the number of positive cells was quantified as mentioned above.

3.11 Colorimetric assays

3.11.1 GAG and DNA assays

In order to quantify the amount of ECM produced, cell pellets were digested with papain (papaya proteinase) overnight at 60°C. The digested pellets were analyzed with GAG and DNA assay kits (Chondrex, WA, USA) and the absorbance was read at 525nm and fluorescence at excitation 360nm/emission 460nm for GAG and DNA respectively with a microplate reader (Synergy HTX, VT, USA). The amount of GAG was normalized to DNA content and the analyses was performed in duplicates with different donor cells. These assays were performed in Studies II-VI.

3.11.2 Cell proliferation assay

Cell counting kit-8 (CCK-8) (Dojindo) was used to quantify cell viability and proliferation in different *in vitro* models used (Studies I-VI). The mode of action behind this assay is that the amount of formazan dye produced by dehydrogenase in the cells is directly proportional to the number of viable cells. In brief, 50µl of prepackaged CCK-8 solution was added to the cells and incubated for 4 hours. The supernatant was collected in duplicates and absorbance measured at 450 nm by a microplate reader. CCK-8 assays were performed at day 7, 14, and 28 before harvesting the cells/tissues.

3.11.3 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) assay (Abcam) was carried out to measure cell death as LDH is released into extracellular space as a by-product of plasma membrane damage due to cell death. Briefly, cell media collected from the pellets with different stimulations were analyzed with LDH assay kit and the absorbance measured at 450nm as mentioned above. Analyses were conducted in duplicates with different donor cells (Study VI).

3.11.4 MMP-1 assay

Disc tissues (1g) were placed on a rotor with 2mL PBS for 24 hours. The samples were centrifuged (200g, 4°C, 5 minutes) and supernatants were collected. The supernatants (Study V) and cell media collected from DC pellets (Study VI) were analyzed with MMP-1 assay kit (Abcam) and the absorbance was measured at 450nm. All analyses were carried out in duplicates and repeated with cells from different donors.

3.12 Mass spectrometry for proteomic analysis

Mass spectrometry (MS) analysis was carried out to identify the constituents of CM derived from hMSCs (Study IV). In Brief, proteins from CM were concentrated and digested with 1% sodium deoxycholate overnight. The digested peptides were then labeled with mass tagging reagents and liquid chromatography-mass spectrometry was carried out by the Proteomics Core Facility, Sahlgrenska Academy, University of Gothenburg. CM was analyzed and compared to MSC media. Tandem-mass tags were used to quantify the relative content of peptides in the CM in comparison to MSC media. Proteome Discoverer v1.4 was used to perform data analysis. MSC media was used as a denominator when calculating fold changes and ratio (Study IV).

3.13 Statistical analysis

All statistical data are illustrated as the mean ± standard error of the mean (SEM) and were analyzed via SPSS v25.0 software. Two-tailed Student's t-test was used for comparing the means of 2 groups (Studies II, IV-VI) and multifactorial analysis of variance (ANOVA) with Tukey post hoc analysis was used for multiple comparisons (Studies III, IV-V). P-value < 0.05 was considered statistically significant. No statistical analysis was conducted for Study I due to low sample size.

3.14 Summary of evaluation techniques used in studies I-VI

Table 4. The different evaluation methods used in Studies I-VI are summarized in the table below-

Study	*In vitro* model	Cells	Assays	Histological evaluation	Other
I	hydrogel	hMSCs	CCK-8	ABvG, Von Kossa, ISH, IHC	-
II	2D, 3D	hMSCs, DCs	CCK-8, GAG, DNA, Annexin V, TUNEL	ABvG, IHC	-
III	3D, co-culture	hMSCs, DCs	CCK-8, GAG, DNA	ABvG, IHC	-
IV	3D	hMSCs, DCs	CCK-8, GAG, DNA	ABvG, IHC	CM collection, MS
V	3D	hMSCs, DCs	CCK-8, GAG, DNA, MMP-1	ABvG, IHC	CM collection, Flow cytometry
VI	3D	hMSCs, DCs	CCK-8, GAG, DNA, LDH, TUNEL, MMP-1,	ABvG, IHC	UCF, TEM, NTA, Flow cytometry, WB

CCK-8: Cell counting kit 8, TUNEL: TdT- mediated dUTP Nick End Labeling, GAG: glycosaminoglycan, LDH: lactate dehydrogenase, ABvG: Alcian blue van Gieson, ISH: *in situ* hybridization, IHC: immunohistochemistry, CM: conditioned media, MS: mass spectrometry, UCF: ultracentrifugation, TEM: transmission electron microscopy, NTA: nanoparticle tracking analysis, WB: western blotting.

4 SUMMARY OF RESULTS

4.1 Study I

The aim of Study I was to explore the effects of BMP-3, IL-1β and their combined effect (Pre-treatment) on hMSCs in a hydrogel model both in the presence and absence of TGF-β. The effects were investigated in terms of cell proliferation, differentiation and ECM production over 28 days period in TGF-β containing chondrogenic media and MSC media. The experimental set up was carried out as illustrated in figure 12. Human mesenchymal stem cells from three LBP patients were used in this study.

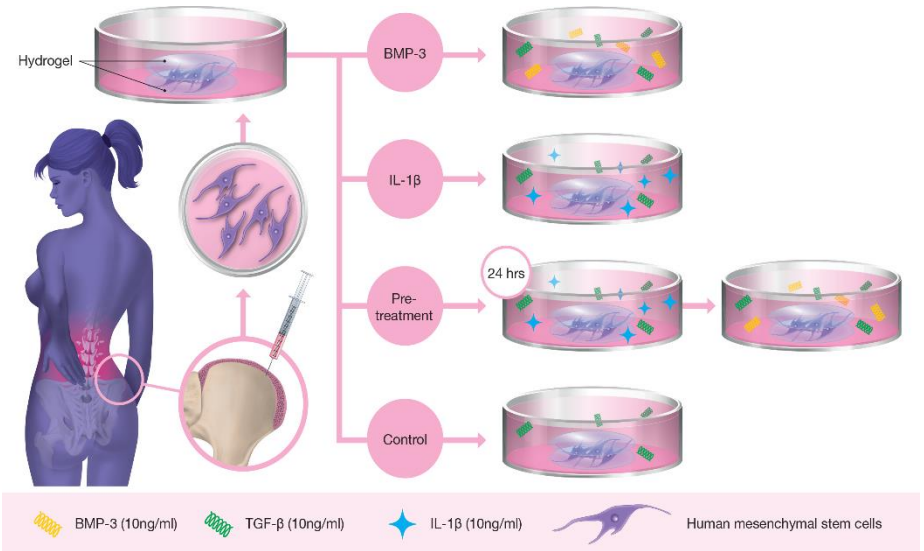

Figure 12. Graphical illustration of experimental set up for chondrogenic group in Study I. MSC media was used for non-chondrogenic group.

A higher level of proteoglycan accumulation was observed with alcian blue van Gieson (ABvG) staining in chondrogenic media group compared to the ones stimulated with MSC media. Among the chondrogenic media group, the highest level of proteoglycan accumulation was observed in the pre-treatment (PRE-T) group compared to BMP-3 and IL-1β stimulations while none was detected in control (figure 13). CCK-8 assay also detected the highest cell viability in the PRE-T group. The highest expression of the OCT4 gene was observed at day 7, thereafter, the level of expression diminished over time. COL2A1 gene and SOX9 protein expressions were observed in all the samples from both media groups. The presence of these markers confirmed that the chondrogenic differentiation of hMSCs was achieved in this hydrogel model

in both media used. In summary, these findings indicate that pre-exposure of hMSCs with pro-inflammatory cytokine prior to chondrogenic induction with growth factors enhanced chondrogenesis in hMSCs.

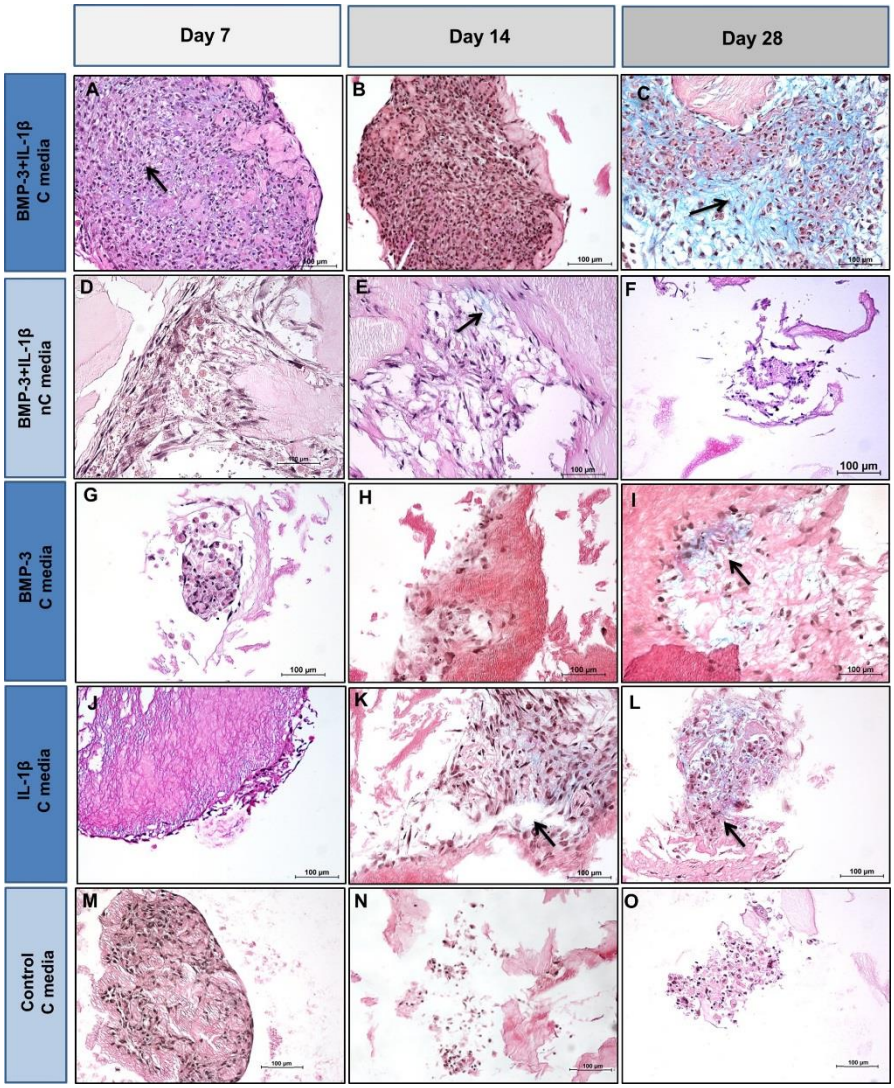

Figure 13. Representative images of Alcian blue van Gieson staining of hMSCs encapsulated in hydrogel stimulated with BMP-3, IL-1β or PRE-T at day 7, 14, and 28 in chondrogenic (C) and MSC (nC) media. Blue structure indicated by black arrows represents proteoglycans while pink represents collagen. The black dots are the nuclei of the cells. Images presented with permission from Mary Ann Liebert, Inc. (Hingert et. al, 2017).

4.2 Study II

In study II, the impact of pain-induced stress on the progression of IVD degeneration was investigated by studying the effects of stress hormone cortisol on DCs and hMSCs obtained from LBP patients in both 2D and 3D *in vitro* models as illustrated in figure 14. The experiments were carried out at two concentrations, a physiological (150ng/mL) and an increased (300ng/mL) concentration. Disc cells and human mesenchymal stem cells from four LBP patients were used in this study.

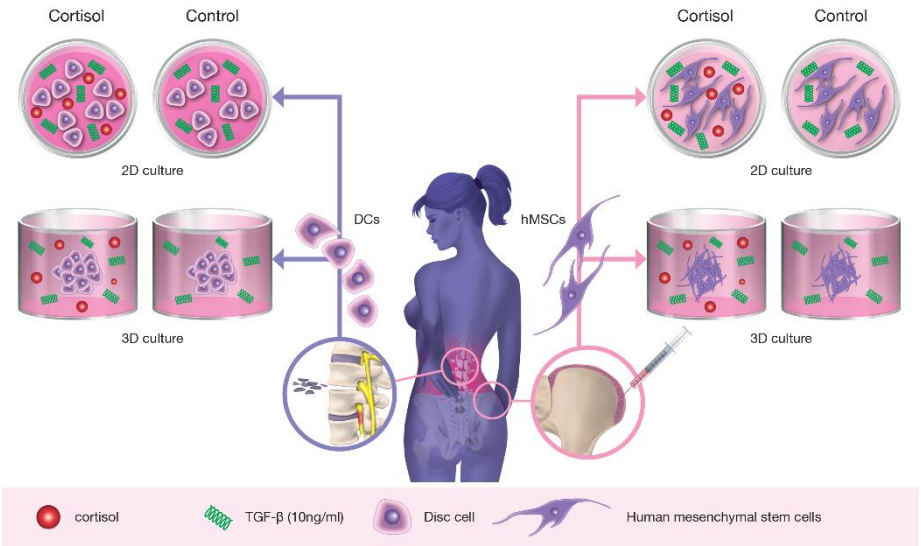

Figure 14. Graphical illustration of the experimental set up of Study II displaying 2D and 3D models used to stimulate both DCs and hMSCs with cortisol. Two sets of cortisol treatments with 150ng/mL and 300ng/mL were carried out.

CCK-8 assay detected lower cell viability in both hMSCs and DCs with cortisol stimulation especially at 300ng/mL in both 2D and 3D cultures compared to controls (figure 15A-B). Annexin V and TUNEL assays also detected increased apoptotic activity with cortisol treatment in 2D and 3D cultures respectively in both cell types. Further, DNA quantification of the pellets revealed a significant drop ($p < 0.05$) in DNA content in cortisol treated pellets compared to controls in both DCs and hMSCs. ECM production was also found to be inhibited by exposure to cortisol at both concentrations (figure 15C-D). In hMSC pellets, OCT4 expression was detected at all time points indicating the presence of undifferentiated hMSCs, which suggests delayed chondrogenesis. Moreover, elevated levels of cytokine and chemokine receptors were also detected in pellets treated with cortisol especially at

300ng/mL concentration. To sum up, the findings from this study indicated that constant exposure to the stress hormone, cortisol causes detrimental effects at cellular levels for both DCs and hMSCs, even at physiological concentration.

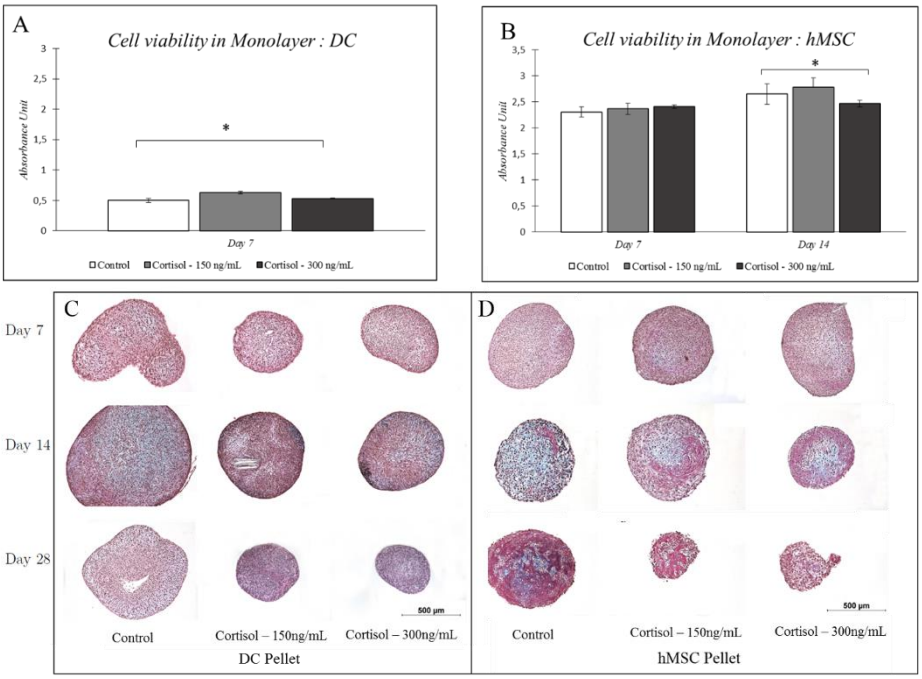

Figure 15. Disc cells and human MSCs treated with cortisol at 150ng/mL and 300ng/mL. A-B: Cell viability of DCs and hMSCs stimulated with cortisol in 2D monolayer culture. C-D: Alcian blue van Gieson staining of DC and hMSC pellets stimulated with cortisol from day 7 to 28 in 3D pellet culture. Blue structure represents proteoglycans while pink represents collagen. The black dots are the nuclei of the cells. Images presented with permission from S. Karger AG, Basel (Hingert et. al, 2019).

4.3 Study III

Study III was a follow-up study of Study I where the effects of BMP-3, IL-1β, and PRE-T were investigated in the 3D pellet model on hMSCs, DCs and co-culture of DCs and hMSCs in a 1:1 ratio as shown in figure 16. Additionally, the investigation was carried out in two concentrations, 1ng/mL and 10ng/mL. Disc cells and hMSCs from six and three LBP patients respectively were used for this study.

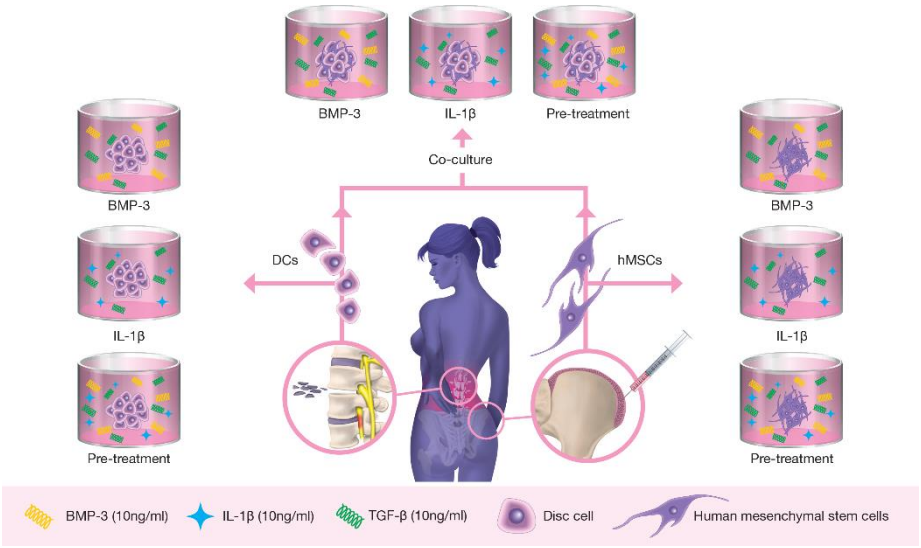

Figure 16. Graphical illustration of experimental set up for Study III displaying BMP-3, IL-1β, and PRE-T stimulations of DC, hMSC and co-culture pellets. The experiment was carried out with the stimulants at 1ng/mL and 10ng/mL concentrations.

The results from this study revealed that stimulation at 10ng/mL promoted chondrogenesis in all cell types compared to that of 1ng/mL concentration. CCK-8 assay detected higher cell viability with PRE-T stimulation in the hMSCs group, while the level was similar between all stimulations in DCs group, and in the co-culture group, BMP-3 stimulation yielded the highest cell viability. ABvG staining showed a higher level of proteoglycan accumulation in hMSC and co-culture pellets compared to DC pellets. However, similar levels were detected when comparing between different stimulants in each cell type.

Nonetheless, quantitative analysis of GAG content through glycosaminoglycan (GAG) assay disclosed that PRE-T resulted in significantly higher GAG production in hMSCs. No significant difference was observed between the stimulations in DCs, however, in co-culture pellets,

BMP-3 stimulation promoted significantly higher GAG production compared to the other stimulations. Immunohistochemistry (IHC) detected the presence of SOX9 and COLIIA1 expressions in the pellets confirming the occurrence of chondrogenesis in this model. All in all, BMP-3 was found to enhance cell proliferation and ECM production in DC and co-culture pellets at 10ng/mL compared to other stimulants while PRE-T best promoted cell proliferation and chondrogenic differentiation of hMSC pellets, a result in-line with that of Study I. A summary of the results from this study is presented in table 5.

Table 5. Summary of the results for cell proliferation and ECM production of all the pellets on day 28 at 10ng/mL. The tick marks, ✓= low, ✓✓= moderate, ✓✓✓= high, ✓✓✓✓=highest levels. Images reproduced with permission from Mary Ann Liebert, Inc. [21]

Cell type and stimulation (10ng/mL)	Cell proliferation	ECM production
hMSCs		
BMP-3	✓✓	✓✓✓
IL-1β	✓	✓✓✓
PRE-T	✓✓✓	✓✓✓
DCs		
BMP-3	✓✓✓	✓✓✓
IL-1β	✓✓✓	✓✓
PRE-T	✓✓✓	✓✓
Co-culture		
BMP-3	✓✓✓✓	✓✓✓✓
IL-1β	✓✓	✓✓
PRE-T	✓✓	✓✓✓

4.4 Study IV

In study IV, the effects of CTGF and hMSC derived conditioned media (CM) on DCs and hMSCs were investigated in a 3D pellet model. Additionally, the constituent of conditioned media (CM) was analyzed with mass spectrometry (MS) and the soluble peptides in the CM were identified. The experimental set up is shown in figure 17. Disc cells and hMSCs from three LBP patients were used in this study.

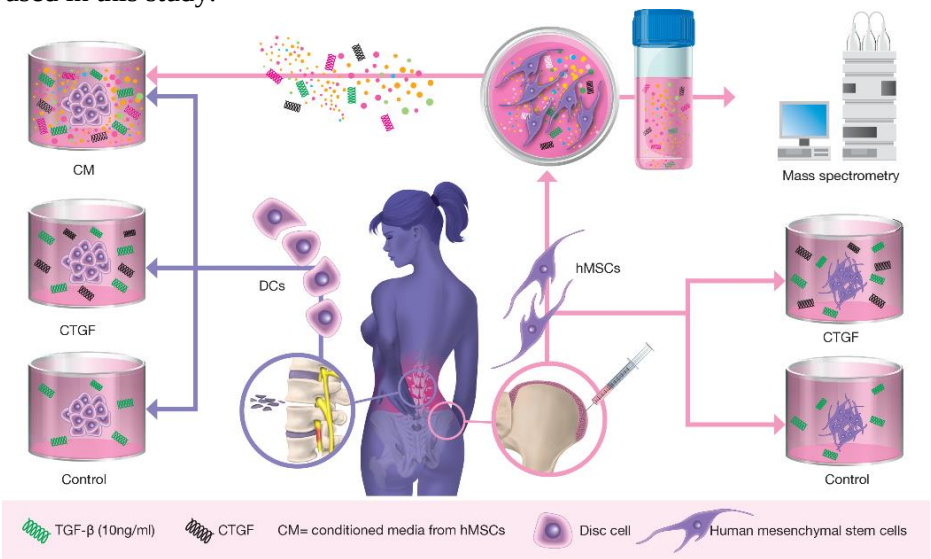

Figure 17. Graphical illustration of the experimental set up of Study IV where DC and hMSC pellets were treated with CTGF, and CM derived from hMSCs. Identification of CM constituent was carried out by mass spectrometry.

CCK-8 assay presented significantly higher cell viability in CM treated DC pellets compared to CTGF and control. No significant difference in cell viability was observed between CTGF and control groups in pellets from both cell types. A higher level of proteoglycan accumulation was detected with ABvG staining in CM treated DC pellets at all time points compared to CTGF and control. A similar pattern of staining was observed in hMSCs groups (figure 18A-B). The quantitative result from the GAG assay also supported the results from ABvG staining (figure 18C-D). The presence of KRT19 and SOX9 in DC and hMSC pellets respectively confirmed the occurrence of chondrogenesis in this model. IHC detected significantly higher ($p < 0.01$) expressions of ACAN and COLIIA1 in CM treated DC pellets. However, no significant difference in the levels was observed between CTGF and control groups in pellets from both cell types.

Further, MS analysis identified more than 700 peptides in the CM of which 129 showed a relative abundance of ≥2 and CTGF was among the peptides identified. The 129 soluble peptides identified consisted of growth factors, enzymes, ECM protein, TIMPs, MMPs, etc. (figure 19). The growth factors identified included CTGF, BMP-1, and vascular endothelial growth factor (VEGF). All in all, the findings from this study suggest that hMSCs derived CM possessed powerful therapeutic effects on DCs and the observed outcome was most likely due to the synergistic effects of multiple proteins.

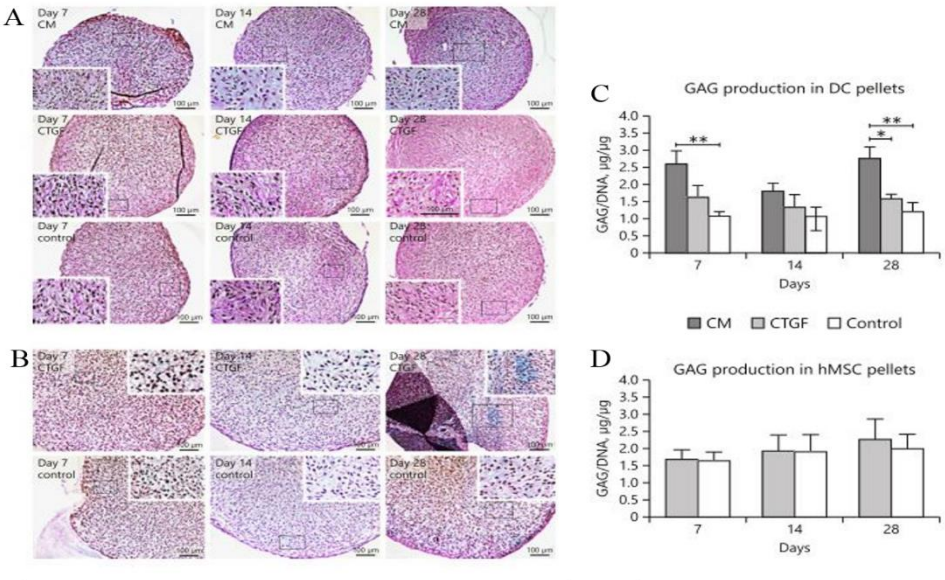

*Figure 18.Alcian blue van Gieson staining of (A) DC and (B) hMSC pellets stimulated with CM and CTGF at day 7-28. Blue structures represent proteoglycan while pink represent collagen. The black dots are the nuclei of the cells. (C-D): Quantitative analysis of GAG production in DC and hMSC pellets. Data presented as the mean ± SEM (*p < 0.05, **p <0.01). Images presented with permission from S. Karger AG, Basel (Hingert et. al, 2020).*

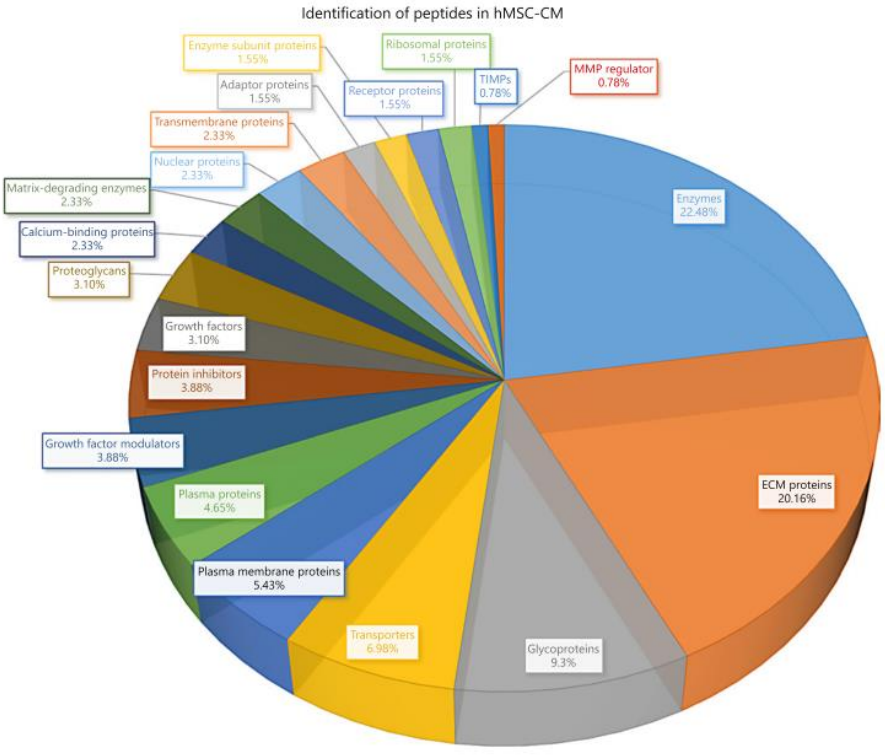

Figure 19. Pie chart demonstrating the composition of different peptide groups identified in CM from hMSCs. Images presented with permission from S. Karger AG, Basel (Hingert et. al, 2020).

4.5 Study V

In this study, physiological levels of MMP-1 in degenerated disc tissues were quantified. Further, the ability of CM in mitigating the catabolic effect of MMP-1 at physiological levels (5, 50 or 100ng/mL) was investigated. The experimental set up for the *in vitro* study is illustrated in figure 20. Degenerated IVD tissues from six LBP patients were used for quantification of MMP-1 level while DCs and hMSCs from three LBP patients were used for the *in vitro* experiments.

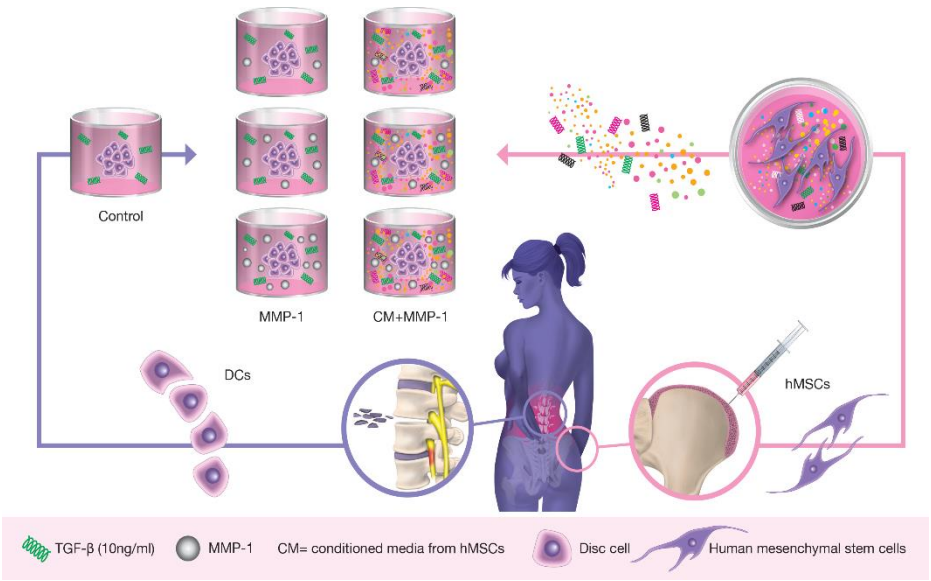

Figure 20. Graphical illustration of the experimental set up of Study V where DC pellets were treated with 5, 50 and 100ng/mL MMP-1 with or without CM.

The mean concentration of MMP-1 in degenerated IVD tissues was 151ng/mL (range 84-254ng/mL) with the highest levels detected in Pfirrmann grade 4/5 disc tissues. As for the *in vitro* study, CCK-8 assay detected high cell viability in DCs stimulated with CM even in the presence of MMP-1 (CM+MMP-1). However, the viability decreased with increasing MMP-1 concentration. Simultaneously, lower cell viability was detected in the MMP-1 group compared to control.

ABvG staining also showed less proteoglycan accumulation with increasing MMP-1 concentration. A higher level of proteoglycan was detected in pellets treated with 5ng/mL MMP-1 compared to 100ng/mL in the CM+MMP-1 group. However, little to no proteoglycan was observed in the MMP-1 group.

The quantitative results from the GAG assay also supported the findings from ABvG staining. However, the level of GAG production at 5ng/mL MMP-1 in the CM+MMP-1 group was significantly higher than that of controls.

Further, significantly higher expressions of ACAN and COLIIA1 were detected with IHC in the CM+MMP-1 group compared to MMP-1 and control groups. However, the level also decreases with increased MMP-1 concentration. All in all, the result suggested that CM has the ability to counteract the matrix degrading ability of MMP-1, especially at lower MMP-1 concentrations.

4.6 Study VI

To further explore the potential of hMSCs secretome, the effect of hMSCs derived small EVs (sEVs) was investigated. In this study, sEVs were isolated from CM of hMSCs and the isolated sEVs were characterized for EV markers, size, and concentration. Disc cell pellets were then stimulated with sEVs $5x10^{10}$ EVs/mL and evaluated for cell proliferation, viability, ECM production, chondrogenesis, and their secretions. The experimental set up is shown in figure 21. CM was collected from hMSCs isolated from one LBP patient's bone marrow aspirates and DCs from five patients were used in this study.

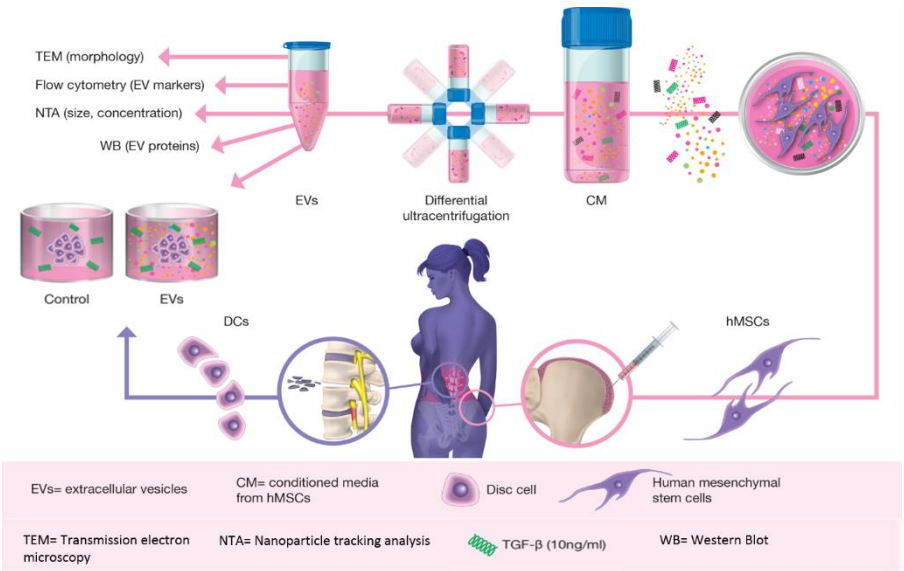

Figure 21. Graphical illustration of the experimental set up of study VI where sEVs were isolated from hMSC CM and further characterized with WB, NTA, FACS, and TEM. The sEVs were then used to stimulate DC obtained from LBP patients.

Characterization of the sEVs isolated showed the presence of EV surface markers and microscopy revealed the presence of sEVs as shown in figure 22. Nanoparticle tracking analysis (NTA) presented that the mean and mode size of the sEVs isolated were 175 ± 6 nm and 144 ± 2 nm respectively suggesting that the sEVs isolated are exosomes or small EV (sEVs). CCK-8 assay and PCNA expressions showed an increase in cell proliferation while lactate dehydrogenase (LDH) and TUNEL assays revealed a decrease in cell death with sEVs stimulation in DC pellets. The presence of KRT19 and SOX9 validated the chondrocyte-like cell characteristics of DCs in the pellets. GAG production was detected with ABvG staining and GAG assay as early as day 7 with sEV stimulation. IHC also revealed the presence of COLIIA1 and ACAN

confirming the occurrence of chondrogenesis. Additionally, a lower level of MMP-1 secretion was detected in pellets stimulated with sEVs compared to control. All in all, the findings from this study suggest that sEVs could be an important player responsible for the therapeutic effects of hMSCs.

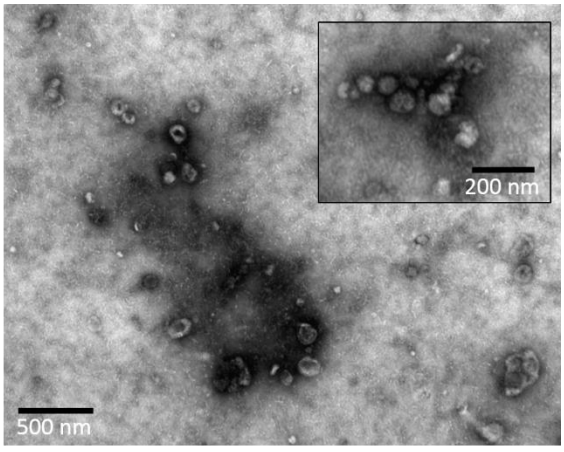

Figure 22. Transmission electron microscopy (TEM) image of sEVs revealed the presence of cup-shaped, vesicle-like structures within 50-150nm in diameter.

5 DISCUSSION

Signaling factors have been suggested by many studies as an alternative to cell therapy for IVD regeneration as they have the ability to promote chondrogenesis [128, 161-163]. The overall ambition of the studies presented in this thesis was to identify the factors that can be applied as a less-invasive therapy for the treatment of IVD degeneration. Additionally, the aim was also to identify factors that have pathological consequences on the progression of disc degeneration.

The studies composing this thesis demonstrated that chondrogenic differentiation of hMSCs can be achieved by PRE-T and BMP-3 stimulation both in a hydrogel (Study I) and a pellet model whereas BMP-3 was found to enhance cell proliferation and ECM production in DC and co-culture (Study III). On the other hand, continuous exposure to the stress hormone cortisol, even within physiological concentration was found to have detrimental effects on DCs and hMSCs (Study II).

A powerful chondrogenic effect was observed in DCs when stimulated with conditioned media (CM) derived from hMSCs (Study IV). In fact, CM mitigated the matrix degrading effect of MMP-1 in DC pellets (Study V). Upon investigating the constituent of CM, over a hundred soluble factors belonging to different peptide families were identified (Study IV). In addition to these soluble molecules, hMSCs also secreted small extracellular vesicles (sEVs), which when added to DC pellets promoted cell viability and expedited ECM production (Study VI).

These results could contribute to the understanding of the role and the function of signaling factors. Although limitations and methodological challenges still exist, the knowledge from these studies can be applied for the development of new treatment strategies for IVD degeneration.

5.1 The effects of the selected signaling factors

The signaling factors investigated in the studies were BMP-3, IL-1β, cortisol, CTGF, CM, and sEVs.

5.1.1 Cellular viability and proliferation

In study I [164], improved cellular viability was observed in hMSCs with BMP-3 stimulation with or without the presence of TGF-β indicating that BMP-3 alone could facilitate the hMSCs to become more viable. The reason why TGF-β was supplemented in chondrogenic/control media in all the studies was because TGF-β is regarded as the main regulator of chondrogenesis and its presence can boost chondrogenic processes [94]. However, IL-1β did not appear to affect cell viability especially in the absence of TGF-β. IL-1β is a powerful pro-inflammatory cytokine found in abundance in degenerated IVDs [14]. Nevertheless, stimulation with IL-1β did not have detrimental effects on the hMSCs. The same was observed in Study III [21] with hMSCs, DCs, and co-culture in the pellet system. The reason behind this observation could be because the concentration of the cytokine used (10ng/mL) in the studies might have been lower than the concentration in degenerated discs. To the best of the author's knowledge, the concentration of IL-1β in degenerated IVD tissues has never been reported, instead, the presence of IL-1β was conveyed in terms of gene and protein expressions in the literature. Pre-treatment of hMSCs with IL-1β followed by BMP-3 stimulation (PRE-T) resulted in higher cell proliferation compared to stimulation with BMP-3 or IL-1β alone both in hydrogel and pellet models. Pre-treatment with IL-1β has been reported to trigger the immunosuppressive and migratory abilities of hMSCs [165, 166]. The observation of enhanced cell viability in the PRE-T group could be because IL-1β exposure activated the hMSCs and the added exposure to BMP-3 further intensified the reciprocity to drive cellular proliferation as discussed in studies I and III.

Similar cell proliferation pattern in hMSCs was also observed in studies I and III where a rapid cell proliferation was observed from day 7 to 14 followed by a drop from day 14 to 28. MSCs are known to divide rapidly after an initial lag phase *in vitro*, however, the doubling time of the population depends on seeding density and patient variation [81]. The cell proliferation patterns of DCs stimulated with BMP-3, IL-1β or PRE-T were also observed to share similarities where an initial decrease was detected followed by a slight increase over time. The initial speculation for this result was that the DCs were hard to influence as they were mature cells from degenerated IVDs, which have been exposed to an inflammatory environment. Nonetheless, stimulation of these DCs with CM in study IV [167] suggests otherwise as a constant increase in cell proliferation was observed from day 7 to 28. In fact, in Study V, stimulation of DCs with CM even in the presence of MMP-1 (5, 50, 100ng/mL) resulted in higher cell proliferation than in the control group with TGF-β. The observation could be on the grounds that CM possessed multiple factors that

could facilitate the cells to be viable and proliferative. CTGF stimulation, follow the same proliferation pattern as seen with BMP-3, IL-1β and PRE-T in DCs. CTGF did not have a strong effect on hMSCs. On the other hand, treatment with sEVs showed a rapid and continuous increase in cell proliferation in DCs proving that these mature cells can be influenced given the right signaling factors. Exosomes-derived from MSCs have also been reported to promote cell proliferation in chondrocytes [25]. Based on these studies, CM and sEVs derived from hMSCs seemed to contain powerful signaling factors that could bring about the regeneration of the degenerated DCs.

As for co-culture, stimulation with BMP-3 resulted in higher cell proliferation and viable cells compared to stimulations with IL-1β or PRE-T suggesting that BMP-3 can boost the proliferation of hMSCs and DCs in co-culture. Studies have reported that BMP-3 promotes hMSC proliferation [168, 169] however, in this case, it could either be BMP-3 or the paracrine signaling of the hMSCs within the environment of the co-cultured DCs. To sum up, BMP-3 best promoted cell proliferation in co-culture while PRE-T did so in hMSCs.

In contrast, exposure to stress hormone cortisol even at physiological concentration restricted cell proliferation in both DCs and hMSCs in Study II [170] where a lower amount of DNA was also detected compared to control. Similarly, exposure to pharmacological doses of cortisol has been reported to inhibit the proliferation of chondrocytes in rat models [171]. Likewise, low cell proliferation was detected in DCs treated with MMP-1 in Study V. This could be because of the degradation of the collagen matrix in the pellets by MMP-1 as the viability of the cells depends on the availability of the matrix and nutrients [42, 172].

5.1.2 Apoptosis and cell death

Programmed cell death or apoptosis is described as a morphologically distinct form of cell death [173]. The loss of viable nuclear pulposus (NP) cells due to apoptosis plays an important role in IVD degeneration [174]. The degenerative environment in the IVD by itself was also reported to promote cellular apoptosis [175]. A higher number of apoptotic cells were detected in DCs and hMSCs treated with cortisol compared to controls. Corticosteroids including cortisol have been previously reported to induce apoptosis in articular cartilage cells [16] and other cell types [34], however at a much higher concentration than the ones used in Study II [171]. This finding

Research question #1 addressed: What effect does cortisol have on hMSCs and DCs on a cellular level?

indicates that the release of cortisol due to pain-induced stress could aggravate the pathogenesis of IVD degeneration by mediating the loss of viable cells due to apoptosis.

Contrary to the negative influence of cortisol on the cells, treatment of DCs with sEVs from hMSCs in Study VI resulted in lower cell death and apoptotic cells. This finding was supported by other studies that exosomes hinder apoptosis in cartilage cells [25, 176]. Besides, exosomes from MSCs were also reported to inhibit IL-1β and ischemia-induced chondrocyte apoptosis [177, 178]. Detection of low cell death and apoptotic cells in DCs treated with sEVs confirmed that sEVs could potentially be responsible for enhanced proliferative effect in DCs.

5.1.3 Chondrogenic differentiation of human MSCs

The initial purpose of incorporating MSCs in cell therapy strategies was so that MSCs could differentiate into chondrocyte-like cells of the IVD for optimization before transplantation into the site of degeneration [18]. To achieve this, the right stimulant is necessary to drive these multipotent hMSCs into the cell lineage of interest. In Study I, BMP-3 and PRE-T stimulations induced differentiation of hMSCs approximately around day 14 where a drop in proliferating cells was observed from day 14 to 28 as mentioned previously. The reason behind the drop in cell number could be because most of the cells have differentiated while some might either died or might be unable to proliferate and differentiate simultaneously [137]. No mineralization was observed in the MSC media group indicating that the hMSCs did not differentiate into osteogenic lineage. Moreover, the gene and protein expressions of COL2A1 and SOX9 respectively in all stimulation groups confirmed the chondrogenic differentiation of the cells. The expression of OCT4, the most vital transcription factor that mediates multipotency in MSCs was observed at day 7 where the gene expression decreased as time progressed [179, 180]. This loss in multipotency overtime confirmed differentiation of the hMSCs. However, a prolonged multipotency was observed in hMSCs treated with IL-1β and PRE-T even though the expressions of COL2A1 and SOX9 were observed at day 28. This could be due to the presence of IL-1β as hMSCs tend to secrete anti-inflammatory factors as an immunomodulatory response either for survival or as a response to the pro-inflammatory environment [181]. This hypothesis is supported by the results in Study III where higher expression of SOX9 was observed in IL-1β stimulated hMSCs and co-culture compared to

Research question #2 addressed: What effect does pre-exposure to pro-inflammatory cytokine have on hMSCs' differentiation and chondrogenesis?

other groups reflecting that the cells were either still in the early chondrogenic state or chondrogenic differentiation was delayed. However, it is reasonable to speculate that, this prolonged multipotency could be a strategic approach as the cells needed a pool of hMSCs to exert the above-mentioned effects while some of them differentiate. Similarly, exposure of hMSCs to cortisol in Study II also resulted in delayed differentiation. In this case, the expression of OCT4 was observed until day 28 indicating that exposure to stress hormone cortisol even at physiological level compromised chondrogenic differentiation of hMSCs. However, SOX9 expression was observed suggesting that chondrogenesis was compromised but not entirely terminated. Studies have also reported that stress induced secretion of corticosteroid suppresses differentiation and repair potential of MSCs *in vivo* [12, 14]. In Study IV, stimulation of hMSCs with CTGF resulted in chondrogenic differentiation based on the observation of proteoglycan accumulation and SOX9 expression. However, the impact of CTGF was not as powerful as that of PRE-T or BMP-3.

5.1.4 Chondrogenesis in disc cells

Disc cells used in the studies were isolated from degenerated IVD tissues comprising of a mixture between annulus fibrosus (AF) and nucleus pulposus (NP) tissues. Chondrocyte-like cells are native to the NP and in order to ensure the cells investigated in the studies have chondrocyte-like/NP cells characteristics, the expressions of chondrocyte-like/NP cells markers were investigated. The main suggested markers are keratin-19 (KRT19), aggrecan (ACAN), collagen type II (COLIIA1), SOX9. [35, 36, 182]. The expression of SOX9 protein in DCs pellets treated with cortisol in Study II confirmed that the cells were chondrocyte-like cells, however, the decrease in the expression level from day 7 to 28 showed that exposure to cortisol compromised chondrogenesis in DCs. The expression of COLIIA1 in DC pellets in Study III confirmed that the cells used were chondrocyte-like cells, however, BMP-3 stimulation resulted in a stronger expression of this protein compared to other stimulations. The DCs studied in Study IV and V also expressed KRT19, ACAN, and COLIIA1 regardless of stimulation exhibiting chondrocyte-like cell characteristics. However, DCs stimulated with CM produced higher levels of ACAN and COLIIA1 while CTGF shared similar levels with control. This observation indicated that CM possessed a powerful tool that could drive the DCs from degenerated discs towards chondrogenesis. The expressions of chondrocyte-like cell markers were also detected in DCs stimulated with sEVs

Research question #3 addressed: Can growth factors be used to enhance chondrogenesis in mesenchymal stem cells and disc cells from LBP patients *in vitro*?

in Study VI. To sum up, the DCs isolated from degenerated IVDs used in all the studies exhibited chondrocyte-like/NP cell markers confirming the characteristic of chondrocyte-like cells and the occurrence of chondrogenesis. However, different growth factors had different impacts on the expression level of these markers.

5.1.5 Extracellular matrix production

In addition to the expression of certain markers, the production of extracellular matrix (ECM) acts as an indicator of chondrocyte-like cells as only chondrocytes and chondrocyte-like cells can produce proteoglycan and other components of ECM [72]. ACAN and COLIIA1 are vital components of the ECM as the cells interact with these components to further produce proteoglycans [30, 41]. Additionally, the cells maintain their viability through interacting with the ECM structure [42]. In hMSCs, proteoglycan accumulation was observed as early as in day 7 with PRE-T where it was observed on day 28 with BMP-3 in Study I. In the pellet model in Study III, there was no significant difference in the amount of GAG produced between the different stimulations in hMSCs. However, in DCs, BMP-3 stimulated pellets possessed a higher level of GAG production along with the expression of COLIIA1 compared to other groups. This means that BMP-3 at 10ng/mL could influence the DCs to produce matrix, however, the effect of BMP-3 on DCs alone was not as powerful as its effect on co-culture. Co-culture pellets produced the highest level of GAG when stimulated with BMP-3. This finding allows the speculation that BMP-3 provided an important signal to the DCs and the enhanced effect observed could be coming from the hMSCs through cell-cell contact or cross-talk between the cells as mentioned previously. Stimulation of DCs with CM resulted in significantly higher proteoglycan accumulation and GAG production compared to stimulation with CTGF in Study IV. The rationale behind selecting CTGF for investigation was due to the report that CTGF promotes cell viability of NP [22] and articular chondrocytes [183]. Hence, it is possible that the results did not verify the hypothesis because the concentration of CTGF used in the study might have been too low. The motivation for using 10ng/mL CTGF was due to the presence of TGF- β in chondrogenic media, which was suggested to induce the efficacy of CTGF [184, 185]. In study V, stimulation of DCs with CM even in the presence of MMP-1 up to a concentration of 50ng/mL yielded a high level of GAG production, ACAN, and COLIIA1 expressions. However, the effectiveness of CM was found to be limited to the concentration of MMP-1 in the environment as the levels of ECM

Research question #4 addressed: Positive effects seen on disc cells in co-culture with hMSCs – What causes these effects?

proteins decreased with increasing concentration of MMP-1. This suggests that CM could potentially be used for the treatment of early stages of IVD degeneration where the levels of MMPs are still manageable. Furthermore, exposure of DCs to the sEVs also resulted in the early production of GAG as well as exhibited high levels of ACAN and COLIIA1 expressions. Exosomes from MSCs have been reported to increase chondrogenic genes such as COL2A1 and aggrecan [177]. Small EVs have the potential for therapeutic application as they can mediate several biological actions of hMSCs. On the other hand, stimulation of DCs and hMSCs with cortisol not only restricted cell proliferation but also suppressed accumulation of proteoglycan and ECM production, suggesting that constant exposure to the stress hormone cortisol has detrimental effects on cellular levels.

5.2 The content of hMSCs' secretion

The secretion of hMSCs has been shown to play a critical role in tissue regeneration as they carry out biological actions of the hMSCs through paracrine signaling [186]. hMSCs tend to secrete a wide range of bioactive molecules as a response to stimuli from the local microenvironment [102]. Study IV revealed more than 120 constituents of the CM including growth factors, anti-apoptotic factors, modulators of growth factors, MMP inhibitors, and multiple ECM proteins. CTGF was identified among the growth factors. CTGF has been reported to promote ECM production in notochordal cells as it plays a role in the development of cartilage and IVD in the developmental stage of the spine [22, 163]. BMP-1 growth factor, another member of the TGF-β superfamily, as well as vascular endothelial growth factor (VEGF) was also identified. CTGF facilitates physical interactions with BMP-1 and TGF-β ligands while VEGF promotes cartilage regeneration and repair [161, 162, 187, 188]. The presence of this network of proteins illustrates the complexity of the signaling mechanisms behind maintaining homeostasis in the IVD environment. Further explanation for not seeing the powerful effect of CTGF on DCs in the study could be because CTGF alone was not responsible for the repair mechanism as it seemed to collaborate with other molecules in *in vivo* setting. Several plasma and ECM proteins identified in CM are also known to mediate cellular proliferation and hinder apoptosis [189, 190]. This maybe the reason why the stimulation of DCs with CM increased cell viability and proliferation. Tissue inhibitor of MMP-1 (TIMP-1) was also identified in the CM clarifying why CM could restrict matrix degradation ability of MMP-1 in Study V. Moreover, the presence of ECM proteins such as peroxidasin and plasminogen activator inhibitor-1, which regulates assembling and remodeling

of ECM [191-195] maybe the reason for why a powerful chondrogenic effect was observed in DCs with CM stimulation.

Besides signaling peptides, hMSCs also secrete extracellular vesicles of various sizes [104]. The sEVs isolated from hMSCs in Study VI were between 100 and 150nm in size and indicated that they were small EVs with the characteristics of exosomes. Extracellular vesicles are described as the mediators of cell-cell communication as they possess remarkable features of transferring proteins and active genetic material such as microRNA to other cells [112, 196]. The observation of enhanced cell viability and early ECM production of DCs in Study VI could be due to the fact that these sEVs were internalized by the DCs [176] or that they delivered bioactive factors that could directly influence the cells [197]. These findings suggest that the therapeutic effects of hMSCs were most probably generated by the secreted bioactive substances that worked synergistically to bring about chondrogenic effects in DCs.

Research question #5 addressed: How do hMSCs exert their therapeutic effects on DCs? What do they secrete and what is the outcome of stimulating disc cells with secretome of hMSCs?

5.3 Tissue level of MMP-1

Matrix metalloproteinase 1 (MMP-1) is a collagenase responsible for the degradation of collagen structure within the IVD as mentioned in 1.1.3. In Study V, the tissue level of MMP-1 was quantified. The concentration of MMP-1 in degenerated IVD tissues was found to be between 74 to 254ng/mL where higher concentration was observed in tissues with Pfirrmann Grade 4 or 5. Studies on MMP-1 level in blood serum have also reported a higher level of MMP-1 in patients with severe IVD degeneration compared to mild and moderate scales [45, 198, 199]. These reports confirmed the involvement of MMP-1 in the progression of disc degeneration. In the IVD environment, synthesis and degradation of ECM are kept in balance by MMPs and TIMPs [43, 44]. However, an elevation in the level of MMP-1 disrupts the homeostasis where TIMPs can no longer keep the balance, hence, the process of degeneration advances.

5.4 Expression of catabolic factors

Disc cells have been reported as sources of cytokines and chemokines within the IVD tissues and their sequence of expression changes with the progression of IVD pathology [200]. It has been suggested that the assessment of these

cells could provide critical information regarding the state of the disc environment [200]. IL-1β is known as the master regulator of IVD degeneration that signals the participation of a plethora of cytokines and chemokines [201]. Hence, in Study II the expressions of IL-1β receptor (IL-1R) and chemokine receptor 2 (CXCR2) were investigated and it was found to be elevated in both DCs and hMSCs exposed to cortisol. It was expected to observe the presence of these receptors in hMSCs as hMSCs are known to take part in immunomodulatory responses [181]. However, detection of IL-1R and CXCR2 in DCs suggests an autocrine or paracrine signaling mechanism in DCs [8]. This may account for why regenerative effects were observed in DCs when stimulated with sEVs. Nonetheless, elevated expressions of IL-1R and CXCR2 were reported in degenerated IVD tissues compared to the non-degenerated ones [201]. In addition, the expressions of IL-1R and CXCR2 trigger the release of MMPs, which leads to catabolic processes in the IVDs [202].

The presence of MMP-1 at elevated levels is one of the indicators of IVD degeneration as discussed in 5.3. However, in Study VI, DCs treated with sEVs released a lower level of MMP-1 when compared to DCs in the control group. An overall decrease in MMP-1 secretion was also observed from day 4 to day 28. The speculation behind this finding could be due to the cross-talk between the DCs and the sEVs. As mentioned previously, DCs have the capacity to participate in paracrine signaling while sEVs could transfer protein and other bioactive molecules to other cells. It is, therefore, reasonable to further speculate that sEVs were providing the DCs with vital signaling material needed to reduce the secretion of MMP-1 overtime. Studies on MSC-exosomes also reported a decrease in MMPs level with exosomes stimulation in arthritis models *in vivo* [177, 203].

Furthermore, higher levels of IL-6 and IL-8 were detected in sEVs treated DCs compared to controls in Study VI, which was believed to be secreted by the DCs. However, sEVs are also known for transporting cytokine and both IL-6 and IL-8 are reported to be associated with secretory vesicles [204-206]. Therefore, it was unclear if these cytokines were derived from the DCs or the EVs. Hence, further investigations are warranted in order to elucidate the mechanism behind this result.

5.5 Methodological consideration and challenges

5.5.1 Cell type and sources

Human MSCs have emerged as a key candidate for regenerative medicine especially in the field of musculoskeletal degeneration due to their inherent ability to differentiate into chondrocyte-like cells, thereby providing numerous potential for cell therapy strategies. Accumulating evidence indicates a paradigm shift suggesting the therapeutic effects of hMSCs may not be solely due to their self-renewal properties, but also due to their paracrine ability [207]. Thus, hMSCs and their secretions have been one of the main focus of the studies in this thesis. As the hMSCs used in the studies were isolated from patients tissues, it is therefore of importance to establish a reliable isolation protocol and to determine the characteristic of hMSCs. Likewise, DCs have also been one of the main focus of the studies in order to better understand how hMSCs and signaling proteins could affect DCs in degenerated IVDs. The DCs used in the studies were obtained from degenerated IVD tissues from patients diagnosed with LBP. However, the disc tissue harvested was a mixture of AF and NP tissues as mentioned above. It was not possible to discriminate between the differences due to the disintegration of the tissue types in the obtained disc material.

The challenges were the availability of the patient samples, and patient variation due to small sample sizes. However, the main advantage of the studies in this thesis was that the cells investigated were obtained from clinically relevant samples where the outcomes could be directly translated into physiological or clinical settings.

5.5.2 *In vitro* models

In this thesis, a few *in vitro* models were employed to study the effects of different signaling factors on DCs and hMSCs. Hydrogels of various types are being intensively studied as they facilitate minimal invasive transplantation of the cells into the site of degeneration in the IVDs [18]. 3D hydrogel model was used in Study I and was proven effective as it allowed survival, proliferation, and differentiation of the hMSCs into chondrocyte-like cells of the IVD. Hydrogels have also been reported to facilitate cell survival for at least 6 months after transplantation in mini pig models [208]. However, the limitations of this model lie in the fact that it was difficult to obtain the same thickness of the sections as the hydrogel tends to break apart. Also, it was difficult to obtain sections with a similar number of cells as the encapsulated cells tend to be distributed unequally. On the other hand, the pellet model used in Studies II-VI was proven effective as they allow chondrogenic

differentiation of hMSCs as well as ECM production within the pellets. 3D pellet cultures were employed in most of the studies as it mimics the *in vivo* micro-environment.

In addition to the model used, the dosage of signaling molecules used could also affect the outcome of the results as the result may differ from *in vivo* studies. For instance, in Study II exposure of DCs to cortisol demonstrated a detrimental effect, however, in this case, the cells were constantly exposed to 150-300ng/mL cortisol for 28 days whereas, in the body, the serum level of cortisol fluctuates between 10-250ng/mL in 24 hours [209]. The rationale behind setting up the experiment in this manner was to investigate the effect of pain-induced stress on the progression of IVD degeneration. IVDs obtain nutrient supplies through diffusion from capillaries surrounding the endplates [7]. We speculate that small protein (hormone) like cortisol should be able to reach the vicinity of the IVD through diffusion as estrogen has been reported to be detected in NP tissue [210]. More investigation needs to be carried out to confirm the presence and the level of cortisol in IVD tissues.

5.6 Clinical relevance

Musculoskeletal disorder such as chronic low back pain (LBP) is one of the leading causes of disability globally with a high life-time prevalence. In recent years, the tissue engineering approach which includes biomaterials, cellular and molecular strategies have been recognized as a promising treatment scheme. The cell therapy approach aims to regenerate the IVD through autologous and exogenous cell transplantation as well as endogenous cell stimulation and recruitment [68, 211]. The clinical relevance of the studies presented in this thesis lies in the fact that the results contribute to increasing the knowledge regarding endogenous cell stimulation through various signaling factors tested. Moreover, the enhanced chondrogenic effects in co-culture suggest that endogenous cell recruitment is a key mechanism that needs to be tackled in *in vivo* settings. An important feature of degenerated IVD is a decline in the number of viable and functional cells, with a substantial population of cells in a senescent state [212, 213]. Hence, this approach of transplanting viable cells such as IVD cells or MSCs could activate and replenish the cells in the IVD enhancing self-repair and regeneration. Results from the studies in this thesis demonstrated that endogenous stimulation of cells with growth factors or pre-treatment of hMSCs with IL-1β cytokine could boost the recruitment and regeneration processes in the IVDs. Emerging evidence from preclinical models has also shown the potential applications of MSCs in cell therapy [73, 214]. Animal models and clinical studies have

further demonstrated the efficacy of MSC therapy in cartilage repair and regeneration [214, 215]. However, the results presented in this thesis along with ones from recent studies have revealed that the benefits of MSC therapy are mainly due to the secretion of multiple bioactive factors by the MSCs, which may regulate key biological processes [85, 216]. Studies presented in this thesis revealed the constituent of hMSCs' secretome, which consisted of signaling peptides and extracellular vesicles. The studies further demonstrated that the factors isolated were capable of driving the degenerated DCs into producing new ECM. This knowledge can pave the ways for the development of cell-free therapies, which could complement the surgical treatment methods of today. However, the next stage would be to identify an appropriate factor or a combination of different peptides and confirm the results in animal models before clinical usage. The development of a cell-free therapy can resolve safety issues associated with transplantation of living cells, immune complication, mal-differentiation of the cells, and transmission of infections. These derivatives of hMSCs' secretome could provide considerable advantages over cell therapy in terms of manufacturing, handling, dosage, product shelf-life, storage and their potential to be manufactured as a pharmaceutical ready-to-use product. Moreover, it is more economical and practical for clinical administration compared to the invasive treatments or invasive cell collection methods. Lastly, these bioactive factors could be further engineered to tailor cell-specific effects.

6 SUMMARY AND CONCLUSION

The studies presented in this thesis contribute to the knowledge in the field of regenerative medicine by identifying signaling molecules that can influence the degenerative disc cells towards matrix production and regeneration as well as towards detrimental consequences.

- ✓ Pre-treatment of hMSCs with pro-inflammatory cytokine, IL-1β, prior to chondrogenic induction with BMP-3 growth factor enhanced chondrogenesis in hMSCs.
- ✓ Exposure to cortisol even at physiological concentrations suppressed cellular proliferation, differentiation, and chondrogenesis in hMSCs and DCs.
- ✓ BMP-3 alone enhances chondrogenesis in DCs, and co-culture of degenerated DCs with hMSCs where an enhanced cell proliferation and matrix production was detected in co-culture.
- ✓ CM from hMSCs improved chondrogenesis of degenerated DCs most likely due to the synergistic effects of multiple peptides and EVs identified in the CM.
- ✓ Conditioned media derived from hMSCs has the ability to drive degenerated disc cells towards regeneration while extinguishing the effects of MMP-1.
- ✓ Extracellular vesicles (exosomes) from hMSCs expedited regeneration processes in degenerated DCs and could be one of the key signaling systems contributing to the therapeutic abilities of the hMSCs.

7 FUTURE PERSPECTIVES

It would be of interest to further explore the effects of PRE-T *in vivo* where hMSC activation with IL-1β could be performed prior to transplantation into degenerated IVDs followed by administration of BMP-3. This would answer the question if the same effect can be observed *in vivo* settings. Moreover, tissue levels of IL-1β should also be investigated in degenerated IVD tissues in order to understand at which concentration IL-1β induces the pathologies of the IVDs.

Multiple potent signaling peptides were identified in the CM that showed the potential of their involvement in the enhanced chondrogenic effect observed. Hence, a few relevant peptides could be selected to develop a cock-tail media that could be used to stimulate the disc cells. This would identify the proper combination of the factors responsible for the effects.

Furthermore, it would be interesting to analyze the sEVs/exosomes to identify their contents through proteomic analysis to further reveal the factors responsible for the regenerative effects on the disc cells. Newly emerged isolation techniques can also be incorporated to increase the purity of the particles. Human MSCs are known to secrete factors as a response to stimuli in their microenvironment. Therefore, it would be relevant to compare the content of sEVs secreted from hMSCs cultured in a pro-inflammatory environment to the ones in normal growth media to further confirm the notion of paracrine signaling. This will also allow the manipulation of hMSCs to produce desired factors that could signal for regeneration in the IVD environment. Additionally, it is advisable to investigate the effects of sEVs in animal models to confirm the findings observed in the studies presented in this thesis. Future studies should also focus on unraveling the mechanism behind the regenerative effects of these factors.

Whether or not the stress hormone cortisol makes it to the vicinity of the IVDs through diffusion still remained to be confirmed. Therefore, it would be interesting to investigate the expression or the presence of glucocorticoid receptors that bind to cortisol and other glucocorticoids in IVD tissues. More investigations on pain-induced stress are needed as stress has been shown to worsen the progression of many diseases.

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