T.C.

REPUBLIC OF TURKEY

HACETTEPE UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

ASSESSMENT OF THE EFFECTS OF MELATONIN ON THE FUNCTIONAL DEFICITS INDUCED BY CELLULAR STRESS IN OBESE DONOR DERIVED MESENCHYMAL STEM CELLS

Ece Gizem POLAT (BSc)

Stem Cell Program

MASTER THESIS

ANKARA

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Assoc. Prof. Fatima S. F. AERTS KAYA

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APPROVAL PAGE

Assessment of the Effects of Melatonin on the Functional Deficits Induced by Cellular Stress in Obese Donor Derived Mesenchymal Stem Cells

Ece Gizem POLAT

Supervisor: Assoc. Prof. Fatima AERTS KAYA

This thesis study has been approved and accepted as a MSc. dissertation in "Stem Cell" Program by the assessment committee, whose members are listed below, on 22.05.2024.

Chairman of the Committee:	*Prof. Dr. N. Dilara ZEYBEK* *Dept of Stem Cell Sciences*	*(Signature)*
Advisor of the Dissertation:	*Assoc. Prof. Dr. Fatima AERTS KAYA* *Dept of Stem Cell Sciences*	*(Signature)*
Member:	*Assist. Prof. Dr. Parisa SHARAFİ* *TOBB University, Dept of Medical Biology*	*(Signature)*

This dissertation has been approved by the above committee in conformity to the related issues of Hacettepe University Graduate Education and Examination Regulation.

Prof. Dr. Müge YEMİŞCİ ÖZKAN
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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in the data set. In the case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except for cited references. It was produced by myself in consultation with my supervisor Assoc. Prof. Dr. Fatima AERTS KAYA and written according to the rules of thesis writing at Hacettepe University Graduate School of Health Sciences.

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ABSTRACT

POLAT, E.G. Assessment of the effects of Melatonin on the functional deficits induced by cellular stress in obese donor derived mesenchymal stem cells. Hacettepe University Graduate School of Health Sciences Stem Cell Master Thesis, Ankara, 2024. Obesity negatively affects different types of stem cells and BM-MSCs from individuals with a high BMI display a severely impaired differentiation capacity, reduced proliferation and increased ER stress. However, the role of oxidative stress (OS) in the development of obesity-related loss-of-stemness and the potential protective role of melatonin (MT) is currently unknown. Here, we evaluated ER stress, OS and senescence in BM-MSCs obtained from healthy (BMI 20-25), obese (BMI of 25-30) and morbid obese (BMI>30) donors. We assessed the proliferative and three-lineage differentiation potential of BM-MSCs in relation with BMI and we investigated whether treatment with MT with or without TUDCA can modulate ER stress, OS and improve cellular functions of obese donor-derived BM-MSCs. BM-MSCs obtained from all donors were morphologically and phenotypically similar. Obese and morbid obese donor-derived BM-MSCs display signs of increased ER stress and moderate OS, resulting in accelerated senescence, decreased proliferation, increased levels of ROS and decreased differentiation capacity. TUDCA partially reversed the effects of obesity-related loss-of-stem cell function. MT stimulated proliferation of healthy BM-MSCs, but was not able to recover loss-of-stemness. Neither TUDCA nor MT alone or in combination were sufficient to completely rescue the differentiation potential of obese donor-derived BM-MSCs. In conclusion, obesity-related loss-of-stem cell function can be contributed to increased ER stress and moderate OS, that can be partially treated with TUDCA.

Keywords: Melatonin, TUDCA, obesity, endoplasmic reticulum stress, oxidative stress

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ÖZET

POLAT, E.G. Melatonin'in Obez Donor Mezenkimal Kök Hücrelerinde Hücresel Strese Bağlı Olarak Oluşan Fonksiyon Bozuklukları Üzerindeki Etkilerinin Değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Kök Hücre Yüksek Lisans Tezi, Ankara, 2024. Mezenkimal stromal/kök hücreler (MKH'ler), multipotent özelliklere ve kendini yenileme yeteneklerine sahip iğ şeklindeki hücrelerdir. MKH'ler hücre kültür kaplarına yapışır ve kondrositlere, osteositlere ve adipositlere farklılaşabilmektedirler. ISCT (International Society for Cell and Gene Therapy) kriterlere göre kemik iliği (Kİ) kaynaklı MKH'ler (Kİ-MKH) hücre yüzeyi belirteçleri olan CD73, CD90 ve CD105 için pozitif; CD45, CD34, CD14, CD11b ve HLA-DR belirteçleri için ise negatiflerdir (1). Hücreler hem kolayca izole edilebilmekte hem de düşük immünojeniteye sahiptirler. MKH'ler hem Kİ, yağ dokusu gibi yetişkin dokulardan hem de plasenta, göbek kordonu ve amniyotik sıvı gibi fetal dokulardan izole edilebilmektedirler (1). MKH'ler yüksek proliferatif kapasiteye sahip olmaları, göç için kemotaksik sinyallere yanıt vermeleri ve yaralanma bölgelerine yönelmelerinin yanı sıra önemli immünomodülatuar özelliklere de sahiptirler (2). Bu özelliklerinden dolayı MKH'ler rejeneratif tıp alanında ve (oto)immün hastalıkların tedavisinde özellikle önemli hale gelmişlerdir. Son yirmi yıldır MKH'ler çeşitli klinik çalışmalarda test edilmiş ve klinik kök hücre tedavisi için hem etkili hem de güvenli kaynaklar oldukları gösterilmiştir (3). Kök hücreler yaşam boyunca hem *in vivo* hem de *in vitro* koşullar altında çeşitli stres durumlarıyla karşılaşabilmektedir. Hücrelerin strese karşı verdikleri tepki; hücre tipine, stres kaynağına ve stresin süresine bağlı olarak değişebilmektedir. Stres faktörlerine yanıt olarak hücreler, hayatta kalmalarını desteklemek veya yeni bir dengeyi yeniden kurmak için çok çeşitli koruyucu mekanizmaları etkinleştirebilmektedir. Kronik stres durumlarında veya strese karşı koruyucu mekanizmalar stresi azaltmak için yeterli olmadığında apoptotik veya otofaji yolakları aktive olmaktadır. Dolayısıyla hücrenin hücresel stresle nasıl ve ne kadar başa edebildiği, kaderini belirlemektedir. Aşağıda hücrelerin yaşam boyunca karşılaşabileceği farklı stres koşulları ve onlara karşı verdikleri tepkiler tartışılmaktadır. Endoplazmik retikulumu (ER), hem salgılanan ve membrana bağlı

proteinlerin katlanmasında hem de hücre içi kalsiyum homeostazisinin düzenlenmesinde önemli bir rol oynamaktadır. Homeostatik koşullar altında üç ER stres sensörü vardır; PKR benzeri ER kinaz (PERK), İnositol gerektiren enzim 1α (IRE1α) ve aktive edici transkripsiyon faktörü 6 (ATF6). Normal şartlarda bu sensörler aktif değildir ve ER moleküler şaperonu GRP78'e (veya BiP) bağlı durumdadır. Protein sentezi sırasında ER'deki katlanmamış/yanlış katlanmış proteinlerin sayısı artar ve proteinler, uygun şekilde katlanıncaya kadar ER'de bekletilmektedir. Proteinler doğru üçüncül şekillerine uygun şekilde katlanamazsa, proteinler veya peptidler yok edilmek üzere işaretlenmektedir. Protein üretimindeki artış veya protein katlama kapasitesindeki azalma, katlanmamış/yanlış katlanmış proteinlerin birikmesi nedeniyle ER stresine sebep olmaktadır. ER stresine yanıt olarak, katlanmamış protein yanıtı (UPR) adı verilen bir süreç başlatılmaktadır. UPR, katlanmamış/yanlış katlanmış proteinleri ortadan kaldırmak için GRP78'in sensörlerden serbest bırakılmasıyla başlamaktadır. Bu süreç PERK ve IRE1α'nin otofosforilasyonuna ve ATF6'nın ER'den Golgi kompleksine göç etmesine neden olmaktadır. ER stres seviyeleri arttığında, hücreler başlangıçta ER ekspansiyonu ile protein katlama kapasitelerini artırarak, diğer proteinlerin üretimini azaltarak ve şaperon proteinlerinin sentezinde bir artış sağlayarak, katlanmamış/yanlış katlanmış proteinlerin parçalanması veya düzeltilmesiyle sonuçlanan ER stresini azaltmaya çalışılacaktır. PERK'nin aktivasyonu, ökaryotik başlatıcı faktör 2α'nin (eIF2α) aşağı yönde aktivasyonu ve daha sonra çekirdeğe giren ve apoptotik yol C/EBP homolog proteininin (*CHOP*), X-box'ın gen ekspresyonunu artıran transkripsiyon faktörü 4'ün (*ATF4*) aktive edilmesiyle sonuçlanmaktadır. ER stresi sonucunda gerçekleşen *IRE*1α otofosforilasyonuyla sXBP1 proteininde bir artışa ve ATF6 proteininin ER membranından ayrılmasına neden olmaktadır. ATF6 daha sonra Golgi organeline yerleşerek, modifiye edilerek ATF6N olarak çekirdeğe girip; şaperon, lipit ve *XBP*1 üretimini arttırmaktadır. Stres uzun süre devam edilip aşılmaz olduğunda ise UPR, *CHOP*'un aktivasyonuyla proapoptotik bir yanıtı aktive edebimektedir. Sonunda ER stresi yeterince azaltılamadığında hücre otofaji veya apoptotik yolların aktivasyonu yoluyla yok edebilmektedir. Oksidatif stres (OS), reaktif oksijen ve nitrojen türlerinin

(ROS ve RNS) düzensiz üretimi ve/veya temizlenememesi ile karakterize edilmektedir. Yüksek ROS düzeyleri hücresel hasara ve işlev bozukluğuna neden olurken, düşük bazal ROS düzeylerinin hücre için gerekli olduğu ve hücresel çoğalmayı, farklılaşmayı ve hayatta kalmayı sürdürdüğü düşünülmektedir. ROS'un yaklaşık %90'ı mitokondriyal elektron taşıma sistemleri (ETS) tarafından endojen olarak üretilmektedir (4). Radyasyon, ultraviyole ışık, hipoksi ve düşük sıcaklık gibi dış faktörler de ROS üretiminin artmasına neden olmaktadır. ROS hücre içi sinyalleşme ve iletişimde önemli bir rol oynamaktadır. Hücrelerin fizyolojik çoğalması ve farklılaşması için belirli seviyelerde ROS gerekli olmasına rağmen, aşırı ROS'un MKH'lerde fonksiyonel hasara neden olduğu gösterilmiştir (5). Sürekli artan ROS seviyeleri, biyolojik makromoleküllerde (DNA/RNA) kırıkların meydana gelmesi yoluyla hücrede ciddi hasara neden olabilmekle birlikte genomik dengesizliğe yol açabilmektedir. Kök hücreler, güçlü antioksidan/DNA onarım mekanizmaları nedeniyle diğer olgun hücrelere göre OS'ye nispeten daha dayanıklıdır. Ayrıca kök hücreler, oksidatif fosforilasyon yerine glikolize bağımlı olmaları nedeniyle bu hücreler OS'ye karşı daha az duyarlı olup, bu da onları OS'ye karşı kendilerini korumak için olgun hücrelere göre daha avantajlı kılmaktadır. Ancak savunma mekanizmaları yetersiz kalırsa OS, ER stresine benzer şekilde kronikleşebilmektedir. Devam eden OS, nörodejeneratif ve metabolik hastalıklar gibi çeşitli insan hastalıklarının başlangıcına ve ilerlemesine neden olduğu gösterilmiştir. Antioksidan moleküller OS'yi azaltır ve hücreyi uzun süreli strese karşı koruyabilmektedir. Artan katalaz ekspresyonu, hücrenin OS'ye karşı direncini artırmaktadır. ER stresine benzer şekilde, XBP1 ve hedef genleri OS'ye yanıt olarak aktive edilerek DNA hasarı onarımını ve redoks homeostazisini düzenlemektedir. *XBP1*'in baskılanmasının, artan ROS seviyeleri ve kalıcı p38 fosforilasyonu yoluyla OS'yi arttırdığı gösterilmiştir (6). *XBP1*'in silinmesi veya susturulması, süperoksit dismutaz 1 (*SOD1*) ve tioredoksin redüktaz 1 (*TXNRD1*) dahil olmak üzere antioksidan genlerin ekspresyonunun azalmasına neden olmaktadır (7). Yaşlanan hücreler, yaşlanma fenotipini diğer hücrelere yayarak ve inflamatuar proteinlerin salgılanmasına neden olarak komşu hücrelerin davranışını etkileyen yaşlanmayla ilişkili salgı fenotipi (SASP) kazanmaktadır (8). Mekanik olarak

yaşlanma, proliferatif uyaranların varlığına rağmen geri döndürülemez bir hücre döngüsü durması ile karakterize edilmekte ve eksojen/endojen mutajenlerin neden olduğu replikasyon hatalarının bir sonucu olarak biriken DNA hasarından kaynaklanmaktadır. DNA hasar tepkisi (DDR) sırasında p53 stabilizasyonu ile mutasyonları tespit edilmekte ve p21 aktivasyonu yoluyla hücre döngüsünü zorla durdurarak DNA onarımını başlatmaktadır. Ancak DNA hasarı devam ederse; p38-mitojenle aktifleştirilen protein kinaz (MAPK) aktivasyonu, mitokondriyal fonksiyon bozukluğuna, CDK inhibisyonuna ve Rb1 aktivasyonuna neden olarak hücresel yaşlanmayı tetikleyebilmektedir. Ek olarak, artan ROS seviyeleri telomer erozyonunu hızlandırabilmekte ve mitokondriyal metabolizmayı daha da azaltabilmektedir. Ayrıca bu durum kök hücrelerin hücre döngüsünden kalıcı olarak çıkmasına sebep olmaktadır (9). Telomerler tüm ökaryotik kromozomların uçlarında bulunur ve kısa nükleotid TTAGGG tekrarlarından oluşmaktadır. DNA replikasyonu sırasında DNA'nın uç kısmı kopyalanmadığından dolayı her replikasyon döngüsünde telomerler kısalmaktadır. Biriken DNA hasarı ve telomerlerin kısalması sonucunda MKH'lerin kendini yenileme ve rejeneratif potansiyeli azalarak, hücreler replikatif yaşlanmaya girmektedir. Yaş, obezite ve sistemik inflamasyon gibi diğer faktörlerin de bu yaşlanma sürecini hızlandırarak kök hücre fonksiyonunu daha fazla negatif yönden etkilediği gösterilmiştir (10). Yaşlanmanın bir başka biyolojik belirteci yaşlanmayla ilişkili b-galaktosidazdır (SA-β-Gal). Sağlıklı hücrelerde b-galaktosidaz pH 4.0'da eksprese edilirken, yaşlanmayla ilişkili bu SA-β-Gal'in ifadesi pH 6.0'da olduğu gösterilmiştir ve sağlıklı ve yaşlanmış hücreleri ayırt etmek için kullanılabilmektedir. Obezite küresel bir halk sağlığı sorunu olup, diyabet, kardiyovasküler hastalıklar gibi metabolik hastalıkların olasılığını arttırmaktadır. Hem karbonhidrat hem de lipit metabolizması obeziteden etkilenmektedir. Glikolipotoksisitenin bir sonucu olarak ER fonksiyonunun bozulması; olgunlaşmamış, yanlış katlanmış veya katlanmamış proteinlerin birikmesine neden olmaktadır. ER stresi kronikleşirse hepatik lipogenezi indüklemekte ve pankreatik beta hücrelerinde glukozla uyarılan insülin sekresyonunu bozarak karaciğer ve pankreasta fonksiyonel bozukluklara neden olmaktadır. Yetişkinlerde obezite, vücut kitle indeksi (BMI) kullanılarak ölçülür; burada 20-25'lik

bir BMI sağlıklı bireyleri, BMI ≥ 25 obezleri ve BMI ≥30 morbid obez hastaları temsil etmektedir. Kök hücrelerin rejeneratif tıp amacıyla kullanım alanları hızla genişlemektedir. Genel popülasyonda obezite oranlarının yükselmesi ve obezitenin kök hücre fonksiyonu üzerindeki olası (olumsuz) etkilerinin aydınlatılması hızla önem kazanmıştır: kök hücre ve doku onarım fonksiyonlarının obeziteden etkileyebildiği için tedavi hedefi olarak kullanılabilmektedir, ek olarak kök hücrelerin fonksiyonel olarak bozulmuş olabilmesi hücrelerin kullanılabilecek tedavi alanlarını sınırlandırmaktadır. Yüksek BMI'ye sahip insan donörlerden elde edilen Kİ-MKH'lerin ciddi derecede bozulmuş osteojenik, azalmış adipogenik farklılaşma, azalmış proliferasyon oranları ve artan yaşlanma ile birlikte ER stresi ile ilişkili genler olan *ATF4* ve *CHOP*'un artan ekspresyonunu sergiledikleri gösterilmiştir (11). Benzer şekilde obez bireylerden elde edilen yağ dokusu kaynaklı kök hücrelerin farklılaşması bozulmakta ve ömürleri kısalmaktadır (12). Obezite ayrıca kök hücre nişini etkileyerek ve bozarak hematopoietik kök/progenitör hücreler (HPKH'ler), iskelet kası kök hücreleri ve germ hücreleri dahil olmak üzere diğer birçok kök hücre tipinin fonksiyonlarını hem doğrudan hem de dolaylı olarak olumsuz etkiler. Örneğin, Kİ adipositlerinde obeziteye bağlı bir artış, HPKH fonksiyon bozukluğuna ve bağışıklık yetersizliğine neden olabilmektedir. Memelilerde Melatonin (N-asetil-5-metoksitriptamin, MT); günün karanlık evrelerinde başlıca epifiz bezindeki pinealositler tarafından üretilmektedir. MT sentezi; Kİ, yumurtalık, testis, bağırsak, plasenta ve karaciğer gibi birçok farklı dokuda gerçekleşmektedir. MT esas olarak sirkadiyen ritmin düzenleyicisi olmasına rağmen antioksidan savunma mekanizmalarının düzenlenmesinde, bağışıklık sisteminin modülasyonunda, kanserin önlenmesinde ve osteoproteksiyonda da önemli bir rol oynamaktadır (13). MT'nin sirkadiyen etkisine ana sirkadiyen genler *CLOCK* ve *BMAL1* aracılık etmektedir. Sirkadiyen ritmin çevresel bozulması obezitenin gelişimini tetiklemekte veya hızlandırmaktadır. Memelilerde MT etkilerini dört farklı mekanizma aracılığıyla gösterir: 1) yüzey MT reseptörlerine bağlanma, 2) nükleer reseptörlere bağlanma, 3) hücre içi proteinlere bağlanma ve 4) doğrudan antioksidan etki yoluyla (14). Bu MT reseptörleri beyin, retina, kardiyovasküler sistem, karaciğer ve safra kesesi, pankreas, böbrek, bağışıklık sistemi

hücreleri, trombositler, prostat ve meme epiteli gibi vücudun farklı bölgelerinde bulunabilmektedir. MT1 aktivasyonu, Protein Kinaz A'nın (PKA) inhibisyonuna, forskolin (FSK) ile uyarılan cAMP oluşumunun inhibisyonuna yol açmakta ve hem pAKT hem de pERK1/2'nin forsorilasyonu ve aktivasyonu sağlamaktadır. MT2 reseptörü G-proteinlerine bağlanarak hem adenilat siklaz inhibisyonuna hem de guanilil siklaz yolunun inhibisyonuna, aynı zamanda FSK ile uyarılan cAMP oluşumunun inhibisyonuna neden olmaktadır. MT2 sinyal yollarının aktivasyonuna PKC'nin aktivasyonu ve pERK1/2'nin fosforilasyonu aracılık ederken, pAKT'nin aktivasyonu aracılık etmemektedir. MT reseptörü 3/Kinon redüktaz 2 enzimi (MT3), redüktaz grubuna aittir ve kinonların elektron transfer reaksiyonlarını inhibe ederek OS'den korunmada rol oynar(15). MT3 reseptörü, MT'ye olan düşük afinitesi nedeniyle MT1 ve MT2 bağlanmasından daha hızlı ligand birleşme/ayrılma kinetiği sergilemektedir. MT3/QR2 detoksifikasyonda önemli bir rol oynamaktadır. MT3 reseptörün bağlanma bölgesinin membrana bağlı iken, QR2 enzimi sitozolde bulunmaktadır. MT'nin bilinen tek nükleer reseptörü, Retinoid ilişkili yetim nükleer hormon reseptörüdür (RZR/RORα) ve hem periferik sinir sisteminde hem merkezi sinir sisteminde bulunmaktadır. MT'nin ROR'un ligand bağlama domeynine bağlanmasından sonra, RZR/RORα'un reseptör DNA bağlanma domeynine, hedef genlerindeki bir ROR yanıt elemanına (RORE) bağlanarak transkripsiyonu başlatmaktadır. RZR/RORα'nun MT aktivasyonu, immün modülasyondan, hücresel büyümeden ve periferdeki kemiğin farklılaşmasından sorumludur. MT'nin nükleer reseptörüyle etkileşimi, protein kinaz C'nin (PKC) aktivasyonuyla sonuçlanır ve mononükleer hücrelerde IL-2 ve IL-6 üretimini indüklemektedir. MT'nin doğrudan etkileri, hücre içi proteinler kalmodulin, kalretikulin ve tubulin ile etkileşim yoluyla modüle edilmektedir. Kalmodulin, hücre içi ikincil haberci olarak görev yapmaktadır. MT, kalsiyumun kalmodulin'e bağlanmasını doğrudan antagonize etmekte ve MT'nin kanser gelişimi üzerindeki anti-proliferatif etkisine bu yolak aracılık edebileceği düşünülmektedir. Yüksek lipit çözünürlüğü nedeniyle MT, reseptörleriyle olan etkileşimlerinden bağımsız olarak hücre zarından serbestçe geçerek güçlü bir oksijen radikal temizleyicisi olarak görev yapabilmektedir. Memelilerde, OS altındaki

hücrelerin dolaşımdaki MT'yi periferik kandan hızla temizlediği gösterilmiştir. MT daha sonra oligopeptit taşıyıcılar PEPT1 ve PEPT2 yoluyla mitokondriye girerek antioksidan olarak işlev görmekte ve lipitleri, proteinleri ve DNA'yı oksidatif hasardan korumaktadır. MT'nin mitokondriyal üretimi ayrıca oksijen radikallerinin temizlenmesine yardımcı olmaktadır. MT'nin etkilerini gösterdiği diğer mekanizmalar, metallerin bağlanması ve OH radikallerinin azaltılması, süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx) ve glutatyon redüktaz (GR) gibi antioksidan enzim sistemlerinin aktivasyonu yoluyladır (16). MT'nin kök hücreler üzerindeki etkileri kök hücre tipine göre değişmektedir. Birçok çalışma, MT'nin MKH'lerin osteojenik ve kondrojenik farklılaşmasını indüklerken, adipogenik farklılaşmayı baskıladığını göstermiştir. Bu etkilerin çoğuna Wnt/b-Katenin sinyal yolunun aktivasyonu ile başlatılmaktadır. İnflamasyon sırasında MT'nin proinflamatuar sitokinlerin üretimini baskılayarak hücresel canlılığı koruduğu gösterilmiştir. Ayrıca MT bu hücreleri apoptozdan korduğu ve hücresel stresi azaltarak canlılığı arttırabildiği gösterilmiştir. Ek olarak, MKH'lerin MT ile ön muamelesinin, böbrek iskemisinin tedavisinde kullanıldığında terapötik etkinliğini arttırdığı gösterilmiştir (17). Her ne kadar Kİ-MKH'lerde MT reseptörlerinin varlığı doğrudan doğrulanmamış olsa da MT antagonistlerinin kullanımı, MKH'lerde sinyallemenin MT2 reseptörlerinin aktivasyonu yoluyla gerçekleştiğini göstermektedir. Kİ nişindeki MT'nin, ROS üretiminin baskılanması ve NF-kB sinyallemesinin inhibisyonu yoluyla iltihaplanma yoluyla HKPH bakımını desteklediği de gösterilmiştir. Embriyonik kök hücreleri (EKH'ler) ve indüklenmiş pluripotent kök hücreleri (uPKH'ler) içeren MT çalışmaları nadir olmasına rağmen, MT'nin büyük ölçüde MT1 ile etkileşim yoluyla bu kök hücreleri de etkilediği gösterilmiştir (18). MT aynı zamanda MT reseptörüne bağımlı N6-metiladenosin (m6A) RNA düzenlemesi yoluyla uzun süreli kültürlenmiş EKH'lerin pluripotensitesini de arttırmıştır. MT; nöronal kök ve progenitör hücrelerde (NKPH'ler) farklılaşma, çoğalma ve hayatta kalma yollarında önemli bir rol oynamaktadır. MT kan-beyin bariyerini geçebildiğinden ve aynı zamanda hem lipitte hem de suda çözünebildiğinden, NKPH'lerin çoğalmasını ve hayatta kalmasını modüle etmek için kullanılabilmektedir

(19). NKPH'lerde MT, etkilerini MT1 ve MT2 reseptörlerine bağlanarak, hem ERK/MAPK hem de PI3K/AKT yollarını aktive ederek göstermektedir. MT; hasarlı, işlevsiz NPKH'lerin onarımını destekleyerek NPKH'lerin hayatta kalması, çoğalması ve farklılaşmasında da önemli bir rol oynamaktadır. MT'nin farklı pluripotent ve doku kök hücreleri üzerindeki etkisinin yanı sıra, kanser kök hücrelerini (KKH'ler) doğrudan etkileyerek kanser önleyici etkilere sahip olduğu da gösterilmiştir (20). Bu anti-kanser etkileri birçok araştırmacı tarafından hem *in vitro* hem de *in vivo* olarak ve birçok farklı kanser türü için doğrulanmıştır. MT'nin, anti-proliferatif etkiler, onkogen ekspresyonunun düzenlenmesi, antioksidan ve anti-anjiyogenik etkiler gibi birçok mekanizma yoluyla kanseri inhibe ettiği gösterilmiştir. MT sinyali aynı zamanda KKH'lerin kendini yenileme ve hayatta kalmasıyla ilişkili anahtar yolları da düzenleyebilmektedir (20). Çoğu zaman kanser hücreleri oksidatif fosforilasyondan aerobik glikolize geçer; bu süreç Warburg etkisi olarak adlandırılmaktadır (21). Kanser hücreleri bu adaptasyon mekanizmasından yararlanarak, hücre büyümesi için gerekli olan nükleotidlerin, lipitlerin ve proteinlerin yeni üretimi için glikozu bir karbon kaynağı olarak kullanmaktadırlar. MT kullanımının kanser hücresi metabolizmasını aerobik glikolizden uzaklaştırdığı, böylece kanser hücresi büyümesini inhibe ettiği gösterilmiştir (22). MT ilk olarak glikoz ve lipit metabolizmasının düzenlenmesinde yer alan endojen bir hormon olarak işleviyle ve daha sonra bir antioksidan rolüyle tanınmıştır. Uykudan mahrum bırakılan hayvanların kilo alımına duyarlı olduğu gösterilmiş; bu da epifiz bezi, MT üretimi ve sağlıklı kilonun korunması arasında doğrudan bir ilişkiye işaret etmiştir. İlk olarak, hipotalamik nöronlar ve suprakiazmatik çekirdeklerde (SCN) eksprese edilen MT ve MT1 arasındaki etkileşim, norepinefrin (NE) üretimi yoluyla sempatik sinir sistemi aktivitesini arttırmakta ve eşleşmeyen protein 1 (UCP1), PPARG ve PGC1'in uyarılmasına ve gen ekspresyonunun artmasına neden olmaktadır (23). İkinci olarak, MT doğrudan BAT üzerinde etki ederek hücre içi cAMP'de bir azalmaya, PKA aktivitesinde azalmaya, cAMP'ye duyarlı element bağlayıcı proteinin (CREB) fosforilasyonuna ve hem MT1 hem de MT2 reseptörlerinin aktivasyonu yoluyla UCP1 ekspresyonunun yukarı regülasyonuna neden olmaktadır. Üçüncü olarak, MT'nin uygulanması, BAT

mitokondrisinde MT ve metaboliti olan N1-asetil-N2-formil5-metoksiknuramin (AFMK) ile birlikte organeli OS'den korumakta ve Kahverengi adipoz doku (BAT)'ın termojenik fonksiyonunu geliştirmektedir. Zucker Diabetic Fatty (ZDF) farelerine uzun süreli MT uygulamasının, Beyaz adipoz doku (WAT)'nun bej yağ dokusuna geçişini ve UCP1 ekspresyonunun yukarı regülasyonunu teşvik ettiği, böylece termojenez ve kilo kaybına katkıda bulunabildiği gösterilmiştir (23). Bu nedenlerden dolayı BAT enerji kullanımını iyileştirmek için MT kullanımının obezitenin tedavisi için potansiyel bir terapötik hedef sağlayabileceği öne sürülmüştür. Obezitede yağ dokusu tarafından üretilen proinflamatuar sitokinler ROS üretmektedir. Etkili bir serbest radikal temizleyici olarak görev yapan MT'nin uygulanması, daha sonra endojen antioksidan enzimler SOD, katalaz (CAT), GPx ve GR ile iş birliği yaparak hücrelerin doğrudan ROS'u temizleyerek OS'ye karşı korumasını sağlamaktadır. MT ayrıca glutatyon metabolizmasıyla ilgili antioksidan enzimleri aktive ederek ROS'u dolaylı olarak azaltmakta ve böylece mitokondriyal hasarı inhibe etmektedir. Ayrıca MT, OS'yi azaltan geçiş metallerini şelatlayarak hidroksil radikallerinin oluşumunu azaltabilmektedir. MT'nin obeziteye bağlı OS'yi azaltmasının bir başka yolu, hücre içi bir bozunma yolu olan otofajinin düzenlenmesi (aktivasyonu) yoluyladır. IL-1β varlığında MT'nin hem ROS üretimini azalttığı hem de SOD düzeylerini artırarak serbest radikalleri ortadan kaldırdığı, böylece genel hücre sağkalımını koruduğu gözlemlenmiştir. MT'nin anti-apoptotik etkisine, hem pPERK, GRP78 ve p-eIF2α gibi ER stresiyle ilişkili proteinlerin aşağı regüle edilmesiyle ER stresinin azaltılması hem de oksidatif ve nitrosatif stres, hücreleri apoptoza karşı daha az duyarlı hale getirmektedir. MT'nin OS'yi iyileştirdiğine dair ikna edici kanıtlar hem aşırı kilolu hayvanlarda hem de insanlarda bulunmuştur. MT'nin fare Kİ-MKH'lerinde ER stresini baskılayabildiği gösterilmiştir. Bir antiinflamatuar ajan olarak MT, proinflamatuar yanıtı baskılayarak obezite dahil bazı kronik hastalıkların iyileşmesini destekleyebilmektedir. MT'nin hem klinik ortamda hem de günlük yaşamda ticari olarak temin edilebilen gıda takviyesi olarak kullanımı arttığından, insanlarda kullanımının güvenliği ilgi konusu olmuştur. Ek olarak, mevcut klinik veriler, eksojen MT uygulamasının ciddi bir yan etkisinin olmadığını göstermektedir. Kolesterol, insan

karaciğerinde birincil safra asitleri olan kolik asit ve kenodeoksikolik asitten (CDCA) sentezlenmektedir. Ursodeoksikolik asit (UDCA) taurin ile konjuge edilerek tauroursodeoksikolik asite (TUDCA) dönüştürülmektedir (24). TUDCA ve UDCA, G-protein bağlı reseptör 5 (TGR5), sfingozin-1-fosfat reseptörü 2 (S1PR2) ve nükleer reseptör Farnesoid X Reseptörü (FXR) gibi membran reseptörlerinin ligandlarıdır. Ek olarak TUDCA ve UDCA, a5β1 integrini bağlayıp aktive ederek Na+/taurokolat ortak taşıyıcı peptidi (NTCP) aracılığıyla doğrudan hücre içine taşınabilmektedirler. Ek olarak, Mineralokortikoid reseptörü (MR) ve glukokortikoid reseptörü (GCR) yoluyla da hücreye girebilmektedirler. FXR, safra asidi sentezinin düzenlenmesi, safra asidi sekresyonu, bağırsakta safra asidi emilimi ve safra asitlerinin hepatik alımı gibi çeşitli süreçlerden sorumludur. TGR5 sinyali çoğunlukla ekstrahepatik bölgelerde, enerji homeostazisini düzenlediği ve insülin salgısının uyarıldığı bölgelerde işlev görmektedir. BAT ve iskelet miyositlerinde TGR5 sinyallemesi ile CREB aktivasyonu sağlamakta ve obeziteyi ve insülin direncini önlemek için immünsüpresyona, enerji ve glukoz homeostazisine aracılık etmektedir. Enflamasyon sırasında FXR aktivasyonu, aktivatör protein 1 (AP-1) ve sinyal ileticileri ve transkripsiyon 3 aktivatörleri (STAT3) ile etkileşime girerek hücresel sinyal yollarını inhibe etmektedir. Ek olarak, FXR, NF-kB gibi immün yanıtların anahtar genlerinin ekspresyonunun inhibe edilmesinde de rol oynamaktadır (25). TUDCA; GRP78 ifadesinin düşürülmesi ve PERK/JNK fosforilasyonunu azaltarak ve CHOP ifadesinde bir azalma sağlayarak ER stresi baskılamaktadır. Aynı zamanda TUDCA'nın ROS üretimi yoluyla yüksek glukoza maruz kalan beyaz adipositlerde anti-apoptotik etkiyi indükleyebildiği gösterilmiştir. TUDCA'nın tip 2 diyabetik farelerde GRP78 ve ATF4 gibi ER stres belirteçlerini azalttığını, endotel fonksiyonunda iyileşmeye ve aterosklerozda azalmaya yol açtığını göstermiştir. Obezitenin birçok farklı kök hücre türü üzerinde olumsuz etkisi olduğu gösterilmiştir. Daha önce yüksek BMI'li bireylerden izole edilen Kİ-MKH'lerin ciddi derecede bozulmuş osteojenik ve azalmış adipogenik farklılaşma, azalmış proliferasyon oranları ve ER stresiyle ilişkili genler *ATF4* ve *CHOP*'un artan ekspresyonunu sergilediğini göstermiştik (26). ER stresini azaltmak için TUDCA veya 4-Fenilbütirat (4-PBA) ile tedaviye maruz bırakılan Kİ-MKH'lerin, kök hücre

fonksiyonunda kısmi iyileşme gözlenmiş, bu da osteojenik ve adipogenik farklılaşmanın artmasına neden olmuştur. Ancak, BMI'nin kondrojenik farklılaşma üzerindeki etkisi, OS'nin obeziteye bağlı kök kaybı gelişimindeki rolü ve MT'nin ER stresini, OS'yi ve diğer yolları azaltarak potansiyel koruyucu rolü daha önce değerlendirilmemiştir. Bu nedenle çalışma hipotezimiz şu şekildedir: "Kİ-MKH'lerde görülen ve artan BMI'e bağlı olarak gelişebilen köklülük kaybı MT ve TUDCA uygulamaları ile düzeltilebilir". Bu hipotezi test etmek için bu yüksek lisans tezi kapsamında: 1) Sağlıklı donörlere (BMI 20-25), obez donörlere (BMI 25-30) ve morbid obez donörlere (BMI>30) sahip donörlerden elde edilen Kİ-MKH'lerde ER stresi, OS ve yaşlanmayı değerlendirmesi; 2) Kİ-MKH'lerin çoğalma ve farklılaşma potansiyelini BMI ile ilişkili olarak değerlendirmesi; 3) TUDCA ile veya TUDCA olmadan MT tedavisinin ER stresini, OS'yi modüle edip edemeyeceğini ve obez donörden türetilen Kİ-MKH 'lerin hücresel fonksiyonlarını iyileştirip iyileştiremeyeceği araştırılması planlanmıştır. Kİ-MKH'ler yetişkin sağlıklı (vücut kitle indeksi/BMI 20-25), obez (BMI 25-30) ve aşırı obez (BMI>30) donörlerden elde edilmiş ve karakterizasyon için 3. pasaja (P3) ve devam deneyleri için 6. pasaja kadar kültürlenmiştir. Yapılan deney sonuçlarında elde edilen verilere göre sağlıklı, obez ve morbid obez donör Kİ-MKH'ler morfolojik olarak birbirlerine benzerdir. Sağlıklı, obez ve morbid obez donörlerden alınan Kİ-MKH/P3, CD29, CD45, CD73, CD105, CD90 ve HLA-DR'ye karşı antikorlarla boyanmıştır. Tüm donörlerden alınan hücreler, CD29 (>%93), CD73 (>%93), CD90 (>97) ve CD105 (>%85) CD45 (<%3) ve HLA-DR (<%5) ekspresyonlarına ve düşük ekspresyonlarına dayanarak Kİ-MKH'ler olarak sınıflandırılabilir. Bu çalışmada her grupta karma cinsiyet popülasyonları kullanılmıştır. Ayrıca yaşın etkisini de değerlendirmek amaçlandığı için BMI ile yaş arasındaki korelasyon (Pearson korelasyonu: r=0,35) hesaplanmıştır. Çalışma grubunda yaş arttıkça BMI'da istikrarlı bir artış saptansa da yaş ile BMI arasında anlamlı bir korelasyon (p>0,05) bulunmamıştır. Bu nedenle, bu tez kapsamında sağlıklı, obez ve morbid obez Kİ-MKH donörlerinin ileri analizlerinde yaş, bir değişken olarak göz ardı edilmiştir. Sağlıklı (n=3), obez (n=3) ve morbid obez (n=3) hastalardan alınan Kİ-MKH'ler/P6, OS gen ekspresyonun değerlendirilmesi için *SOD1* ve *TXNRD1* gen ekspresyonuna; ER stresin/

UPR aktivasyonunun değerlendirilmesi için *sXBP1, XBP1, ATF4* ve *CHOP* gen ekspresyonlarına bakılmıştır. Önceki çalışmalarda, *ACTB* ekspresyonunun Kİ-MKH'lerde osteojenik ve adipogenik soya doğru farklılaşma sırasında kararsız olduğu bulunduğu için Kİ-MKH farklılaştırmalarındaki kullanımı uygun değildir. Bu nedenle burada 3 farklı " *house-keeping* " genleri; *ACTB, RPLP0* ve *GAPDH*, farklı Kİ-MKH donörlerinde ekspresyonun stabilitesi açısından test edilmiştir. Farklı donörlerin CT değerlerine göre en az değişkenlik *RPLP0* ifadesinde gözlenmiş ve bu nedenle bu tez boyunca bu ‘’*house-keeping’’* gen olarak kullanılmıştır. Sağlıklı, obez ve morbid obez donörlerden elde edilen Kİ-MKH'ler adipojenik, osteojenik ve kondrojenik soylara göre farklılaştırılmıştır. Sonuçlar hem histolojik boyama hem de RT-PCR kullanılarak değerlendirilmiştir. 3 haftalık farklılaşmanın ardından değerlendirilen Kİ-MKH'lerin adipojenik ve osteojenik soylara yönelik farklılaşma kapasitesi artan BMI ile azalırken, ancak bu veriler grupların küçük olduğundan istatistiksel olarak anlam kazanamamıştır. Farklılaşma analizlerinin başlatılmasından bir hafta sonra değerlendirilen erken adipojenik ve osteojenik farklılaşma ile ilişkili genlerin ekspresyonu, sağlıklı donör Kİ-MKH'leri tarafından obez ve morbid-obez donörden elde edilenlere kıyasla adipojenik (*SCD*) ve osteojenik (*ALPL*) yönünde daha iyi farklılaşmayı ortaya çıkarmıştır. Kondrojenik farklılaşma, yalnızca farklılaşmadan 3 hafta sonra değerlendirilmiştir ve adipojenik ve osteojenik farklılaşmanın aksine, kondrojenik farklılaşma ile ilişkili *COL2* artan BMI ile ekspresyonu artmıştır. Obez ve morbid obez donörlerde ER stresi ve OS ile ilişkili gen ekspresyonu, sağlıklı donörden türetilen Kİ-MKH'lerdeki seviyelerle karşılaştırılmıştır. Sağlıklı, obez ve morbid obez donör Kİ-MKH'lerde temel hücresel stres seviyeleri değerlendirildiğinde, morbid obez grupta yalnızca *ATF4* seviyelerinin anlamlı ölçüde arttığı bulunmuştur. Adipojenik, osteojenik ve kondrojenik soylara doğru farklılaşma sonrasında hücresel stres belirlendiğinde hem ER stresi hem de OS belirteçleri genel olarak düşük kalmıştır, ancak osteojenik farklılaşma sırasında obez ve morbid obez donörlerde *sXBP1* ekspresyonundaki artış dikkat çekicidir. Sağlıklı, obez ve morbid obez donör Kİ-MKH’lerde ROS seviyeleri ölçülmüştür. ROS yüzdeleri (H2DCFDA pozitif hücreler) BMI ile birlikte artmasına rağmen bu veriler anlamlı bulunmamıştır ($r=0,45$, $p>0,05$).

Kültürlerdeki yaşlanan hücrelerin yüzdeleri ile BMI arasındaki doğrudan korelasyon da anlamlı bulunmamıştır (r=0.78, p>0.05). Bununla birlikte, veriler grup başına katmanlara ayrıldığında yaşlanan hücrelerdeki artışlar oldukça anlamlı bulunmuştur (sağlıklı vs obez, p<0,01; sağlıklı vs morbid obez, p<0,001). MT'nin hücresel büyüme ve canlılık üzerindeki potansiyel olumsuz etkilerini değerlendirmek için sağlıklı donör Kİ-MKH'ler kullanılmıştır. MT'nin sağlıkı Kİ-MKH'lerin proliferasyonu desteklediğini ve önemli bir toksisite göstermediği bulunmuştur. Bu verilere ve literatürde standart olarak kullanılan MT doz aralıklarına dayanarak, diğer tüm deneyler 30 nM MT dozuyla gerçekleştirilmiştir. MT, TUDCA ve 4-PBA kullanımının potansiyel sinerjistik etkilerini değerlendirmek için bu kimyasallar, hücre çoğalmasına ilişkin WST-1 analizi kullanılarak sağlıklı donör Kİ-MKH'ler üzerinde test edilmiş ve hücre büyüme analizleri 4. gün, 7. gün ve 11. günde ve 14. günde yapılmıştır. Grubumuzun önceki verilerine göre MT 30 nM, TUDCA 50 uM ve 4-PBA 1 mM konsantrasyonda kullanılmıştır. MT ve TUDCA'nın tek kullanımı en iyi çoğalma destekleme kapasitelerini gösterirken, 4-PBA tek başına veya kombinasyon halinde hiçbir zaman standart kullanılan mediyum DMF10'unu geçememiştir. Bu nedenle sonraki deneylerde 4-PBA kullanımı dahil edilmemiştir. MT'nin akış aşağı sinyal aktivasyon yolları değerlendirilmiştir. MT1'in aktivasyonunun hem AKT hem de ERK'nin aşağı yönde fosforilasyonuyla sonuçlandığı, oysa MT2'ye bağlanmanın yalnızca ERK'nin fosforilasyonuyla sonuçlandığı gösterilmiştir. MT ve/veya TUDCA'nın obez ve morbid obez donörden türetilen Kİ-MKH'lerin farklılaşması üzerindeki etkisi gözlemlenmiştir. İşlevsel olarak bozulmuş Kİ-MKH/P6'nın obez ve morbid obez donörlerden farklılaşma kapasitesini geliştirmek için hücreler, MT ve/veya TUDCA varlığında adipogenik, osteojenik ve kondrojenik soylara farklılaştırılmıştır. Her ne kadar TUDCA'nın eklenmesi adipogenik, osteojenik ve kondrojenik yöne doğru farklılaşmasını desteklerken bu etki sadece obezlerde gözlenirken morbid obezler de gözlenememiştir. Bu da hücrelerin stres seviyelerinin çok yüksek olduğu takdirde artık ne TUDCA ne de MT etkilerine duyarlı olamayacağı göstermektedir. MT'nin eklenmesi, obez donörden türetilmiş Kİ-MKH'lerde yalnızca kondrojenik *SOX9* ifadesini geliştirmiştir. TUDCA'nın MT ile hiçbir sinerjistik etkisi gözlenmemiştir. Ayrıca TUDCA ve MT ile kombine tedavi, üç soyun tamamında genel

olarak bastırılmış farklılaşma kapasitesiyle sonuçlanmıştır. Obez ve morbid obez donörden türetilmiş Kİ-MKH'lerde MT ve/veya TUDCA'nın hücresel stres düzeyleri üzerindeki etkisine de bakılmıştır. Öncelikle sağlıklı, obez ve morbid obez bir donörden farklılaştırılması sırasında ilk olarak OS ve ER stresinin hücresel stres belirteçlerinin gen ekspresyonu değerlendirilmiştir. Obez ve morbid obez donör Kİ-MKH'leirndeki *sXBP1* ekspresyonunda bir artış gözlenmiş; bu da IRE1α yolunun ve daha az ölçüde PERK yolunun aktivasyonunu göstermiştir. Bu noktada da OS, Kİ-MKH'lerin obez ve morbid obez donörlerden kaynaklanan fonksiyon kaybında önemli bir rol oynamadığı gözlemlenmiştir. Daha sonra, Kİ-MKH'leri obez ve morbid obez donörlerden ayırmada hücresel stres belirteçlerini baskılayan ve bu hücrelerin farklılaşma kapasitesini artıran MT (OS üzerinde etkili olan) ve TUDCA'nın (ER stresini baskılayan) etkileri değerlendirilmiştir. Ne TUDCA ne de MT, obez ve morbid obez donörden türetilen Kİ-MKH'lerin farklılaşma potansiyelini tamamen kurtarmak için yeterli olmamış, ancak özellikle MT tedavisinden sonra osteojenik farklılaşma üzerinde etkileri gözlemlenmiştir. Sonuç olarak tüm donörlerden elde edilen BM-MSC'ler morfolojik ve fenotipik olarak benzerdir. Obez ve morbid obez donörden türetilen Kİ-MKH'ler, artan ER stresi ve orta derecede OS belirtileri gösterir, bu da yaşlanmanın hızlanmasına, çoğalmanın azalmasına, ROS seviyelerinin artmasına ve farklılaşma kapasitesinin azalmasına neden olur. TUDCA, obeziteye bağlı kök hücre fonksiyonu kaybının etkilerini kısmen tersine çevirmiştir. MT, sağlıklı Kİ-MKH'lerin çoğalmasını uyarmış, ancak köklülük kaybını telafi edememiştir. Ne TUDCA ne de MT tek başına veya kombinasyon halinde, obez donörden türetilen Kİ-MKH'lerin farklılaşma potansiyelini tamamen kurtarmak için yeterli değildir. Sonuç olarak, obeziteye bağlı kök hücre fonksiyonu kaybı, TUDCA ile kısmen tedavi edilebilecek olan artan ER stresine ve orta düzeyde OS'ye katkıda bulunabilir.

Anahtar Kelimeler: Melatonin, TUDCA, obezite, endoplazmik retikulum stres, oksidatif stres

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SYMBOLS and ABBREVIATIONS

ADSC: ADIPOSE DERIVED STEM CELL

AFMK: N1-ASETİL-N2-FORMİL5-METOKSİKNURAMİN

ATF4: ACTIVATING TRANSCRIPTION FACTOR 4

ATF6: ACTIVATING TRANSCRIPTION FACTOR 6

ATP: ADENOSINE TRIPHOSPHATE

BAT: BROWN ADIPOSE TISSUE

BIP: BINDING IMMUNOGLOBULIN PROTEIN

BM: BONE MARROW

BMAL1: BRAIN AND MUSCLE ARNT-LIKE PROTEIN-1

CAMP: CYCLIC ADENOSINE MONOPHOSPHATE

CAT: CATALASE

CDCA: CHENODEOXYCHOLIC ACID

CHOP: C/EBP HOMOLOGOUS PROTEIN

CREB: CAMP RESPONSE ELEMENT-BINDING PROTEIN

CSC: CANCER STEM CELL

DBD: DNA BINDING DOMAIN

DDR: DNA DAMAGE RESPONSE

EMT: EPITHELIAL MESENCHYMAL TRANSITION

ELF2α: EUKARYOTIC TRANSLATION INITIATION FACTOR 2A

ER: ENDOPLASMIC RETICULUM

ESC: EMBRYONIC STEM CELL

ETS: ELECTRON TRANSPORT SYSTEM

FSH: FOLLICLE-STIMULATING HORMONE

FSK: FORSKOLIN

FXR: FARNESOID X RECEPTOR

GADD34: GROWTH ARREST AND DNA DAMAGE-INDUCIBLE PROTEIN

GCR: GLUCOCORTICOID RECEPTOR

GLUT: GLUCOSE TRANSPORTER

GnRH: GONADOTROPIN-RELEASING HORMONE

GPx: GLUTATHIONE PEROXIDASE

GR: GLUTATHION REDUCTASE

GRP78: GLUCOSE REGULATED PROTEIN 78

HSPC: HEMATOPOIETIC STEM/PROGENITOR CELL

IL1-β: INTERLEUKIN-1 BETA

IL-2: INTERLEUKIN-2

IL-6: INTERLEUKIN-6

IPSC: INDUCED PLURIPOTENT STEM CELL

IRE1α: INOSITOL-REQUIRING ENZYME 1 A

ISCT: INTERNATIONAL SOCIETY FOR CELL AND GENE THERAPY

LBD: LIGAND BINDING DOMAIN

MAPK: MITOGEN-ACTIVATED PROTEIN KINASE

M6A: N6-METHYLADENOSINE

MR: MINERALOCORTICOID RECEPTOR

MSC: MULTIPOTENT STROMAL/STEM CELL

MT: MELATONIN

MT1/2/3: MELATONIN RECEPTOR 1/2/3

NE: NOREPINEPHRINE

NFκB: NUCLEAR FACTOR KAPPA B

NR: NUCLEAR RECEPTOR

NSC: NEURAL STEM CELL

NSPC: NEURONAL STEM AND PROGENITOR CELLS

NTCP: NA+/TAUROCHOLATE COTRANSPORTER PEPTIDE

OS: OXIDATIVE STRESS

OXPHOS: OXYDATIVE PHOSPHORYLATION

pAKT: PHOSPHORYLATED AKT

PEPT1/2: PEPTIDE TRANSPORTERS 1/2

pERK: PHOSPHORYLATED ERK

PGC1: PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-GAMMA COACTIVATOR

PI3K: PHOSPHOINOSITIDE 3-KINASE

PKA: PROTEIN KINASE A

PPARγ: PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA

RNS: REACTIVE NITROGEN SPECIES

RORE: ROR RESPONSE ELEMENT

ROS: REACTIVE OXYGEN SPECIES

RZR/RORα: NUCLEAR RECEPTOR

SLC2: SOLUTE CARRIER FAMILY 2

STAT3: SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3

SOD: SUPEROXIDE DISMUTASE

SOD1: SUPEROXIDE DISMUTASE 1

SASP: SENESCENCE-ASSOCIATED SECRETORY PHENOTYPE

SA-β-GAL: SENESCENCE ASSOCIATED β GALACTOSIDASE

SCN: SUPRACHIASMATIC NUCLEUS

S1PR2: SPHINGOSYNE-1-PHOSPHATE RECEPTOR 2

SXBP1: SPLICED X-BOX BINDING PROTEIN 1

TGR5: TAKEDA G PROTEIN-COUPLED RECEPTOR 5

TXNRD1: THIOREDOXIN REDUCTASE 1

TUDCA: TAUROURSODEOXYCHOLIC ACID

TNF: TUMOR NECROSIS FACTOR

UPR: UNFOLDED PROTEIN RESPONSE

UCP: UNCOUPLED PROTEIN 1

UDCA: URSODEOXYCHOLIC ACID

ZDF: ZUCKER DIABETIC FATTY

XBP1: X-BOX BINDING PROTEIN 1

QR2: QUINONE REDUCTASE 2

FIGURES

TABLES

1. INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) are spindle-shaped cells with multipotent properties and self-renewal abilities. In addition to having a high proliferative capacity, responding to chemotaxic signals for migration, and homing to injured sites, MSCs also have important immunomodulatory properties. Because of these properties, MSCs have become particularly important in the field of regenerative medicine and in the treatment of (auto)immune diseases. Over the last two decades, MSCs have been tested in various clinical studies and have been shown to be both effective and safe sources for clinical stem cell therapy.

During protein synthesis, the number of unfolded/misfolded proteins in the ER increases and the proteins are kept in the ER until they are folded appropriately. If proteins fail to fold into their correct tertiary shape, the proteins or peptides are marked for destruction. An increase in protein production or a decrease in protein folding capacity causes ER stress due to the accumulation of unfolded/misfolded proteins. In response to ER stress, a process called the unfolded protein response (UPR) is initiated. Eventually, when ER stress cannot be reduced sufficiently, the cell is destroyed through autophagy or activation of apoptotic pathways.

Oxidative stress (OS) is characterized by the dysregulated production and/or failure to clear reactive oxygen species (ROS). While high ROS levels cause cellular damage and dysfunction, low basal ROS levels are thought to be essential to the cell and maintain cellular proliferation, differentiation and survival. ROS play an important role in intracellular signaling and communication. Although certain levels of ROS are required for physiological proliferation and differentiation of cells, excessive ROS has been shown to cause functional damage in MSCs. Constantly increased ROS levels can cause serious damage to the cell through breaks in biological macromolecules (DNA/RNA) and lead to genomic instability.

Senescent cells acquire a senescence-associated secretory phenotype (SASP), which affects the behavior of neighboring cells by spreading the senescent

phenotype to other cells and causing the secretion of inflammatory proteins. Mechanistically, aging is characterized by an irreversible cell cycle arrest despite the presence of proliferative stimuli and is caused by accumulated DNA damage as a result of replication errors caused by exogenous/endogenous mutagens.

Obesity is a global public health problem and increases the likelihood of metabolic diseases such as diabetes and cardiovascular diseases. Impairment of ER function causes the accumulation of immature, misfolded or unfolded proteins. Obesity in adults is quantified using body mass index (BMI), where a BMI of 20–25 represents healthy individuals, BMI ≥ 25 represents obese individuals, and BMI ≥30 represents morbidly obese patients.

The use of stem cells for regenerative medicine is rapidly expanding. The rise in obesity rates in the general population and the elucidation of the possible (negative) effects of obesity on stem cell function have rapidly become important: since stem cell and tissue repair functions can be affected by obesity, they can be used as a treatment target, in addition, the fact that stem cells may be functionally impaired limits the therapeutic areas that the cells can be used for.

Melatonin (N-acetyl-5-methoxytryptamine, MT) is produced mainly by pinealocytes in the pineal gland during the dark phases of the day and exerts its effects through four different mechanisms: 1) binding to surface MT receptors, 2) binding to nuclear receptors, 3) binding to intracellular proteins, and 4) through a direct antioxidant effect. TUDCA suppresses ER stress by reducing GRP78 expression and reducing PERK/JNK phosphorylation, causing a decrease in CHOP expression.

Within the scope of this master's thesis we aimed to investigate 1) ER stress & OS in BM-MSCs obtained from healthy donors (BMI 20-25), obese donors (BMI 25-30) and morbidly obese donors (BMI>30); 2) the proliferation and differentiation potential of BM-MSCs in relation to BMI; 3) whether MT treatment with or without TUDCA can modulate ER stress, OS, and improve the cellular functions of obese donor-derived BM-MSCs.

2. GENERAL INTRODUCTION

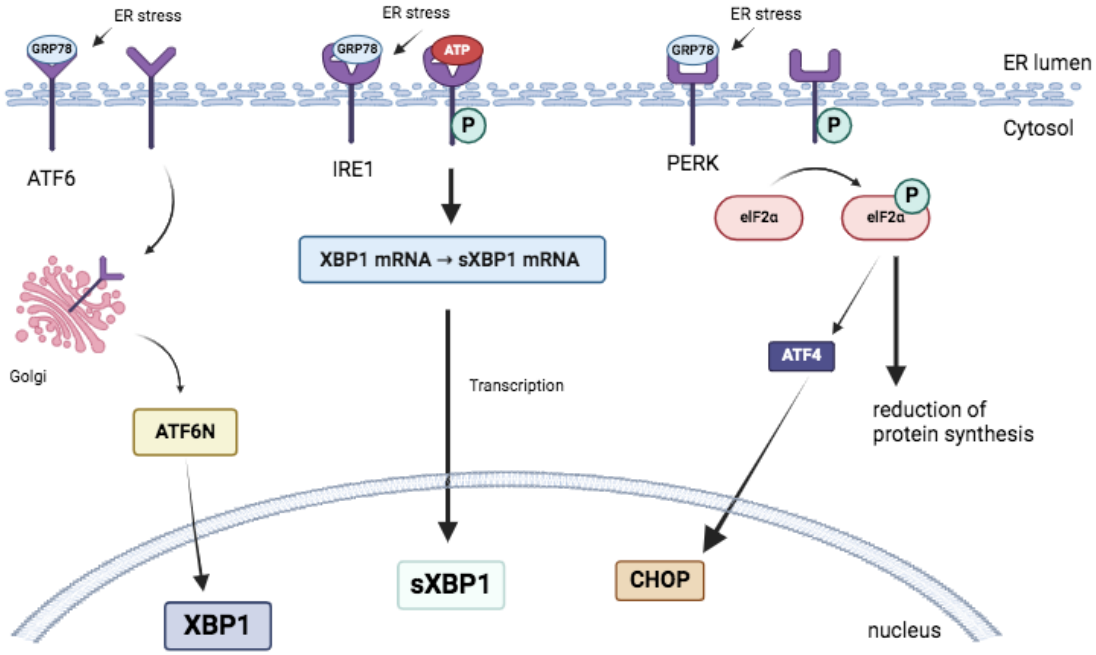
2.1. Multipotent mesenchymal stromal/stem cells

Mesenchymal stromal/stem cells (MSCs) are spindle-shaped cells with multipotent properties and self-renewal abilities. MSCs are adherent and can differentiate into chondrocytes, osteocytes and adipocytes (1, 27). Cells are positive for markers CD73, CD90 and CD105 and negative for CD45, CD34, CD14, CD11b and HLA-DR (1). They can be obtained easily and are known to show low immunogenicity (28). MSCs can be isolated from adult tissues, including bone marrow (BM) and fatty tissue (29) and fetal tissues, including placenta, umbilical cord and amniotic fluid (3). Furthermore, MSCs have a high proliferative capacity, respond to chemotaxic signals for migration and homing to sites of injury and possess important immunomodulatory properties (2). Because of these properties, MSCs have become especially important in the field of regenerative medicine and in the treatment of (auto)immune diseases. Over the last two decades, MSCs have been tested in various clinical studies and have been shown to be both effective and safe sources for clinical stem cell therapy (3).

2.2. Stem cells and stress

Stem cells may encounter various stress situations throughout life in both *in vivo* and *in vitro* conditions. Responses of cells to stress may vary depending on the cell type, the stress source and the duration of stress. In response to stress factors, cells can activate a wide range of protective mechanisms to promote their survival or to reinstate a new balance. In cases of chronic stress or when stress-induced protective mechanisms are not sufficient to reduce stress, apoptotic or autophagy pathways are activated may be activated. Thus, how (well) the cell copes with/responds to cellular stress may determine its fate. Below, different stress conditions that cells may encounter throughout life and their cellular stress responses are discussed (Figure 2.1).

A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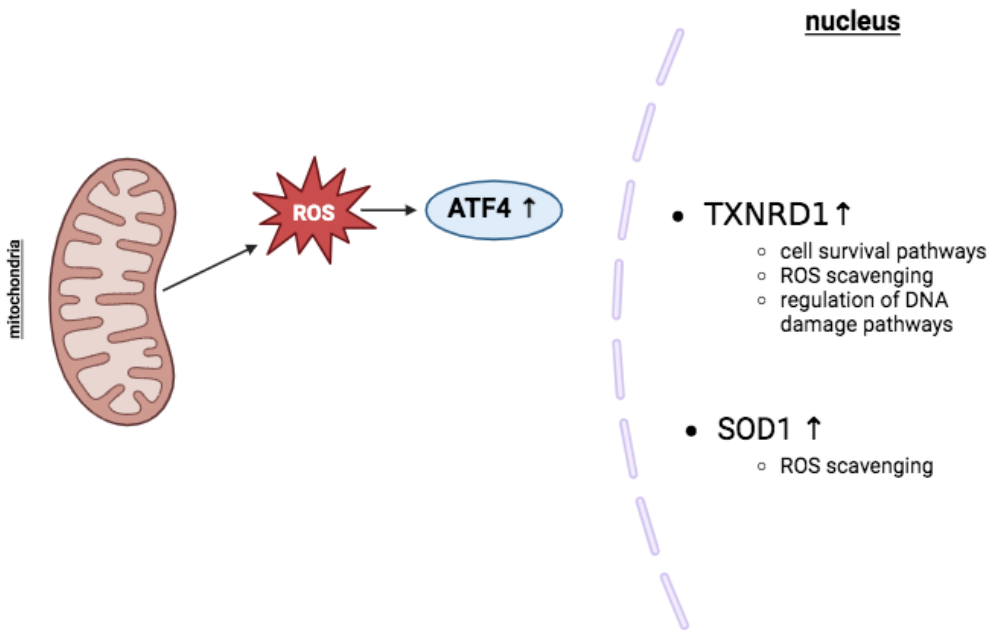
nucleus
mitochondria
ROS
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• TXNRD1 ↑
◦ cell survival pathways
◦ ROS scavenging
◦ regulation of DNA damage pathways
• SOD1 ↑
◦ ROS scavenging

C

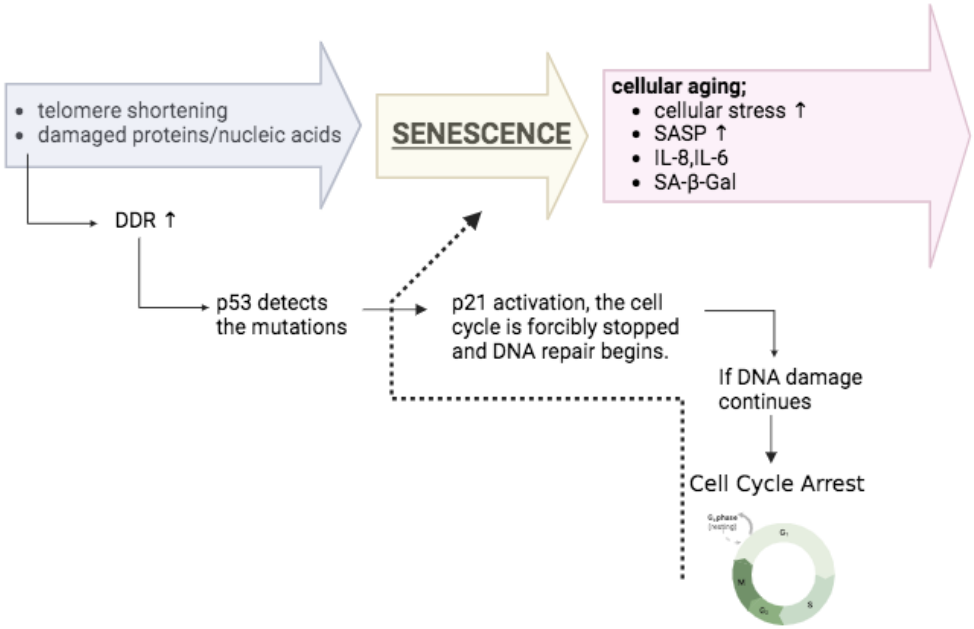

Figure 2.1. Stem cells and stress pathways. A: ER stress caused by the accumulation of un/misfolded proteins leads to separation of the molecular chaperone GRP78 and causes autophosphorylation of the IRE1a, ATF6 and PERK ER stress sensor proteins, activating downstream repair mechanisms that include ER expansion, increased gene expression of molecular chaperone genes and induction of apoptotic and autophagy pathways. B: Increased ROS production in the cells activates *SOD* and *TXNRD1* antioxidant and ROS scavenging pathways and increases expression of *XBP1* and *ATF4* to activate cell rescue pathways. C: Senescence is the combined result of telomere shortening and accumulation of DNA damage. Activation of the DNA damage response (DDR), results in p53 stabilization and p21 activation causing cell cycle arrest allowing detection and repair of mutations. Inability to repair the damage results in irreverible cell cycle arrest, induction of a senesence-associated secretory profile (SASP) and increased expression of senescence-associated b-galactosidase.

2.2.1. Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) plays an important role in the folding of secreted and membrane-bound proteins (30) and in the regulation of intracellular calcium homeostasis. Under homeostatic conditions the three ER stress sensors, i.e. PKR-like ER kinase (PERK), Inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6) are inactive and bound to the ER molecular chaperone GRP78. During protein synthesis, the number of un/misfolded proteins in the ER increases and proteins are retained in the ER until they are folded properly. If proteins cannot be folded properly, the proteins or peptides are labeled for destruction. An increase in protein production or a decrease in protein folding capacity result in ER stress due to accumulation of un/misfolded proteins and the initiation of the unfolded protein response (UPR). The UPR begins when GRP78 is released from the sensors in order to capture un/misfolded proteins. This process causes autophosphorylation of PERK and IRE1α and migration of ATF6 from the ER to the Golgi complex. When ER stress levels increase, cells initially try to reduce ER stress by increasing their protein folding capacity, decreasing the production rate of other proteins along with an increase in synthesis of chaperone proteins, resulting in breaking down or correction of unfolded/misfolded proteins. Activation of PERK results in downstream activation of eukaryotic initiator factor 2α (eIF2α) and activating transcription factor 4 (ATF4), which then enters the nucleus and increases gene expression of apoptotic pathway C/EBP homologous protein (*CHOP*), X-box binding protein 1 (*XBP1*) and growth arrest and DNA damage inducible gene (*GADD34)*. ER stress further results in IRE1α autophosphorylation, which causes an increase in spliced *sXBP1* transcripts and the separation of the ATF6 protein from the ER membrane. ATF6 then settles in the Golgi organel, where it is modified and enters the nucleus as ATF6N and increases the production of chaperones, lipids and *XBP1*. When stress is severe and insurmountable, the UPR can activate a pro-apoptotic response by activation of *CHOP*. Eventually, when ER stress cannot be reduced

sufficiently, the cell is eliminated by autophagy or through activation of apoptotic pathways (4, 30).

2.2.2. Oxidative Stress

Oxidative stress (OS) is characterized by dysregulated production and/or failure to clear reactive oxygen and nitrogen species (ROS and RNS). While high ROS levels cause cellular damage and dysfunction, low basal ROS levels are essential for normal cellular proliferation, differentiation and survival (31, 32). Approximately 90% of ROS is produced endogenously by mitochondrial electron transport systems (ETS). External factors such as radiation, ultraviolet light, hypoxia and low temperature may cause increased ROS production (4). Although certain levels of ROS are required for physiological proliferation and differentiation of cells, excess ROS causes functional damage in MSCs (4). OS resulting from continuously increased ROS levels can cause severe damage to the cell through induction of breaks in biological macromolecules (DNA/RNA), leading to genomic instability. Stem cells are relatively resilient to OS due to their powerful antioxidant/DNA repair mechanisms and their dependence on glycolysis rather than oxidative phosphorylation. However, if defense mechanisms fail, OS can become chronic, similar to ER stress, causing the onset and progression of various human diseases, such as neurodegenerative and metabolic diseases (33).

Antioxidant molecules reduce OS and protect the cell from prolonged stress. Increased catalase expression increases the cell's resistance to OS. Similar to ER stress, *XBP1* and its target genes are activated in response to OS, regulating DNA damage repair and redox homeostasis. Suppression of *XBP1* has been shown to increase OS through increased ROS levels and persistent p38 phosphorylation. Deletion or silencing of *XBP1* results in decreased expression of antioxidant genes, including superoxide dismutase 1 (*SOD1*) and thioredoxin reductase 1 (*TXNRD1*) (5). *SOD1* is found distributed in the cytosol, nucleus, peroxisomes and mitochondrial membranes (6). *TXNRD1* is an oxidoreductase and plays an important role in intracellular redox balance by scavenging ROS (6). The SOD family of enzymes scavenges oxygen radicals through a series of oxidation/reduction cycles (34). Also,

activation of *ATF4* plays a role in various rescue pathways to maintain cell survival (35).

2.2.3. Senescence

Senescent cells acquire a senescence-associated secretory phenotype (SASP) that affects the behavior of neighboring cells by propagating the aging phenotype to other cells and causing secretion of inflammatory proteins (7). Mechanistically aging is characterized by an irreversible a cell cycle arrest despite the presence of proliferative stimuli and is caused by DNA damage that has accumulated as a result of replication errors caused by exogenous/endogenous mutagens. During the DNA damage response (DDR) p53 stabilization detects mutations and initiates DNA repair by forcibly interrupting the cell cycle through p21 activation. However, if DNA damage continues to persist; p38-mitogen-activated protein kinase (MAPK) activation may cause mitochondrial dysfunction, CDK inhibition and Rb1 activation (36, 37), inducing cellular aging. In addition, increased levels of ROS can accelerate telomere erosion and further decrease mitochondrial metabolism. In addition, the persistant OS further forces the stem cells to permanently exit the cell cycle (8). Senescent MSCs exhibit reduced autophagy, self-renewal and regenerative potential, eventually resulting in replicative exhaustion (38). Decreased autophagy causes loss of proteostasis and increases mitochondrial activity, further aggravating OS in MSCs (39). Other factors, such as a age, obesity and systemic inflammation have also been shown to affect stem cell function through accelerating senescence (9, 40).

Telomere shortening is considered an universal biomarker for aging. Telomeres are found at the ends of all eukaryotic chromosomes and consist of short nucleotide TTAGGG repeats. During DNA replication, the end portion of the DNA is not replicated and therefore telomeres shorten with each replication cycle. Telomere length therefore provides information about the cell's proliferation history. Another biomarker of senescence is senescence-associated β-galactosidase (SA-β-Gal). Although β-galactosidase is also expressed in non-senescent, healthy cells at pH 4.0,

SA-b-Gal has been shown to have an optimal working pH of 6.0 and can therefore be used to distinguish between healthy and senescent cells (41, 42).

2.3. Obesity

Obesity has become a global public health problem and increases the likelihood of metabolic diseases, such as diabetes and cardiovascular diseases (43, 44). Both the carbohydrate and lipid metabolism are affected by obesity. As a result of the glycolipotoxicity, malfunction of the ER results in the accumulation of immature, misfolded or unfolded proteins. If ER stress becomes chronic, it induces hepatic lipogenesis and impairs glucose-stimulated insulin secretion in pancreatic beta cells, causing functional disorders of the liver and pancreas (45-47).

Obesity is quantified in adults using the body mass index (BMI), where a BMI of 20-25 represents healthy individuals, BMI ≥ 25 represents obese and BMI ≥30 represents morbidly obese patients. The fields of use of stem cells for regenerative medicine purposes have been expanding. However, due to the elevated rates of obesity in the general population, it has become increasingly important to elucidate the possible (negative) effects of obesity on stem cell function (10) for two reasons: Firstly, if stem cell and tissue repair functions are affected by obesity, this could be used as a treatment target; secondly, the autologous use of stem cells for regenerative medicine is rapidly increasing, and infusion or use of functionally impaired autologous stem cells may not result in optimal regenerative/immune modolatory effects. Indeed, it has been shown by multiple groups, including ours, that obesity causes functional impairment of bone marrow (BM)-derived MSCs from humans and mice and reduces their regenerative potential (10, 48). BM-MSCs obtained from human donors with a high BMI have also been shown to exhibit severely impaired osteogenic and reduced adipogenic differentiation, reduced proliferation rates, and increased expression of *ATF4* and *CHOP*, genes associated with increased aging and ER stress (10). Similarly, the differentiation of adipose

tissue-derived stem cells (ADSCs) derived from obese individuals is impaired and their lifespan is reduced (11). Additionally, p16, p21, and IL6 gene expression in ADSCs has been shown to correlate with BMI (46). Obesity also has a negative impact on the in vitro functions of MSCs and extracellular vesicles (EV) derived from these cells (49, 50). Obesity also negatively affects the functions of many other stem cell types, including hematopoietic stem/progenitor cells (HSPCs), skeletal muscle stem cells and germ cells (11, 51-54) both directly and indirectly by affecting and disrupting the stem cell niche. For example, an obesity-related increase in BM adipocytes may cause HSPC dysfunction and immune deficiency (55).

2.4. Melatonin

2.4.1. Functions and effects of Melatonin

In mammals, Melatonin (N-acetyl-5-methoxytryptamine, MT) is produced in the pineal gland (56) during the night, as well as in many different tissues, such as BM, ovary, testis, intestine, placenta and liver (57). Although MT is mainly a regulator of circadian rhythm, it also plays an important role in the regulation of antioxidant defense mechanisms, modulation of the immune system, cancer prevention and osteoprotection (12). The circadian effect of MT is mediated by the main circadian genes *CLOCK* and *BMAL1*. Environmental disruption of the circadian rhythm triggers or accelerates the development of obesity (58). Exposure to acute blue light reflected from phones, televisions or tablets before bedtime affects circadian rhythm, inhibits fat oxidation and suppresses MT levels, indicating that long-term exposure to blue light at night may cause obesity through disruption of the MT-regulated circadian rhythm (59). It has also been shown that MT regulates memory storage by directly affecting hippocampal neurons and is related to body posture and balance control (13). Use of MT has been shown to improve symptoms of Parkinson's disease, Alzheimer's disease and traumatic brain injury, depression, cerebral ischemia, hyperhomocysteinuria and glioma (60). MT has an important function in maintaining hormonal balances and controls the production of GnRH (gonadotropin-releasing

hormone), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In addition, MT suppresses estrogen receptor expression and activation by stimulating progesterone secretion from granulosa cells (60).

2.4.2. Melatonin-receptor interactions and downstream signaling

In mammals, effects of MT have been shown to be mediated through different four mechanisms: 1) binding to MT receptors on the plasma membrane, 2) binding to nuclear receptors, 3) binding to intracellular proteins and 4) through a direct antioxidant effect (13). MT and its receptor interactions are described sequentially below and depicted schematically in Figure 2.2.

MT surface membrane receptors

In humans, MT has four different receptors, of which three are membrane receptors and one functions as a nuclear receptor for MT (13). MT receptors are found in the brain, retina, cardiovascular system, liver and gallbladder, colon, cecum, skin, pancreas, kidney, immune system cells, platelets, brown and white adipocytes, prostate and breast epithelial cells, ovarian/granulosa cells, myometrium and placenta (13). The MT receptor 1 (MT1) is located on human chromosome 4, consists of 351 amino acids and creates adenylate cyclase inhibition by binding to G-proteins (13). MT1 activation leads to inhibition of forskolin (FSK)-stimulated cAMP formation along with inhibition of Protein Kinase A (PKA). Activation of MT1 signaling further results in the activation of the PI3K pathway, with downstream phosphorylation and activation of both pAKT and pERK1/2 (61, 62).

The MT receptor 2 (MT2) is located on human chromosome 11 and consists of 363 amino acids (13). MT2 binds to various G-proteins, causing both adenylate cyclase inhibition and inhibition of the soluble guanylyl cyclase pathway, as well as inhibition of FSK-stimulated cAMP formation (13). Activation of MT2 signaling pathways is mediated through activation of PKC and downstream phosphorylation of

pERK1/2, but not activation of pAKT. Therefore assessment of the downstream signaling pathways may shed a light on the presence of the different MT receptors expressed by stem cells (61, 62).

The MT receptor 3/Quinone reductase 2 enzyme (MT3) belongs to the reductase group and is involved in protection from OS by inhibiting electron transfer reactions of quinones (14). The gene for MT3/QR2 is located on chromosome 6, and encodes a physiological homodimer of 230 aminoacids and 25,8 kDA (63). The receptor was identified based on pharmacological grounds and displays more rapid ligand association/dissociation kinetics than MT1 and MT2 binding due to its lower affinity for MT. MT3/QR2 plays an important role in detoxification. MT3 can be found in the liver, kidney, heart, lung, intestine, muscle and BAT (13). Interestingly, while the MT3-binding site has been described to be membrane-bound, the QR2 enzyme is found in the cytosol (64).

MT nuclear receptor

The only known nuclear receptor of MT is the Retinoid-related orphan nuclear hormone receptor (RZR/RORα) and is found both in the peripheral nervous system and in the brain (13). The RZR/RORα receptor belongs to the nuclear receptor (NR) superfamily, which consists of 48 members (65). The gene for RORα is located on chromosome 15 and encodes a 59 kDa protein, consisting of 523 aminoacids. After binding of MT to the ligand binding domain of ROR, the receptor DNA binding domain of RZR/RORα binds to a ROR response element (RORE) on its target genes and initiates transcription (66). MT activation of RZR/RORα is responsible for immunomodulation, cellular growth, and differentiation of bone in the periphery (13). Interaction of MT with its nuclear receptor results in activation of protein kinase C (PKC) and induces IL-2 and IL-6 production in mononuclear cells (67).

Interactions of MT with intracellular proteins

MT has been shown to bind to a wide range of different proteins, including specific and orphan receptors (discussed above), several enzymes, including quinone reductase 2, metalloprotease-9, pepsin and protein phosphatase 2, mitochondrial pore proteins, PEPT1/2 and GLUT transporter proteins, and others (68). Direct effects of MT are modulated by interaction with the intracellular proteins calmodulin, calreticulin and tubulin (67). Calmodulin serves as an intracellular second messenger. MT directly antagonizes the binding of calcium to calmodulin (67) and it is thought that the anti-proliferative effect of MT on cancer development may be mediated through this pathway. In addition, MT has been shown to bind to serum albumin, which serves as a carrier of MT in plasma, but does not affect its function or biological activity (68).

Antioxidant effects of MT

Due to its high lipid solubility, MT can independently from interactions with its receptors, freely diffuse through the cell membrane and serve as a potent oxygen radical scavenger (69). In mammals, cells under OS have been shown to rapidly clear circulating MT from the peripheral blood (70). MT then enters the mitochondria through oligopeptide transporters PEPT1 and PEPT2, where it functions as an antioxidant, protecting lipids, proteins and DNA from oxidative damage (70). Mitochondrial production of MT further aids in the scavenging of oxygen radicals. In addition, not only MT, also metabolites of MT have been shown to have an antioxidant function (71). Other mechanisms through which MT exerts its effects are through binding of metals and reducing ^{o}OH radicals, activation of antioxidant enzyme systems, such as superoxide dismutase (SOD), glutathion peroxidase (GPx) and glutathion reductase (GR) (15).

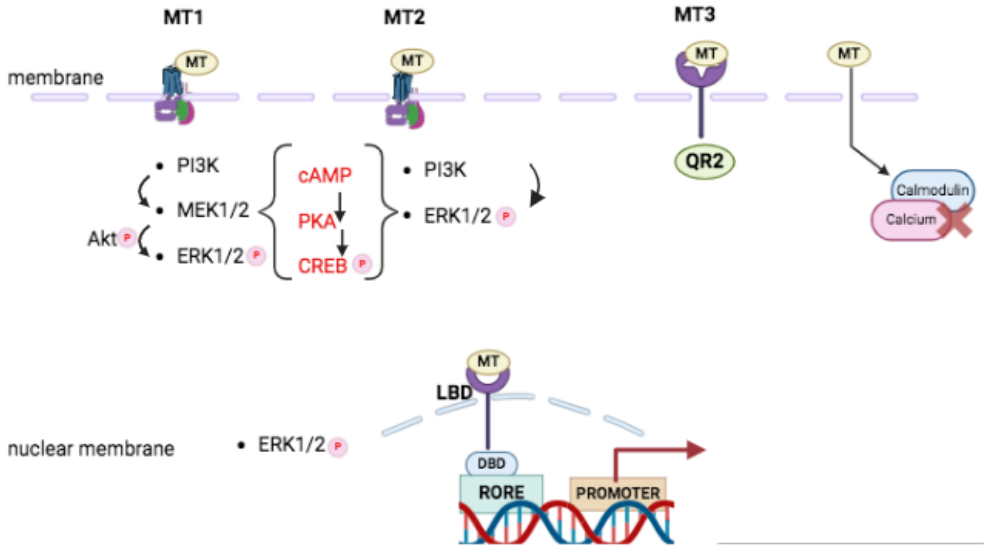

Figure 2.2. Working mechanism of Melatonin. Melatonin (MT) has three membrane receptors (MT1, MT2, and MT3) and one nuclear receptor (RZR/RORα). The working mechanism of the receptor RZR/RORα, melatonin binds to the Ligand Binding Domain (LBD) and through this interaction, the DNA Binding Domain (DBD) binds to the RORE region and activates the promoter and initiates gene expressin of downstream genes. Whereas RZR/RORα signaling is responsible for immuno-modulation, cellular growth and bone differentiation, activation of MT1 and MT2 receptors is involved in the regulation of antidepressant activity, sleep disorders, anxiety and nociception. Whereas the MT3-binding site has been described to be membrane-bound, the Quinone Reductase 2 (QR2) enzyme is found primarily in the cytosol. Activation of MT3 is mainly involved in protection of cells against OS and detoxification.

2.4.3. Effects of MT on stem cells

The effects of MT on stem cells vary depending on the stem cell type. Multiple studies have shown that whereas MT induces osteogenic and chondrogenic differentiation of MSCs, it suppresses adipogenic differentiation. Most of these effects are mediated through activation of the Wnt/b-Catenin signaling pathway (72). During inflammation, MT has been shown to protect cellular viability by suppressing the production of pro-inflammatory cytokines. In addition, MT protects these cells from apoptosis and increases viability by reducing cellular stress.

Furthermore, preconditioning of MSCs with MT has been shown to increase their therapeutic (proangiogenic/mitogenic) efficiency when used for the treatment of renal ischemia (16). Although the presence of MT receptors have not been directly confirmed on BM-MSCs, the use of MT antagonists indicate that signaling in MSCs occurs through activation of MT2 receptors (73). Although MT receptor expression studies in BM-MSCs are lacking, in ADSCs expression of both MT1 and MT2 receptors has been confirmed (74).

HSPCs express both the MT1 and MT2 receptors (17). In HSPCs, the onset of darkness-induced production of MT results in a decrease in BM norepinephrine (NE) and TNF, metabolically reprogramming HSCs, increasing their short- and long-term repopulation potential (75). MT in the BM niche has also been shown to support HSPC maintenance through suppression of ROS production and inflammation via inhibition of NF-kB signaling.

Although MT studies involving embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are rare, MT has been shown to affect these stem cells as well, largely through interaction with MT1. MT also restored the pluripotency of long-term cultured ESCs through MT receptor-dependent N6-methyladenosine (m6A) RNA-regulation (76). In addition, MT improved reprogramming efficiency of murine iPSCs in a secondary inducible system (77). In fact, MT also been shown to improve reprogramming of skin fibroblasts directly to a neural cell fate without the intermediate of the iPSC phase (77)

MT also plays an important role in the differentiation, proliferation, and survival pathways in neuronal stem and progenitor cells (NSPCs). Because MT can cross the blood-brain barrier and is also both lipid- and water-soluble, it can be used to modulate the proliferation and survival of NSPCs, for example in a hypoxia model, MT was shown to protect NSPCs and increases cell viability *in vitro* (18). In NSCs, MT exerts its effects through binding to the MT1 and MT2 receptors (78), activating both the ERK/MAPK and PI3K/AKT pathways (79, 80). MT also plays an important role in

the survival, proliferation and differentiation of NSPCs by supporting repair of damaged, dysfunctional NSPCs (81).

In addition to its effect on different types of pluripotent and tissue stem cells, MT has also been shown to have anti-cancer effects by directly affecting cancer stem cells (CSCs). These anti-cancer effects have been confirmed by many researchers both *in vitro* and *in vivo* and for multiple different types of cancer (19). MT has been shown to inhibit cancer through multiple mechanisms, such as anti-proliferative effects, regulation of oncogene expression, antioxidant and anti-angiogenic effects. MT signaling can also regulate key pathways associated with the self-renewal and survival of CSCs (20). Direct uptake of MT via the SLC2/GLUT1 family of glucose transporters, has been shown to attenuate prostate cancer cell growth through competition with glucose (82). In addition, oligopeptide transporters, PEPT1/2, of human breast cancer cells actively transfer melatonin from outside the cell into the cytosol and from the cytosol into the mitochondria. The active transport process allows for higher concentrations of melatonin in the mitochondria relative to the cytosol or nucleus. Often cancer cells switch from oxydative phosphorylation (OXPHOS) to aerobic glycolysis, a process called the Warburg effect (21). Cancer cells benefit from this adaptation mechanism since it allows the cells to use glucose as a carbon source for the *de novo* production of nucleotides, lipids and proteins needed for increased cell growth (22). The use of MT has been shown to reverse cancer cell metabolism away from aerobic glycolysis, thus inhibiting cancer cell growth (83). In addition, MT has been shown to inhibit the invasive properties of ovarian cancer stem cells via suppression of proliferation and epithelial mesenchymal transition (EMT) related gene expression. Furthermore, MT was shown to inhibit ovarian CSC migration through modulation of PI3K and MAPK signaling pathways in receptor-dependent and independent ways (84). An overview of the known effects of MT on different stem cells is provided in Figure 2.3.

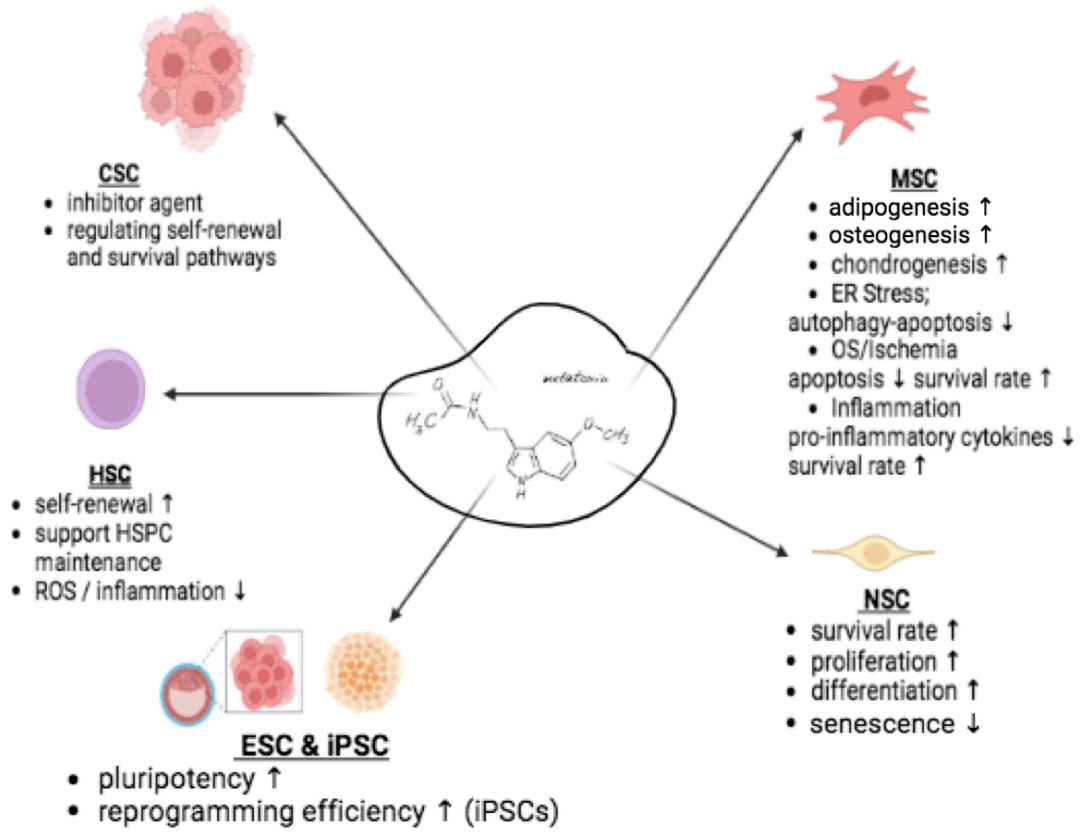

Figure 2.3. Effect of MT on different stem cell types. The effect of melatonin (MT) varies depending on the cell type. CSC: cancer stem cells, MSC: mesenchymal stem cells, HSC: hematopoetic stem cells, ESC: embryonic stem cells, iPSC: induced pluripotent stem cells and NSC: neural stem cells.

2.5. Relationship between MT and obesity

MT was first recognized for its function as an endogenous hormone, taking part in the regulation of glucose and lipid metabolism, and only later on for its role as an antioxidant. Sleep deprived animals were shown to be sensitive to weight gain, indicating a direct relationship between the pineal gland, MT production and maintenance of healthy weight (85). Indeed, suppletion of MT can be used to decrease body weight in overweight rats, rabbits and hamsters (23). Firstly, interaction between MT and MT1, expressed by hypothalamic neurons and in the suprachiasmatic nuclei (SCN), increases sympathetic nervous system activity through

production of NE, resulting in stimulation and increased gene expression of uncoupling protein 1 (UCP1), PPARG, PGC1 in metabolically active brown adipose tissue (BAT) (23). Secondly, MT acts directly on BAT, causing a decrease in intracellular cAMP, a decreased PKA activity, phosphorylation of cAMP-responsive element binding protein (CREB), and upregulation of UCP1 expression through activation of both MT1 and MT2 receptors (86). Thirdly, administration of MT results in accumulation of MT in BAT mitochondria, where together with its metabolite N1-acetyl-N2-formyl5-methoxyknuramine (AFMK) it protects the organel from OS and improves the thermogenic function of BAT (23). Long-term MT administration to Zucker Diabetic Fatty (ZDF) mice has been shown to promote WAT to beige adipose tissue transition and upregulation of UCP1 expression, thus contributing to thermogenesis and weight loss (23). For these reasons it has been proposed that the use of MT to improve BAT energy use may provide a potential therapeutic target for the treatment of obesity (86) (Figure 2.4).

In obesity, pro-inflammatory cytokines produced by the adipose tissue generate ROS. Administration of MT, which serves as an effective free radical scavenger, cooperates with endogenous SOD, catalase (CAT), GPx and GR to protect cells against OS by directly clearing ROS (13, 87). MT also indirectly reduces ROS by activating antioxidant enzymes related to glutathione metabolism, thus inhibiting mitochondrial damage (59, 88). Furthermore, MT can reduce the formation of hydroxyl radicals by chelating transition metals decreasing OS (89). Another way through which MT can decrease OS related to obesity is through regulation (activation) of autophagy, an intracellular degradation pathway (90). In the presence of IL-1β, MT both reduces ROS production and eliminates free radicals by increasing SOD levels, thus protecting overall cell survival (91). The anti-apoptotic effect of MT is mediated through both reduction of the ER stress by downregulating the ER stress-related proteins, such as p-PERK, GRP78 and p-eIF2α (57), and by reduction of oxidative and nitrosative stress, rendering the cells less sensitive to apoptosis (92). Compelling evidence that MT improves OS has been found in both overweight animals and humans (87, 93). MT can suppress ER stress in mouse BM-MSCs (94). As

an anti-inflammatory agent, MT can promote the recovery of some chronic diseases, including obesity, by suppressing the pro-inflammatory response (95, 96) (Figure 2.4).

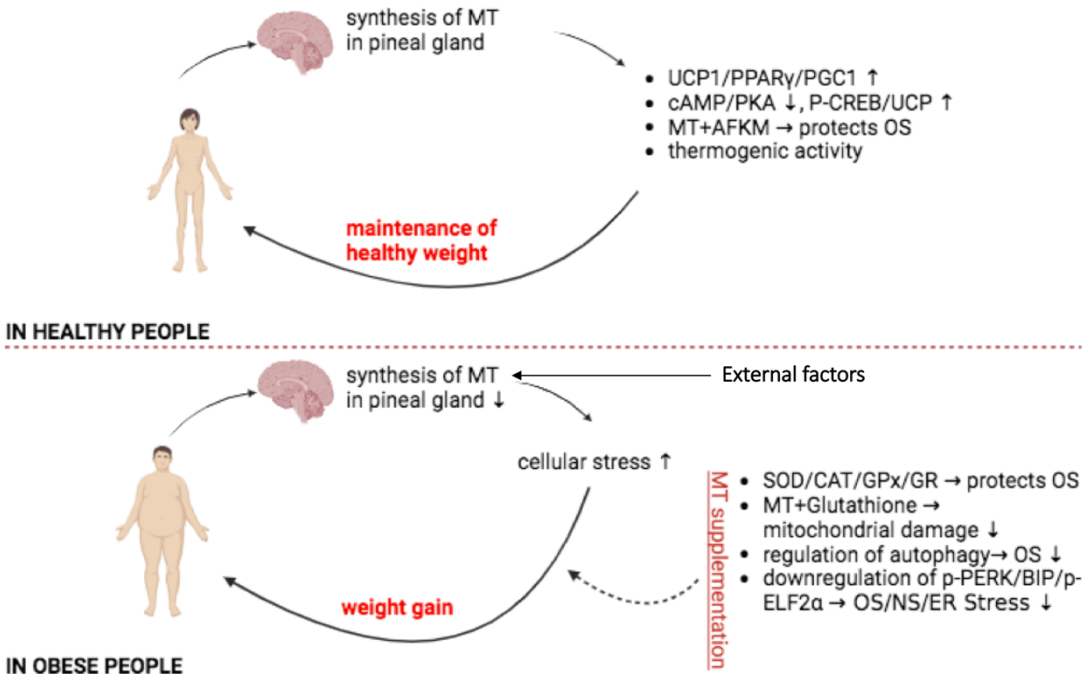

Figure 2.4. Role of MT in the development and potential treatment of obesity. In healthy humans, MT is responsible for regulating thermogenic activity while protecting cells from oxidative stress. Increased weight results in decreased MT production, causing loss of cellular damage control mechanisms, further increasing weigth gain. Suppletion of MT may serve as a potential treatment target to decrease cellular stress levels, reduce mitochondrial damage and control weight.

2.6. Clinical use and safety of melatonin

Since the use of MT, both in a clinical setting and in daily life, as commercially available food supplement, has been increasing, the safety of its use in humans has been an issue of interest. Studies using MT alone or as complementary therapy have reported that short-term use in adults is safe, but data on pregnant and breastfeeding women are currently not available. At this point, there are no data indicating that exogenously administered MT causes any serious side effects, although it may cause

mild side effects such as dizziness, headache and nausea (97). In addition, clinical side effects of short-term use of MT in pediatric patients have been shown to be mild (97) and restricted to a single case of migraine and one of an epileptic insult (98). Furthermore, use of long-term MT treatment of children and adolescents with autism spectrum disorders and insomnia has been demonstrated to be safe and effective, with the most commonly occurring side effects being fatigue, drowsiness and mood changes. No harmful effects on growth and/or pubertal development have thus far been observed (98). Mild headaches, increased sleepiness and skin rashes have been reported in severely ill patients and the elderly after taking MT (60). Overall, available clinical data indicate that MT administration has no serious side effects (60).

2.7. TUDCA

Cholesterol is synthesized in the human liver from the primary bile acids cholic acid and chenodeoxycholic acid (CDCA) and secreted in bile as taurocholic acid, glycocholic acid, glycochenodeoxycholic acid and taurochenodeoxycholic acid. Secondary bile acids, such as ursodeoxycholic acid (UDCA), are produced following the processing of primary bile acids by the gut microbiota (24, 99). UDCA is conjugated with taurine and converted to tauroursodeoxycholic acid (TUDCA) (24) and returns to the liver via enterohepatic circulation. TUDCA and UDCA are ligands of membrane receptors, such as G-protein coupled receptor 5 (TGR5), sphingosine-1-phosphate receptor 2 (S1PR2) and the nuclear receptor Farnesoid X Receptor (FXR). TUDCA and UDCA can be directly transported into the cell via the Na+/taurocholate cotransporter peptide (NTCP) through binding and activating α5β1 integrin (100). They can also enter the cell via the mineralocorticoid receptor (MR) and glucocorticoid receptor (GCR) (Figure 2.5) (100). FXR is responsible for various processes, such as regulation of bile acid synthesis, bile acid secretion, intestinal bile acid absorption, and hepatic uptake of bile acids (101-105). TGR5 is expressed in Kupffer cells but not hepatocytes and extrahepatic TGR5 signaling regulates energy homeostasis and stimulation of insulin secretion (106). TGR5 signaling in BAT and

myocytes enables the conversion of ATP to cAMP. This way, downstream CREB activation mediates immunosuppression, energy and glucose homeostasis to prevent obesity and insulin resistance (107). FXR activation during inflammation inhibits cellular signaling pathways by interacting with activator protein 1 (AP-1) and signal transducers and activators of transcription 3 (STAT3). FXR also inhibits the expression of NF-kB (25). TUDCA prevents the upregulation of GRP78/BiP causing a decrease in CHOP expression, thus reducing ER stress.

TUDCA has been shown in multiple studies to directly affect ER stress and through both inhibition of ER stress and ROS production is able to induce an anti-apoptotic effect in white adipocytes exposed to high glucose (108). Studies have further shown that TUDCA reduces the ER stress markers GRP78 and ATF4 in type 2 diabetic mice, resulting in improved endothelial function and a decrease in atherosclerosis (26). In addition, we have previously shown that TUDCA is able to decrease ER stress in BM-MSCs from individuals with a high BMI and can partially correct differentiation effects in obese donor BM-MSCs (10). Interactions with MT and/or 4-PBA may further enhance these effects (Figure 2.6).

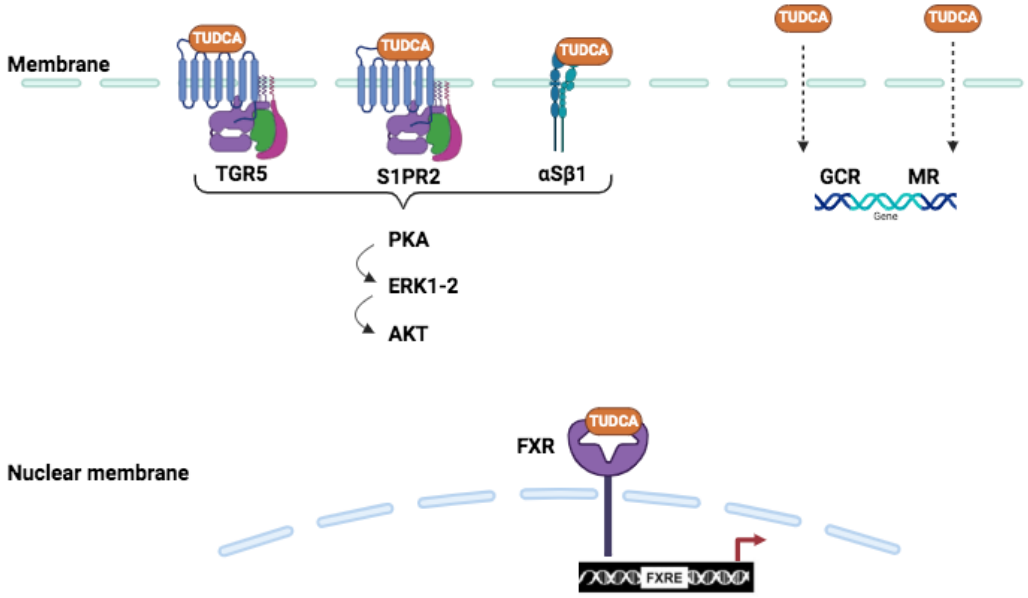

Figure 2.5. TUDCA receptors. TUDCA has three membrane receptors (TGR5, S1PR2 and αSβ1 and one nuclear receptor (FXR). These membrane receptors are responsible for modulation of apoptosis, decrease OS, ER stress and inflammation, and increase cell survival and proliferation. The nuclear receptor FXR is responsible for regulation of thermogenic activity. Binding of TUDCA causes FXR to bind to FXR response elements (FXREs) on the DNA, resulting in activation of target gene expression. TUDCA can also enter the cell via the mineralocorticoid receptor (MR) and glucocorticoid receptor (GCR).

2.8. Hypothesis and aim of the study

Obesity has been shown to have a negative effect on many different types of stem cells. We previously showed that BM-MSCs isolated from individuals with a high BMI display a severely impaired osteogenic and reduced adipogenic differentiation, reduced proliferation rates and an increased expression of the ER stress-related genes *ATF4* and *CHOP* (10). BM-MSCs exposed to treatment with TUDCA or 4-Phenylbutyrate (4-PBA) to decrease ER stress showed partial recovery of stem cell function, resulting in increased osteogenic and adipogenic differentiation. However, the effect of BMI on chondrogenic differentiation, the role of OS in the development of obesity-related loss of stemness and the potential protective role of MT, through decreasing ER stress, OS and other pathways has not been assessed before. Therefore, our working hypothesis is "Loss of stemness in BM-MSCs is associated with

increasing BMI and can be attenuated using administration of MT and TUDCA". In order to test this hypothesis we aimed to:

1) Evaluate ER stress, OS and senescence in BM-MSCs obtained from donors with a healthy donors (BMI 20-25), obese donors (BMI of 25-30) and morbid obese donors (BMI>30);

2) Evaluate the proliferative and differentiation potential of BM-MSCs in relation with BMI;

3) Investigate whether treatment with MT with or without TUDCA can modulate ER stress, OS and improve cellular functions of obese donor-derived BM-MSCs.

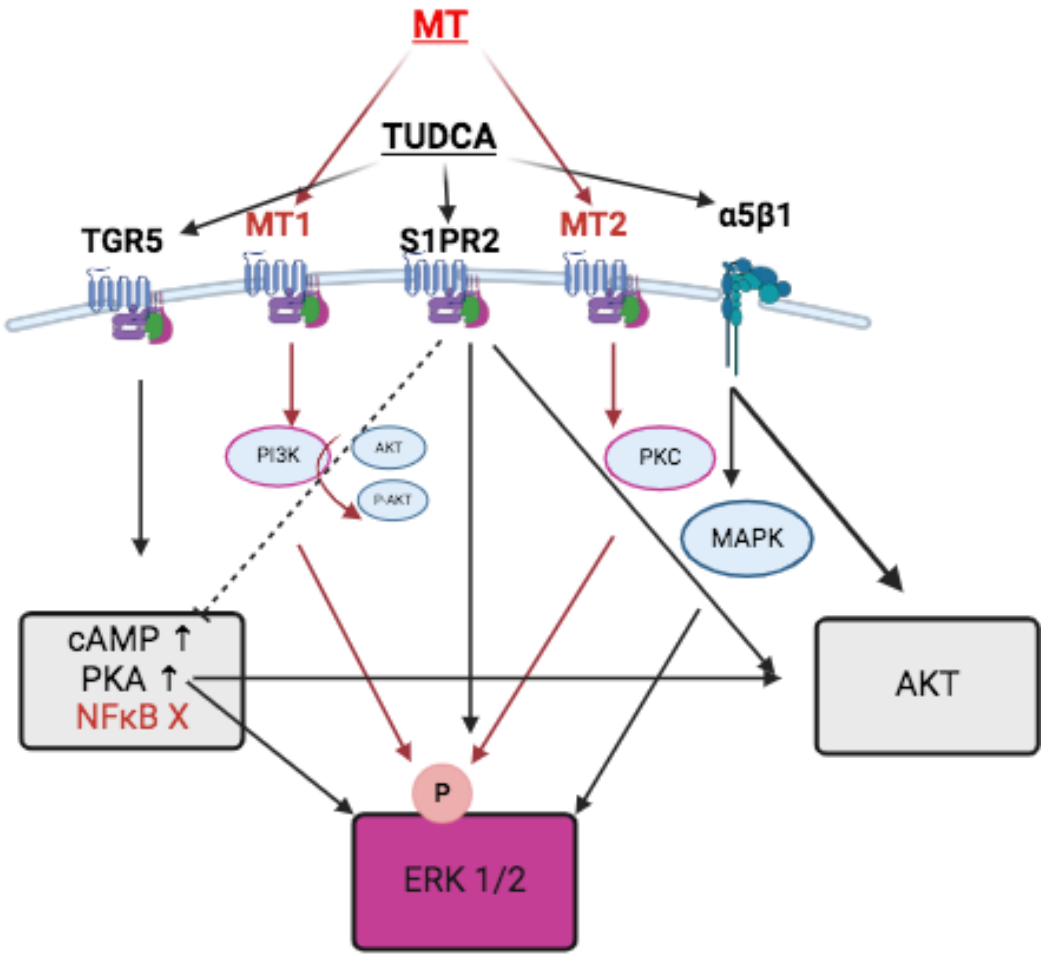

Figure 2.6. Working mechanisms of MT and TUDCA. After TUDCA binds to TGR5, the NFKB pathway is suppressed, while cyclic adenosine monophosphate/ protein kinase A (cAMP/PKA) increases. The increase in PKA results in ERK1/2 phosphorylation. Activation of S1PR2 results in direct phosphorylation of ERK1/2; $\alpha 5\beta 1$ activates the AKT pathway and causes ERK1/2 phosphorylation through MAPK activation. Binding of MT to MT1 causes AKT phosphorylation by PI3K, which then causes ERK 1/2 phosphorylation, whereas MT2 exerts its effect by directly phosphorylating ERK 1/2 via Protein kinase C (PKC), without the need for AKT phosphorylation.

3. MATERIALS & METHODS

3.1. Bone Marrow Mesenchymal Stem Cell Isolation and Characterization

BM-MSCs were isolated from BM donors who applied to the Pediatric Hematology Bone Marrow Transplantation Unit after approval of Hacettepe University Non-Interventional Ethics Committee (2022/11-68). Briefly, BM was collected from adult (>18 years old); healthy (body mass index/BMI 20-25, n=3), obese (BMI 25-30, n=3) and morbid obese (BMI>30, n=3) donors. 2-5 mL of BM was diluted 1:1 with phosphate buffered saline (PBS, HP0050-570, Cegrogen) and layered on Ficoll (ρ=1.077, D604, Diagnovum). Cells were centrifuged at 1500 rpm and the mononuclear cell (MNC) fraction was collected. MNCs were counted with Turk's dye, consisting of 50 mg crystal violet (42555) diluted in 495 mL MQ water and 5 mL glacial acetic acid (302011, Carlo/Erba). Cells were plated in T-75 culture plates in a basal medium mixture consisting of 40% DMEM-LG (31885-049; Life Technologies, UK) and 60% MCDB-201 (M6770-1L; Sigma, Saint Louis, MO), supplemented with 10% Fetal Calf Serum [FBS] (10270-106, Gibco, UK), 1% Penicillin/Streptomycin [P/S] (A2213; Biochrom AG, Germany), 2 mM L-Glutamine (Biochrom AG), or briefly DMF10, at $3x10^5$ cells/plate. Cells were cultured at 37^{o}C, 5% CO_2 and medium of the cells was changed every 3-4 days. When confluency reached 80%, the cells were detached with the 0.25% Trypsin (25200-072, Gibco) /EDTA (25200-072, Gibco) [T/E], prepared as follows: 0.35 g $NaHCO_3$, %1 1 mL phenol red, 8.09 g NaCl, 0.4 g KCl, 0.35 g glucose ($C_6H_{12}O_6$, 50997, Merck), 2.5 g trypsin and 0.372 gram EDTA in 1000 mL MQ water at an pH of 7.4. For trypsinization of the cells, the medium in the flasks was discarded and flasks were washed once with 1X PBS. T/E was added according to flask size (Table 3.1) and cells were incubated 37^{o}C for 10 minutes. Digestion was stopped by addition of two volumes of DMF10 medium. Cells were collected in tubes and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and cells were resuspended in 1 mL DMF10 and counted with 0.4% Trypan blue. $2x10^5$ cells were seeded into fresh T-75 flasks and expanded up till passage 3 (P3) for detailed immunophenotyping and characterization of the cells, as described before (10).

Table 3.1. Characteristics of cell culture plates and culture conditions

Culture plate	Area (cm^2)	T/E (mL)	DMF-10 (mL)
6-well	9,6 cm^2	1 mL	2 mL
T25	25 cm^2	2 mL	5 mL
T75	75 cm^2	3 mL	10 mL

3.2. Immunophenotyping

For immunophenotyping, BM-MSC/P3 cells were collected using T/E, as described above. For each tube $1x10^5$ cells were used. Cells were stained with directly labeled antibodies against CD29, CD73, CD105, CD90, HLA-DR and CD45 (Table 3.2).

Table 3.2. Specifics of antibodies used

Antibody	Fluorochrome	Catalog nr	Clone	Company
CD29	APC	559883	MAR4	BD Pharmingen
CD45	APC	345808	2D1	Becton Dickinson
CD73	FITC	550257	AD2	Becton Dickinson
CD90	FITC	555596	5E10	Becton Dickinson
CD105	PE	121057	SN6	eBioscience
HLA-DR	PE	347403	2D1	Becton Dickinson

Incubations were performed in presence of PBS/0.5% BSA/0.05% NaN3 (PBN), supplemented with 2% human AB serum for 15 minutes at room temperature, in the dark. After incubation, the cells were washed twice with PBN, assessed using a BDAccuri flow cytometer and analyzed with BD CSampler software. In follow-up experiments, cells that passed quality control (i.e. >85% expression of CD29, CD90, CD73, CD105 and <5% expression of CD45 and HLA-DR) were expanded until P6 and used to evaluate cellular stress.

3.3. Cell Freezing & Thawing

3.3.1. Cryopreservation of BM-MSCs

Cells were collected using T/E dissociation, transferred to 50 mL tubes, centrifuged and resuspended in 1 mL DMF-10. Cells were counted using 0,4% Trypan blue and frozen at a concentration of $5x10^5$-$1x10^6$ cells/vial after addition a same volume of ice cold 2X Freezing Medium. 2X Freezing Medium was prepared as follows: 4 mL DMEM-LG, 2 mL DMSO (67685, PanReac AppliChem) and 4 mL FBS-HI were mixed at room temperature and cooled down to 4°C before use. Cryovials were placed into a Mr. Frosty container (Nalgene) and stored at -80°C. They were placed in the nitrogen tank for long-term storage within 48 hours.

3.3.2. Thawing of BM-MSCs

Cells were stored in a nitrogen tank for use in subsequent studies and to minimize cell loss. For thawing, cryovials obtained from the nitrogen tanks were immediately placed on ice. Cells were thawed by transferring the vials to a 37°C water bath until a small piece of ice remained in the vial. All subsequent procedures were performed on ice. The contents of the cryovials were transferred to a 15 mL tube containing 9 mL of PBS and 10% FBS. After centrifugation at 1500 rpm for 5 minutes at 4°C, the supernatant was discarded and the pellet was resuspended in 1 mL DMF-10 for counting. Cells were plated in flasks (see Table 3.1) and placed in the incubator.

3.3.3. Mycoplasma test

Mycoplasma tests were performed to when cells 48-72 hours after removal from long-term storage in liquid N2 and after prolonged culture to prevent possible cross contamination. Briefly, the Mycoplasma spectrophotometric test (Lonza, MycoAlert) or the EZ-PCR Mycoplasma Test Kit (Biological Industries #20-700-20) were used to check the presence of the most common mycoplasma strains.

Preferrably, freshly thawed cells were tested using the Lonza test 3 days after thawing. However, in case of borderline results or when assessing important samples the mycoplasma PCR kit was used. For spectrophotometric measurements, 1 mL of the test sample medium/supernatant was transferred to fresh 1 mL eppendorf tubes. After centrifugation at 200*xg* for 5 minutes, 100 uL of the supernatant was transferred to 5 mL polystyrene tubes with sterile caps. 100 uL of Reagent solution was added, incubated for 5 minutes, and measured using the luminometer (Reading A). Then, 100 uL of the Reagent substrate was added, the tubes were incubated at room temperature for 10 minutes and measured on the luminometer a second time (Reading B). The ratio (Reading B/Reading A) between the first and second measurements is indicative for the presence or absence of Mycoplasma contamination, where a ratio of <1 indicates negative for Mycoplasma; 1-1.2: borderline or suspect; >1.2 Mycoplasma contamination.

For mycoplasma PCR, samples were centrifuged at 16.000*xg* for 5 minutes. The supernatant was discarded and the pellet was resuspended with 20 µL of buffer included in the kit. Samples were denaturated at 95°C for 3 minutes. Separate tubes were prepared for positive controls, negative controls and samples, as described in Table 3.3. After pipetting of the controls and samples, the PCR was set up, as shown in Table 3.4.

Table 3.3. Mycoplasma PCR setup

	Test sample	**Pos control**	**Neg control**
Reaction mix	4 uL	4 uL	4 uL
Internal Control DNA	0,4 uL	0,4 uL	0,4 uL
Internal Control Primer	2 uL	2 uL	2 uL
Test Sample/Positive sample DNA	2 uL	0,4 uL	0,0 uL
dH_2O	11,6 uL	13,2 uL	13,6 uL
Total volume	20 uL	20 uL	20 uL

The prepared tubes were placed in the PCR device (Authorized thermal cycler, Eppendorf) and the PCR was run as follows: 94°C 1', 60°C 2', 35 cycles of 72°C 1', 94°C 30'', 60°C 2' and 5' 72 °C. A 2% agarose gel was prepared in 1X TBE buffer solution (For 1L 5X TBE Buffer; 54 gram Tris, 27,5 gram Boric acid, 20 mL EDTA, 980 mL dH_2O) until PCR was completed. The mixture was heated for 2 minutes (Authorized thermal cycler, Eppendorf). After cooling down, 10 µl Ethidium Bromide (EtBr, AppliChem, 008734) was added and the gel was poured into the gel tank to polymerize. After the PCR was completed, 8 uL of PCR product was mixed with 2 uL of 6X Loading dye and 10 uL was loaded into the wells of the gel. Electrophoresis was performed for 20 minutes at 120 V and the agarose gel image was viewed under UV. The presence of mycoplasma contamination is determined in comparison with the positive and negative DNA sample controls.

3.2. Differentiation assays

For adipogenic and osteogenic differentiation healthy, obese and morbid obese donor BM-MSC/P3 cells were plated in 6-well culture dishes at a concentration of $5x10^4$ and $2.5x10^4$ cells/well, respectively. To assess the effects of MT, all assays were performed in the presence and absence of MT.

Adipogenic differentiation medium consisted of DMEM-LG, 10% FBS, 1 µM dexamethasone (Invitrogen, Paisley, Scotland, UK), 60 µM indomethacin (Sigma, Saint Louis, MO), 500 µM isobutylmethylxanthine (Sigma, Saint Louis, MO) and 5 µg/mL insulin (Sigma, Saint Louis, MO), as described before (10). Adipogenic differentiation was evaluated after 1 week of differentiation using RT-qPCR for expression of the adipogenic differentiation markers *PPARG* and *SCD*. *RPLP0* was used as a housekeeping gene (109). After 21 days of differentiation, the presence of lipids was evaluated with Oil Red O (ORO) staining. Oil Red O dye stock was prepared by dissolving 420 mg Oil Red O (1320-065, Sigma) in 120 mL absolute isopropanol (1367431, Fisher) overnight. ORO working solution was diluted prepared by diluting the stock 4:3 in ddH_2O and filtering through a Whatmann paper to remove

undissolved dye remnants. Briefly, wells were washed twice with PBS and fixated using 4% PFA (50000, JT Baker) for 15 minutes at room temperature. Cells were then stained by incubation with ORO for 10 minutes at room temperature and multiple washes using running tap water to remove excess dye. Photographs were taken using an Olympus inverted microscope (CKX41) at 10 and 20X magnification. For semiquantitative analysis, stained wells were incubated with 1mL 2% NP40 (9002-93-1, Sigma) in Isopropanol for 15 minutes, after which the solution was collected and assessed spectophotometrically at 492 nm using a microplate reader (Tecan, Sunrise).

Osteogenic differentiation medium was prepared as follows: 10% FBS, 100 nM dexamethasone, 10 mM betaglycerophosphate (Applichem, Germany) and 0.2 mM L-ascorbic acid (Sigma, Saint Louis, MO) in DMEM-LG (10) Osteogenic differentiation was evaluated after 1 week of differentiation using RT-qPCR for the osteogenic differentiation markers *ALPL* and *RUNX2*. After 21 days of differentiation, cells were stained with Alizarin Red stain (ARS13-22-03, AppliChem). ARS was prepared by dissolving 2 grams of alizarin red in 100 mL ddH_2O. pH was adjusted to 4.2 using 0.5% ammonium hydroxide (318612, Fluka Analytical). For staining of calcium phosphates, the medium in the 6-well culture dishes was discarded and wells were washed with 1 mL of PBS. Wells were fixed with 4% PFA for 10 minutes and then stained with 1 mL of ARS dye for 10 minutes at room temperature. Wells were rinsed well with running tapwater and then assessed for using microscopy. Photographs were taken at 4X, 10X and 20X magnification. To assess calcium content in the wells, 1 mL of 0,6 M HCl (07102, Sigma) was added to control and positive wells and incubated for 4 hours at room temperature to dissolve Calcium. Calcium content was assessed using the Quantichrom Calcium assay kit (BioAssay systems, DICA500). Briefly, kit reagents A and B were brought to roomtemperature and mixed 1:1. 5 uL of samples and calcium standards was pipetted into wells of a flat bottom 96-well plate and 95 uL of the Reagent A/B mixture was added. Color changes were assessed spectophotometrically 10 minutes later using measurements at an optical density of 620 nm, with reference measurements at 0 nm.

For chondrogenic differentiation, $2x10^5$ cells were transferred to 15 mL polypropylene tubes. Cells were spun down and pellets were left overnight at 37°C, 5% CO_2 and allowed to form small spheres. The next day media were replaced with chondrocyte differentiation medium, consisting of DMEM-HG (20958-22, Gibco), 1 mM Sodium Pyruvate (SML0309, Sigma), 100 nM dexamethasone, 50 µg/ml L-Ascorbic acid, 10 ng/mL TGF-β3 (002-066), 1X ITS (I-1884, BD Biosciences). Medium of the free floating pellets was changed twice a week and after three weeks the medium was discarded and cell spheres were washed with 1 mL of PBS and used for RT-qPCR. Chondrogenic differentiation was assessed using the cartilage-specific gene markers *SOX9* and *COL2*.

3.4. Evaluation of Cellular Stress in BM-MSCs

3.4.1. Evaluation of ROS Levels

To investigate the effects of obesity on oxidative stress in BM-MSCs, intracellular ROS levels were evaluated in BM-MSCs from healthy, obese and morbid obese donors. H2DCFDA (2',7'-dichlorodihydrofluorescein-diacetate) (Thermofisher Scientific, cat no D399) which can freely cross the cell membrane but is not fluorescent, becomes fluorescent in the presence of ROS, which cleaves acetate groups and converts it to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, passage 6 (P6) BM-MSCs were trypsinized and 150.000 cells were used for analysis. Negative control BM-MSCs and test sampels were incubated in DMF10 only. Positive controls were incubated in DMF-10 with 100 uM H_2O_2 (H1009, Sigma) for 2 hours at 37°C, 5% CO_2, centrifuged at 1500 rpm for 5 minutes and washed 2 more times with PBS. Then the samples were incubated with 10 uM H2DCFDA in pre-warmed PBS for 30 minutes, spun down at 1500 rpm for 5 minutes, and washed 3 times with ice-cold PBS. After the last wash, 100 µL of PBS was added and intracellular ROS levels were evaluated on a BDAccuri flow cytometer (110). Gating strategies were determined based on negative and positive control samples.

3.4.2. Optimization of PCR conditions

Gradient PCRs were established to determine the optimal temperature for primers, as described in Table 3.4.

Table 3.4. Gradient PCR constituents

Constituent	1X (uL)	20X (uL)
10X Buffer	1	20
$MgCl_2$	0,6	1,2
cDNA	2	40
Taq Polymerase	0,1	2
RNAse/DNAse free dH_2O	4,1	82
40 uM dNTP	0,2	4
Forward primer	1	20
Reverse primer	1	20

A common mix was prepared without Forward (F) and Reverse (R) primers and divided over the PCR tubes. After addition of the relevant gene F and R primers, mixes were thoroughly vortexed, spun down in a centrifuge and placed in the PCR device (Eppendorf, Authorized Thermal Cycler) using the following test conditions (Table 3.5).

Table 3.5. Gradient PCR test conditions

Cycle	Duration	Temperature (^oC)
1	4 minutes	95
	30 seconds	94
30-35	30 seconds	57
	30-60 seconds	72
1	5 minutes	72

A 1.5% agarose gel was prepared by dissolving 1.5 g of agarose (154662, Fisher Bioreagents) in 100 mL of 1X TBE Buffer (For 1L 5X TBE; 54 gram Tris, 27,5 gram Boric acid, 20 mL 0,5 M EDTA and top up to 1L)). 15 uL EtBr was added and the liquids were poured into the gel tank to polymerize. After the PCR products were prepared they were placed on ice. 2 uL of 6X Loading Dye (2220) was added to the mixtures and 10 uL sample was loaded onto the gels. 10 uL of a 100 bp Ladder (2171, Nepenthe) was loaded into the first and last wells. Depending on the degree of band separation, the gel was run at 150 V for approximately 30-45 minutes and images were taken using a Fluorchem.

3.4.3. RT-qPCR

The expression of the OS-related genes, *SOD1* and *TXNRD1* was assessed in BM-MSCs from healthy, obese and morbid obese donors using RT-qPCR (111). To assess ER stress expression of *sXBP1, XBP1, ATF4* and *CHOP* was examined. Briefly, BM-MSCs/P6 were collected in Qiazol (57201570, Qiagen) and diluted 1:1 in absolute ethanol to obtain total RNA. The mixture was passed through a column by centrifugation for 30 seconds at 16000x*g* and subjected to DNase I treatment using 400 uL RNA wash buffer (Zymo, R1003-3-48), 35 uL DNA digestion buffer (E1010-1-16) and 5 uL DNAse I per sample (total RNA isolation kit, Zymo, R2062). The mixture was washed twice with 400 uL pre-wash buffer (R2050-2-160) and once with 700 uL wash buffer. 17 uL of pre-heated DNase/RNase free water was added, centrifuged at maximum *g* for 1’ and RNA concentrations were determined using the Nanodrop (Spectrofotometer, ND-100) by measuring purity at A260/A280. Forward and reverse sequences of primers used in this thesis are given in Table 3.6.

Table 3.6. Primers used for RT-qPCR

Gene	Forward	Reverse	Marker
PPARG	CAGCACCACCGATCAGAAGA	TCCCATTTCCGAGGAGGGAT	**Adipogenic differentiation**
SCD	CCCCTGCTTACTTGGTGAGG	TGTTCAGCAGGGTTTGTGGT	**Adipogenic differentiation**
RUNX2	CGCCTCACAAACAACCACAG	TCACTGTGCTGAAGAGGCTG	**Osteogenic differentiation**
ALPL	GCTATCCTGGCTCCGTGC	CACTGTGGAGACACCCATCC	**Osteogenic differentiation**
COL2	GGTCCTGCAGGTGAACCC	CTCTGTCTCCTTGCTTGCCA	**Chondrogenic differentiation**
SOX9	AGACAGCCCCCTATCGACTT	CGGCAGGTACTGGTCAAACT	**Chondrogenic differentiation**
sXBP1	GCTGAGTCCGCAGCAGGT	CTGGGTCCAAGTTGTCCAGAAT	**ER stress/UPR**
XBP1	CAGACTACGTGCACCTCTGC	CTGGGTCCAAGTTGTCCAGAAT	**ER stress/UPR**
CHOP	GCTCAGGAGGAAGAGGAGG	TCCTGCTTGAGCCGTTCATT	**ER stress/UPR**
ATF4	TTAAGCCATGGCGCTTCTCA	GGTCGAAGGGGGACATCAAG	**ER stress/UPR**
SOD1	CTCACTCTCAGGAGACCATT	CCACAAGCCAAACGACTTCCAG	**Oxidative stress**
TXNRD1	GTTACTTGGGCATCCCTGGT	CGCACTCCAAAGCGACATAGGA	**Oxidative stress**
RPLP0	TGGTCATCCAGCAGGTGTTC	ACAGACACTGGCAACATTGCGG	**Housekeeping gene**

All samples were adjusted according to the sample containing the lowest amount of RNA and mRNAs were converted into cDNA using the Quantitect Reverse Transcription Kit (00314158, Applied Biosystems) (Table 3.7).

Table 3.7. Ingredients used for cDNA synthesis

Ingredients	**1X (uL)**
dH_2O	3,2 uL
10X rxn.	2 uL
Random Primers	2 uL
dNTP mix.	0,8 uL
RT	1uL
RNase inhibitor	1uL
Total volume	10 uL

The prepared tubes were placed in the PCR device (Authorized thermal cycler, Eppendorf) and the PCR was run, as follows: 25°C 10', 37°C 120', 35 cycles of 85°C 5'. Mixes were prepared for each primer as required (1X SYBR: 5 uL 2X mastermix, 2 uL ddH_2O, 0.5 uL F and 0.5 uL R primer) and distributed to 384 well plates, using 8 µL per well. 2 uL cDNA of the test samples was added to the wells and analyzed using a LightCycler 480-II (Roche). *RPLP0* gene expression was used as a normalizer in all experiments after confirming its expression was more stable than expression of *ACTB* during different physiological conditions (109). All experiments were performed using 3 technical replicates and 3 different donors.

3.5. Senescence test

Differences in expression of senescence-associated β-galactosidase in healthy, obese and morbidly obese BM-MSC/P6 cells were measured using the senescence-associated- β-galactosidase kit (SA-β-Gal, Cayman Chemical Company. BM-MSC/P6 were cultured in 6 well plates at 25000 cells/well and when subconfluent the medium was removed and wells were washed with 2 mL 1X PBS. Cells were fixed in 1 mL of fixation solution for 15 minutes at room temperature. Then, wells were washed twice with 2 mL 1X PBS and 1 mL of β-Gal staining solution at pH 5.9-6.1 was added to the wells for overnight incubation at 37°C. Images were taken using an inverted light microscope, showing blue (β-Gal positive) stain as an indicator for senescent cells and evaluated with ImageJ software (10).

3.6. Effects of MT, TUDCA and 4-PBA on cellular stress in BM-MSCs

3.6.1. Assessment of the dose-effect range of Melatonin

The effects of different doses of MT were tested on healthy MSCs using apoptosis and cell viability tests. First a 1 mg/mL stock of MT was prepared by dissolving MT in pure ethanol and further dilution in dH_2O. To prepare working solution 1, stock was diluted 1:100 in DMEM-LG (Working Solution 1, 10 ug/mL). Then

Working Solution 2 was prepared by diluting Working Solution 1, 1:10 in DMEM-LG (Working Solution 2, 1 ug/mL). Healthy donor BM-MSCs were plated in 6-wells and exposed to different concentrations of MT (0-300 nM) for 24 hours. After this period, cells were collected using Trypsin/EDTA and centrifuged once. Cells were reconstituted in Annexin Binding Buffer (BB; 0,01 M HEPES, 0,14 M NaCl, 2,5 mM $CaCl_2$) consisting of 300.000 cell and divided into 3 tubes: 1) negative control, Annexin BB only; 2) Annexin BB + Ann-V-FITC (Ann-V, BioLegend, 640906); and 3) Annexin BB + Ann-V-FITC + Propidium Iodide (PI, Sigma-Aldrich). Cells were mixed carefully to prevent cell death due to procedure-related issues and left to incubate in the dark for 15 minutes. Before measurement, Annexin BB was added and apoptosis was evaluated with the BDAccuri flow cytometer within 15 minutes of staining and without further washing to minimize centrifugation steps. Doses used of TUDCA (50 uM) and 4-PBA (1 mM) in this thesis were based on a previous study (10).

3.6.2. Assessment of Melatonin downstream pathway activation

Healthy donor BM-MSCs/P3 were trypsinized and spun down at 1500 rpm for 5 min. The supernatant was removed and pellets were resuspended in 1 mL of PBS. 30 nM MT was added to all tubes except for the negative control tube and incubated at 37^{o}C, 5% CO_2. Cells were collected before (0') and 15', 30', 45', 60' and 90' minutes after application of MT. Directly after collection, cells were fixated in 500 uL Fix Buffer 1 (557870, BD Bioscience) at 37^{o}C and for 10 minutes. The cells were washed twice with 2 mL PBN and 500 µL of ice-cold methanol was added, while cells were kept on ice for 30 minutes. For staining of phosphoproteins, cells were washed twice with 2 mL PBN, and incubated with 2.5 µL of anti-pERK-Alexa Fluor 488 (675508, Biolegend) and 10 µL of anti-pAkt-PE (560378, BD Bioscience) antibodies in 100 µL of PBN + AB Serum for 15 minutes at room temperature. Cells were washed twice with 2 mL PBN, and assessed using a BDAccuri flow cytometer.

3.6.3. Assessment of the effects of MT&TUDCA&4-PBA on cell proliferation

The effects of TUDCA, MT and 4-PBA were tested on healthy MSCs using WST-1(11644807001, Roche). 2000 cells were added per well of a 96 well flat bottom microplate. The doses used in the WST-1 analysis were as follows; TUDCA: 100 uM, 4-PBA: 4mM and MT: 30 nM. On days 1, 4, 7 and 10, 1:10 WST-1 was added the wells and incubated for 4 hours at 37°C, 5% CO_2. Microplates were assessed using a Tecan Sunrise Microplate Reader at 450 nm.

3.7 Statistical Analysis

Where necessary, significant differences between two groups were calculated with Student's t-test in Excel for Mac version 14.6.9 software. $P<0.05$ is considered significant. Expression changes that occur due to variables in RT-PCR experiments were calculated using the ($\Delta\Delta$Ct method) quantitation (112). Correlations were calculated with the Pearson's test.

4. RESULTS

4.1. BM-MSC characteristics from healthy and obese donors

4.1.1. Healthy and obese donor BM-MSCs are morphologically similar

BM-MSCs were obtained from adult healthy (body mass index/BMI 20-25), obese (BMI 25-30) and morbid obese (BMI>30) donors and cultured until the 3rd passage (P3) for characterization and until the 6th passage (P6) for experiments (Table 4.1).

Table 4.1. Clinical data and characteristics of BM-MSC donors used

Group	Donor	Sex	Age	BMI
Healthy	GP	K	35,00	21,10
Healthy	EE	K	18,00	21,48
Healthy	EA	E	26,00	22,02
Average ± Std. Deviation			26,33 ± 6,94	21,53± 0,38
Obese	GE	K	18,00	27,34
Obese	NK	K	27,00	29,33
Obese	HHA	E	30,00	29,39
Average ± Std. Deviation			25,00± 5,10	28,69± 0,95 *
Morbid Obese	ŞÇ	K	34,00	30,85
Morbid Obese	YY	K	39,00	32,53
Morbid Obese	HK	K	30,00	40,37
Average ± Std. Deviation			34,33± 3,68	34,58± 4,15 *

*p<0,05 in comparison to the Healthy Group.

At the end of Passage 3, cells were evaluated morphologically (Figure 4.1). However, no apparent differences were observed during this period, in terms of shape or size.

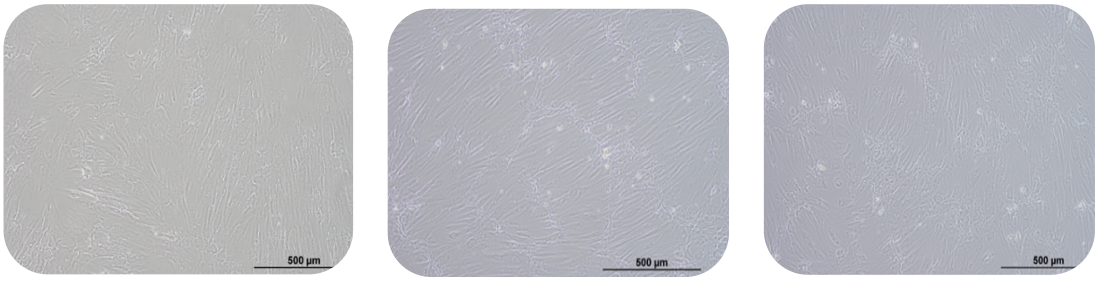

Figure 4.1. Morphology of healthy, obese and morbid obese donor BM-MSCs. Morphologically no apparent differences were detected in terms of size or shape between healthy (left), obese (middle) and morbid obese (right) donor BM-MSCs in at P3, 4X Olympus inverted microscope (CKX41). Light microscopy pictures of representative samples.

4.1.2. Healthy and obese donor BM-MSCs are phenotypically similar

For immunophenotyping, BM-MSCs/P3 from healthy, obese and morbid obese donors (n=3, per group) were stained with antibodies against CD29, CD45, CD73, CD105, CD90 and HLA-DR (Figure 4.2). As expected, cells from all donors could be classified as BM-MSCs, based on their expression of CD29 (>93%), CD73 (>93%), CD90 (>97) and CD105 (>85%) and low expression of CD45 (<3%) and HLA-DR (<5%). No statistical differences were found in expression of the antigens ($p>0,05$).

In order to prevent confounding effects in this study, we used mixed gender populations in each group. Since we also wanted to assess the presence of any interference of age on BMI, we calculated the correlation (Pearson's correlation: $r=0,35$) between BMI and age (Figure 4.3). Although a steady increase was found in BMI with increasing age, there was no significant correlation ($p>0,05$) between age and BMI in our study group. Therefore, age has been discarded as a confounding variable in further analyses of healthy, obese and morbid obese BM-MSC donors within this thesis.

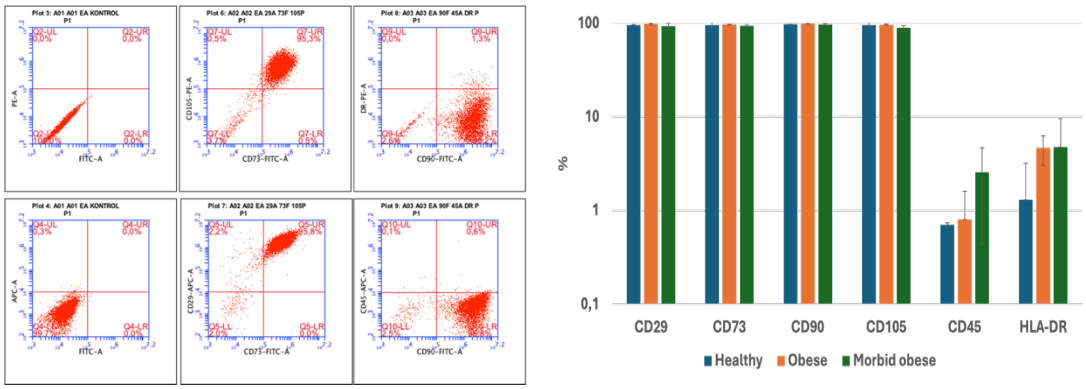

Figure 4.2. **Immunophenotype of BM-MSCs from healthy and obese donors** Upper panel: dot plots of a representative BM-MSC sample. Lower panel: BM-MSC/P3 cells from healthy, obese and morbid obese donors (n=3 per group) were stained with antibodies against CD29, CD45, CD73, CD105, CD90 and HLA-DR. Shown are average ± standard deviations.

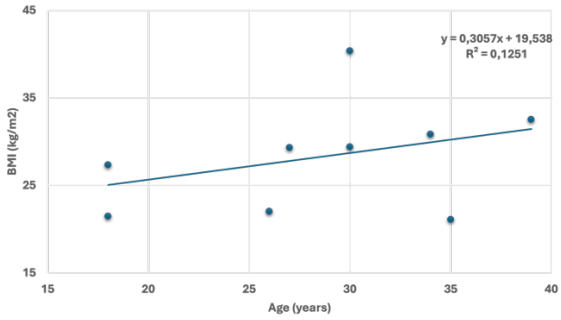

Figure 4.3. **Relationship between BMI and age** Data from 9 BM-MSC donors are provided with the X-axis representing age in years and the Y-axis the body mass index (BMI) in kg/m^2. No significant correlation was found between age and BMI (r=0.35, p>0.05).

4.1.3. Optimization of RT-PCR conditions

BM-MSCs/P6 from healthy (n=3), obese (n=3) and morbid obese (n=3) patients were used to measure *SOD1* and *TXNRD1* gene expression to assess OS and gene expression of *sXBP1* (spliced) and unspliced *XBP1*, *ATF4* and *CHOP* were used as indicators of ER stress/UPR activation. Previously, *ACTB* expression was found to be unstable in BM-MSCs during differentiation towards osteogenic and adipogenic lineage, making it an inadequate "house keeping" gene in differentiating BM-MSCs. Therefore, here 3 different "house keeping" genes, i.e. *ACTB, RPLP0* and *GAPDH* were

tested for stability of expression in different BM-MSC donors (Table 4.2). Based on the CT values of different donors, the least variability was observed in *RPLP0* expression and therefore this house keeping gene was used throughout this thesis. A gradient PCR was used to determine the temperature at which the primers used worked with the highest efficiency (Figure 4.4).

Table 4.2. Expression of "house keeping" genes by BM-MSCs

Donor	BMI (kg/m^2)	*GAPDH (CT)*	*RPLP0 (CT)*	*ACTB (CT)*
HK	40,37	33,71	26,69	31,27
YY	32,53	34,19	25,51	28,29
ŞÇ	30,85	29,68	24,51	25,77
HHA	29,39	27,26	25,95	28,07
EA	22,20	28,86	25,51	26,97

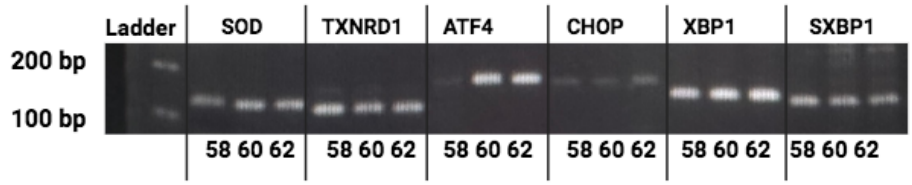

Figure 4.4. **Gradient PCR gel image for cellular stress gene primers** Gradient PCRs were used to determine optimal temperature (58, 60 and 62°C) for the primers of the following genes: SOD1(length:168), TXNRD1(length:171), ATF4(length:168), CHOP(length:161), XBP1(length:167) and sXBP1(length:164).

4.1.4. Obesity affects differentiation of BM-MSCs

BM-MSCs from healthy, obese and morbid obese donors were differentiated towards adipogenic, osteogenic and chondrogenic lineage. Results were assessed using both histological staining and RT-PCR (Figure 4.5). Whereas the differentiation capacity of BM-MSCs towards adipogenic ($p>0,05$) and osteogenic ($p<0,05$) lineage assessed after 3 weeks of differentiation decreased with increasing BMI, these data were not all significant. Expression of early adipogenic and osteogenic differentiation-related genes assessed at one week after initiation of differentiation assays revealed better differentiation towards adipogenic (*SCD*) and osteogenic (*ALPL*) by healthy donor BM-MSCs, in comparison to obese and morbid-obese donor-derived BM-MSCs. Chondrogenic differentiation was assessed at 3 weeks after differentiation only and interestingly, in contrast to adipogenic and osteogenic differentiation, chondrogenic differentiation-related *COL2*, but not *SOX9*, expression increased with increasing BMI, with the highest expression found in morbid obese donor-derived BM-MSCs, indicating a skewed differentiation pattern diverting from adipogenic and osteogenic towards a chondrogenic phenotype.

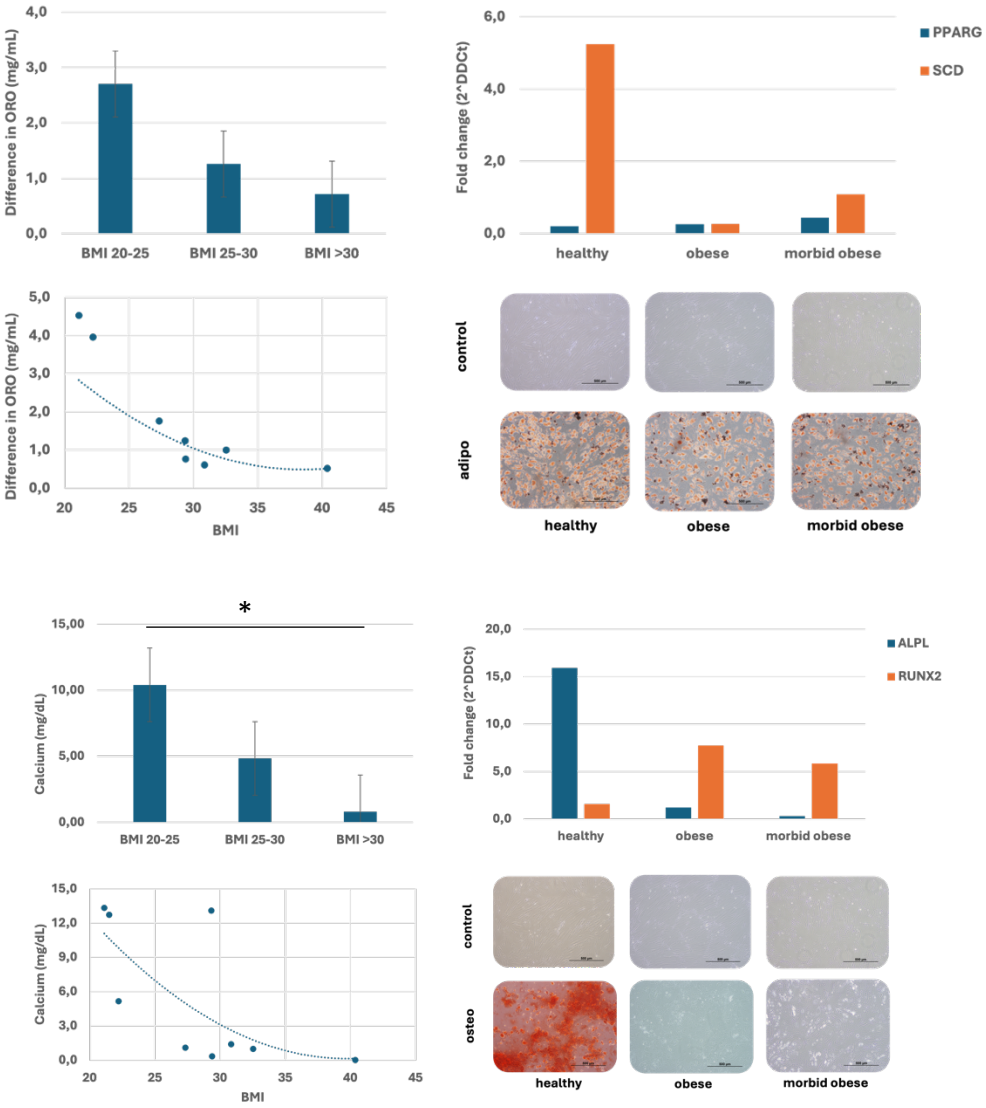

Figure 4.5. **Differentiation of BM-MSCs from healthy, obese and morbid obese donors.** Upper panel: Semi-quantitative analyses of adipogenic differentiation using measurements of intracellular Oil Red O (ORO), plotted against groups (healthy: BMI 20-25; obese: BMI 25-30 or morbid obese: BMI >30) or individual data plotted against BMI. Quantitative measurements of adipogenic differentiation were assessed using RT-qPCR for the adipogenic marker genes *PPARG* and *SCD*. Lower panel: Semi-quantitative analyses of osteogenic differentiation using measurement of Calcium phosphate depositions in culture wells ($p<0,05$), plotted against groups or individual BMI data. RT-qPCR data are comprised of a single healthy, obese and morbid obese donor.

4.2. Cellular stress in healthy, obese and morbid obese donor BM-MSCs

4.2.1. Cellular stress in BM-MSCs from obese and morbid obese donors

ER stress and OS-related gene expression in obese and morbid obese donors was compared with levels in healthy donor-derived BM-MSCs (Figure 4.6). When baseline levels of cellular stress in healthy, obese and morbid obese donor BM-MSCs were assessed, only the levels of *ATF4* in the morbid obese group were found to be significantly increased, although both markers of OS and ER stress appear to be increased in obese and morbid obese donor-derived BM-MSCs. When cellular stress was determined after differentiation towards adipogenic, osteogenic and chondrogenic lineage, both ER stress and OS markers overall remain low, although the increase in *sXBP1* expression by obese and morbid obese donors during osteogenic differentiation is noticeable. However, since the differentiation data were generated using single donors significance cannot be calculated.

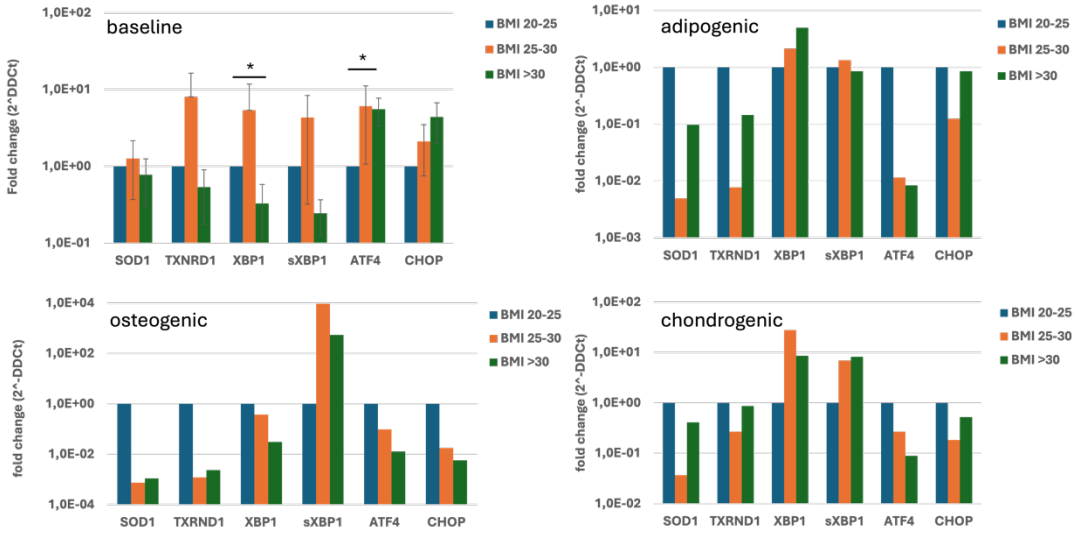

Figure 4.6. Cellular stress in healthy, obese and morbid obese donor-derived BM-MSCs during baseline levels and differentiation Cellular stress markers in BM-MSCs from healthy (BMI 20-25), obese (BMI 25-30) and morbid obese (BMI >30) donors at baseline, n=3 donors per group, * $p<0.05$ (upper left); after differentiation towards adipogenic lineage (upper right, n=1); after differentation towards osteogenic lineage (lower left, n=1); and chondrogenic differentiation (lower right, n=1).

4.2.2. Presence of senescent BM-MSCs and ROS levels increase with BMI

ROS levels were determined in BM-MSCs from healthy, obese and morbid obese donor BM-MSCs. Although ROS percentages (H2DCFDA positive cells) increased with BMI, these data were not significant (r=0.45, p>0.05), (Figure 4.7). The direct correlation between percentages of senescent cells in cultures vs BMI was also not significant (r=0.78, p>0.05). However, increases in senescent cells were highly significant if data were stratified per group (healthy vs obese, p<0.01; healthy vs morbid obese, p<0.001), (Figure 4.8).

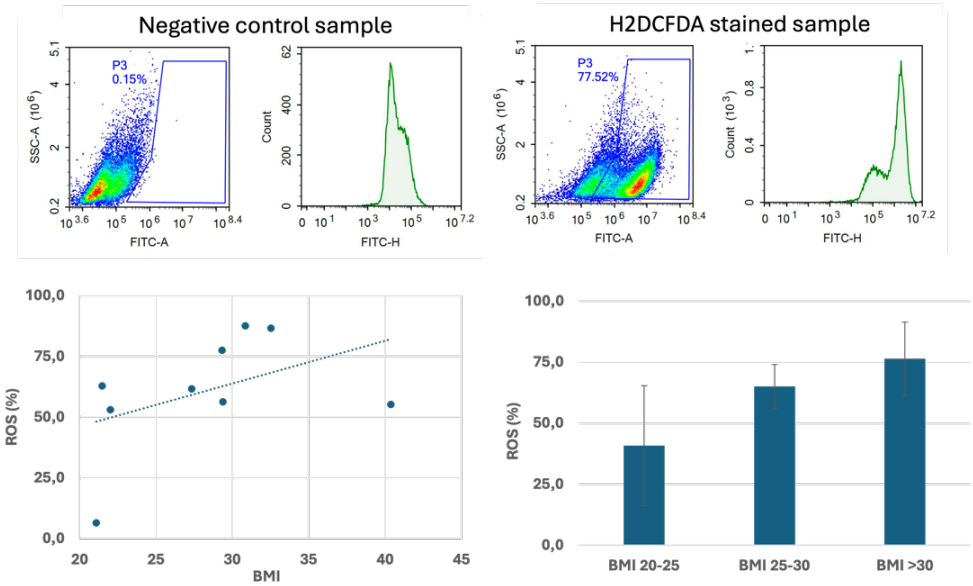

Figure 4.7. ROS levels increase with BMI Upper panel: Dot plots and histograms analyses of ROS using H2DCFDA staining (left: negative control; right: positive sample). Lower panel: ROS percentages and correlation (r: 0,45) with BMI (left) and stratified per group of healthy (BMI 20-25), obese (BMI 25-30) and morbid obese (BMI >30) BM-MSCs (right).

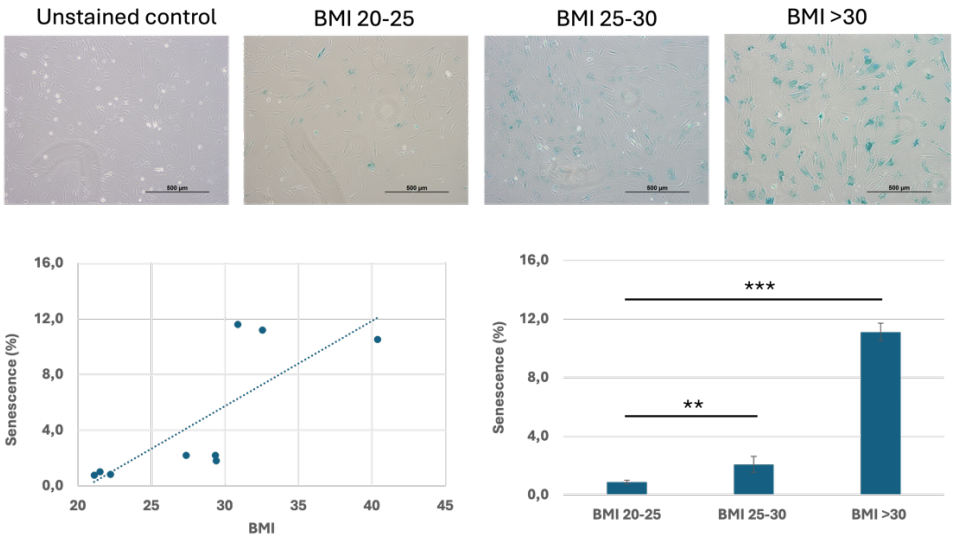

Figure 4.8. **Relationship between ROS and BMI** Upper panel: BM-MSCs/P6 from healthy (BMI 20-25), obese (BMI 25-30) and morbid obese (BMI >30) individuals were stained with SA-b-Gal. Lower panel: Senescence was determined by calculating the percentage of senescent cells per culture. Although the correlation with BMI was not significant (left), differences were highly significant when senescence was determined per group (right). ** p<0.01; *** p<0.001.

4.3. Effects of Melatonin and TUDCA on BM-MSCs

4.3.1. Melatonin toxicity and dose determination

Healthy donor BM-MSCs were used to assess potentially negative effects of MT on cellular growth and viability. Although different doses of MT ranging from 3 to 300 nM were tested, none showed any significant toxicity. Based on these data and the standardly used dose ranges of MT in literature, all further experiments were carried out with a dose of 30 nM MT (Figure 4.9).

To assess potential synergistic effects of the use of MT, TUDCA and 4-PBA, these chemicals were tested on healthy donor BM-MSCs using WST-1 analysis of cell proliferation and cell growth analyses were done on day 4, day 7, day 11 and day 14 (Figure 4.10). MT was used at a dose of 30 nM, TUDCA was used at 50 uM and 4-PBA was used at a concentration of 1 mM, based on previous data from our group (13). Single use of MT and TUDCA showed the best proliferation supporting capacities,

whereas 4-PBA alone or in combination never exceeded DMF10 only. Therefore, in further experiments use of 4-PBA was not incorporated.

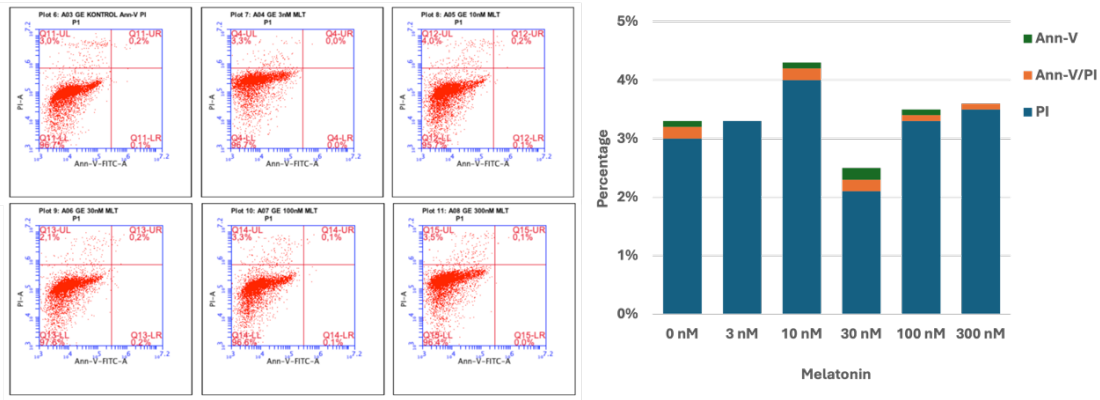

Figure 4.9. **Effects of Melatonin on BM-MSC viability** Left: Dot plots showing Propidium Iodide (PI) on the Y-axis and Annexin-V-FITC (Ann-V-FITC) staining on the X-axis. Right: Percentage of early apoptotic cells (Ann-V), late apoptotic cells (Ann-V/PI) and necrotic cells (PI) after treatment with MT at doses of 3-300 nM.

4.3.2. Healthy donor BM-MSCs cell proliferation index after treatment with MT&TUDCA&4-PBA

In line with the WST-1 analyses, it was decided to use MT and TUDCA in follow-up experiments because the group using 4-PBA was below the control group as seen in the figure 4.10. Therefore, 4-PBA was not used in subsequent experiments.

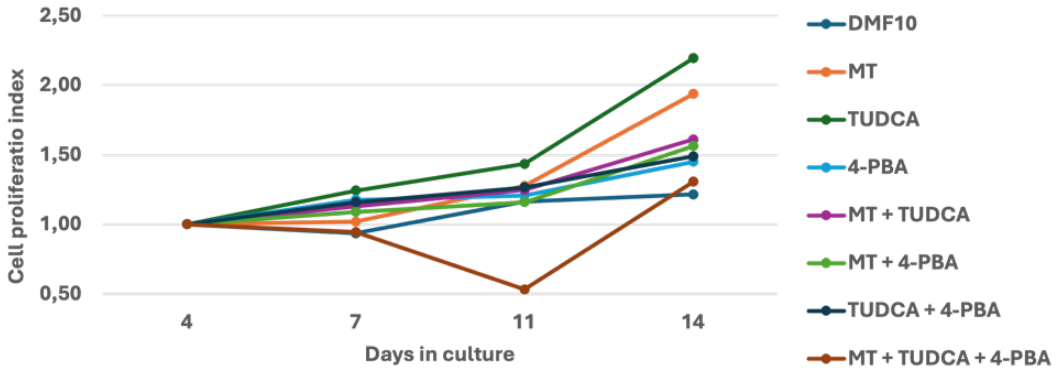

Figure 4.10. **Cell proliferation index of healthy donor BM-MSCs after treatment with MT, TUDCA and/or 4-PBA** Cells were cultured in DMF10 (complete medium) supplemented with 30 nM MT, 50 uM TUDCA and/or 1 mM 4-PBA and incubated with WST-1 at day 4, 7, 11 and 14 (X-axis). The relative cell proliferation in comparison to day 4 index is given on the Y-axis.

4.3.3. Assessment of downstream activation pathways of MT

Activation of MT1 has been shown to result in downstream phosphorylation of both AKT and ERK, whereas binding to MT2 results in phosphorylation of ERK only. To assess which MT receptor is the main receptor for signal transduction bu BM-MSCs, cells were exposed to MT for 15-90 minutes and phosphorylation of AKT and ERK was assessed using anti-pAKT-PE and anti-pERK-FITC antibodies (Figure 4.11). Increased expression of pERK only indicates that the effects of MT are largely mediated by interaction with the MT2 receptor.

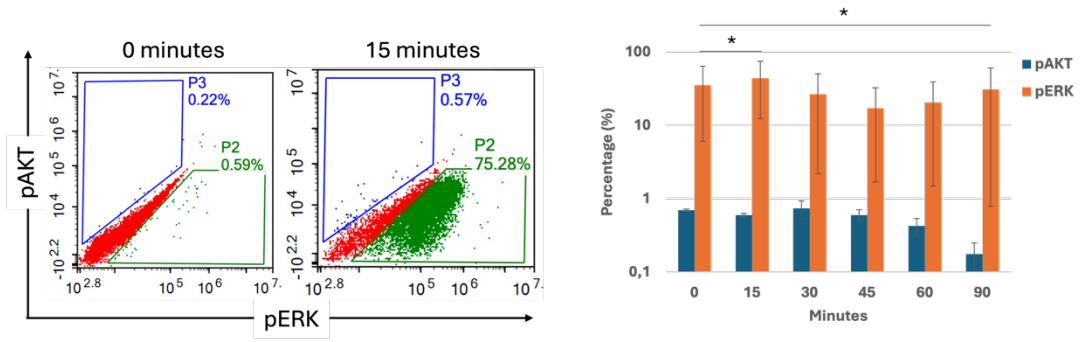

Figure 4.11. Expression of pAKT and pERK after Melatonin exposure Left: Dot plots showing pERK (X-axis) and pAKT (Y-axis) gating before and after addition of 30 nM MT, representative sample. Right: percentages (Y-axis) of pAKT (blue) and pERK (orange) after exposure to MT for 15-90 minutes (X-axis), average ± standard deviation of n=3 experiments using healthy donor BM-MSCs (p<0,95).

4.3.4. Effect of MT and/or TUDCA on differentiation of obese and morbid obese donor-derived BM-MSCs

In order to improve differentiation capacity of functionally impaired BM-MSCs/P6 from obese and morbid obese donors, cells were differentiated in presence of MT and/or TUDCA into adipogenic, osteogenic and chondrogenic lineage (Figure 4.12). Although addition of TUDCA appears to be beneficial for differentation towards

adipogenic, osteogenic and chondrogenic direction, this effect is only observed in the obese, but not in the morbid obese donor-derived BM-MSCs, indicating that stress levels may be too severe to fully compensate with TUDCA. Addition of MT only improved chondrogenic *SOX*9 expression in the obese donör-derived BM-MSCs. No synergistic effects of TUDCA with MT were observed, evenmore, combined treatment with TUDCA and MT resulted in an overall suppressed differentiation capacity into all three lineages.

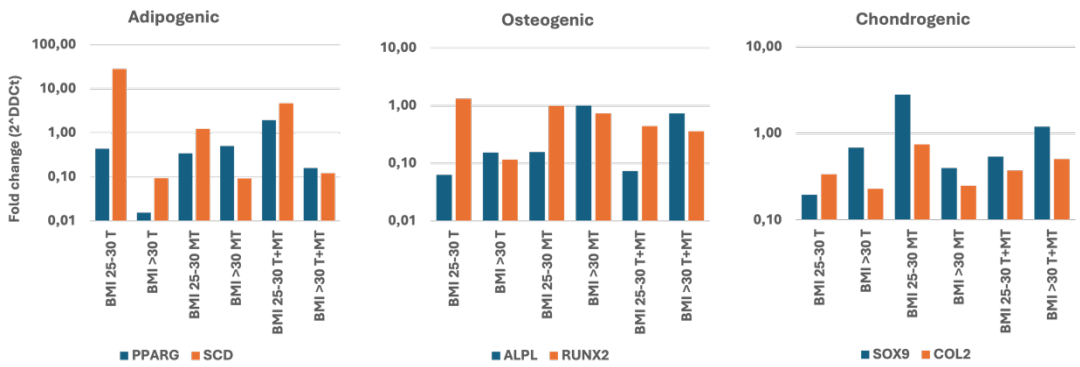

Figure 4.12. Effect of MT and/or TUDCA on differentiation of obese BM-MSCs BM-MSCs/P6 from an obese (BMI 25-30) and a morbid obese (BMI >30) donor were subjected to adipogenic (left), osteogenic (middle) and chondrogenic (right) differentiation protocols in the presence of TUDCA (T), Melatonin (MT) or both (T+MT). Gene expression of tissue-dependent genes was assessed and fold change ($\Delta\Delta$Ct) was calculated with respect to BM-MSCs differentiated in the absence of TUDCA or MT.

4.3.5. Effect of MT and/or TUDCA on cellular stress levels in obese and morbid obese donor-derived BM-MSCs

Firstly gene expression of cellular stress markers of OS and ER stress was assessed during differentiation of BM-MSCs from a healthy, obese and morbid obese donor (Figure 4.13). An increase was observed in expression of *sXBP1* in BM-MSCs from the obese and morbid obese donor, indicating activation of the *IRE1* pathway, and to a lesser extent the *PERK* pathway. OS does not appear to play a major role in the loss-of-function of BM-MSCs from obese and morbid obese donors.

Next the effects of MT (acting on OS) and TUDCA (suppressing ER stress) to suppress markers of cellular stress in differentiating BM-MSCs from obese and morbid obese donors and increase differentiation capacity of these cells were assessed (Figure 4.14). Neither TUDCA nor MT were sufficient to completely rescue differentiation potential of obese and morbid obese donor-derived BM-MSCs, although encouraging effects are observed on osteogenic differentiation, particularly after treatment with MT.

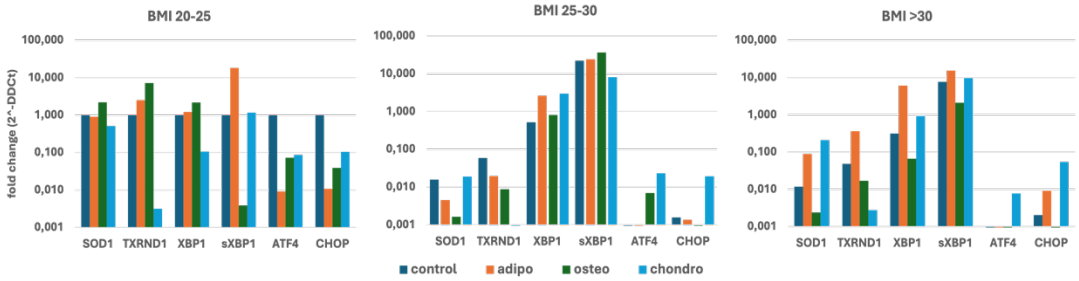

Figure 4.13. Gene expression of cellular stress markers in BM-MSCs from healthy, obese and morbid obese donors during differentiation Gene expression of OS markers *SOD1* and *TXRND1*, as well as ER stress indicators *XBP1, sXBP1, ATF4* and *CHOP* were assessed before (dark blue) and after differentiation towards adipogenic (orange), osteogenic (green) and chondrogenic (light blue) direction in BM-MSCs from a healthy (left, BMI 20-25, n=1), obese (middle, BMI 25-30, n=1) and morbid obese (right, BMI >30, n=1) donor.

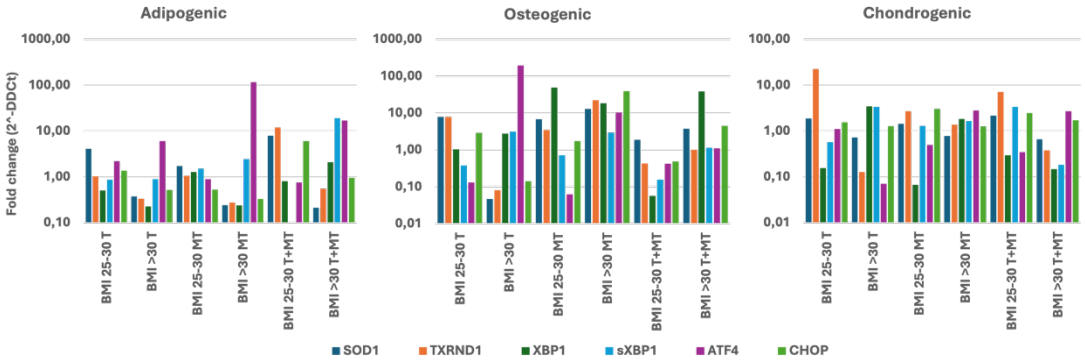

Figure 4.14. Effect of TUDCA and MT on gene expression of cellular stress markers in BM-MSCs from an obese and morbid obese donor during differentiation BM-MSCs from an obese (n=1) and a morbid obese (n=1) donor were differentiated towards adipogenic (left), osteogenic (middle) and chondrogenic (right) lineage and treated with TUDCA (T), Melatonin (MT) or both (T+MT) and gene expression of OS markers *SOD1* and *TXRND1*, as well as ER stress indicators *XBP1, sXBP1, ATF4* and *CHOP* was assessed. Fold change in expression was calculated relative to the differentiated, but non-treated (no TUDCA, no MT) control cultures.

5. DISCUSSION

Obesity has become a global public health problem because it increases the likelihood of developing a wide range of metabolic diseases, such as diabetes and cardiovascular diseases and increases the risk for co-morbidity (43). Due to the limited effectiveness of current treatments, new treatment strategies are needed. Obesity not only negatively affects major organ systems, but it can also directly and indirectly (through effects on the stem cell niche) affect the functions of adult stem cells (44). These stem cells are tissue resident cells that are necessary for the survival, maintenance and repair of these tissues and organs. Under physiological conditions these stem cells remain largely silent, but they can actively participate in tissue repair when needed.

It was previously shown that elevated levels of ER stress in BM-MSCs from high BMI donors resulted in loss of stem cell function, which becomes apparent by a decreased proliferative capacity, decreased differentiation capacity, particularly in osteogenic lineage, and increased or early senescence (30). Treatment with TUDCA, an ER stress inhibitor, showed promiss as a candidate drug to promote osteogenic differentiation in BM-MSCs derived from obese donors. In addition, 4-PBA showed a similar effect on suppression of ER stress, but was less effective than TUDCA.

Within the framework of this thesis we aimed to explore the mechanisms behind the loss-of-stemness observed in BM-MSCs from obese individuals. In addition to our previous work, we wanted to assess the role of oxidative stress in the development of stem cell failure and the potential use of Melatonin, as a powerful antioxidant drug, to suppress and decrease the negative effects of OS on stem cell function. We furthermore wanted to broaden our insights into the exact extent of the loss-of-stemness by assessing the relationship between obesity, BM-derived MSCs and chondrogenic differentiation.

Here, we used data obtained from 9 BM-MSC donors, with BMIs ranging from 21.1 to 40.4 in the age groups of 18-39 years. In order to exclude age and sex as confounding factors we tried to match all study groups as well as possible. In addition, we showed that there was no correlation between age and BMI in this study. We then proceeded to assess the relationship between increasing BMI and BM-MSC function. As expected we found that BMI does not directly affect BM-MSC morphology or immunophenotype. However, there is a strong indication that obesity directly increases intracellular ROS levels, accelerates senescence, severely decreases osteogenic differentiation and to a lesser extent interferes with adipogenic and chondrogenic differentiation.

Next we sought out to reveal to what extent oxidative stress and endoplasmic reticulum stress play a role in the malfunctioning of (morbid) obese donor-derived BM-MSCs. For this we used RT-qPCR to assess baseline levels of the cellular stress markers *SOD1* and *TXNRD1*, which are typically upregulated in response to OS (30) and *CHOP, ATF4* and (unspliced*) XBP1* and spliced *XBP1* as markers of ER stress(4). We found that under these baseline conditions, *ATF4* was significantly increased in obese and morbid obese donor BM-MSCs. In addition, *XBP1/sXBP1* expression levels were increased during in particularly osteogenic differentiation. In contrast, *SOD1* and *TXNRD1* expression as markers of OS were not elevated, although direct measurement of intracellular ROS levels suggest at least low level OS that steadily increased proportionally to BMI. Nevertheless, these data indicate that one of the main mechanisms involved in obesity-related loss-of-stemness may be increased ER stress, rather than acute/chronic OS. The direct effect of obesity on stem cell senescence is striking and highly significant, further underlining the potential clinical risks related to increased body mass.

In order to assess whether it is possible to abrogate the negative effects of obesity on stem cell function we wanted to test the efficacy of Melatonin treatment. MT has long been known to have positive effect on the modulation/suppression of both oxidative stress and ER stress (23). In addition, MT has an anti-inflammatory

effect and decreases OS by scavenging free radicals (4). It has further been shown that MT application protects cell survival, reduces ROS production in the presence of IL-1β, and can eliminate free radicals by increasing *SOD* levels (91). In addition, MT has a strong protective effect by inhibiting OS-induced apoptosis in MSCs (57). It has also been shown that MT has an antiapoptotic effect by reducing the expression of ER stress-related proteins such as *p-PERK, BIP* and *p-eIF2α* (57).

TUDCA has been shown in multiple studies to directly affect ER stress and through both inhibition of ER stress and ROS production possesses a strong anti-apoptotic effect (108). Studies have further shown that TUDCA reduces the ER stress markers *GRP78* and *ATF4 in vivo* (26). We previously published that TUDCA is able to decrease ER stress in BM-MSCs from individuals with a high BMI and can partially correct differentiation effects in obese donor-derived BM-MSCs(26). However, since TUDCA can only partially correct the differentiation defects, we wanted to assess whether targeting both increased OS and ER stress through simultaneous treatment with Melatonin and TUDCA can further improve the correction of stem cell function.

The appropriate MT dose was determined using healthy donor BM-MSCs, the doses used of TUDCA had been determined before in a similar setting. When supplied separately, both TUDCA and MT stimulated the proliferative potential of healthy donor BM-MSCs. However, simultaneous use resulted in suppression, rather than support of proliferation. 4-PBA, which is also known to be a potent suppressor of ER stress (113) was tested as well, but since both TUDCA and MT outperformed the effects of 4-PBA, we further focused on the use of these chemicals in follow-up experiments. Similar to the effects observed on proliferation of BM-MSCs, simultaneous use of TUDCA and MT did not show any advantage in improving adipogenic, osteogenic or chondrogenic differentiation, although separate application of TUDCA improved adipogenic and osteogenic differentiation in obese donor-derived BM-MSCs, but not morbid obese donor-derived BM-MSCs. In contrast, MT improved chondrogenic differentiation in obese donor-derived BM-MSCs, but not morbid obese donor-derived BM-MSCs. These data indicate that although both

TUDCA and MT have a positive effect on differentiation of BM-MSCs, advanced cellular stress in morbid obese donor-derived BM-MSCs cannot be aleviated using these drugs. In addition, TUDCA and MT do not show additive or synergistic effects, and in some experiments may even interfere with each others effects.

Expression of baseline levels of the respective OS and ER stress markers *SOD1, TXNRD1*, and *CHOP, ATF4, XBP1* and *sXBP1* was low in healthy BM-MSCs, but in particular during osteogenic differentiation of obese and morbid obese donor-derived BM-MSCs, an increase in *XBP1* and *sXBP1* was observed. Treatment with TUDCA resulted in normalization of *XBP1* expression in obese donor-derived BM-MSCs, but not could not suppress *XBP1* or *ATF4* in morbid obese donor-derived BM-MSCs, indicating that extended ER stress cannot be sufficiently modulated with TUDCA treatment alone. Lack of major involvement of OS in the dysfunctioning of obese/morbid obese donor-derived BM-MSCs may have been the reason that the effects of MT in this setting and at this dose were minimal.

MT has been shown to bind and function through several different receptors, of which the most well known are the surface receptors MT1 and MT2, the membrane/cytosolic receptor MT3 and the nuclear receptor RZR/RORα (114). The downstream signaling pathways of MT through binding of MT to these receptors is well described in literature and although we were not able to directly measure the presence of MT receptors, we believe that actions of MT in BM-MSCs are largely modulated through interaction with MT2. These data are in line with the use of MT antagonists that previously indicated that signaling in MSCs occurs most likely through activation of MT2 receptors (73). We therefore believe that the relative absence of major effects of MT on stimulation or recovery of stem cell functions in BM-MSCs derived from obese and morbid obese donors may be related to the fact that OS plays a minimal role in the development obesity-related loss-of-stemness.

The increase in ATF4 and CHOP expression in obese and morbid obese donor-derived BM-MSCs, as well as the increase in *XBP1* and *sXBP1* during osteogenic differentiation indicates activation of both the *PERK* and *IRE1*α pathways of the UPR. Therefore, targeting these pathways with either TUDCA or another additional ER

stress inhibitor may be more advantageous in recovering stem cell function than by focusing on suppression or inhibition of OS, although it cannot be excluded that targeting both mechanisms will not result in a synergistic effect.

Nevertheless, here we show that 1) obesity severely interferes with stem cell function, as apparent from decreased proliferative capacity, increased senesence and decreased or skewed differentiation potential; 2) increased BMI will result in accelerated senescence and loss-of-stemness; 3) ER stress is the main mechanism involved in the obesity-related loss-of-stemness; 4) treatment with TUDCA can partially correct stem cell function, but only if the obesity is moderate: severe obesity results in irreversible changes in BM-MSC function that can no longer be corrected; 5) melatonin shows a positive effect on healthy BM-MSCs by improving proliferative capacity, but in obesity-related loss-of-stemness, the effects are minimal; 6) simultaneous use of TUDCA and MT interferes with the positive effects of either drug, resulting in decreased proliferation and differentiation.

In this thesis we used 9 BM donors to assess the effects of obesity on BM-MSC function. Although we observed a clear trend with increasing BMI resulting in decreased differentiation, decreased proliferation, increased senescence, increased ROS levels, increased ER stress levels and moderately affected OS, many of the results we obtained were not significant due to the small group size. Therefore, increasing the group sizes remains imperative to be able to translate these data directly to the clinic. Nevertheless, this thesis strongly supports the idea that obesity by itself is sufficient to severely disturb stem cell function and the higher the BMI, the larger the effect on stem cell function, until eventually the loss-of-stem cell function becomes irreversible and the cells enter early senescence or activate apoptotic pathways. At this point, none of the treatments will be able to reverse the detrimental effects on the cell, furthermore underlining the importance of prevention of obesity, rather than focusing on recovery of lost stem cell functions.

6. CONCLUSION

In the framework of this master thesis we hypothesized that “Loss of stemness in BM-MSCs is associated with increasing BMI and can be attenuated using administration of MT and TUDCA”. We have now confirmed that loss of stemness in BM-MSCs from obese and morbid obese donors is indeed associated with increasing BMI and can be partially attenuated using administration of TUDCA to suppress ER stress, but only if the degree of obesity is moderate. OS does not appear to play a leading role in the development of obesity-related loss-of-stemness in BM-MSCs, although it remains a contributing factor that when protracted may further affect remaining cellular functions. Treatment with MT or other anti-oxidant agents may have a supporting role here.

This thesis underlines that obesity severely affects stem cell function and that its detrimental effects at max can be partially suppressed once the point of no return has not been passed. Therefore, prevention of obesity is more likely to have a lasting positive effect on stem cell function than treatment with ER suppressing drugs that can only partially recover stem cell function. Further research focusing on these topics is therefore needed to be able to cope with the current obesity epidemic.

In conclusion, obesity-related loss-of-stem cell function can be contributed to increased ER stress and moderate OS. Increased BMI is correlated with increased senescence and most likely related to loss-of-stemness, although larger study groups will have to provide final confirmation. Treatment with TUDCA will result in partial gain-of-function in moderate obesity, but does not synergistically work with MT.

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8. SUPPLEMENTS

Supplement-1. Approval of ethical commitee

T.C.
HACETTEPE ÜNİVERSİTESİ
Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16960557-[illegible]
Konu : **ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU**

Toplantı Tarihi : 21 HAZİRAN 2022 SALI
Toplantı No : 2022/11
Proje No : GO 22/651 (Değerlendirme Tarihi: 21.06.2022)
Karar No : 2022/11-68

Üniversitemiz Tıp Fakültesi Kök Hücre Bilimleri Anabilim Dalı öğretim üyelerinden Doç. Dr. Fatima Aerts KAYA'nın sorumlu araştırmacı olduğu, Ece Gizem POLAT'ın yüksek lisans tezi olan, GO 22/651 kayıt numaralı ***"Melatonin'in Obez Donor Mezenkimal Kök Hücrelerinde Hücresel Strese Bağlı Olarak Oluşan Fonksiyon Bozuklukları Üzerindeki Etkilerinin Değerlendirilmesi"*** başlıklı proje önerisi araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş olup, 22 Haziran 2022 – 22 Haziran 2024 tarihleri arasında geçerli olmak üzere etik açıdan **uygun bulunmuştur.** Çalışma tamamlandığında sonuçlarını içeren bir rapor örneğinin Etik Kurulumuza gönderilmesi gerekmektedir.

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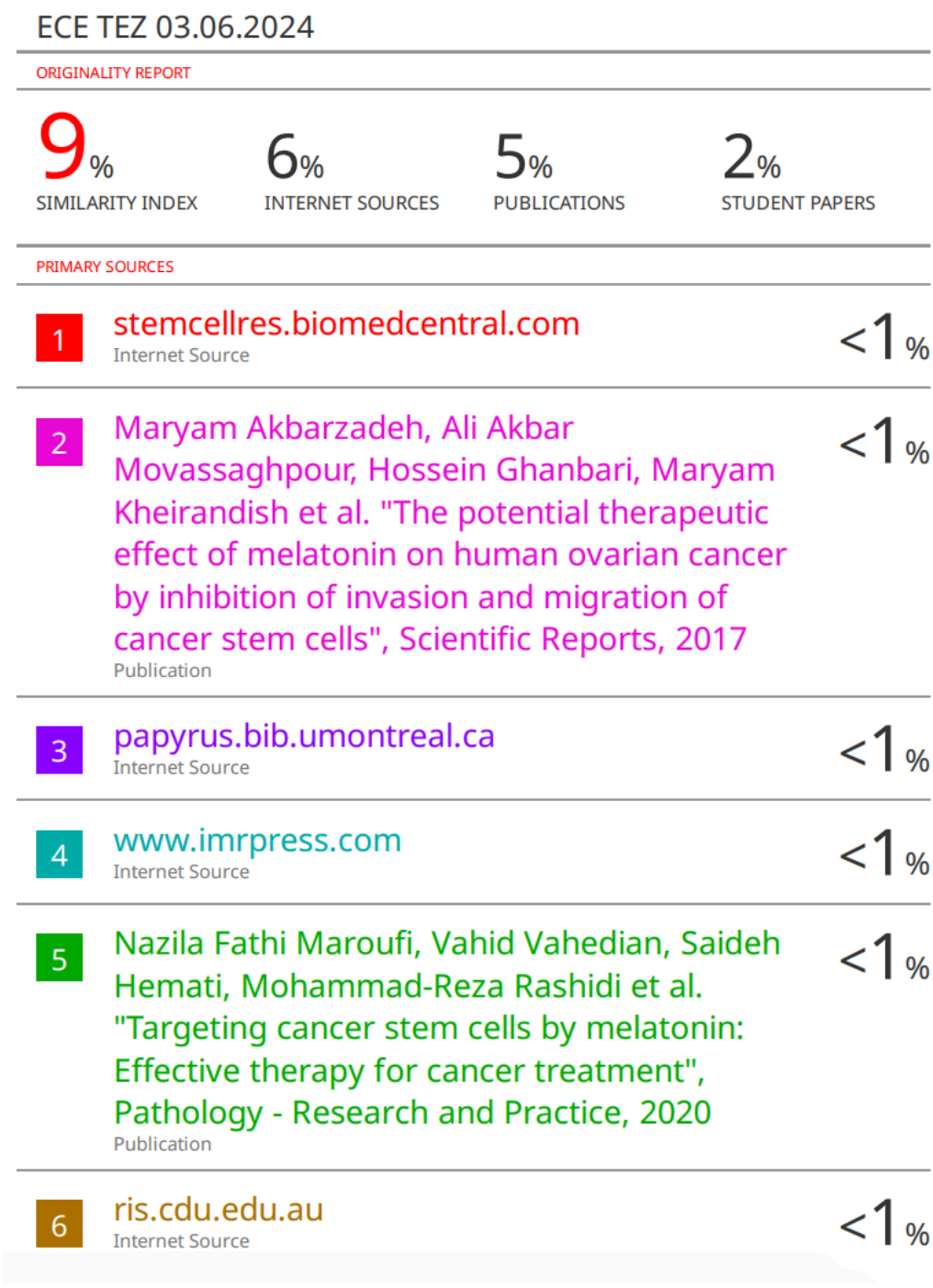

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1. INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) are spindle-shaped cells with multipotent properties and self-renewal abilities. In addition to having high proliferative capacity, responding to chemotaxis signals for migration, and homing to injury sites, MSCs also have important immunomodulatory properties. Because of these properties, MSCs have become particularly important in the field of regenerative medicine and in the treatment of [auto]immune diseases. Over the last two decades, MSCs have been tested in various clinical studies and have been shown to be both effective and safe sources for clinical stem cell therapy.

During protein synthesis, the number of unfolded/misfolded proteins in the ER increases and the proteins are kept in the ER until they are folded appropriately. If proteins fail to fold into their correct tertiary shape, the proteins or peptides are marked for destruction. An increase in protein production or a decrease in protein folding capacity causes ER stress due to the accumulation of unfolded/misfolded proteins. In response to ER stress, a process called the unfolded protein response (UPR) is initiated. Eventually, when ER stress cannot be reduced sufficiently, the cell is destroyed through autophagy or activation of apoptotic pathways.

Oxidative stress (OS) is characterized by the dysregulated production and/or failure to clear reactive oxygen species (ROS). While high ROS levels cause cellular damage and dysfunction, low basal ROS levels are thought to be essential to the cell and maintain cellular proliferation, differentiation, and survival. ROS play an important role in intracellular signaling and communication. Although certain levels of ROS are required for physiological proliferation and differentiation of cells, excessive ROS has been shown to cause functional damage in MSCs. Constantly increasing ROS levels can cause serious damage to the cell through breaks in biological macromolecules (DNA/RNA) and lead to genomic instability.

Senescent cells acquire the senescence-associated secretory phenotype (SASP), which affects the behavior of neighboring cells by spreading the senescent

9. CURRICULUM VITAE