EFFECT OF MELATONIN ON OSTEOGENIC, NEUROGENIC, ADIPOGENIC, CHONDROGENIC, MYOGENIC AND ODONTOGENIC DIFFERENTIATION OF HUMAN TOOTH GERM STEM CELLS

by

Nurullah Aydoğdu

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APPROVED BY:

Prof. Dr. Fikrettin Şahin .....

Assoc. Prof. Dilek Telci .....

Prof. Dr. Ertuğrul Kılıç .....

DATE OF APPROVAL: ..../..../....

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ABSTRACT

EFFECT OF MELATONIN ON OSTEOGENIC, NEUROGENIC, ADIPOGENIC, CHONDROGENIC, MYOGENIC AND ODONTOGENIC DIFFERENTIATION OF HUMAN TOOTH GERM STEM CELLS

Stem cell based applications have become a popular and promising approach for therapy of a number of disorders including neurodegenerative disorders, diabetes, degenerative muscle diseases and osteoporosis as well as trauma, inflammations, burns and injuries. Human tooth germ stem cells are an adult stem cell source, which have mesenchymal stem cell properties and show high proliferative and differentiation capacity. Melatonin, a hormone produced mainly by pineal gland in humans, are demonstrated to regulate differentiation of human and mouse mesenchymal stem cells into various cell lineages besides its other functions in the body. In this study, the effects of melatonin on osteogenic, neurogenic, adipogenic, chondrogenic, myogenic and odontogenic differentiation of human tooth germ stem cells have been investigated. The results showed that melatonin increases the viability of cells. It significantly increases osteogenic, neurogenic, chondrogenic, myogenic and odontogenic of the cells whereas it decreases adipogenic differentiation capability. These results suggest that melatonin has a great potential to increase differentiation capacity of human tooth germ stem cells and might be used in regenerative therapy applications involving stem cell differentiations besides defining potential treatments for obesity because of its suppressor effects on adipogenesis.

ÖZET

MELATONİNİN İNSAN DİŞ GERM KÖK HÜCRELERİNİN KEMİK, NÖRON, YAĞ, KIKIRDAK, KAS VE DİŞ HÜCRELERİNE FARKLILAŞMASI ÜZERİNE OLAN ETKİSİ

Kök hücre uygulamalarının, nörodejeneratif hastalıklar, diyabet, dejeneratif kas hastalıkları, osteoporoz, yanık, inflamasyon, travma ve yaralanmalar gibi birçok hastalığın tedavisi için popüler ve umut verici bir yaklaşım olduğu gösterilmiştir. Mezenkimal kök hücre özelliklerine sahip olan insan diş germ kök hücrelerinin, yüksek çoğalma ve farklılaşma kapasitesine sahip olan önemli bir yetişkin kök hücre kaynağı olduğu kanıtlanmıştır. İnsanda beyin epifizi tarafından üretilen melatoninin, vücuttaki diğer birçok işlevinin yanı sıra insan ve fare mezenkimal kök hücrelerinin çeşitli hücre hatlarına farklılaşması üzerinde de etkili olduğu gösterilmiştir. Bu çalışmada melatoninin insan diş germ kök hücrelerinin kemik, nöron, yağ, kıkırdak, kas ve diş hücrelerine farklılaşması üzerindeki etkisi araştırılmıştır. Melatoninin bu hücrelerin canlılığını artırdığı tespit edilmiştir. Elde ettiğimiz sonuçlara göre melatonin insan diş germ kök hücrelerinin kemik, nöron, kıkırdak, kas ve diş hücrelerine farklılaşma yeteneğini artırırken bu hücrelerin yağ hücresine dönüşme yeteneğini azaltmaktadır. Sonuçlar melatoninin insan diş germ kök hücrelerinin farklılaşması için önemli bir potansiyele sahip olduğunu ve adipojenez üzerindeki etkilerinden dolayı obezite tedavisinde kullanılabilmesinin yanı sıra yenileyici tedavi uygulamalarında da melatoninden yararlanılabileceğini göstermektedir.

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αP2	Adipocyte protein 2
α-SMA	α-smooth muscle actin
ACTA2	Alpha-actin-2
ALP	Alkaline phosphatase
BMP-2	Bone morphogenetic protein 2
C/EBP	CCAAT/enhancer-binding protein
cDNA	Complementary deoxyribonucleic acid
COL1A	Collagen type 1 a
COL2A	Collagen type 2 a
DAPI	4',6-diamidino-2-phenylindole
DFPCs	Dental follicle precursor cells
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPSCs	Dental pulp stem cells
DSCs	Dental stem cells
DSP	Dentin sialoprotein
DSPP	Dentin sialophosphoprotein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
g	Gram
$GABA_A$	Type A γ-butyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
HSC	Hematopoietic stem cell
HTGSCs	Human tooth germ stem cells
IBMX	3-isobutyl-1-methylxanthine

IFN-γ	Interferon-γ
IL-2	Interleukin-2
IL-6	Interleukin-6
IPDSCs	Immature dental pulp stem cells
ITS	Insulin-Transferrin-Selenium
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LPL	Lipoprotein lipase
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Mikromolar
MAP2	Microtubule-associated protein 2
mg	Milligram
mM	Milimolar
nm	Nanometer
nM	Nanomolar
mL	Milliliter
mmol	Millimole
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MTS	Methyl tetrazolium salt
NCBI	The National Center for Biotechnology Information
NeuroD1	Neurogenic differentiation 1
NF	Neurofilament
NSE	Neuron-specific enolase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDLSCs	Periodontal ligament stem cells
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PSA	Penicillin streptomycin amphotericin B
RNA	Ribonucleic acid
rpm	Rotation per minute

Runx2	Runt-related transcription factor 2
SCAP	Stem cells from apical papilla
SCN	Suprachiasmatic nuclei
SHED	Stem cells from human exfoliated deciduous teeth
TGF-β1	Transforming growth factor beta 1
TH	Tyrosine hydroxylase
UV	Ultraviolet

1. INTRODUCTION

1.1. GENERAL VIEW OF STEM CELLS

Stem cells are undifferentiated cells that have the capacity of proliferation, differentiation, self-renewing and tissue regeneration. When a stem cell divides, one of the daughter cells self-renew to remain as a stem cell while the other one becomes a differentiated specialized cell [1].

Stem cells are divided into five groups based on their sources: embryonic stem cells, embryonic germ cells, fetal stem cells, umbilical cord blood stem cells and adult stem cells (Figure 1.1). Embryonic stem cells (ESCs) are pluripotent stem cells derived from inner cell mass of a 5 or 6 day old human blastocyst [2]. ESCs are capable to differentiate into primordial germ layers (ectoderm, endoderm and mesoderm). However, they have a number of limitations. A limited amount of ESCs can be produced even with the use of feeder layers [3]. ESCs are likely to acquire genetic mutations and transform into tumor cells since they lack a fully developed G1 check point [4-5]. Moreover, despite their great differentiation potential, usage of ESCs raises ethical, religious and political debates since the isolation of ESCs involves the destruction of the embryo. Embryonic germ cells are pluripotent stem cells which are derived from 5-9 week old fetuses and can differentiate into all three primordial germ layers [6]. Fetal stem cells are originated from the organs of fetuses [2]. Umbilical cord blood is another stem cell source including mesenchymal stem cells [7] which are similar to bone marrow-derived mesenchymal stem cells with respect to cellular properties and multi-lineage differentiation capacity [8-9].

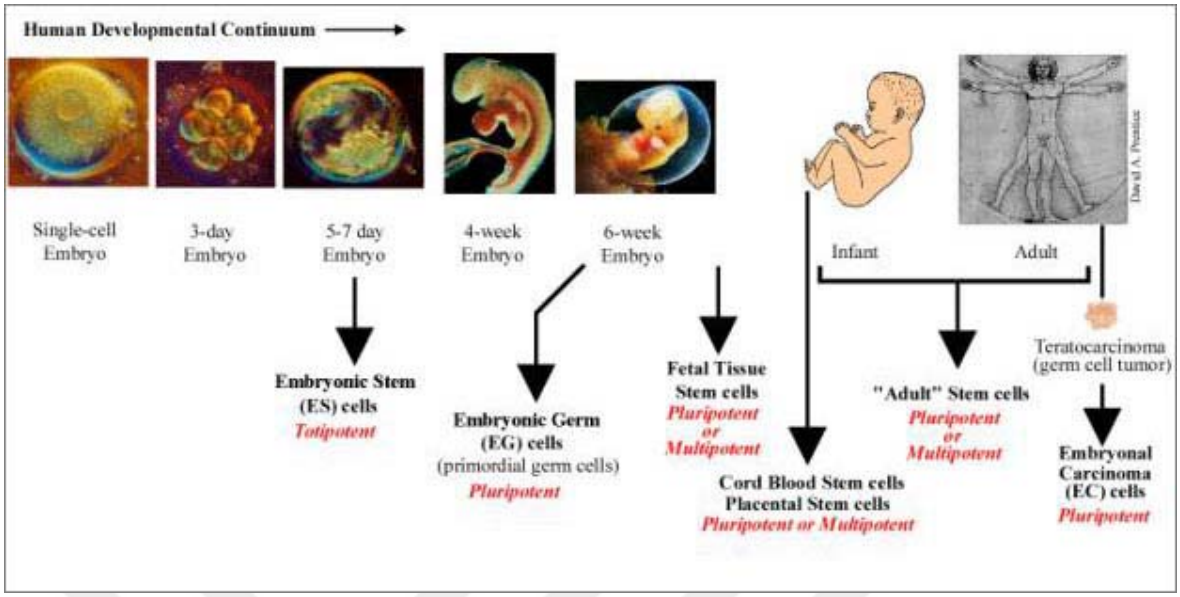

Figure 1.1. Stem cell sources [10]

Adult stem cells are found in various tissues of the body after embryonic development and provide homeostasis by replacing dying cells and regenerating damaged tissues as well as maintain their existence by self-renewal. In the human body, there are different types of adult stem cells such as hematopoietic, mesenchymal, bone marrow, adipose, endothelial, neural and intestinal stem cell. Adult stem cells are only able to give rise to cell types of tissues which they belong to. Hematopoietic stem cells, the first and best described stem cells, are derived from bone marrow and can differentiate into other blood cell types. They are demonstrated to be converted to cell types other than those of blood system such as liver cells, endothelial cells and stromal cells under certain conditions [11]. Bone marrow stem cells also have the ability to differentiate into a number of cell lineages and express tissue-spesific proteins in a number of organs such as liver, brain, skeletal muscle, skin, heart, bone, cartilage and fat [12-17]. The existence of stem cells are also shown in adipose tissue, which are referred to as adipose stem cells. Adipose stem cells are similar, but not identical to the bone marrow msenchymal stem cells. They have been demonstrated to differentiate into a broad range of cell types including bone, liver, cartilage, neural and endothelium cells [18-19]. Endothelial progenitor cells contribute to development of vascular network systems by proliferating and differentiating into mature endothelial cells [20]. Neural stem cells can be isolated from central nervous system or developing brain, and exhibit capacity to generate neurons, astrocytes and oligodendrocytes [21]. Intestinal

stem cells maintain the homeostasis of adult intestinal epithelium by differentiating into cell lines including enterocytes, Paneth cells and goblet cells [22].

Mesenchymal stem cells (MSCs) are nonhematopoietic stem cells derived from the bone marrow, but bone marrow is not the only source of MSCs. They can also be obtained from brain, spleen, liver, lung, muscle, pancreas, thymus, adipose tissue and human umbilical cord [23].

1.2. BIOLOGY OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells are multipotent stromal cells which have high potential of differentiation into a variety of cell types. MSCs have a distinct interest among other stem cells since they are easily isolated, cultured and manipulated. MSCs were first identified by colony-forming unit-fibroblast assay in bone marrow of guinea pig by Friedenstein in 1970 [24]. MSCs are characterized by different methods. They can be easily characterized by analyzing their antigenic surface markers by flow cytometry. In this method, MSCs are positive for CD73, CD90 and CD105 whereas they are negative for hematopoietic antigens such as CD45, CD34, CD14 or CD19, HLA-DR or CD11b and CD79α surface markers [25].

Mesenchymal stem cells show multipotent differentiation capacity which means they differentiate into multiple, but limited cell types under certain *in vitro* and *in vivo* conditions. MSCs from various species such as human, mouse, rat, rabbit and canine are have the capacity to develop into terminally differentiated cell lineages including bone [26-27], muscle [28-29], tendon [30-31], cartilage [32] and adipose tissue [33-34]. Moreover, it was recently shown that MSCs can also be converted to unrelated cell types by the induction of certain factors. Takahashi *et al.* showed the generation of induced pluripotent stem cells from adult human dermal fibroblasts via the induction of Oct3/4, Sox2, Klf4 and c-Myc [35]. In another study, human umbilical cord blood stem cells were differentiated into human neural cells [36]. This differentiation ability of MSCs into different connective tissue cell types has made them an ideal clinical candidate cell source for tissue regeneration studies, especially regeneration of bone [37], cartilage [38] and tendon [30].

Although molecular mechanisms that underlie MSC differentiation have not been completely understood, Baksh *et al.* proposed a model for the regulation of stem cell differentiation, which involves two continuous yet distinct compartments shown in Figure

1.2. In the ‘stem cell compartment’, MSCs undergo transcriptional changes and give rise to new precursor cells. Stem cells are quiescent until they are stimulated. After stimulation, for instance, by the presence of growth factors, they divide asymmetrically and generate one daughter cell, the exact copy of the mother cell, and one daughter cell to become a precursor cell with a limited pluripotency capacity. These precursor cells continue dividing symmetrically and generate tripotent and bipotent precursor cells which share morphological similarity to MSCs, but differ at the transcriptional level. In the ‘commitment compartment’, precursor cells continue to divide symmetrically and form unipotent progenitor cells. These cells are simultaneously converted to fully committed mature cells with cell line specific properties [39]. Growth factors, transcription factors, cytokines and various molecules in extracellular matrix are involved in the commitment and differentiation of MSCs into mature cells types [40-41].

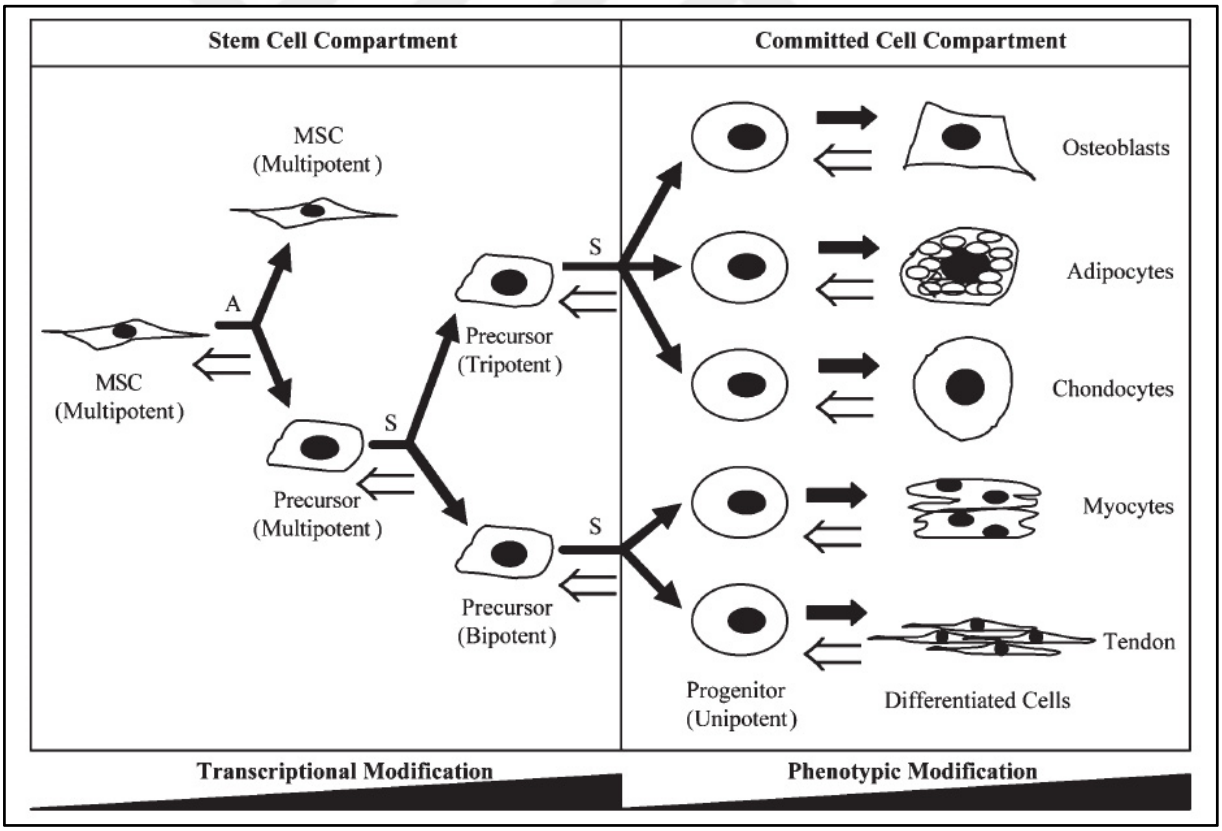

Figure 1.2. Model of adult stem cell differentiation [39]

Traditionally, it has been accepted as central dogma that stem cells are able to differentiate into cell lineages from the tissue of origin. This is called orthodox plasticity of the stem cells. For instance, neural stem cells give rise to certain nerve cell types present in the brain. But this information has changed over the years since numerous studies challenge this central dogma by demonstrating that adult stem cells can be converted to unrelated cell types under specific microenvironmental conditions. For example, bone marrow stem cells are able to generate neural cells [42] and hepatocytes [11]. This concept is called unorthodox plasticity.

1.3. DENTAL STEM CELLS

Dental stem cells have recently gained a big interest among the other adult stem cell types. Dental stem cells are isolated from adult third molars, exfoliated deciduous teeth and adult periodontal ligament [43-44]. These dental stem cells show mesenchymal stem cell characteristics and have the ability to differentiate into several cell types including neurogenic, adipogenic and odontogenic cells [45].

Dental pulp is a connective tissue surrounded by dentine and harbors nerve and blood vessels. Dental pulp has four distinct areas from the outer layer to the inner layer: The first part is odontoblastic layer which contains odontoblasts that produce dentin. The second part is cell free zone rich in nerves and capillaries, and the third part, also named ‘cell rich zone’, contains fibroblasts as well as the undifferentiated progenitor cells with pluripotential properties. The inner part harbors nervous network and vascular supply [46].

Post-natal dental pulp tissue contains stem cells functional in dentin formation. Adult dental pulp stem cell is first identified by Gronthos *et al.* in 2000 [47]. Dental pulp stem cell and bone marrow stem cells are found to express the same stem cell markers including CD44, CD106, CD146 and STRO-1 [45]. The expression of STRO-1 in dental tissues is suggested to be characterized with multilineage potential [48].

Dental pulp stem cells produce mineral tissue related matrix proteins such as osteocalcin and alkaline phosphatase. Besides their dentinogenic differentiation potential, dental pulp stem cells are also able to differentiate into adipogenic and neurogenic cells [49]. Recently, it was shown that dental pulp stem cells can be converted to osteogenic, chondrogenic and myogenic cells [50]. Moreover, dental pulp tissue harbors stem cells which have odontogenic differentiation capacity [51]. Dental stem cells isolated from human exfoliated

deciduous teeth (SHED), also known as immature dental pulp stem cells (IDPSCs), are shown to be highly proliferative and express ESCs markers including Oct4, Nanog, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 [52]. Dental follicle cells are derived from dental follicle, the ectomesenchymal tissue containing precursor cells which are able to generate periodontum, periodontal ligament, alveolar bone and cementum [53]. Dental follicle contains the developing tooth, surrounds the enamel organ and dental papilla and organizes the tooth eruption [54]. Periodontal ligament stem cells are located in periodontal ligament of the tooth which also contains cementoblasts (cementum-forming cells) and osteoblasts (bone-forming cells), and are functional in tissue regeneration and homeostasis of periodontal tissue [55-56]. Dental papilla has been known to contribute to tooth formation and convert to pulp tissue. Sonoyama *et al.* has discovered a new type of stem cells (SCAP) in the apical papilla which seem to be a different population from dental pulp stem cells. SCAP shows a higher proliferation rate and odonto/osteogenic differentiation capacity [57-58]. Furthermore, some studies report the neurogenic and adipogenic differentiation capacity of SCAP [57-59].

1.4. HUMAN TOOTH GERM STEM CELLS (HTGSCs)

Human tooth germ stem cells (HTGSCs) are derived from human third molar tooth germs of young individuals at the age of 10 to 16. The tooth germ is the aggregate of progenitor cells which lead to formation of a tooth. It is composed of the dental papilla, dental follicle and enamel organ. HTGSCs have been discovered to show mesenchymal stem cell properties. They are derived from dental pulp and dental follicle tissues (Figure 1.3) [60]. Third molar tooth germs develop after the age of six, but still keeps its undifferentiated state before this time. This could be the reason that MSCs derived from third molar tooth germs such as HTGSCs are likely to have high proliferation and multipotent differentiation capacity [52].

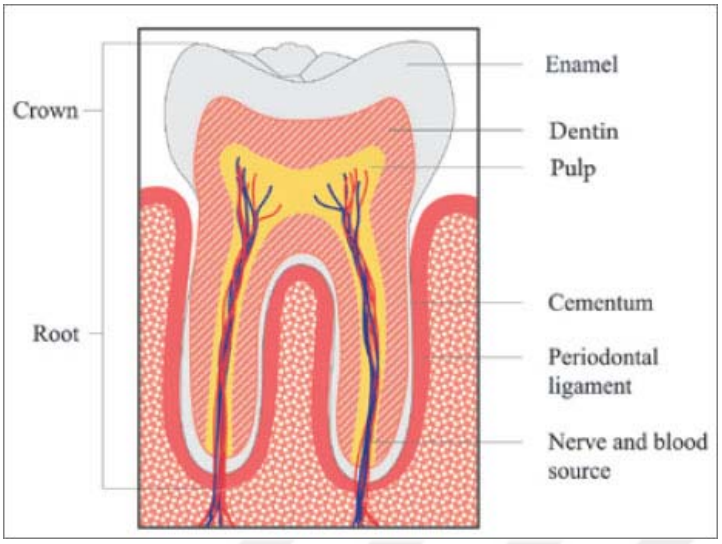

Figure 1.3. Structure of a tooth [61]

Moreover, stem cells found in tooth germs are demonstrated to have ability to differentiate into various cells lines of all of the three primordial germ layers [62].

1.5. MELATONIN

Melatonin, also known as *N*-acetyl-5-methoxytryptamine, is an indoleamine hormone mainly produced in the pineal gland [63] as well as in many other tissues including the eye [64], skin [65], gastrointestinal tract [66-67], lymphocytes [68] and thymus [69]. The production of melatonin is inhibited by light. Most of its biological effects are mediated through the activation of melatonin receptors while others are suggested to be due to the chemical antioxidant nature of indoleamine and occur at higher, non-physiological concentrations [70]. In humans, there are two well-characterized melatonin receptors (MT_1 and MT_2) which are G protein-coupled proteins with high affinity to melatonin [71-72]. Moreover, an additional melatonin receptor subtype MT_3 has been discovered in birds and amphibians [73]. Melatonin exhibits its pleiotropic effects via two molecular mechanisms: binding to its high-affinity receptors on membrane and interacting with intracellular targets to regulate signal transduction pathways or scavenging of free radicals [74].

Melatonin has a number of functions in the human body. It functions as part of the regulation system of circadian rhythm by being synthesized during the dark phase of the light/dark cycle and being delivered to the body through the blood stream. Although

suprachiasmatic nuclei (SCN) is the main region in the brain which controls circadian rhythms through its hormonal and neuronal activities, the daily rhythm of melatonin is used as a mediator by SCN clock to convey circadian message to target tissues and organs possessing melatonin receptors [75].

Several studies suggest that melatonin can change the release of various neurotransmitters such as dopamine, serotonin, norepinephrine and acetylcholine in rats [76-79]. In addition, melatonin regulates the function of type A γ-aminobutyric acid ($GABA_A$) receptors through the activation of its receptors [80]. It has been suggested that, in humans, sedative, analgesic, anticonvulsive and anxiolytic effects of melatonin might be connected to its interaction with $GABA_A$ [81].

Melatonin has been shown to interact with the immune system although the mechanisms remain yet to be completely unveiled. It is of great importance to boost the immune response of elderly individuals and patients having immunocompromised conditions [82-83]. It was demonstrated that melatonin *in vitro* enhances the cytokine production including interleukin (IL)-2, IL-6 and interferon-γ (IFN-γ) in human mononuclear cells [84]. Furthermore, it enhances IFN-γ production and natural killer cell activity by increasing the cytokine production [83-85]. These effects of melatonin may occur through the MT_1 and MT_2 membrane receptors since they have been identified in various tissues of immune system including lymphocytes, spleen and thymus.

Melatonin is also an antioxidant molecule with the ability to cross cell membrane and the blood-brain barrier [86-88]. It was first discovered to be a potent hydroxyl radical scavenger in 1993 [89]. The usage of oxygen in cells causes the production of reactive free radical species such as hydrogen peroxide, superoxide anion, nitric oxide and hydroxyl radical, which are highly toxic and deleterious, and damage macromolecules like DNA, proteins and lipids, hence triggering apoptosis. The antioxidant ability of melatonin to protect cells from oxidative damage was proven in numerous publications [90-94]. It can help the cell at all levels in terms of protecting from oxidative/nitrosative damage by radicals and radical-related molecules. It stimulates various antioxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase that convert reactive oxygen and nitrogen species into harmless products [95-98] as well as enhancing the production of another antioxidant, glutathione [99-100]. Moreover, it reduces free radical formation in the mitochondria, a process named radical avoidance [87, 101]. Melatonin also inhibits the

activity of nitric oxide synthase which produce nitric oxide, one of the free radicals [102-103].

1.6. EFFECTS OF MELATONIN ON STEM CELL DIFFERENTIATION

Beside its several physiological processes in human body, melatonin is also discovered to induce the stem cell differentiation into various types of cells. It was demonstrated that melatonin promotes osteoblast differentiation and bone formation in mice [104], enhances osteogenesis of human mesenchymal stem cells by increasing the expression of runt-related transcription factor 2 (Runx2), a key transcription factor involved in osteoblast differentiation [105], regulates osteoblast differentiation [106] and bone growth [107-108].
Melatonin modulates the survival of newborn neurons by stimulating the dendrite maturation which is a necessary process for the existence of new neurons [109-110]. Researchers also found that melatonin promotes neurogenesis in the hippocampus of mice after pinealectomy, the removal of pineal gland, which mimics the withdrawal of melatonin [111]. Furthermore, it augments neurogenic differentiation of neural stem cells at pharmacological concentrations (1-100 μM) in humans [112] and rats the increasing the expression of dopaminergic neuron marker tyrosine hydroxylase [113].
In contrast to enhanced osteogenic and neurogenic differentiation by melatonin, an inhibitory effect on adipogenesis was reported. It inhibits adipogenic differentiation of human mesenchymal stem cells through the suppression of peroxisome proliferator-activated receptor (PPAR)-γ, a nuclear receptor which regulates adipocyte differentiation [105]. Moreover, melatonin decreases the expression of glucose transporter GLUT4 and glucose uptake of adipocytes [114] and inhibits triglyceride accumulation in the osteoblast-like cells of rats [115].
It was reported that melatonin treatment enhances cartilage matrix synthesis and the expression of chondrogenic markers including collagen II, Sox 9 and aggrecan [116] and shows protective and anti-inflammatory effects in chondrocytes [117]. Although melatonin's effect on odontogenic differentiation is still unclear, it may play an important role in the regulation of tooth development since MT_1 receptor has been found in the tooth germs of human and mice [118]. Researchers also discovered that melatonin plays a protective role on the survival of myocytes against damage by doxorubicin [119] and reduces the hypoxic damage in cardiomyocytes [120].

1.7. AIM OF THE STUDY

In this study, we aimed to show the efficiency of melatonin on osteogenic, neurogenic, adipogenic, myogenic, chondrogenic and odontogenic differentiation of HTGSCs. By testing this, it is expected that melatonin might be used as a therapeutic agent in the treatment of a number of disorders such as osteoporosis, osteoarthritis, neurodegenerative and cardiovascular diseases.

2. MATERIALS AND METHODS

2.1. ISOLATION OF HTGSCs

Human tooth germs were collected from the 3rd molar teeth of a 14 year old patient. The collected tooth germs were harvested and cut into small pieces. The tooth germ tissue was plated in six well plates (BIOFIL, TCP, Switzerland) and grown to confluency in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) PSA (10.000 units/mL potassium penicillin, 10.000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B) (Invitrogen, Gibco, UK). The harvested cells expanded and started to appear after 3-4 days, and covered the surface after 8 days (Figure 2.1). Thereafter, the cells were trypsinized using 0.25% (v/v) trypsin/EDTA (Invitrogen, Gibco, UK). Medium was added to detached cells to inhibit the activity of trypsin. After the cells were centrifuged at 300 x g for 5 minutes at room temperature, the pellet was dissolved in fresh medium and seeded on a T-75 flask (Zelkultur Flaschen, Switzerland). The cells were maintained at 37ºC and 5% CO_2 in a humidified incubator. Cells from passages 1~6 were used in all experiments [121].

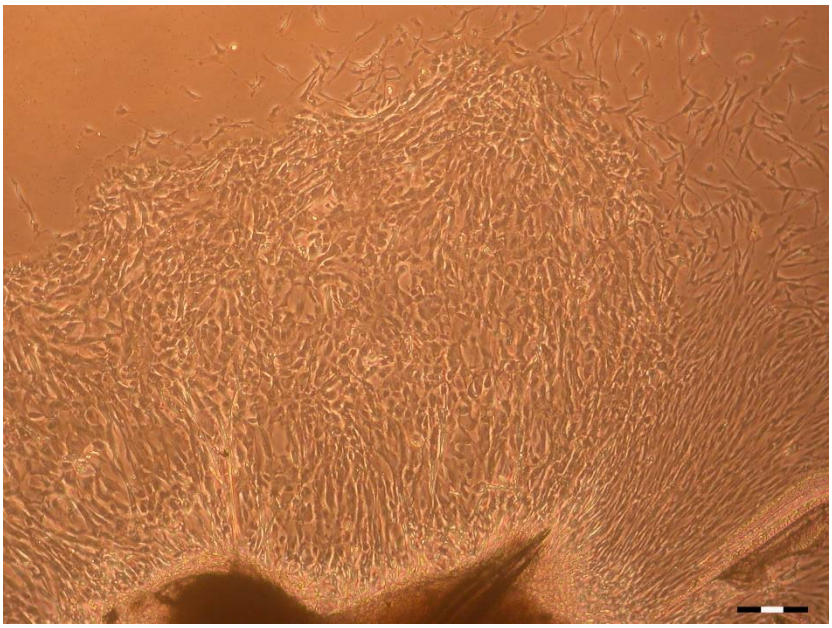

Figure 2.1. Isolated human tooth germ stem cells at day 8. Scale bar: 400 µm

2.2. CHARACTERIZATION OF HTGSCs

The procedure previously published by our group was done for characterization of HTGSCs [121]. Cells were trypsinized and incubated with the primary antibodies which were prepared in PBS. For characterization, primary antibodies against CD29 (cat #BD556049), CD34 (cat #SC-51540), CD45 (cat #SC-70686), CD90 (cat #SC-53456), CD105 (cat #SC-71043), CD133 (cat #SC-65278), CD166 (cat #SC- 53551) (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) and CD73 (cat # BD550256) (Zymed, San Francisco, CA, USA) were used. The cells were washed with PBS to remove the excess primary antibodies. Thereafter, the cells were incubated with fluorescein-iso-thio-cynate (FITC)-conjugated secondary antibody (cat #SC-2989) at 4^{o}C for one hour except CD29 phyco-erythrin (PE)-red light-harvesting protein containing chromophore-conjugated monoclonal antibody was used. The flow cytometry analysis of the cells was done using Becton Dickinson FACS Calibur flow cytometry system (Becton Dickinson, San Jose, CA, USA). 5000 cells were counted in each sample.

2.3. TOXICITY ASSAY FOR VARIOUS CONCENTRATIONS OF MELATONIN

Melatonin (MP Biomedicals LLC, USA) at different concentrations (50 nM, 100 nM and 150 nM) were prepared in DMEM with 10% (v/v) FBS and 1% (v/v) PSA. Cells were seeded on a 96 well plate (BIOFIL, TCP, Switzerland) at a density of 5000 cells per well. The following day cells were treated with different concentrations of melatonin and 20% (v/v) DMSO. Cell viability was measured by the MTS assay (CellTiter96 Aqueous One Solution, Promega, UK) according to the manufacturer's instructions. MTS (3-(4, 5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) is a yellow tetrazolium salt which is catabolized to formazan by dehydrogenase enzyme in mitochondria of the living cells. Formazan is a purple compound and the detection in this assay is based on the measurement of formazan compounds by an ELISA plate reader. After incubating the cells with melatonin for 24, 48 and 72 hours, 10 µl MTS reagent and 100 µl DMEM was given to the cells followed by the incubation for 2 hours. Thereafter, the absorbance at 490 nm was measured by an ELISA plate reader (BioTek Instruments, Inc., VT, USA).

2.4. DIFFERENTIATION PROCESS

HTGSCs were induced to differentiate into osteogenic, neurogenic, adipogenic, chondrogenic, odontogenic and myogenic cells. The cells were seeded on a six well plate for RNA isolation and 48 well plate (BIOFIL, TCP, Switzerland) for immunocytochemistry at a density of 150.000 cells/well and 10.000 cells/well respectively for each differentiation. For osteogenic differentiation the cells were seeded on six well plates followed by the addition of osteogenic differentiation medium, which was consisted of DMEM, 10% (v/v) FBS, 0.1 μM dexamethasone (AppliChem, Germany), 10 mM *β*-glycerol phosphate (Sigma, USA) and 50 μg/mL ascorbic acid (Sigma, USA), with or without 100 nM melatonin. The medium was changed every other day and the cells were treated with the osteogenic differentiation medium for 14 days [105]. For adipogenic differentiation, the cells were induced to differentiate into adipocytes using a previously published method [122]. The cells were seeded on a six well plate and given adipogenic differentiation medium, which is composed of DMEM, 10% (v/v) FBS, 1 μM dexamethasone, 100 μM indomethacin (Sigma, USA), 500 μM IBMX (Calbiochem, Merck Millipore, Germany) and 0.01 mg/mL insulin (Gibco, UK), with or without melatonin. Adipogenic differentiation lasted 10 days in the adipogenic differentiation medium that was changed every other day. In the neurogenic differentiation experiments, a modification of a previously published method was used for neural induction [123]. HTGSCs were treated with the medium including DMEM, 10% (v/v) FBS, 200 μM butylated hydroxyanisole (Sigma, USA), 5 mM KCl, 2 μM valproic acid (Sigma, USA) and 5 g/mL insulin for 14 days with the medium change every 3 days with or without melatonin. A modification of previous method was used for chondrogenic differentiation [124]. The chondrogenic differentiation medium was consisted of DMEM supplemented with 10% (v/v) FBS, 0.1 μM dexamethasone, 10 ng/mL TGF-β1 (Sigma, USA), 50 mg/mL ITS (Gibco, UK), 5.35 mg/ml linoleic acid (Acros Organics, Belgium) and 6.25 μg/mL insulin, with or without melatonin. The chondrogenic differentiation lasted 3 weeks changing the medium in every 2-3 days. The odontogenic differentiation was conducted by treating the cells with the differentiation medium containing DMEM, 10% (v/v) FBS, 10^{-8} M dexamethasone, 5 mmol/L KH_2PO_4 and 50 μg/mL ascorbic acid, with or without melatonin [50]. Eventually, myogenic differentiation was done with the DMEM medium supplemented with 5% (v/v) horse serum, 0.1 μM dexamethasone and 50 μM

hydrocortisone (Sigma, USA) [18, 125]. The myogenic differentiation took 3 weeks with medium changed in every 2-3 days. Positive control groups were treated with the respective differentiation mediums while negative control groups were cultured only in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) PSA.

2.5. IMMUNOCYTOCHEMISTRY ANALYSIS

At the end of the differentiation procedures of HTGSCs, the cells were incubated with 2% (w/v) paraformaldehyde for 30 minutes at 4ºC for fixation. The cells were washed with PBS three times for 5 minutes by shaking the plates on a plate shaker. Later on, the cells were permeabilized by incubating with 0.1% (v/v) Triton X-100 (in PBS) for 5 min at room temperature followed by washing three times for 5 min. with PBS. The cells were incubated with 2% (v/v) goat serum (Sigma, USA) for 20 minutes at 4ºC for preventing non-specific binding of primary antibodies. The cells were again washed three times with PBS. The cells were incubated overnight with primary antibodies at 4ºC. For osteogenic differentiation collagen type 1a (COL1A) (ab292, Abcam, UK) and osteocalcin (sc-30044, Santa Cruz Biotechnology, TX, USA), for neurogenic differentiation enolase (sc-59536, Santa Cruz Biotechnology, TX, USA), nestin (sc-20172, Santa Cruz Biotechnology, TX, USA), neurofilament (NF) (sc-20013, Santa Cruz Biotechnology, TX, USA) and tyrosine hydroxylase (TH) (sc-14007, Santa Cruz Biotechnology, TX, USA), for adipogenic differentiation adipocyte protein 2 (αP2) (sc-30088, Santa Cruz Biotechnology, TX, USA) and PPAR-γ (ab8934, Abcam, UK), for chondrogenic differentiation collagen type 2a (COL2A) (ab3092, Abcam, UK), for myogenic differentiation actin (sc-1616, Santa Cruz Biotechnology, TX, USA), α-smooth muscle actin (α-SMA) (ab5694, Abcam, UK), desmin (ab15200, Abcam, UK) and MyoD (sc-377460, Santa Cruz Biotechnology, TX, USA), and for odontogenic differentiation COL1A and dentin sialoprotein (DSP) (sc-33586, Santa Cruz Biotechnology, TX, USA) were used. The cells were washed three times with PBS to remove the excess antibody after incubating with primary antibodies. Thereafter, the cells were treated with secondary antibodies (Goat anti rabbit IgG Alea Fluor 488, Goat anti mouse IgG Alea Fluor 488) and incubated for 1 hour at 4ºC followed by rinsing three times with PBS. DAPI (AppliChem, Germany) was used to stain the nuclei of the cells by incubating for 20 minutes at 4ºC. The cells were then rinsed three times with PBS and observed under fluorescence microscope (Nicon Eclipse TE200).

2.6. TOTAL RNA ISOLATION AND QUANTITATIVE RT-PCR

Primers for alkaline phosphatase (ALP), nestin, CD133, Sox 9, aggrecan, dentin sialophosphoprotein (DSPP), COL1A, bone morphogenetic protein 2 (BMP-2), desmin and alpha-actin-2 (ACTA2) were designed using nBLAST online software of The National Center for Biotechnology (NCBI). The other primers sequenced were used as previously described in the literature (Table 2.1).

Table 2.1. Primers used in this study

Primer	Sense (5'-3')	Antisense (5'-3')	Ref.
Osteocalcin	GTGCAGAGTCCAGCAAAGGT	TCAGCCAACTCGTCACAGTC	[126]
ALP	GACATCGCCTACCAGCTCAT	TCACGTTGTTCCTGTTCAGC	
Nestin	GGAGTCCTGGATTTCCTTCC	GCCCTGACCACTCCAGTTTA	
CD133	GCCAGCCTCAGACAGAAAAC	CCAAGCCTTAGGAGCATCTG	
MAP2	CCATTTGCAACAGGAAGACAC	CAGCTCAAATGCTTTGCAACTAT	[127]
Adiponectin	TATCCCCAACATGCCCATTCG	TGGTAGGCAAAGTAGTACAGCC	[105]
PPARγ	CCTATTGACCCAGAAAGCGATT	CATTACGGAGAGATCCACGGA	[105]
LPL	ACAAGAGAGAACCAGACTCCAA	AGGGTAGTTAAACTCCTCCTCC	[105]
αP2	AACCTTAGATGGGGGTGTCCT	TCGTGGAAGTGACGCCTTTC	[105]
Sox 9	GAACGCACATCAAGACGGAG	TCTCGTTGATTTCGCTGCTC	
Aggrecan	ACTGCTGCAGACCAGGAGGT	TCCTCGGGGGTGACGATGCT	
COL2A	GTGTGGAAGCCGGAGCCCTG	GGTCCTGGTTGCCCACTGGC	[128]
DSPP	CAGTACAGGATGAGTTAAATGCC	TACTTCTGCCCACTTAGAGCC	
COL1A	CCACGCATGAGCGGACCCTAA	ATTGGTGGGATGTCTTCGTCTTGG	
BMP-2	CCACCATGAAGAATCTTTGGAAGA	ATTAAGCCACTACCTTTGACGATAAC	

Desmin	GGAGAGCCGGATCAATCTCCCCA	ACGACCTCCCCATCCCGTGT	
Myogenin	TAAGGTGTGTAAGAGGAAGTCG	CCACAGACACATCTTCCACTGT	[129]
ACTA2	AGACATCAGGGGGTGATGGT	ATCTTTTCCATGTCGTCCCAGTTG	
MyoD	AAGCGCCATCTCTTGAGGTA	GCGCCTTTATTTTGACC	[125]
GAPDH	TGGTATCGTGGAAGGACTCA	GCAGGGATGATGTTCTGGA	[130]

Total RNA isolation after differentiation was performed using High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. cDNA synthesis from isolated RNA samples were done using High Fidelity cDNA Synthesis Kit (Roche, Germany). Real time PCR was performed using Maxima SYBR Green/ROX (Fermentas, USA) for the determination of expression levels of marker genes after differentiations. cDNAs of the differentiated cells incubated with or without melatonin were used as template and were mixed with primers and Maxima SYBR Green/ROX qPCR Master Mix (2X). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the house keeping gene for the normalization of the data. All the real time PCR experiments were performed using iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA).

2.7. ALKALINE PHOSPHATASE ACTIVITY ASSAY

Alkaline phosphatase activity was determined to demonstrate osteogenic differentiation. After the process of osteogenic differentiation, medium was removed and cells were treated with 0.2% (w/v) Triton X-100 (BioBasicInc., Canada, cat # 9002-93-1) for lysis. Cells were collected from plates and mixed by vortex for 20 minutes at room temperature. Thereafter, 10 µL of cell lysate was added to each well of a 96 well plate followed by the addition of 90 µL of ALP solution (BioAssay Systems, USA, cat # DALP-250). After the incubation for 15 minutes, the absorbance at 405 nm was measured by an ELISA plate reader.

2.8. VON KOSSA STAINING

von Kossa staining is a method based on the detection of calcium or calcium salt deposits. Cells were seeded on 6-well plates at a concentration of 50.000 cells/well. At the end of both osteogenic and odontogenic differentiation, the medium from cells was removed and the cells were washed three times with PBS. Then the cells were incubated with paraformaldehyde for 20 minutes for fixation. After the addition of 3% (w/v) silver nitrate, the cells were exposed to UV light for 1 hour at room temperature. After washing the cells three times with PBS, 5% (w/v) sodium thiosulfate was added to each well followed by incubation for 2 minutes at room temperature. After the incubation, the cells were again rinsed three times with PBS. Thereafter the cells were incubated with nuclear fast red for 5 minutes at room temperature. After washing with PBS, the cells were observed under the light microscope.

2.9. ALCIAN BLUE STATINING

1 gram alcian blue dye (Sigma, USA) was dissolved in 100 mL of 3% (w/v) acetic acid to prepare alcian blue staining solution. The cells were fixed with 2% paraformaldehyde for 30 minutes and incubated with alcian blue staining solution for 30 min. After the incubation, the cells were washed three times with PBS and the samples were observed under the light microscope [18].

2.10. OIL RED OIL STAINING

Oil red oil solution was prepared by dissolving 0.5 gram oil red oil (Sigma, USA) in 100 mL isopropanol. The cells were then fixed with 2% (w/v) paraformaldehyde for 30 minutes followed by wash three times with PBS. The cells were then incubated with oil red oil solution diluted 6:4 in PBS for 1 hour for staining. Cells were washed with PBS and observed under the light microscope [131].

2.11. STATISTICAL ANALYSIS

All data are shown as the means ± standard errors. Graphics were drawn using GraphPad Prism 5 software. The results of real time PCR data were normalized to the mRNA level of GAPDH. The statistical analysis of the results were performed one-way ANOVA followed by the multiple-comparison Tukey's test using GraphPad Prism 5 software. Statistical significance was determined at $P < 0.05$.

3. RESULTS

3.1. CHARACTERIZATION OF HUMAN TOOTH GERM STEM CELLS

Human tooth germ stem cells were analyzed to be characterized for the surface antigens CD29, CD34, CD45, CD73, CD90, CD105, CD133 and CD166 by flow cytometry. Cells were positive for CD29, CD73, CD90, CD105 and CD166 whereas they were negative for CD34, CD45 and CD133 (Figure 3.1). These data showed that HTGSCs were positive for MSC markers and negative for HSC markers.

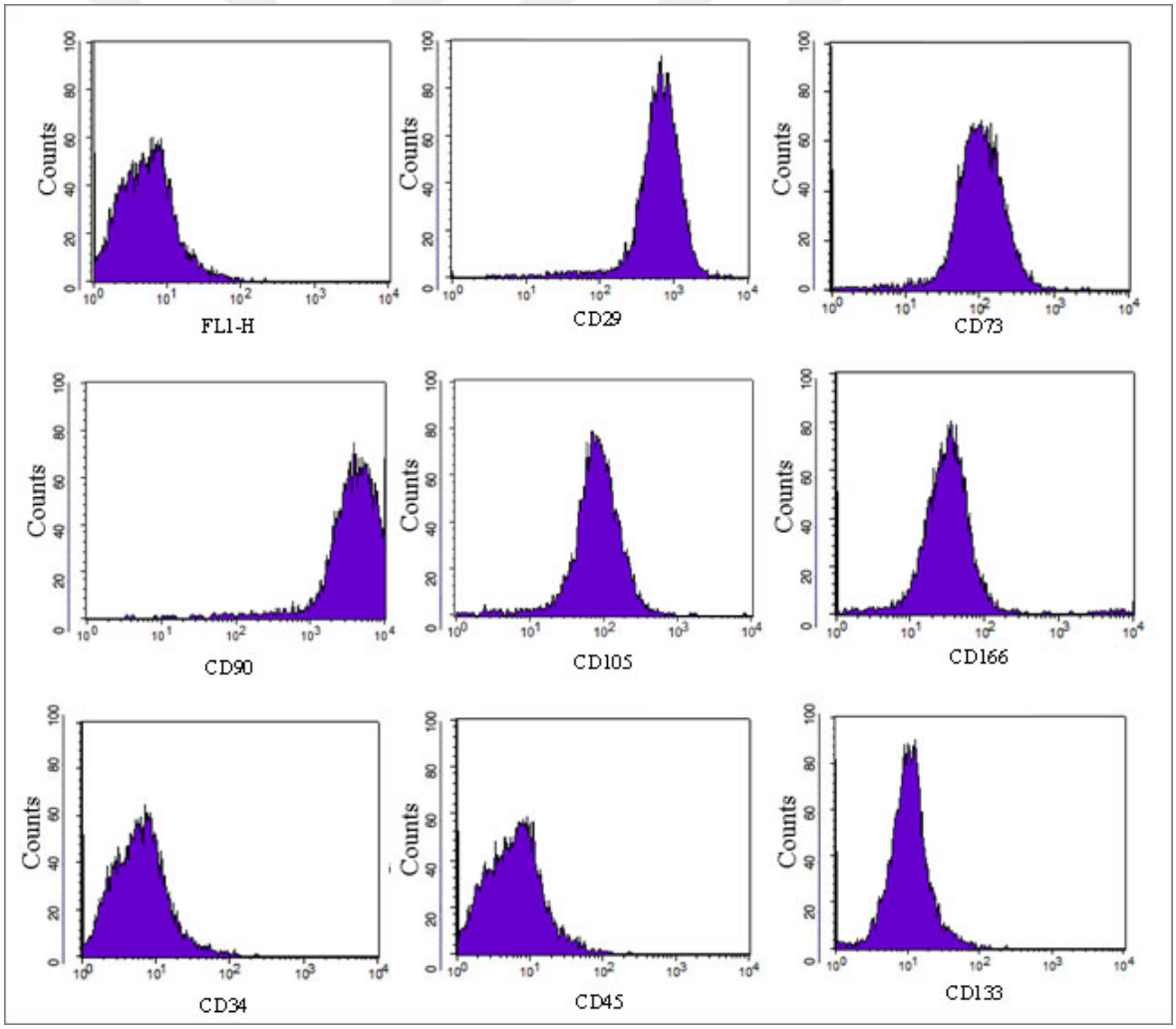

Figure 3.1. Flow cytometry analysis of HTGSCs

3.2. TOXICITY OF MELATONIN

Toxicity of melatonin was measured by MTS assay at various concentrations (50 nM, 100 nM, 150 nM) for day 1, 2 and 3. The results showed that melatonin was toxic to HTGSCs at neither of the concentrations tested (Figure 3.2). On the contrary, it significantly increased the survival of cells at each concentration in each day compared to negative control except 100 nM of melatonin in the 3rd day. 50 nM melatonin increased the survival of cells by 120%, 125% and 110% at day 1, 2 and 3, respectively. For 100 nM melatonin, the survival rate of cells was increased by 125% and 132% at day 1 and 2 while it was not affected at day 3. Finally, 150 nM melatonin raised the survival by 119%, 130% and 110% at day 1, 2 and 3, respectively.

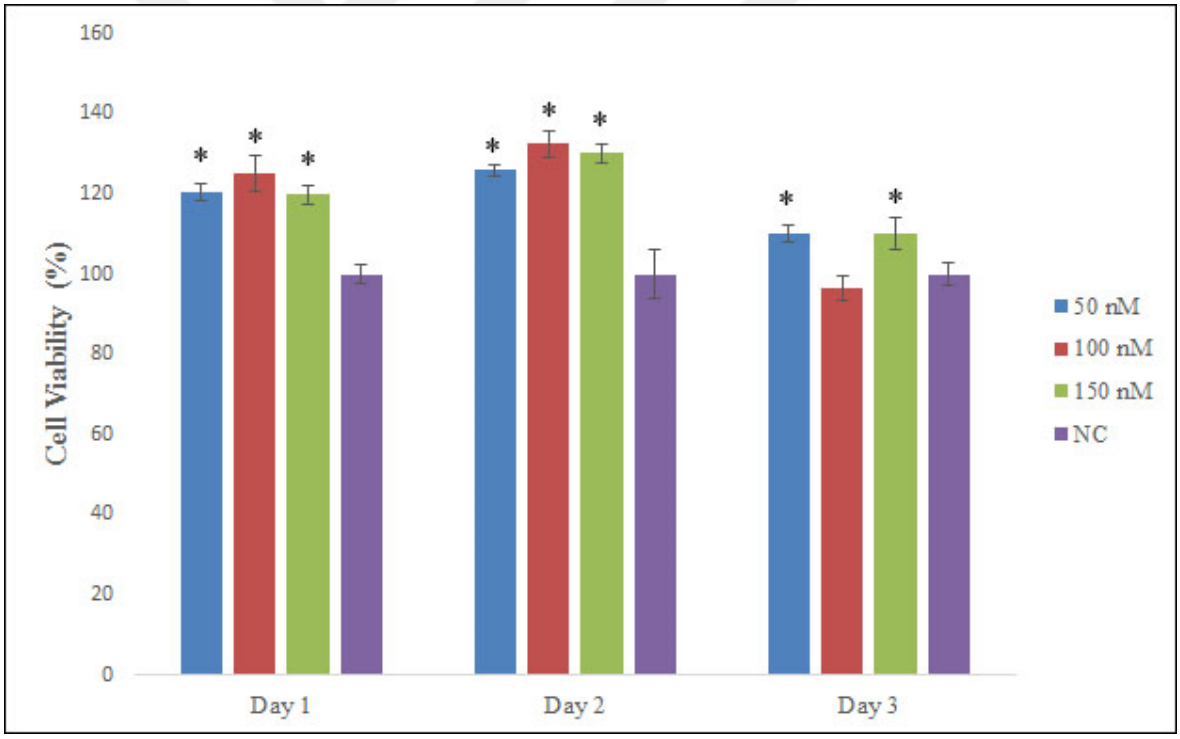

Figure 3.2. Toxicity results of different concentrations of melatonin. NC: Negative control. *$P < 0.05$ versus the negative control

3.3. OSTEOGENIC DIFFERENTIATON

3.3.1. Effect of Melatonin Treatment in Osteogenic Marker Genes

mRNA levels of osteogenic marker genes, osteocalcin, ALP and COL1A, were detected by real time PCR. Osteocalcin is a non-collagenous bone specific protein found in bone and dentin tissues [132]. ALP is a type of hydrolase enzyme which removes phosphate groups from various kinds of molecules including nucleotides and proteins. It is found at high concentrations in bone tissue since it is necessary for the deposition of minerals in bones and teeth [133]. COL1A is another markers for osteogenic differentiation. It is one of collagen types found in bone, cartilage and tendon [134]. Our results showed that cells treated with melatonin have the highest expression levels of these genes. Furthermore, mRNA levels of the marker genes are higher in the positive control compared to the negative control group (Figure 3.3). The expression levels of osteocalcin were 640- and 240-fold elevated in melatonin group and positive control, respectively, compared to the negative control. ALP expression levels were detected 125 and 67 times higher than the negative control in melatonin group and positive control respectively. COL1A expression level was 5.5-fold increase in melatonin groups while positive control was measured 1.8-fold higher than the negative control.

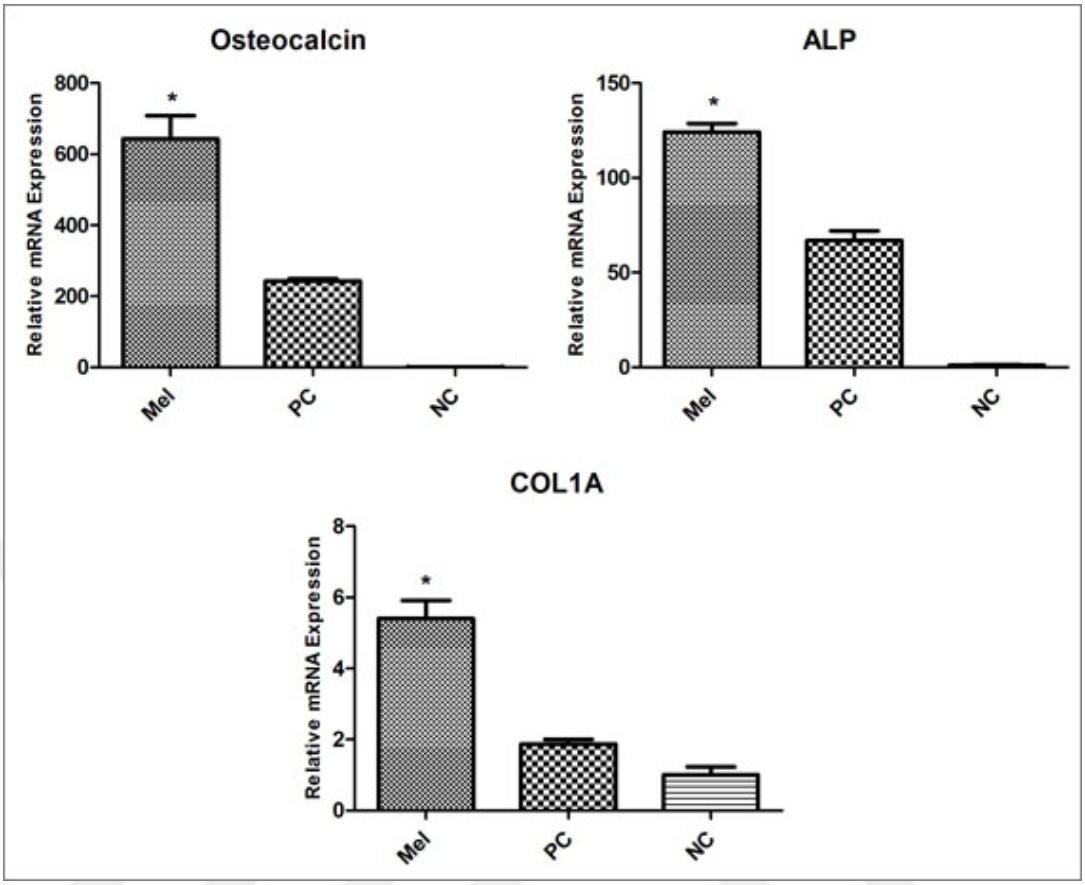

Figure 3.3. Osteocalcin, ALP and COL1A expressions in melatonin and control groups. Mel: Melatonin, PC: Positive control, NC: Negative control. *$P < 0.05$ versus the positive control

3.3.2. Immunocytochemical Analysis of COL1A and Osteocalcin

The expression of osteocalcin and COL1A were visualized by immunocytochemistry. The data showed expressions of osteocalcin and COL1A were up-regulated in melatonin compared with positive control groups whereas there was no expression in the negative control (Figure 3.4).

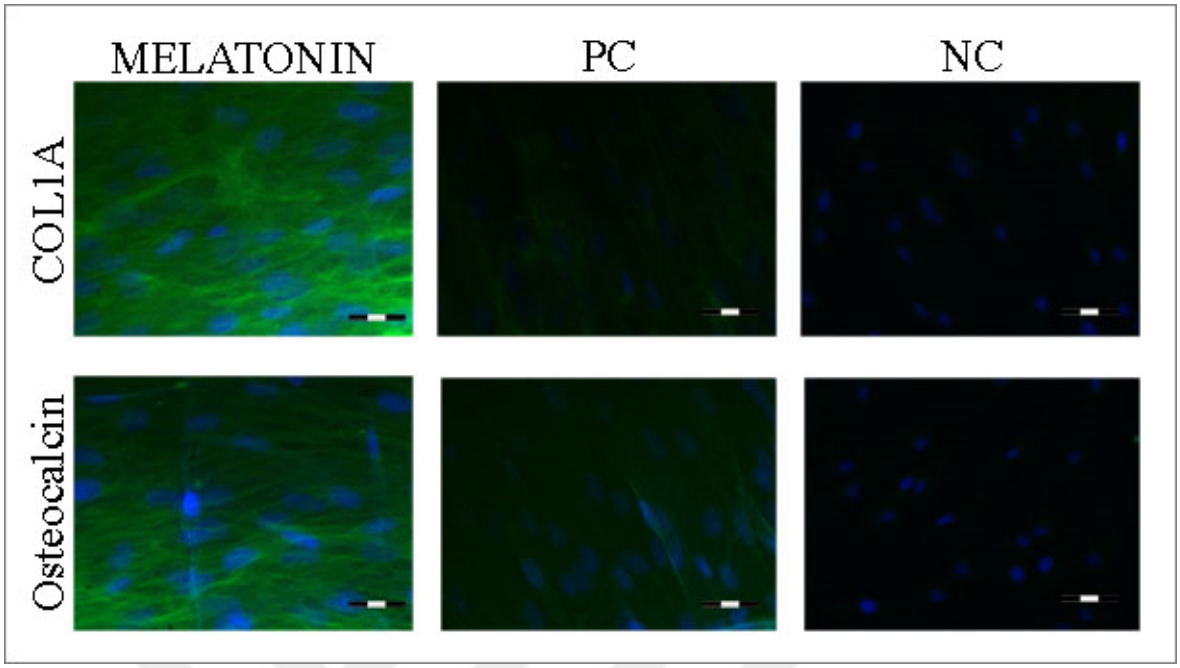

Figure 3.4. Immunostaining of COL1A and osteocalcin in melatonin and control groups. PC: Positive control, NC: Negative control. Scale bar: 100 μm

3.3.3. ALP Activity for Osteogenic Differentiation

ALP activity of differentiated HTGSCs is another marker for osteogenic differentiation. ALP is found in dental and bone tissues at high concentrations. Moreover, alkaline phosphatase level is measured in case of bone diseases in clinical applications [133]. ALP activity was measured for the determination of osteogenic differentiation (Figure 3.5).

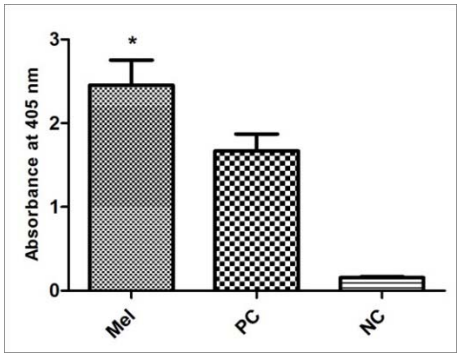

Figure 3.5. ALP activity results of melatonin and control groups. Mel: Melatonin, PC: Positive control, NC: Negative control. *$P < 0.05$ versus the positive control

Melatonin group was found to have the highest level (16-fold) of ALP activity compared to the positive and negative control groups. The ALP activity of positive control was measured 11-fold higher than the negative control.

3.3.4. von Kossa Staining for Osteogenic Differentiation

Osteogenic differentiation of HTGSCs was also confirmed by von Kossa staining which is a standard method to show calcium or calcium salt deposits as an indicator of osteogenic differentiation. Calcium depositions were detected by this method in cells treated with melatonin as well as in the positive control, demonstrating the osteogenic differentiation (Figure 3.6).

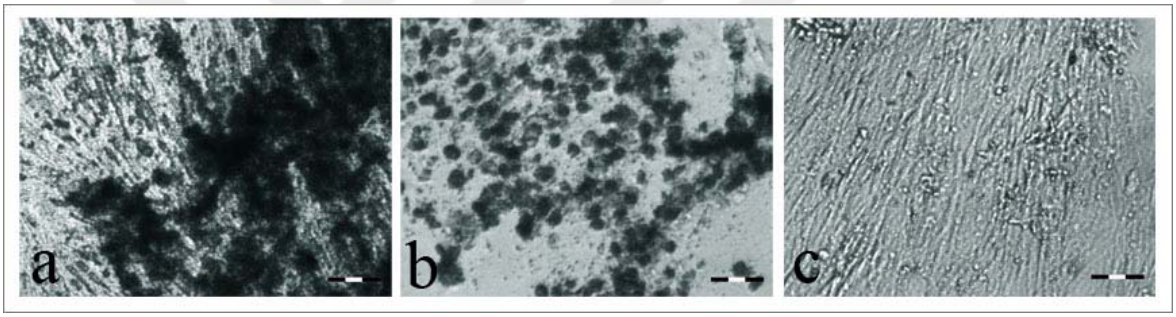

Figure 3.6. von Kossa staining of a. melatonin, b. positive control and c. negative control. Scale bar: 400 μm

3.4. NEUROGENIC DIFFERENTIATION

3.4.1. Effect of Melatonin Treatment on the Neurogenic Marker Genes

CD133 is a transmembrane glycoprotein which is expressed in various tissues including neuronal and glial cells [135]. Nestin is an intermediate filament protein involved in the axon growth of neurons and was found to be expressed in neural precursor cells [136]. Microtubule-associated protein 2 (MAP2) is also another marker for neurogenesis, which is implicated in the microtubule assembly [137]. GAPDH was used as the house keeping gene to normalize the results. The results showed that melatonin group expresses the highest mRNA levels of CD133, nestin and MAP2 compared to positive and negative

controls (Figure 3.7). The expression levels of CD133 were detected 1.7- and 1.3-fold higher in melatonin group and positive control respectively in comparison to the negative control. Melatonin group and positive control showed 2.3- and 1.4-fold higher expression level of nestin compared to the negative control. The expression of MAP2 was elevated up to 3.4- and 1.8-fold in the melatonin group and positive control in comparison to the negative control.

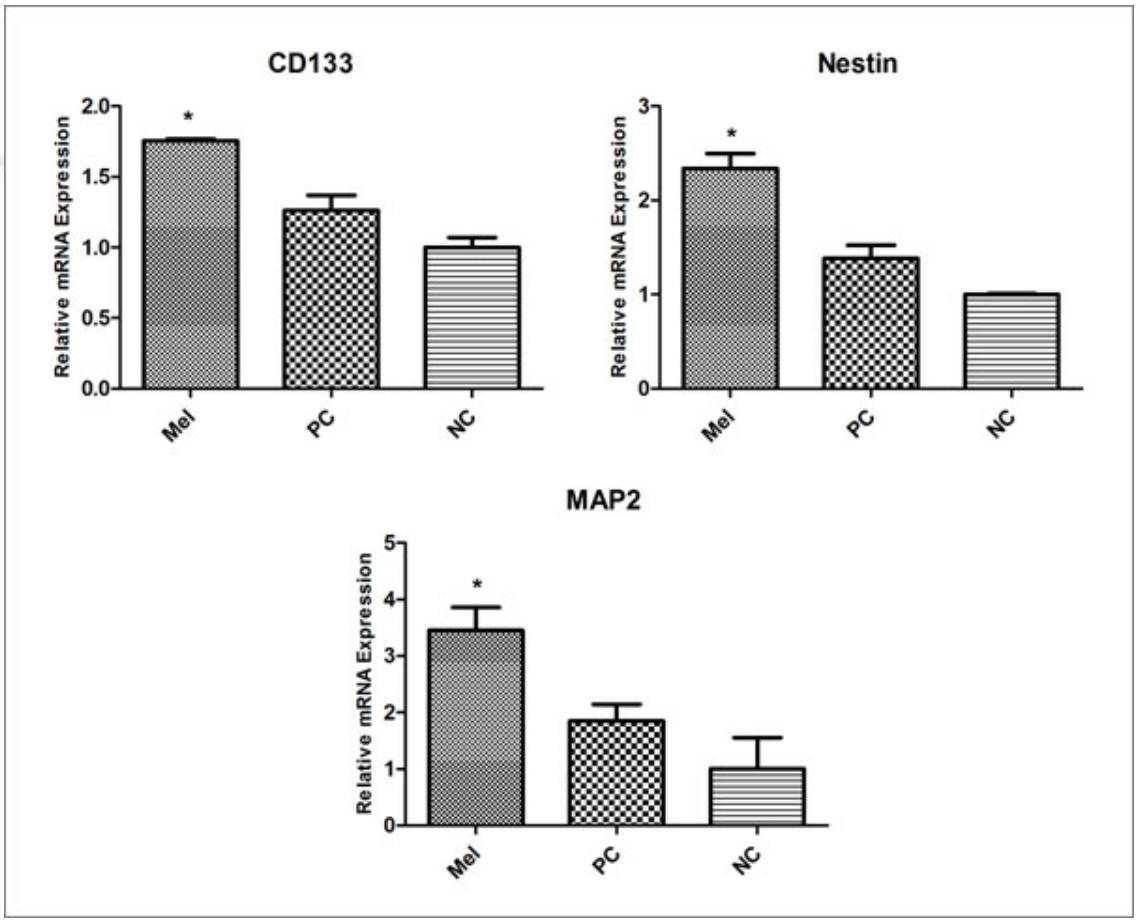

Figure 3.7. CD133, nestin and MAP2 gene expressions of melatonin and control groups. Mel: Melatonin, PC: Positive control, NC: Negative control. *$P < 0.05$ versus the positive control

3.4.2. Immunocytochemical Analysis of NSE, Nestin, NF and TH

Immunocytochemistry assay was performed for neuron-specific enolase (NSE) [50], nestin, NF [58] and TH (Figure 3.8) [138-139]. NSE is found in mature neurons and supposed to serve as a growth factor in neurons [140]. NF is one of the major components of neural cytoskeleton and supports the structure of axons in neurons [141]. TH is an enzyme found in various neural tissues including central nervous system and the adrenal medulla [142]. Melatonin group and the positive control were positively stained for NSE, nestin, NF and TH whereas the negative control does not show expressions of these marker genes.

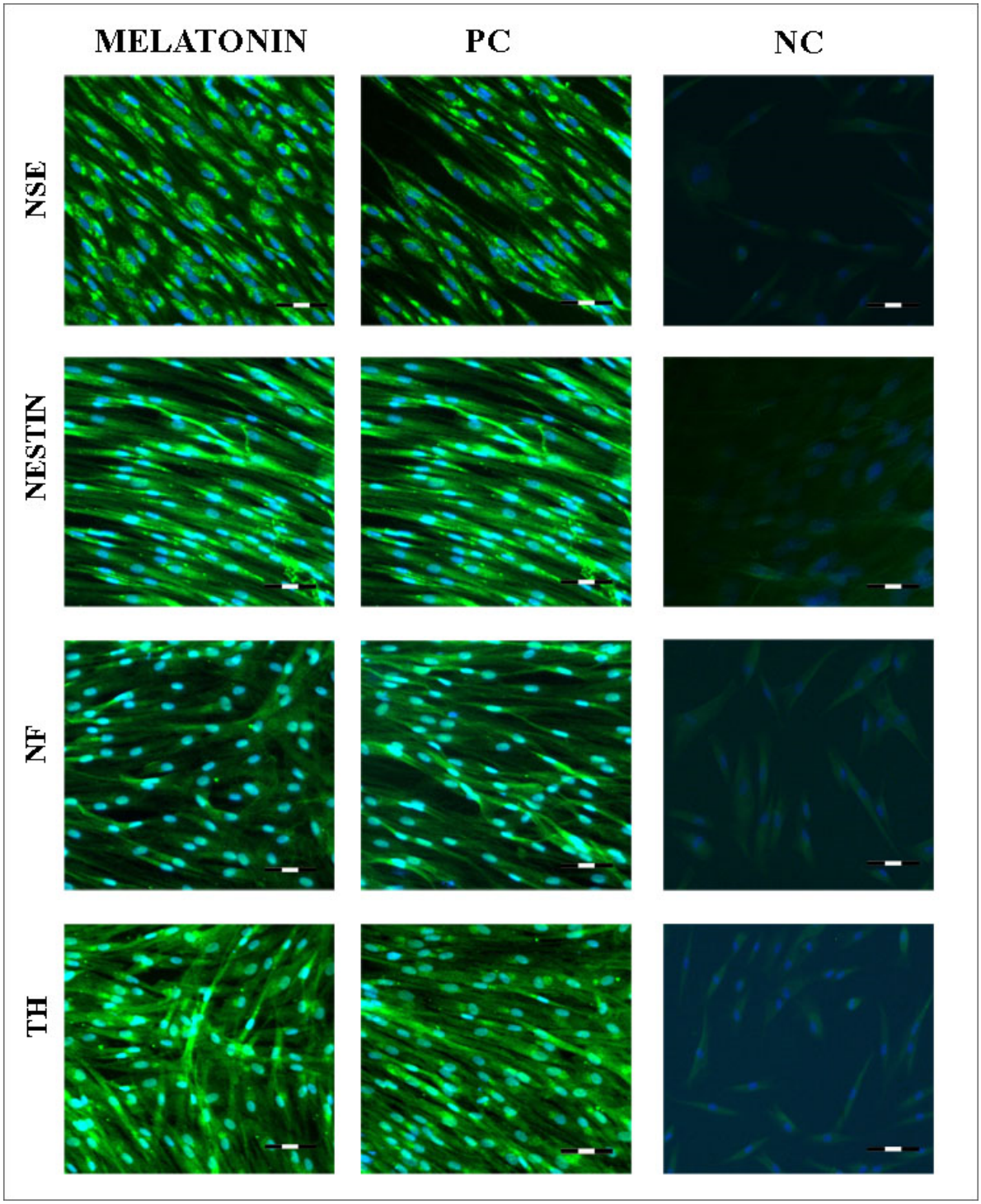

Figure 3.8. Immunostaining of NSE, nestin, NF and TH. PC: Positive control, NC: Negative control. Scale bar: 100 µm

3.5. ADIPOGENIC DIFFERENTIATION

3.5.1. Effect of Melatonin Treatment in Adipogenic Marker Genes

Expression levels of markers for terminal adipocyte differentiation, such as adiponectin, lipoprotein lipase (LPL), αP2 and PPAR-γ were detected by real time PCR. Adiponectin is a hormone mainly secreted by adipose tissue and regulates glucose levels and fatty acid breakdown [143]. LPL is an hydrolytic enzyme which breaks down triglycerides into free fatty acids and monoacylglycerol molecules in adipose tissue [144]. αP2 is a carrier protein of fatty acids in adipocytes [145]. PPAR-γ is a major transcription factor which is found functional in adipocyte differentiation [105]. The expression levels of the marker genes were normalized by dividing by the mRNA level of GAPDH. Cells treated with melatonin during adipogenic differentiation showed the lowest mRNA expressions of the all marker genes compared to positive control groups (Figure 3.9). Melatonin decreased the expression of adiponectin by 4.8-fold, LPL by 2.3-fold, αP2 by 13-fold and PPAR-γ by 6-fold in comparison with the positive control. Moreover, LPL expression level was measured 2.6 times lower in melatonin group compared to the negative control.

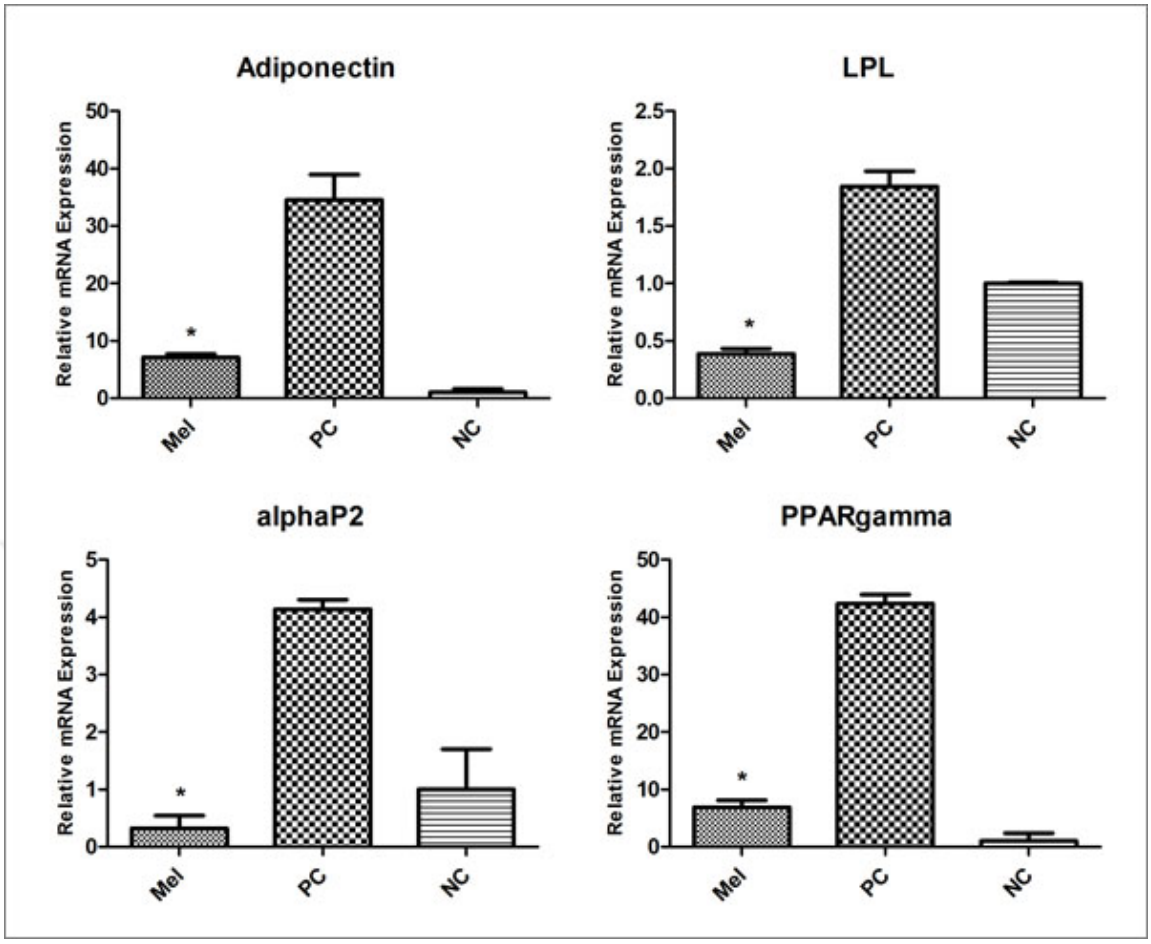

Figure 3.9. Adiponectin, LPL, αP2 and PPAR-γ gene expressions in melatonin and control groups. Mel: Melatonin, PC: Positive control, NC: Negative control. *$P < 0.05$ versus the positive control

3.5.2. Immunocytochemical Analysis of αP2 and PPAR-γ

For adipogenic differentiation, the expression of αP2 and PPAR-γ was checked by immunocytochemistry. Melatonin was found to down-regulate the expression of these two markers and give a similar result to those of the negative control while the positive control groups exhibit higher expressions of αP2 and PPAR-γ (Figure 3.10).

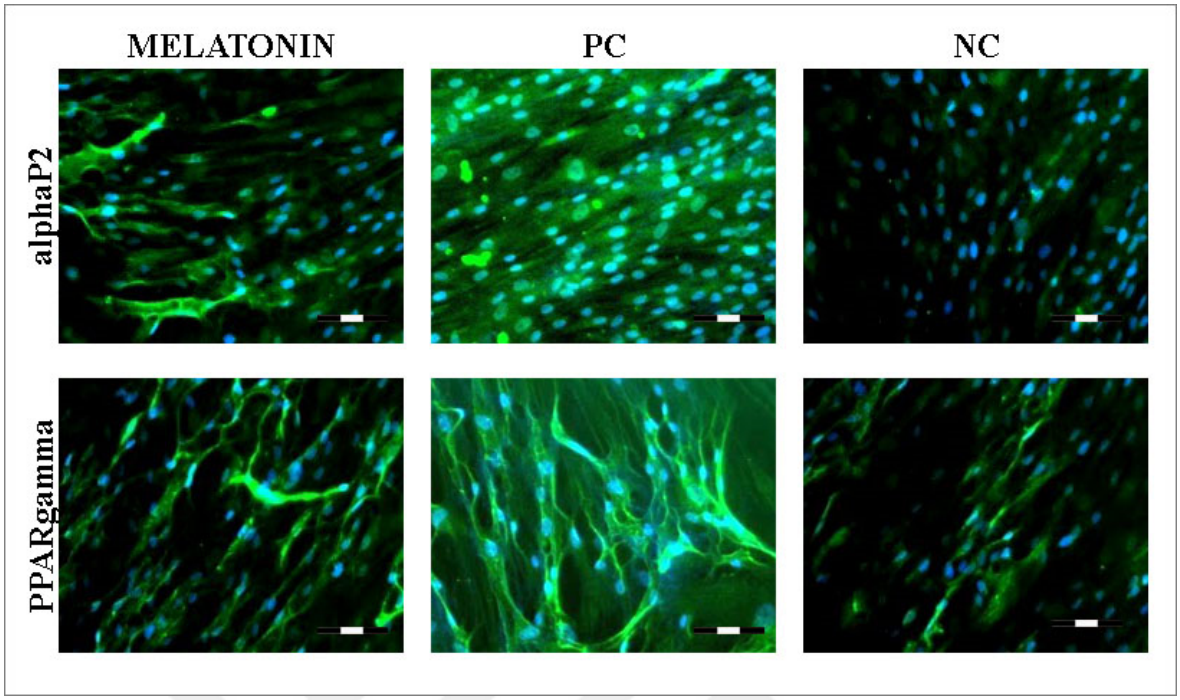

Figure 3.10. Immunocytochemistry results of αP2 and PPAR-γ. PC: Positive control, NC: Negative control. Scale bar: 100 µm

3.5.3. Oil Red Oil Staining for Adipogenic Differentiation

Oil red oil is a lysochrome diazo dye used to stain lipids and neutral triglycerides. Oil red oil staining was performed to visualize the intracellular lipid vesicles after adipogenic differentiation. Lipid vesicles were detected only in the positive control (Figure 3.11).

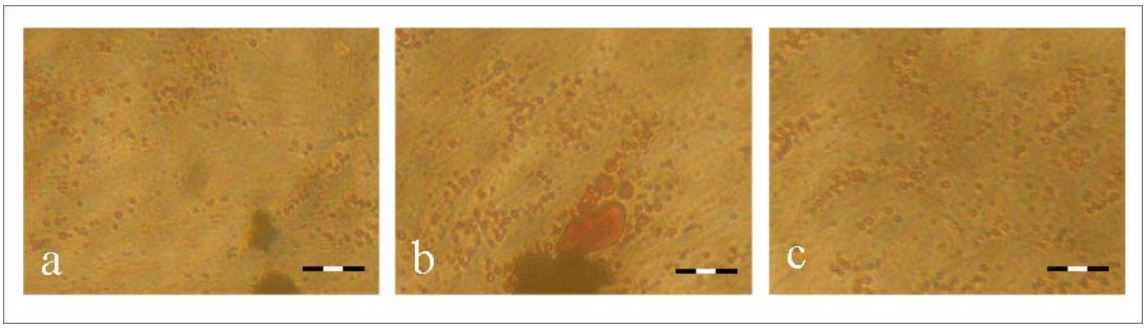

Figure 3.11. Oil red oil staining of a. melatonin, b. positive control and c. negative control. Scale bar: 100 µm

3.6. CHONDROGENIC DIFFERENTIATION

3.6.1. Effect of Melatonin Treatment in Chondrogenic Marker Genes

To confirm chondrogenic differentiation, mRNA levels of Sox 9, aggrecan and COL2A were detected by real time PCR. Sox 9 is a transcription factor that acts during chondrocyte differentiation along with other factors [146]. Aggrecan is one of the compounds of extracellular matrix of cartilage tissue while COL2A is an essential structural protein in cartilage [147-148]. Melatonin groups have much higher mRNA levels of Sox 9, aggrecan and collagen II than the positive control (Figure 3.12). Melatonin increased the expression level of Sox 9 by 2.2-fold, aggrecan by 5.4-fold and COL2A by 7.3-fold compared to the positive control. The expression levels in the positive control were detected as 15-, 25- and 8-fold elevated for Sox 9, aggrecan and COL2A, respectively in comparison with the negative control.

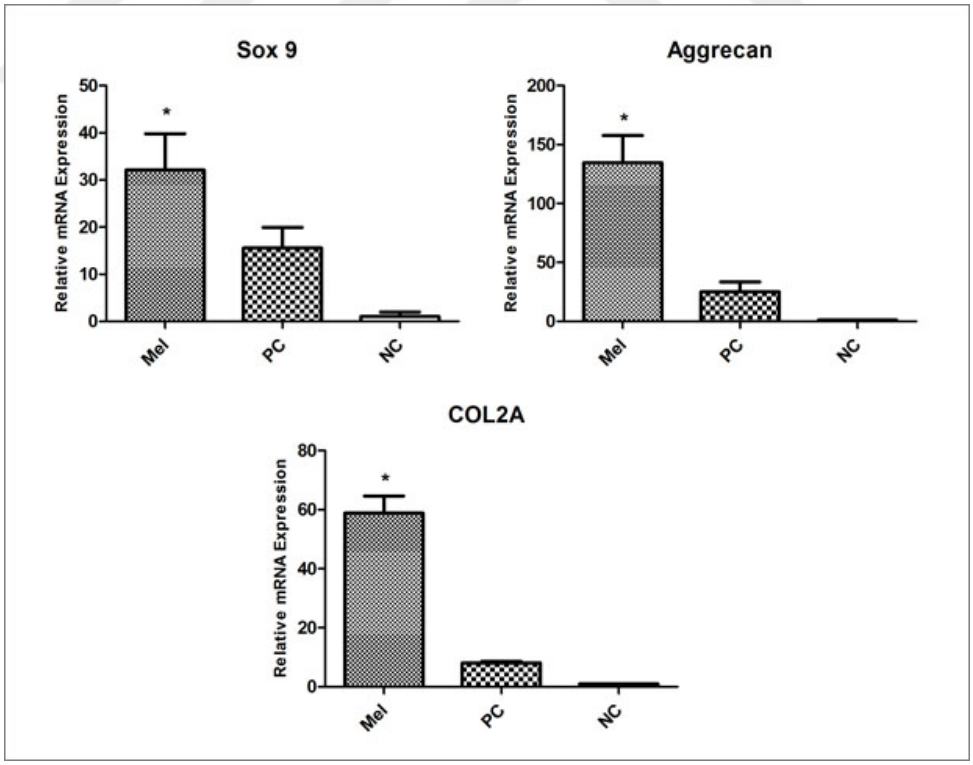

Figure 3.12. Sox 9, aggrecan and COL2A expressions in melatonin and control groups. Mel: Melatonin, PC: Positive control, NC: Negative control. *$P < 0.05$ versus the positive control

3.6.2. Immunocytochemical Analysis of COL2A

COL2A is the main protein of articular and hyaline cartilage tissues. Expression of COL2A was checked with immunocytochemistry. Results showed that melatonin and positive control groups were positively stained whereas the negative control shows almost no expression of COL2A (Figure 3.13).

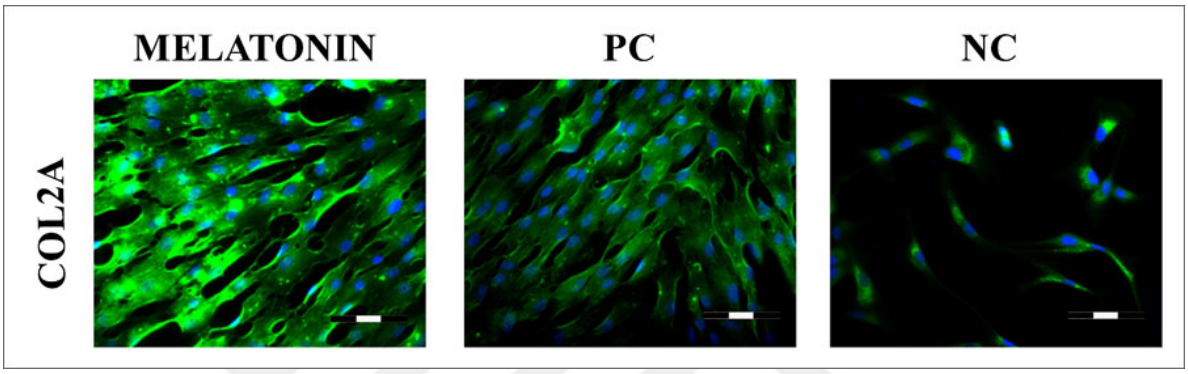

Figure 3.13. Immunostaining of COL2A. PC: Positive control, NC: Negative control. Scale bar: 100 μm

3.6.3. Alcian Blue Staining for Chondrogenic Differentiation

Alcian blue staining was done for the confirmation of chondrogenic differentiation. Melatonin group showed the highest amount of mucopolysaccharides and glycosaminoglycans formed in cells (Figure 3.14).

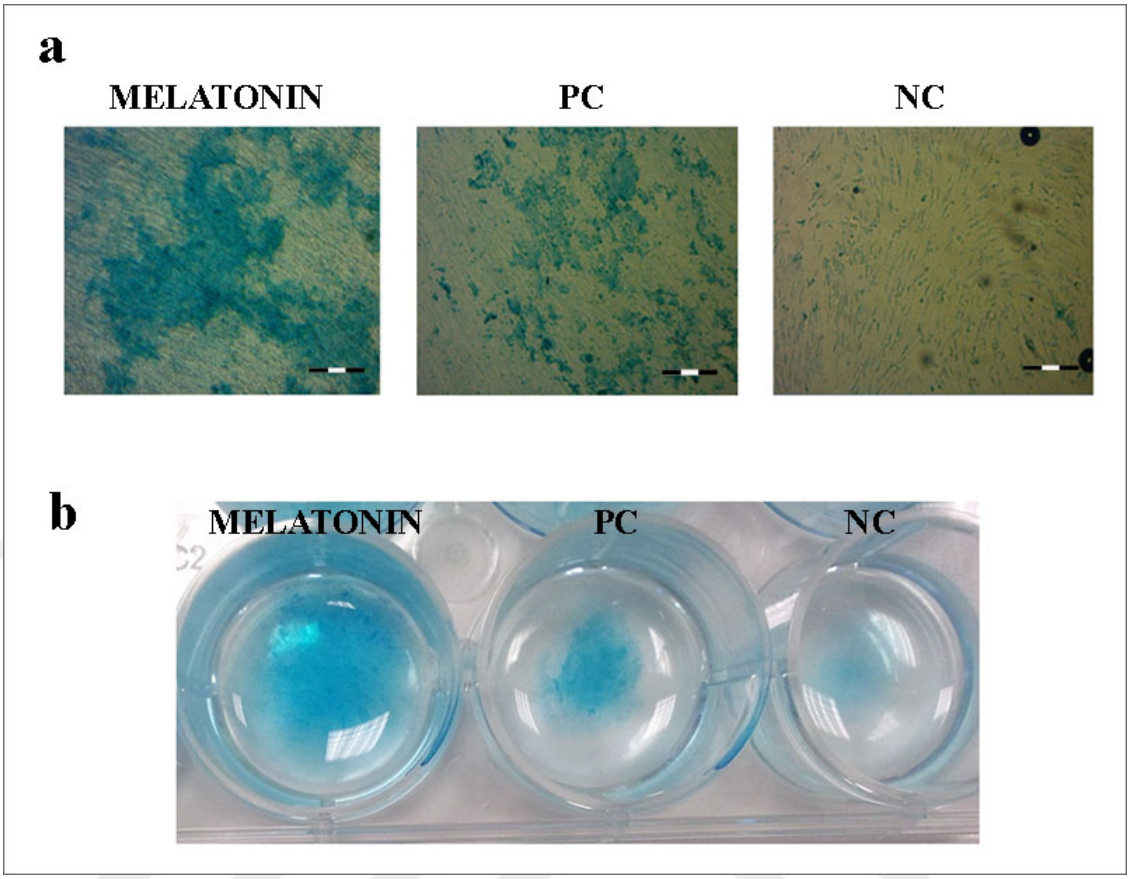

Figure 3.14. a. Alcian blue staining of melatonin and control groups b. General view of stained wells. PC: Positive control, NC: Negative control. Scale bar: 400 µm

3.7. MYOGENIC DIFFERENTIATION

3.7.1. Effect of Melatonin Treatment on the Myogenic Marker Genes

The myogenic markers were analyzed by real time PCR for their expression levels to confirm the myogenic differentiation. Myogenin and MyoD are transcription factors required during myogenesis [149]. Desmin is an intermediate filament protein found in the skeletal muscle, smooth muscle and cardiac muscle tissues [150], and ACTA2 is one of the actin isoforms, which is functional in cell motility, structure and integrity of myofibroblasts [151]. The data from real time PCR was normalized to mRNA level of GAPDH. The results indicate that all of the four marker genes were highly expressed in the melatonin groups (Figure 3.15). Melatonin raised the expression of myogenin by 2.2-fold, MyoD by 1.9-fold, desmin by 7.3-fold and ACTA2 by 1.6-fold compared to the negative

control. The expression levels in the positive control were detected as 1.8-, 1.4-, 4.5- and 1.3-fold elevated for myogenin, MyoD, desmin and ACTA2 respectively, compared to the negative control. Melatonin treatment increased myogenic differentiation compared to the cells given only myogenic differentiation medium or DMEM.

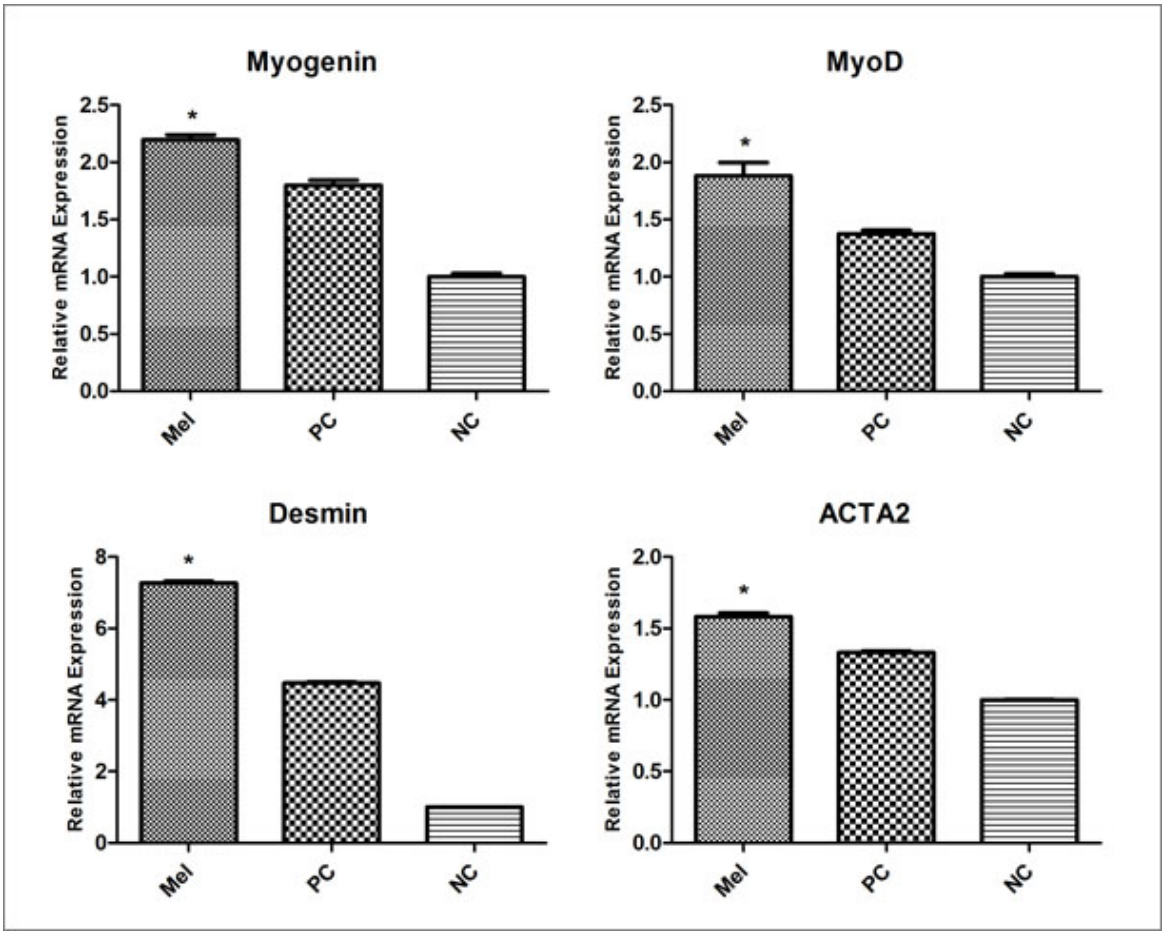

Figure 3.15. Myogenin, MyoD, desmin and ACTA2 gene expressions. Mel: Melatonin, PC: Positive control, NC: Negative control. *$P < 0.05$ versus the positive control

3.7.2. Immunocytochemical Analysis of Actin, α-SMA, Desmin and MyoD

Actin, α-SMA, desmin and MyoD were immunostained for the confirmation of myogenic differentiation of HTGSCs. α-SMA is one of the actin isoforms used as a marker for myofibroblast formation [151]. Immunocytochemistry results indicate that both melatonin group and positive control were positively stained for actin, α-SMA, desmin and MyoD,

demonstrating the myogenic differentiation of the cells. However, the negative control showed less expression levels of these marker genes (Figure 3.16).

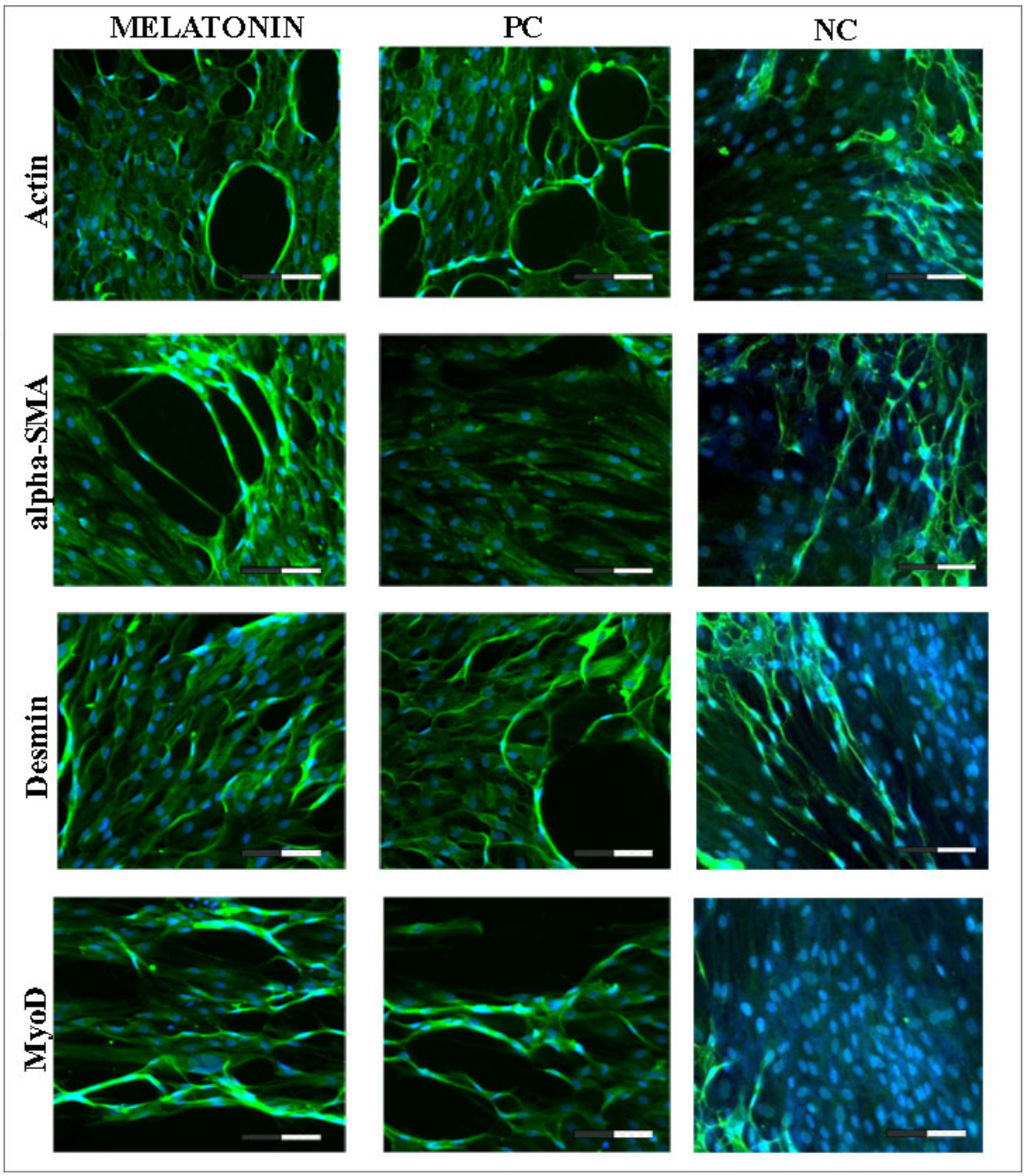

Figure 3.16. Immunostaining of actin, α-SMA, desmin and MyoD. PC: Positive control, NC: Negative control. Scale bar: 100 µm

3.8. ODONTOGENIC DIFFERENTIATION

3.8.1. Effect of Melatonin Treatment in Odontogenic Marker Genes

For odontogenic differentiation, DSPP, COL1A and BMP-2 were chosen as marker genes and mRNA expression levels were detected by real time PCR [50, 152]. Melatonin up-regulated the mRNA expression of DSPP by 1.6-fold, COL1A by 2-fold and BMP-2 by 2.2-fold compared to the positive control (Figure 3.17).

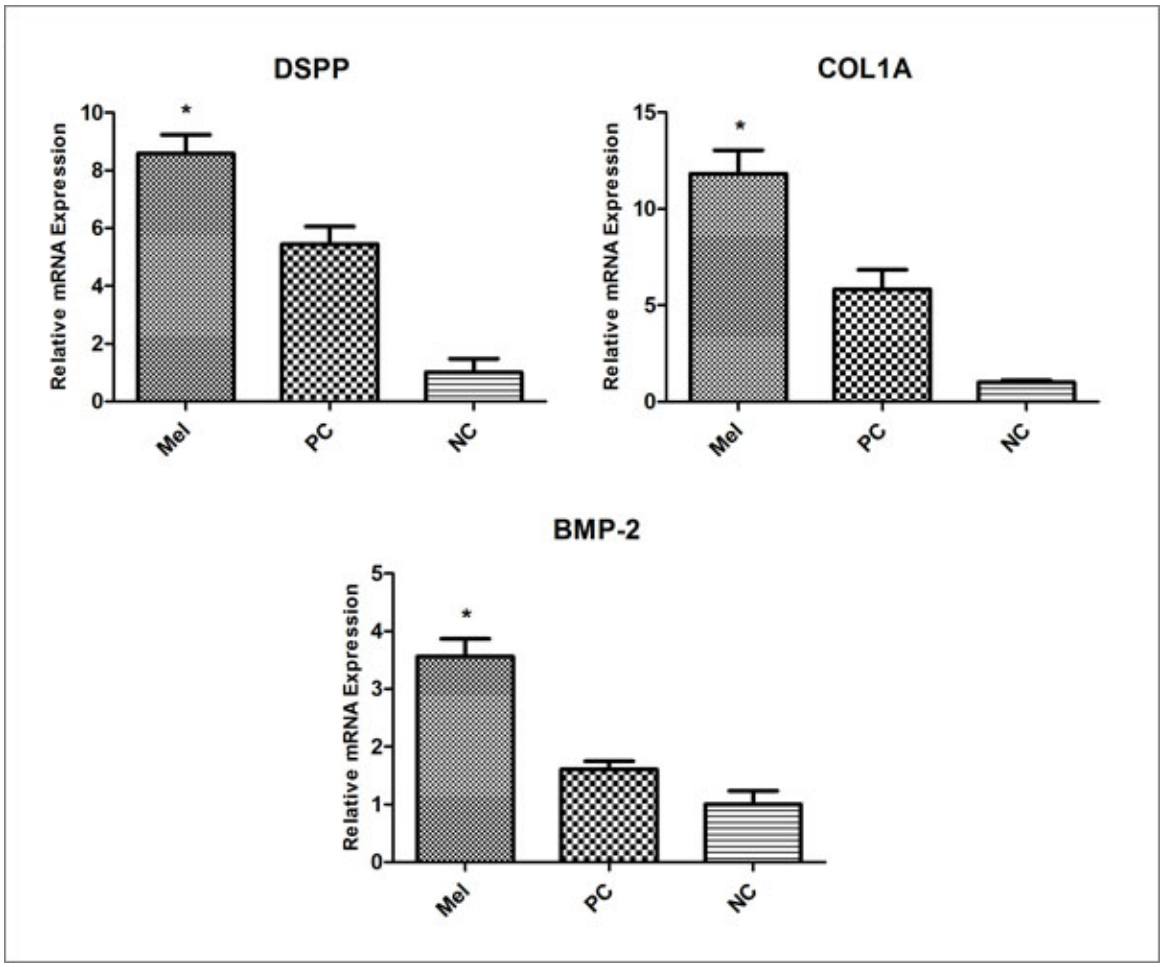

Figure 3.17. DSPP, COL1A and BMP-2 expressions in melatonin and control groups. Mel: Melatonin, PC: Positive control, NC: Negative control. *$P < 0.05$ versus the positive control

3.8.2. Immunocytochemical Analysis of COL1A and DSP

COL1A and DSP were checked in immunocytochemistry to confirm the odontogenic differentiation. DSP is a extracellular matrix protein specific to the dentin tissue [153]. Melatonin group and positive control showed positive results for COL1A and DSP whereas the negative control did not exhibit any expressions for these marker genes (Figure 3.18).

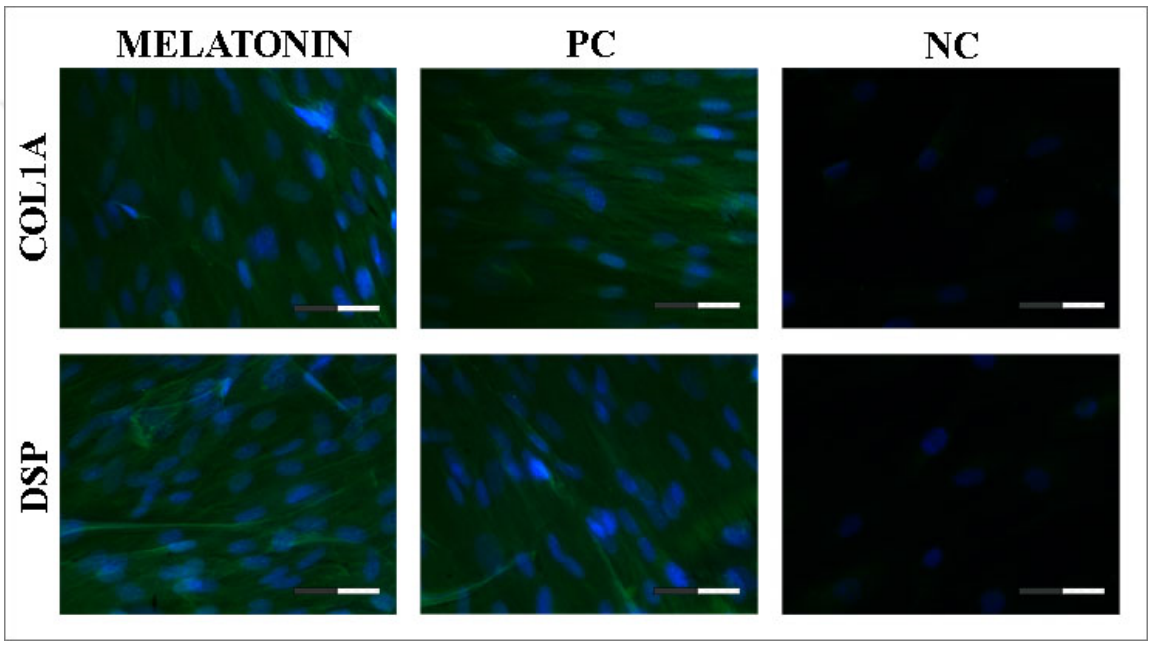

Figure 3.18. COL1A and DSP immunocytochemistry results. PC: Positive control, NC: Negative control. Scale bar: 100 µm

3.8.3. von Kossa Staining

von Kossa staining method was performed to visualize the calcium deposits after odontogenic differentiation. Differentiated cells treated with melatonin as well as the positive control were positively stained whereas the negative control did not give any positive result (Figure 3.19).

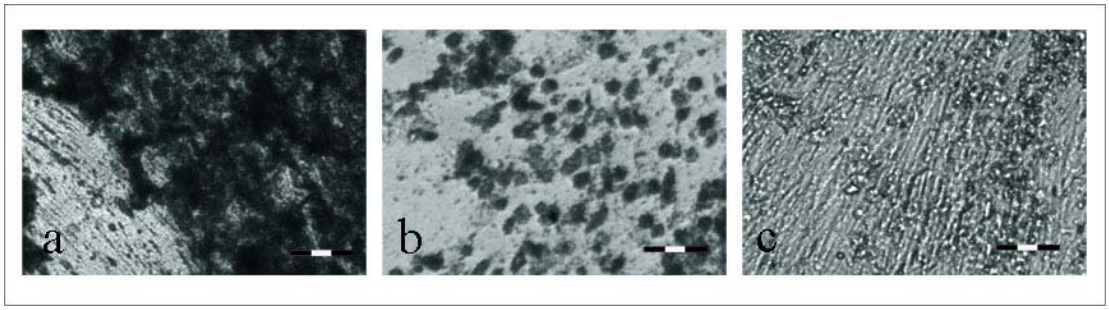

Figure 3.19. von Kossa staining of a. melatonin, b. positive control and c. negative control. Scale bar: 400 μm

4. DISCUSSION

The term “regenerative medicine” is first used by William A. Haseltine in 2000 to describe biomedical approaches involving the replacement or regeneration of cells, tissues or organs to establish normal functions of the damaged ones in the body [154]. Some of the tissues in mammals such as bone marrow, muscle and epithelia go through regeneration to maintain tissue integrity after cell loss in their normal turnover or after an injury. For instance, epithelial tissue can repair and regenerate damaged area throughout life. However, other tissues like cartilage and bone have limited capacity of regeneration while neural and myocardium tissues exert almost no regenerative ability to confer respective tissues. Moreover, organ regeneration is only demonstrated in deer antler among mammals [155]. Because of this limited regenerative capacity of human body, regenerative therapies are very popular and promising for replacing damaged tissues in several conditions such as trauma, burns, inflammations, injuries as well as in many diseases including diabetes, cardiovascular diseases and neurodegenerative disorders.

Stem cells are a good candidate for the regenerative medicine because of their great proliferation and differentiation capacity [156]. The source of stem cell is of great importance, but also one of the major issues to be addressed since adequate number of stem cell is required for therapeutic usage. The number of stem cells in the human body is limited, but numerous studies report that there are some tissues such as bone marrow and dental tissues from which stem cells can be isolated [157]. However, isolation from other sources like nervous system is not likely to be feasible. The ideal concept is to find a source of stem cell at sufficient number and with high proliferation and differentiation ability as well as easy to isolate from the body without any risk for patient and long culture procedure.

All of the requirements above led scientists to search for stem cell sources harboring stem cells with proliferation, self-renewal and differentiation capability. Use of embryonic stem cells are limited due to the ethical concerns in spite of their huge pluripotency and differentiation ability. Instead, adult stem cells have gained interest for stem cell based therapies. Adult stem cells are a good choice since there are several sources in human body. Researchers have characterized various tissues possessing stem cells with the mesenchymal stem cell properties ever since bone marrow was identified as the first

mesenchymal stem cell source [34, 158-161]. Bone marrow mesenchymal stem cells can be easily isolated and cultured *in vitro*. The capacity of self-renewing and differentiation into cells originated from cells with mesenchymal properties rendered them a good candidate for the regenerative therapy [162]. However, surgical procedures are required for the isolation of these cells, therefore it has the potential risks of contamination. The isolation procedure is traumatic and the number of cells obtained is limited. Moreover, bone marrow related disorders are another problem for this procedure [163]. These issues associated with bone marrow mesenchymal stem cells led researchers to seek new adult stem cell sources and isolation methods. Various tissues including skeletal muscle, lung, adipose tissue and dental tissues are demonstrated to have stem cells with the mesenchymal stem cell properties [164].

Dental tissue seems to be a good candidate as an alternative stem cell source. Dental stem cells (DSCs) among others are more easily isolated with simple surgical access and cultivated. They are also mostly waste materials of dental procedures. Furthermore, they do not cause any ethical controversies. Low morbidity in the collecting site after surgical attempt is another advantage of DSCs [165]. DSCs were first isolated by Gronthos *et al.* from pulp tissue and termed as dental pulp stem cells (DPSCs) [47]. Since then, different types of dental stem cells have been identified and characterized from different teeth and different parts of teeth such as exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), dental follicle precursor cells (DFPCs) and stem cells from apical papilla (SCAP) [43, 53, 58, 166]. DSCs were proven to be converted to different lineages: osteogenic, odontogenic, adipogenic and neurogenic cells and were used in some pre-clinical applications [167].

In the current study, we used human tooth germ stem cells obtained from the human third molars (wisdom teeth), which show mesenchymal stem cell characteristics and tested the effect of melatonin on differentiation of these cells into osteogenic, neurogenic, adipogenic, chondrogenic, myogenic and odontogenic cells.

Melatonin is a neurohormone mainly produced by pineal gland and released to the blood stream. It is involved in several physiological functions in the human body. It is part of the system which regulates circadian rhythm and acts as a mediator by transferring the signals from suprachiasmatic nuclei clock to target tissue and organs [75]. It also stimulates immune system by increasing cytokine production and promoting natural killer cell activity [84-85]. There are many publications which reported that melatonin is also a

scavenger of free radicals such as hydrogen peroxide, superoxide anion, nitric oxide and hydroxyl radical [90-94].

Beside all of the functions of melatonin mentioned above, it was demonstrated that melatonin regulates the differentiation efficiency of mesenchymal stem cells. It is showed that melatonin increases osteogenic differentiation in human and mouse mesenchymal stem cells [104-105], stimulates neurogenesis in mice [111] whereas it suppresses the adipogenic differentiation of human mesenchymal stem cells [105].

Before all of the differentiation experiments, toxicity assay was performed to determine which concentration of melatonin to treat cells with. 50, 100 and 150 nM of melatonin was tested to measure its effect on survival of HTGSCs for 3 days. None of the concentrations exhibited toxicity for HTGSCs. We found that all of the three concentrations significantly increased the survival rate of cells apart from 100 nM of melatonin on the 3rd day. In previous studies, melatonin was used at various concentrations ranging from 10 nM to 100 μM and it is found that melatonin shows its effects in a dose dependent manner. For instance, it was demonstrated to increase osteogenesis and decrease adipogenesis as the concentration increases [105]. We chose 100 nM of melatonin to use in the differentiation experiments since it was also used at the concentrations of 50 nM or 100 nM in the differentiation studies [105, 168].

Melatonin was used in the osteogenic differentiation process to see whether it affects the differentiation capacity of HTGSCs. In order to confirm osteogenic differentiation of HTGSCs based on the data of osteocalcin, ALP and COL1A mRNA levels by real time PCR and COL1A and osteocalcin immunostaining was performed. Furthermore, von Kossa staining and ALP activity assays were performed. All the data from these experiments demonstrate that osteogenic differentiation of HTGSCs was increased by melatonin. This result shows similarity to a previous study in which melatonin raises the osteogenic differentiation ability of human mesenchymal stem cells [105].

Neurogenic differentiation of HTGSCs were confirmed by the real time PCR analysis for neurogenic markers CD133, nestin and MAP2 as well as TH, enolase, nestin and MAP2 immunostaining. Cells treated with melatonin during the differentiation were shown to express CD133, nestin and MAP2 higher than positive control, indicating that melatonin increased the conversion of HTGSCs into neurogenic commitment. Immunocytochemistry results of different neurogenic markers such as TH, enolase, nestin and MAP2 also gave positive results for the group treated with melatonin. The results from real time PCR and

immunocytochemistry demonstrate that melatonin treatment raised this conversion into neurons of HTGSCs. Melatonin was previously proved to up-regulate the expression of neurogenic markers such as NeuroD1 [169]. In another study, it was demonstrated that melatonin increased neurogenic differentiation of neural stem cells when it was treated during proliferation period although it decreased the neurogenic differentiation in case of the treatment during the differentiation period [112]. In contrast to this result, we showed that the neurogenic commitment of HTGSCs were raised by melatonin when it was treated during the differentiation process.

Adipogenic differentiation of HTGSCs was confirmed by the detection of mRNA levels of adiponectin, LPL, αP2 and PPAR-γ as well as αP2 and PPAR-γ immunostaining. According to the real time PCR results, positive control shows high expression levels for all of the selected marker genes indicating that adipogenic conversion was successfully done. However, cells treated with melatonin exhibited quite lower mRNA levels of the marker genes compared to the positive control. Moreover, αP2 and PPAR-γ mRNA levels were even less expressed in melatonin group than negative control. Oil red oil staining was also performed to dye and observe lipids and neutral triglycerides produced by the cells as another indicator for adipogenesis. Lipid formation was decreased by melatonin in the melatonin group compared to the positive control. All these results including immunocytochemistry prove that melatonin reduces adipogenic differentiation of HTGSCs. These results are similar to a previous study in which the adipogenic conversion of mesenchymal stem cells were inhibited by melatonin treatment [105]. It was also demonstrated that this inhibition of adipogenic commitment is done through the suppression of PPAR-γ. Similarly, we also found that PPAR-γ was down-regulated at the end of the adipogenic differentiation of HTGSCs.

Chondrogenic differentiation was confirmed by real time PCR analysis of Sox 9, aggrecan and COL2A as well as immunocytochemistry for COL2A. mRNA levels of the genes were found to be expressed much higher in melatonin groups than in the positive control. Importantly, the expression of COL2A, the basic component of articular and hyaline cartilage, was approximately 7-fold higher than the positive control, which is the most increased gene among the others. Real time PCR results show similarity with a previous study in which expressions of these marker genes were up-regulated by melatonin [116]. COL2A was also positively immunostained in melatonin and positive control groups. Alcian blue staining was also performed. Although all groups were positively stained in

this study, staining cells with treated melatonin were much higher than the control groups. All these data prove that melatonin also increases the chondrogenic differentiation of HTGSCs.

Melatonin was also tested for its effect on the myogenic differentiation of HTGSCs. The expression levels of myogenin, MyoD, desmin and ACTA2, which are all myogenic markers, were detected by real time PCR to confirm myogenic differentiation. Immunostaining for desmin, actin, α-SMA and MyoD was also performed for confirmation. Immunocytochemistry results show the expression of those genes in positive control as well as in melatonin groups. Besides, real time PCR analysis proves increased myogenic commitment of HTGSCs driven by melatonin. Although melatonin is discovered to enhance the survival of myocytes, it is still unclear whether it has a potential effect on the myogenic differentiation of mesenchymal stem cells [119-120]. In this study, we show that it raises the myogenic conversion of HTGSCs which have mesenchymal stem cell properties.

Finally, odontogenic differentiation was determined by measuring mRNA levels of DSPP, COL1A and BMP-2 by real time PCR analysis, immunostaining of COL1A and DSP and von Kossa staining to observe calcium depositions. Expressions in real time PCR were found augmented in the melatonin groups in comparison to the positive and negative controls. Melatonin treated cells and positive control group were positively immunostained for the proteins mentioned above. Furthermore, von Kossa staining showed calcium deposits in the melatonin group and positive control. It was concluded that melatonin also raised odontogenic efficiency of HTGSCs. For the first time, melatonin's effect on odontogenic differentiation was shown in this study.

In conclusion, melatonin increased osteogenic, neurogenic, chondrogenic, myogenic and odontogenic differentiation of HTGSCs whereas it inhibits adipogenic differentiation. The osteogenic and adipogenic differentiation of mesenchymal stem cells are depended on certain lineage-specific transcription factors [170-171]. These factors determine whether cells will enter bone or fat cell lineage. PPAR-γ and CCAAT/enhancer-binding protein (C/EBP) family regulate adipogenic differentiation while Runx2 and osterix regulate osteogenic differentiation. The relationship between PPAR-γ and Runx2 is crucial for leading mesenchymal stem cells to osteogenic or adipogenic commitment [172]. It has been discovered that the high expression of PPAR-γ decreases the expression of Runx2 while low expression of PPAR-γ decreases adipogenesis and increase osteogenesis [173-

175]. Our results for osteogenic and adipogenic differentiation are similar to a previous study of which osteogenic differentiation of human mesenchymal stem cells were increased whereas adipogenic differentiation capacity were decreased by melatonin by enhancement of Runx2 and down-regulation PPAR-γ expression respectively [105]. In our study, it was found that PPAR-γ was down-regulated in the treatment of melatonin. However, Runx2 expression in differentiated HTGSCs is also needed to be investigated to better understand whether there is the same regulation system between Runx2 and PPAR-γ. The relationship between osteogenic and adipogenic determination is especially important for osteoporosis in which bone formation is accompanied by an increased adipogenesis in bone marrow. According to our results, melatonin might be a good candidate as a drug for preventing osteoporosis.

Beside its various roles in human body, the use of melatonin increases the possibility of success in regenerative therapy applications since it increases the survival of cells as well as supports the differentiation of them. Moreover, HTGSCs are a good stem cell source for a number of reasons such as having mesenchymal stem cell properties, being a waste material with no contamination risk, causing no ethical issues and having an easy isolation procedure. In this study, we found out that melatonin increases the differentiation abilities of HTGSCs. In the future, it might be possible to use melatonin as a drug due to its promoting effects on stem cells for the treatment of many disorders involving cell or tissue damage such as neurodegenerative diseases, degenerative muscle diseases, diabetes and osteoporosis.

REFERENCES

1. Blau, H. M., T. R. Brazelton, and J. M. Weimann, “The evolving concept of a stem cell: entity or function?”, *Cell*, Vol. 105, no. 7, pp. 829–41, 2001.
2. Bongso, A. and E. Lee, "Stem Cells: Their Definition, Classification and Sources." in Bongso, A. and E. Lee (eds.), *Stem Cells: From Bench to Bedside,* pp. 1–13, World Scientific Publishing, Singapore, 2005.
3. Klimanskaya, I., Y. Chung, L. Meisner, J. Johnson, M. D. West, and R. Lanza, “Human embryonic stem cells derived without feeder cells.”, *Lancet*, Vol. 365, no. 9471, pp. 1636–41, 2005.
4. Krtolica, A., “Stem cell: balancing aging and cancer.”, *The International Journal of Biochemistry & Cell Biology*, Vol. 37, no. 5, pp. 935–41, 2005.
5. Werbowetski-Ogilvie, T. E., M. Bossé, M. Stewart, A. Schnerch, V. Ramos-Mejia, A. Rouleau, T. Wynder, M. J. Smith, S. Dingwall, T. Carter, C. Williams, C. Harris, J. Dolling, C. Wynder, D. Boreham, and M. Bhatia, “Characterization of human embryonic stem cells with features of neoplastic progression.”, *Nature Biotechnology*, Vol. 27, no. 1, pp. 91–7, 2009.
6. Shamblott, M. J., J. Axelman, S. Wang, E. M. Bugg, J. W. Littlefield, P. J. Donovan, P. D. Blumenthal, G. R. Huggins, and J. D. Gearhart, “Derivation of pluripotent stem cells from cultured human primordial germ cells.”, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 95, no. 23, pp. 13726–31, 1998.
7. Kang, X. Q., W. J. Zang, L. J. Bao, D. L. Li, X. L. Xu, and X. J. Yu, “Differentiating characterization of human umbilical cord blood-derived mesenchymal stem cells in vitro.”, *Cell Biology International*, Vol. 30, no. 7, pp. 569–75, 2006.
8. Panepucci, R. A., J. L. C. Siufi, W. A. Silva, R. Proto-Siquiera, L. Neder, M. Orellana, V. Rocha, D. T. Covas, and M. A. Zago, “Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells.”, *Stem Cells*, Vol. 22, no. 7, pp. 1263–78, 2004.
9. Jeong, J. A., S. H. Hong, E. J. Gang, C. Ahn, S. H. Hwang, I. H. Yang, H. Han, and H. Kim, “Differential gene expression profiling of human umbilical cord blood-

derived mesenchymal stem cells by DNA microarray.", *Stem Cells*, Vol. 23, no. 4, pp. 584–93, 2005.

10. Biotron, "Stem Cell and Cell Therapy," [online], http://www.findingtruthmatters.org/articles/stem-cell-debate/images/embryo-development.jpg [retrieved 15 August 2013].
11. Lagasse, E., H. Connors, M. Al-Dhalimy, M. Reitsma, M. Dohse, L. Osborne, X. Wang, M. Finegold, I. L. Weissman, and M. Grompe, "Purified hematopoietic stem cells can differentiate into hepatocytes in vivo.", *Nature Medicine*, Vol. 6, no. 11, pp. 1229–34, 2000.
12. Eglitis, M. A. and E. Mezey, "Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 94, no. 8, pp. 4080–5, 1997.
13. Theise, N. D., M. Nimmakayalu, R. Gardner, P. B. Illei, G. Morgan, L. Teperman, O. Henegariu, and D. S. Krause, "Liver from bone marrow in humans.", *Hepatology*, Vol. 32, no. 1, pp. 11–6, 2000.
14. Krause, D. S., "Plasticity of marrow-derived stem cells.", *Gene Therapy*, Vol. 9, no. 11, pp. 754–8, 2002.
15. Ferrari, G., G. Cusella-De Angelis, M. Coletta, E. Paolucci, A. Stornaiuolo, G. Cossu, and F. Mavilio, "Muscle regeneration by bone marrow-derived myogenic progenitors.", *Science*, Vol. 279, no. 5356, pp. 1528–30, 1998.
16. Hess, D. C., W. D. Hill, A. Martin-Studdard, J. Carroll, J. Brailer, and J. Carothers, "Bone marrow as a source of endothelial cells and NeuN-expressing cells After stroke.", *Stroke*, Vol. 33, no. 5, pp. 1362–8, 2002.
17. Orlic, D., J. Kajstura, S. Chimenti, F. Limana, I. Jakoniuk, F. Quaini, B. Nadal-Ginard, D. M. Bodine, A. Leri, and P. Anversa, "Mobilized bone marrow cells repair the infarcted heart, improving function and survival.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 98, no. 18, pp. 10344–9, 2001.
18. Zuk, P. A., M. Zhu, H. Mizuno, J. Huang, J. W. Futrell, A. J. Katz, P. Benhaim, H. P. Lorenz, and M. H. Hedrick, "Multilineage cells from human adipose tissue: implications for cell-based therapies.", *Tissue Engineering*, Vol. 7, no. 2, pp. 211–28, 2001.

19. Guilak, F., K. E. Lott, H. A. Awad, Q. Cao, K. C. Hicok, B. Fermor, and J. M. Gimble, “Clonal analysis of the differentiation potential of human adipose-derived adult stem cells.”, *Journal of Cellular Physiology*, Vol. 206, no. 1, pp. 229–37, 2006.
20. Rafii, S., “Circulating endothelial precursors: mystery, reality, and promise.”, *The Journal of Clinical Investigation*, Vol. 105, no. 1, pp. 17–9, 2000.
21. Gage, F. H., “Mammalian neural stem cells.”, *Science*, Vol. 287, no. 5457, pp. 1433–8, 2000.
22. Umar, S., “Intestinal stem cells.”, *Current Gastroenterology Reports*, Vol. 12, no. 5, pp. 340–8, 2010.
23. Da Silva Meirelles, L., P. C. Chagastelles, and N. B. Nardi, “Mesenchymal stem cells reside in virtually all post-natal organs and tissues.”, *Journal of Cell Science*, Vol. 119, no. Pt 11, pp. 2204–13, 2006.
24. Friedenstein, A. J., R. K. Chailakhjan, and K. S. Lalykina, “The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells.”, *Cell and Tissue Kinetics*, Vol. 3, no. 4, pp. 393–403, 1970.
25. Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, and E. Horwitz, “Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.”, *Cytotherapy*, Vol. 8, no. 4, pp. 315–7, 2006.
26. Bruder, S. P., N. Jaiswal, and S. E. Haynesworth, “Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation.”, *Journal of Cellular Biochemistry*, Vol. 64, no. 2, pp. 278–94, 1997.
27. Bruder, S. P., A. A. Kurth, M. Shea, W. C. Hayes, N. Jaiswal, and S. Kadiyala, “Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells.”, *Journal of Orthopaedic Research*, Vol. 16, no. 2, pp. 155–62, 1998.
28. Ferrari, G., “Muscle Regeneration by Bone Marrow-Derived Myogenic Progenitors”, *Science*, Vol. 279, no. 5356, pp. 1528–1530, 1998.
29. Galmiche, M. C., V. E. Koteliansky, J. Brière, P. Hervé, and P. Charbord, “Stromal cells from human long-term marrow cultures are mesenchymal cells that

differentiate following a vascular smooth muscle differentiation pathway.", *Blood*, Vol. 82, no. 1, pp. 66–76, 1993.

30. Young, R. G., D. L. Butler, W. Weber, A. I. Caplan, S. L. Gordon, and D. J. Fink, "Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair.", *Journal of Orthopaedic Research*, Vol. 16, no. 4, pp. 406–13, 1998.
31. Awad, H. A., D. L. Butler, G. P. Boivin, F. N. Smith, P. Malaviya, B. Huibregtse, and A. I. Caplan, "Autologous mesenchymal stem cell-mediated repair of tendon.", *Tissue Engineering*, Vol. 5, no. 3, pp. 267–77, 1999.
32. Kadiyala, S., R. G. Young, M. A. Thiede, and S. P. Bruder, "Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro.", *Cell Transplantation*, Vol. 6, no. 2, pp. 125–34, 1997.
33. Dennis, J. E., A. Merriam, A. Awadallah, J. U. Yoo, B. Johnstone, and A. I. Caplan, "A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse.", *Journal of Bone and Mineral Research*, Vol. 14, no. 5, pp. 700–9, 1999.
34. Prockop, D. J., "Marrow Stromal Cells as Stem Cells for Nonhematopoietic Tissues", *Science*, Vol. 276, no. 5309, pp. 71–74, 1997.
35. Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, "Induction of pluripotent stem cells from adult human fibroblasts by defined factors.", *Cell*, Vol. 131, no. 5, pp. 861–72, 2007.
36. Giorgetti, A., M. C. N. Marchetto, M. Li, D. Yu, R. Fazzina, Y. Mu, A. Adamo, I. Paramonov, J. C. Cardoso, M. B. Monasterio, C. Bardy, R. Cassiani-Ingoni, G.-H. Liu, F. H. Gage, and J. C. Izpisua Belmonte, "Cord blood-derived neuronal cells by ectopic expression of Sox2 and c-Myc.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 109, no. 31, pp. 12556–61, 2012.
37. Richards, M., B. A. Huibregtse, A. I. Caplan, J. A. Goulet, and S. A. Goldstein, "Marrow-derived progenitor cell injections enhance new bone formation during distraction.", *Journal of Orthopaedic Research*, Vol. 17, no. 6, pp. 900–8, 1999.
38. Johnstone, B. and J. U. Yoo, "Autologous mesenchymal progenitor cells in articular cartilage repair.", *Clinical Orthopaedics and Related Research*, no. 367 Suppl, pp. S156–62, 1999.

39. Baksh, D., L. Song, and R. S. Tuan, “Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy.”, *Journal of Cellular and Molecular Medicine*, Vol. 8, no. 3, pp. 301–16, 2004.
40. Doi, M., A. Nagano, and Y. Nakamura, “Molecular cloning and characterization of a novel gene, EMILIN-5, and its possible involvement in skeletal development”, *Biochemical and Biophysical Research Communications*, Vol. 313, no. 4, pp. 888–893, 2004.
41. Qi, H., D. J. Aguiar, S. M. Williams, A. La Pean, W. Pan, and C. M. Verfaillie, “Identification of genes responsible for osteoblast differentiation from human mesodermal progenitor cells.”, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 100, no. 6, pp. 3305–10, 2003.
42. Mezey, E., K. J. Chandross, G. Harta, R. A. Maki, and S. R. McKercher, “Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow.”, *Science*, Vol. 290, no. 5497, pp. 1779–82, 2000.
43. Seo, B. M., M. Miura, S. Gronthos, P. M. Bartold, S. Batouli, J. Brahim, M. Young, P. G. Robey, C. Y. Wang, and S. Shi, “Investigation of multipotent postnatal stem cells from human periodontal ligament.”, *Lancet*, Vol. 364, no. 9429, pp. 149–55, 2004.
44. Miura, M., X. Chen, M. R. Allen, Y. Bi, S. Gronthos, B. Seo, S. Lakhani, R. A. Flavell, X. Feng, P. G. Robey, M. Young, and S. Shi, “A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells.”, *The Journal of Clinical Investigation*, Vol. 114, no. 12, pp. 1704–13, 2004.
45. Shi, S., P. M. Bartold, M. Miura, B. M. Seo, P. G. Robey, and S. Gronthos, “The efficacy of mesenchymal stem cells to regenerate and repair dental structures.”, *Orthodontics & Craniofacial Research*, Vol. 8, no. 3, pp. 191–9, 2005.
46. Sinanan, A. C. M., N. P. Hunt, and M. P. Lewis, “Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells.”, *Biotechnology and Applied Biochemistry*, Vol. 40, no. Pt 1, pp. 25–34, 2004.
47. Gronthos, S., M. Mankani, J. Brahim, P. G. Robey, and S. Shi, “Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo.”, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 97, no. 25, pp. 13625–30, 2000.

48. Yang, X., W. Zhang, J. van den Dolder, X. F. Walboomers, Z. Bian, M. Fan, and J. A. Jansen, “Multilineage potential of STRO-1+ rat dental pulp cells in vitro.”, *Journal of Tissue Engineering and Regenerative Medicine*, Vol. 1, no. 2, pp. 128–35, 2007.
49. Gronthos, S., J. Brahim, W. Li, L. W. Fisher, N. Cherman, A. Boyde, P. DenBesten, P. G. Robey, and S. Shi, “Stem cell properties of human dental pulp stem cells”, *Journal of Dental Research*, Vol. 81, no. 8, pp. 531–535, 2002.
50. Zhang, W., X. F. Walboomers, S. Shi, M. Fan, and J. A. Jansen, “Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation.”, *Tissue Engineering*, Vol. 12, no. 10, pp. 2813–23, 2006.
51. Tsukamoto, Y., S. Fukutani, T. Shin-Ike, T. Kubota, S. Sato, Y. Suzuki, and M. Mori, “Mineralized nodule formation by cultures of human dental pulp-derived fibroblasts.”, *Archives of Oral Biology*, Vol. 37, no. 12, pp. 1045–55, 1992.
52. Kerkis, I., A. Kerkis, D. Dozortsev, G. C. Stukart-Parsons, S. M. Gomes Massironi, L. V Pereira, A. I. Caplan, and H. F. Cerruti, “Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers.”, *Cells, Tissues, Organs*, Vol. 184, no. 3–4, pp. 105–16, 2006.
53. Morsczeck, C., W. Götz, J. Schierholz, F. Zeilhofer, U. Kühn, C. Möhl, C. Sippel, and K. H. Hoffmann, “Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth.”, *Matrix Biology*, Vol. 24, no. 2, pp. 155–65, 2005.
54. Wise, G. E., S. Frazier-Bowers, and R. N. D’Souza, “Cellular, molecular, and genetic determinants of tooth eruption.”, *Critical Reviews in Oral Biology and Medicine*, Vol. 13, no. 4, pp. 323–34, 2002.
55. Isaka, J., A. Ohazama, M. Kobayashi, C. Nagashima, T. Takiguchi, H. Kawasaki, T. Tachikawa, and K. Hasegawa, “Participation of periodontal ligament cells with regeneration of alveolar bone.”, *Journal of Periodontology*, Vol. 72, no. 3, pp. 314–23, 2001.
56. McCulloch, C. A. and S. Bordin, “Role of fibroblast subpopulations in periodontal physiology and pathology.”, *Journal of Periodontal Research*, Vol. 26, no. 3 Pt 1, pp. 144–54, 1991.

57. Sonoyama, W., Y. Liu, D. Fang, T. Yamaza, B. M. Seo, C. Zhang, H. Liu, S. Gronthos, C. Y. Wang, S. Wang, and S. Shi, "Mesenchymal stem cell-mediated functional tooth regeneration in swine.", *PLoS One*, Vol. 1, no. 1, p. e79, 2006.
58. Sonoyama, W., Y. Liu, T. Yamaza, R. S. Tuan, S. Wang, S. Shi, and G. T. J. Huang, "Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study.", *Journal of Endodontics*, Vol. 34, no. 2, pp. 166–71, 2008.
59. Abe, S., S. Yamaguchi, and T. Amagasa, "Multilineage Cells from Apical Pulp of Human Tooth with Immature Apex", *Oral Science International*, Vol. 4, no. 1, pp. 45–58, 2007.
60. Liu, H., W. Li, S. Shi, S. Habelitz, C. Gao, and P. Denbesten, "MEPE is downregulated as dental pulp stem cells differentiate.", *Archives of Oral Biology*, Vol. 50, no. 11, pp. 923–8, 2005.
61. Rodríguez-Lozano, F. J., C. L. Insausti, F. Iniesta, M. Blanquer, M. C. Ramírez, L. Meseguer, A. B. Meseguer-Henarejos, N. Marín, S. Martínez, and J. M. Moraleda, "Mesenchymal dental stem cells in regenerative dentistry.", *Medicina Oral, Patología Oral y Cirugía Bucal*, Vol. 17, no. 6, pp. e1062–7, 2012.
62. Ikeda, E., K. Yagi, M. Kojima, T. Yagyuu, A. Ohshima, S. Sobajima, M. Tadokoro, Y. Katsube, K. Isoda, M. Kondoh, M. Kawase, M. J. Go, H. Adachi, Y. Yokota, T. Kirita, and H. Ohgushi, "Multipotent cells from the human third molar: feasibility of cell-based therapy for liver disease.", *Differentiation; Research in Biological Diversity*, Vol. 76, no. 5, pp. 495–505, 2008.
63. Bubenik, G. A., "Thirty four years since the discovery of gastrointestinal melatonin.", *Journal of Physiology and Pharmacology*, Vol. 59 Suppl 2, no. 1, pp. 33–51, 2008.
64. Lundmark, P. O., S. R. Pandi-Perumal, V. Srinivasan, and D. P. Cardinali, "Role of melatonin in the eye and ocular dysfunctions.", *Visual Neuroscience*, Vol. 23, no. 6, pp. 853–62, 2006.
65. Slominski, A., T. W. Fischer, M. A. Zmijewski, J. Wortsman, I. Semak, B. Zbytek, R. M. Slominski, and D. J. Tobin, "On the role of melatonin in skin physiology and pathology.", *Endocrine*, Vol. 27, no. 2, pp. 137–48, 2005.
66. Bubenik, G. A., "Gastrointestinal melatonin: localization, function, and clinical relevance.", *Digestive Diseases and Sciences*, Vol. 47, no. 10, pp. 2336–48, 2002.

67. Konturek, S. J., P. C. Konturek, I. Brzozowska, M. Pawlik, Z. Sliwowski, M. Cześnikiewicz-Guzik, S. Kwiecień, T. Brzozowski, G. A. Bubenik, and W. W. Pawlik, "Localization and biological activities of melatonin in intact and diseased gastrointestinal tract (GIT).", *Journal of Physiology and Pharmacology*, Vol. 58, no. 3, pp. 381–405, 2007.

68. Carrillo-Vico, A., J. R. Calvo, P. Abreu, P. J. Lardone, S. García-Mauriño, R. J. Reiter, and J. M. Guerrero, "Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance.", *FASEB Journal,* Vol. 18, no. 3, pp. 537–9, 2004.

69. Naranjo, M. C., J. M. Guerrero, A. Rubio, P. J. Lardone, A. Carrillo-Vico, M. P. Carrascosa-Salmoral, S. Jiménez-Jorge, M. V Arellano, S. R. Leal-Noval, M. Leal, E. Lissen, and P. Molinero, "Melatonin biosynthesis in the thymus of humans and rats.", *Cellular and Molecular Life Sciences*, Vol. 64, no. 6, pp. 781–90, 2007.

70. Reiter, R., L. Tang, J. J. Garcia, and A. Muñoz-Hoyos, "Pharmacological actions of melatonin in oxygen radical pathophysiology.", *Life Sciences*, Vol. 60, no. 25, pp. 2255–71, 1997.

71. Reppert, S. M., D. R. Weaver, and T. Ebisawa, "Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses.", *Neuron*, Vol. 13, no. 5, pp. 1177–85, 1994.

72. Reppert, S. M., C. Godson, C. D. Mahle, D. R. Weaver, S. A. Slaugenhaupt, and J. F. Gusella, "Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 92, no. 19, pp. 8734–8, 1995.

73. Reppert, S. M., D. R. Weaver, and C. Godson, "Melatonin receptors step into the light: cloning and classification of subtypes.", *Trends in Pharmacological Sciences*, Vol. 17, no. 3, pp. 100–2, 1996.

74. Hardeland, R., J. A. Madrid, D.-X. Tan, and R. J. Reiter, "Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling.", *Journal of Pineal Research*, Vol. 52, no. 2, pp. 139–66, 2012.

75. Cardinali, D. P. and P. Pévet, "Basic aspects of melatonin action.", *Sleep Medicine Reviews*, Vol. 2, no. 3, pp. 175–90, 1998.

76. Cardinali, D. P., C. A. Nagle, F. Freire, and J. M. Rosner, “Effects of melatonin on neurotransmitter uptake and release by synaptosome-rich homogenates of the rat hypothalamus.”, *Neuroendocrinology*, Vol. 18, no. 1, pp. 72–85, 1975.
77. Carneiro, R. C., O. Toffoleto, J. Cipolla-Neto, and R. P. Markus, “Modulation of sympathetic neurotransmission by melatonin.”, *European Journal of Pharmacology*, Vol. 257, no. 1–2, pp. 73–7, 1994.
78. Markus, R. P., W. M. Zago, and R. C. Carneiro, “Melatonin modulation of presynaptic nicotinic acetylcholine receptors in the rat vas deferens.”, *The Journal of Pharmacology and Experimental Therapeutics*, Vol. 279, no. 1, pp. 18–22, 1996.
79. Bucher, B., F. Gauer, P. Pévet, and M. Masson-Pévet, “Vasoconstrictor effects of various melatonin analogs on the rat tail artery in the presence of phenylephrine.”, *Journal of Cardiovascular Pharmacology*, Vol. 33, no. 2, pp. 316–22, 1999.
80. Wan, Q., H. Y. Man, F. Liu, J. Braunton, H. B. Niznik, S. F. Pang, G. M. Brown, and Y. T. Wang, “Differential modulation of GABAA receptor function by Mel1a and Mel1b receptors.”, *Nature Neuroscience*, Vol. 2, no. 5, pp. 401–3, 1999.
81. Golombek, D. A., P. Pévet, and D. P. Cardinali, “Melatonin effects on behavior: possible mediation by the central GABAergic system.”, *Neuroscience and Biobehavioral Reviews*, Vol. 20, no. 3, pp. 403–12, 1996.
82. Pandi-Perumal, S. R., N. Zisapel, V. Srinivasan, and D. P. Cardinali, “Melatonin and sleep in aging population.”, *Experimental Gerontology*, Vol. 40, no. 12, pp. 911–25, 2005.
83. Miller, S. C., S. R. Pandi-Perumal, P. S. R. Pandi, A. I. Esquifino, D. P. Cardinali, and G. J. M. Maestroni, “The role of melatonin in immuno-enhancement: potential application in cancer.”, *International Journal of Experimental Pathology*, Vol. 87, no. 2, pp. 81–7, 2006.
84. Garcia-Mauriño, S., M. G. Gonzalez-Haba, J. R. Calvo, M. Rafii-El-Idrissi, V. Sanchez-Margalet, R. Goberna, and J. M. Guerrero, “Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating CD4+ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes.”, *Journal of Immunology*, Vol. 159, no. 2, pp. 574–81, 1997.
85. Lissoni, P., F. Rovelli, F. Brivio, O. Brivio, and L. Fumagalli, “Circadian secretions of IL-2, IL-12, IL-6 and IL-10 in relation to the light/dark rhythm of the pineal

hormone melatonin in healthy humans.", *Natural Immunity*, Vol. 16, no. 1, pp. 1–5, 1998.

86. Pohanka, M., "Alzheimer's disease and related neurodegenerative disorders: implication and counteracting of melatonin", *Journal of Applied Biomedicine*, Vol. 9, no. 4, pp. 185–196, 2011.
87. Hardeland, R., "Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance.", *Endocrine*, Vol. 27, no. 2, pp. 119–30, 2005.
88. Reiter, R. J., L. C. Manchester, and D. X. Tan, "Neurotoxins: free radical mechanisms and melatonin protection.", *Current Neuropharmacology*, Vol. 8, no. 3, pp. 194–210, 2010.
89. Tan, D., L. Chen, B. Poeggeler, L. Manchester, and R. Reiter, "Melatonin: a potent, endogenous hydroxyl radical scavenger", *Endocrine Journal*, Vol. 1, pp. 57–60, 1993.
90. Bandyopadhyay, D., K. Biswas, U. Bandyopadhyay, R. J. Reiter, and R. K. Banerjee, "Melatonin protects against stress-induced gastric lesions by scavenging the hydroxyl radical.", *Journal of Pineal Research*, Vol. 29, no. 3, pp. 143–51, 2000.
91. Gitto, E., S. Pellegrino, P. Gitto, I. Barberi, and R. J. Reiter, "Oxidative stress of the newborn in the pre- and postnatal period and the clinical utility of melatonin.", *Journal of Pineal Research*, Vol. 46, no. 2, pp. 128–39, 2009.
92. León, J., D. Acuña-Castroviejo, G. Escames, D.-X. Tan, and R. J. Reiter, "Melatonin mitigates mitochondrial malfunction.," *Journal of pineal research*, vol. 38, no. 1, pp. 1–9, Jan. 2005.
93. Poeggeler, B., R. J. Reiter, D. X. Tan, L. D. Chen, and L. C. Manchester, "Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis.", *Journal of Pineal Research*, Vol. 14, no. 4, pp. 151–68, 1993.
94. Reiter, R. J., S. D. Paredes, L. C. Manchester, and D. X. Tan, "Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin.", *Critical Reviews in Biochemistry and Molecular Biology*, Vol. 44, no. 4, pp. 175–200, 2009.
95. Antolín, I., C. Rodríguez, R. M. Saínz, J. C. Mayo, H. Uría, M. L. Kotler, M. J. Rodríguez-Colunga, D. Tolivia, and A. Menéndez-Peláez, "Neurohormone

melatonin prevents cell damage: effect on gene expression for antioxidant enzymes.", *FASEB Journal*, Vol. 10, no. 8, pp. 882–90, 1996.

96. Barlow-Walden, L. R., R. J. Reiter, M. Abe, M. Pablos, A. Menendez-Pelaez, L. D. Chen, and B. Poeggeler, "Melatonin stimulates brain glutathione peroxidase activity.", *Neurochemistry International*, Vol. 26, no. 5, pp. 497–502, 1995.
97. Chang, H. M., Y. L. Huang, C. T. Lan, U. I. Wu, M. E. Hu, and S. C. Youn, "Melatonin preserves superoxide dismutase activity in hypoglossal motoneurons of adult rats following peripheral nerve injury.", *Journal of Pineal Research*, Vol. 44, no. 2, pp. 172–80, 2008.
98. Tomás-Zapico, C., A. Coto-Montes, J. Martínez-Fraga, M. J. Rodríguez-Colunga, R. Hardeland, and D. Tolivia, "Effects of delta-aminolevulinic acid and melatonin in the harderian gland of female Syrian hamsters.", *Free Radical Biology & Medicine*, Vol. 32, no. 11, pp. 1197–204, 2002.
99. Urata, Y., S. Honma, S. Goto, S. Todoroki, T. Iida, S. Cho, K. Honma, and T. Kondo, "Melatonin induces gamma-glutamylcysteine synthetase mediated by activator protein-1 in human vascular endothelial cells.", *Free Radical Biology & Medicine*, Vol. 27, no. 7–8, pp. 838–47, 1999.
100. Winiarska, K., T. Fraczyk, D. Malinska, J. Drozak, and J. Bryla, "Melatonin attenuates diabetes-induced oxidative stress in rabbits.", *Journal of Pineal Research*, Vol. 40, no. 2, pp. 168–76, 2006.
101. López, A., J. A. García, G. Escames, C. Venegas, F. Ortiz, L. C. López, and D. Acuña-Castroviejo, "Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production.", *Journal of Pineal Research*, Vol. 46, no. 2, pp. 188–98, 2009.
102. León, J., G. Escames, M. I. Rodríguez, L. C. López, V. Tapias, A. Entrena, E. Camacho, M. D. Carrión, M. A. Gallo, A. Espinosa, D.-X. Tan, R. J. Reiter, and D. Acuña-Castroviejo, "Inhibition of neuronal nitric oxide synthase activity by N1-acetyl-5-methoxykynuramine, a brain metabolite of melatonin.", *Journal of Neurochemistry*, Vol. 98, no. 6, pp. 2023–33, 2006.
103. Pozo, D., R. J. Reiter, J. R. Calvo, and J. M. Guerrero, "Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin.", *Journal of Cellular Biochemistry*, Vol. 65, no. 3, pp. 430–42, 1997.

104. Roth, J. A., B. G. Kim, W. L. Lin, and M. I. Cho, “Melatonin promotes osteoblast differentiation and bone formation.”, *The Journal of Biological Chemistry*, Vol. 274, no. 31, pp. 22041–7, 1999.

105. Zhang, L., P. Su, C. Xu, C. Chen, A. Liang, K. Du, Y. Peng, and D. Huang, “Melatonin inhibits adipogenesis and enhances osteogenesis of human mesenchymal stem cells by suppressing PPARγ expression and enhancing Runx2 expression.”, *Journal of Pineal Research*, Vol. 49, no. 4, pp. 364–72, 2010.

106. Sethi, S., N. M. Radio, M. P. Kotlarczyk, C. T. Chen, Y.-H. Wei, R. Jockers, and P. A. Witt-Enderby, “Determination of the minimal melatonin exposure required to induce osteoblast differentiation from human mesenchymal stem cells and these effects on downstream signaling pathways.”, *Journal of Pineal Research*, Vol. 49, no. 3, pp. 222–38, 2010.

107. Calvo-Guirado, J. L., G. Gómez-Moreno, L. López-Marí, J. Guardia, J. M. Marínez-González, A. Barone, I. F. Tresguerres, S. D. Paredes, and L. Fuentes-Breto, “Actions of melatonin mixed with collagenized porcine bone versus porcine bone only on osteointegration of dental implants.”, *Journal of Pineal Research*, Vol. 48, no. 3, pp. 194–203, 2010.

108. Calvo-Guirado, J. L., G. Gómez-Moreno, A. Barone, A. Cutando, M. Alcaraz-Baños, F. Chiva, L. López-Marí, and J. Guardia, “Melatonin plus porcine bone on discrete calcium deposit implant surface stimulates osteointegration in dental implants.”, *Journal of Pineal Research*, Vol. 47, no. 2, pp. 164–72, 2009.

109. Ramírez-Rodríguez, G., F. Klempin, H. Babu, G. Benítez-King, and G. Kempermann, “Melatonin modulates cell survival of new neurons in the hippocampus of adult mice.”, *Neuropsychopharmacology*, Vol. 34, no. 9, pp. 2180–91, 2009.

110. Ramirez-Rodriguez, G., L. Ortíz-López, A. Domínguez-Alonso, G. A. Benítez-King, and G. Kempermann, “Chronic treatment with melatonin stimulates dendrite maturation and complexity in adult hippocampal neurogenesis of mice.”, *Journal of Pineal Research*, Vol. 50, no. 1, pp. 29–37, 2011.

111. De Butte, M. and B. A. Pappas, “Pinealectomy causes hippocampal CA1 and CA3 cell loss: reversal by melatonin supplementation.”, *Neurobiology of Aging*, Vol. 28, no. 2, pp. 306–13, 2007.

112. Moriya, T., N. Horie, M. Mitome, and K. Shinohara, “Melatonin influences the proliferative and differentiative activity of neural stem cells.,” *Journal of Pineal Research*, Vol. 42, no. 4, pp. 411–8, 2007.
113. Kong, X., X. Li, Z. Cai, N. Yang, Y. Liu, J. Shu, L. Pan, and P. Zuo, “Melatonin regulates the viability and differentiation of rat midbrain neural stem cells.”, *Cellular and Molecular Neurobiology*, Vol. 28, no. 4, pp. 569–79, 2008.
114. Brydon, L., L. Petit, P. Delagrange, A. D. Strosberg, and R. Jockers, “Functional expression of MT2 (Mel1b) melatonin receptors in human PAZ6 adipocytes.”, *Endocrinology*, Vol. 142, no. 10, pp. 4264–71, 2001.
115. Sanchez-Hidalgo, M., Z. Lu, D. X. Tan, M. D. Maldonado, R. J. Reiter, and R. I. Gregerman, “Melatonin inhibits fatty acid-induced triglyceride accumulation in ROS17/2.8 cells: implications for osteoblast differentiation and osteoporosis.”, *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, Vol. 292, no. 6, pp. R2208–15, 2007.
116. Pei, M., F. He, L. Wei, and A. Rawson, “Melatonin enhances cartilage matrix synthesis by porcine articular chondrocytes.”, *Journal of Pineal Research*, Vol. 46, no. 2, pp. 181–7, 2009.
117. Lim, H. D., Y. S. Kim, S. H. Ko, I. J. Yoon, S. G. Cho, Y. H. Chun, B. J. Choi, and E. C. Kim, “Cytoprotective and anti-inflammatory effects of melatonin in hydrogen peroxide-stimulated CHON-001 human chondrocyte cell line and rabbit model of osteoarthritis via the SIRT1 pathway.”, *Journal of Pineal Research*, Vol. 53, no. 3, pp. 225–37, 2012.
118. Kumasaka, S., M. Shimozuma, T. Kawamoto, K. Mishima, R. Tokuyama, Y. Kamiya, P. Davaadorj, I. Saito, and K. Satomura, “Possible involvement of melatonin in tooth development: expression of melatonin 1a receptor in human and mouse tooth germs.”, *Histochemistry and Cell Biology*, Vol. 133, no. 5, pp. 577–84, 2010.
119. Xu, M. and M. Ashraf, “Melatonin protection against lethal myocyte injury induced by doxorubicin as reflected by effects on mitochondrial membrane potential.”, *Journal of Molecular and Cellular Cardiology*, Vol. 34, no. 1, pp. 75–9, 2002.
120. Salie, R., I. Harper, C. Cillie, S. Genade, B. Huisamen, J. Moolman, and A. Lochner, “Melatonin protects against ischaemic-reperfusion myocardial damage.”, *Journal of Molecular and Cellular Cardiology*, Vol. 33, no. 2, pp. 343–57, 2001.

121. Yalvac, M. E., M. Ramazanoglu, O. Z. Gumru, F. Sahin, A. Palotás, and A. A. Rizvanov, “Comparison and optimisation of transfection of human dental follicle cells, a novel source of stem cells, with different chemical methods and electro-poration.”, *Neurochemical Research*, Vol. 34, no. 7, pp. 1272–7, 2009.

122. Lu, H., L. Guo, M. J. Wozniak, N. Kawazoe, T. Tateishi, X. Zhang, and G. Chen, “Effect of cell density on adipogenic differentiation of mesenchymal stem cells.”, *Biochemical and Biophysical Research Communications*, Vol. 381, no. 3, pp. 322–7, 2009.

123. Safford, K. M., K. C. Hicok, S. D. Safford, Y. D. C. Halvorsen, W. O. Wilkison, J. M. Gimble, and H. E. Rice, “Neurogenic differentiation of murine and human adipose-derived stromal cells.”, *Biochemical and Biophysical Research Communications*, Vol. 294, no. 2, pp. 371–9, 2002.

124. Lee, O. K., T. K. Kuo, W. M. Chen, K. D. Lee, S. L. Hsieh, and T. H. Chen, “Isolation of multipotent mesenchymal stem cells from umbilical cord blood.”, *Blood*, vol. 103, no. 5, pp. 1669–75, 2004.

125. Mizuno, H., P. A. Zuk, M. Zhu, H. P. Lorenz, P. Benhaim, and M. H. Hedrick, “Myogenic differentiation by human processed lipoaspirate cells.”, *Plastic and Reconstructive Surgery*, Vol. 109, no. 1, pp. 199–209; discussion 210–1, 2002.

126. Nam, S., J. E. Won, C. H. Kim, and H. W. Kim, “Odontogenic differentiation of human dental pulp stem cells stimulated by the calcium phosphate porous granules.”, *Journal of Tissue Engineering*, Vol. 2011, p. 812547, 2011.

127. Soleimani, M., S. Nadri, and I. Shabani, “Neurogenic differentiation of human conjunctiva mesenchymal stem cells on a nanofibrous scaffold.”, *The International Journal of Developmental Biology*, Vol. 54, no. 8–9, pp. 1295–300, 2010.

128. Bae, S. E., D. H. Choi, D. K. Han, and K. Park, “Effect of temporally controlled release of dexamethasone on in vivo chondrogenic differentiation of mesenchymal stromal cells.”, *Journal of Controlled Release*, Vol. 143, no. 1, pp. 23–30, 2010.

129. Zuk, P. A., M. Zhu, P. Ashjian, D. A. De Ugarte, J. I. Huang, H. Mizuno, Z. C. Alfonso, J. K. Fraser, P. Benhaim, and M. H. Hedrick, “Human adipose tissue is a source of multipotent stem cells.”, *Molecular Biology of the Cell*, Vol. 13, no. 12, pp. 4279–95, 2002.

130. Lisignoli, G., S. Cristino, A. Piacentini, S. Toneguzzi, F. Grassi, C. Cavallo, N. Zini, L. Solimando, N. Mario Maraldi, and A. Facchini, “Cellular and molecular events

during chondrogenesis of human mesenchymal stromal cells grown in a three-dimensional hyaluronan based scaffold.", *Biomaterials*, Vol. 26, no. 28, pp. 5677–86, 2005.

131. Lillie, R. D. and L. L. Ashburn, "Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degeneration not shown by Herxheimer's technique", *Archives of Pathology*, Vol. 36, p. 432, 1943.
132. Puchacz, E., J. B. Lian, G. S. Stein, J. Wozney, K. Huebner, and C. Croce, "Chromosomal localization of the human osteocalcin gene," *Endocrinology*, Vol. 124, no. 5, pp. 2648–2650, 1989.
133. Singh, S., "Alkaline Phosphatase," Encyclopaedia Britannica Online [online database], http://global.britannica.com/EBchecked/topic/15658/alkaline-phosphatase [retrieved 18 September 2013].
134. De Medeiros, A. M., C. F. Nonaka, H. C. Galvão, L. B. de Souza, and A. Freitas Rde, "Expression of extracellular matrix proteins in ameloblastomas and adenomatoid odontogenic tumors", *Eur Arch Otorhinolaryngol*, Vol. 267, no. 2, pp. 303–310, 2010.
135. Sanai, N., A. Alvarez-Buylla, and M. S. Berger, "Neural stem cells and the origin of gliomas.", *The New England Journal of Medicine*, Vol. 353, no. 8, pp. 811–22, 2005.
136. Gronthos, S., J. Brahim, W. Li, L. W. Fisher, N. Cherman, A. Boyde, P. DenBesten, P. G. Robey, and S. Shi, "Stem cell properties of human dental pulp stem cells.", *Journal of Dental Research*, Vol. 81, no. 8, pp. 531–5, 2002.
137. Przyborski, S. A. and M. A. Cambray-Deakin, "Developmental regulation of MAP2 variants during neuronal differentiation in vitro.", *Brain Research. Developmental Brain Research*, Vol. 89, no. 2, pp. 187–201, 1995.
138. Fu, Y. S., Y. C. Cheng, M. Y. A. Lin, H. Cheng, P. M. Chu, S. C. Chou, Y. H. Shih, M. H. Ko, and M. S. Sung, "Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: potential therapeutic application for Parkinsonism.", *Stem Cells*, Vol. 24, no. 1, pp. 115–24, 2006.
139. Guo, L., F. Yin, H. Q. Meng, L. Ling, T. N. Hu-He, P. Li, C. X. Zhang, S. Yu, D. S. Duan, and H. X. Fan, "Differentiation of mesenchymal stem cells into dopaminergic neuron-like cells in vitro.", *Biomedical and Environmental Sciences*, Vol. 18, no. 1, pp. 36–42, 2005.

140. Mandel, G. and D. McKinnon, “Molecular basis of neural-specific gene expression.”, *Annual Review of Neuroscience*, Vol. 16, pp. 323–45, 1993.
141. Lalonde, R. and C. Strazielle, “Neurobehavioral characteristics of mice with modified intermediate filament genes.”, *Reviews in the Neurosciences*, Vol. 14, no. 4, pp. 369–85, 2003.
142. Nagatsu, T., “Tyrosine hydroxylase: human isoforms, structure and regulation in physiology and pathology.”, *Essays in Biochemistry*, Vol. 30, pp. 15–35, 1995.
143. Díez, J. J. and P. Iglesias, “The role of the novel adipocyte-derived hormone adiponectin in human disease.”, *European Journal of Endocrinology*, Vol. 148, no. 3, pp. 293–300, 2003.
144. Wang, C. S., J. Hartsuck, and W. J. McConathy, “Structure and functional properties of lipoprotein lipase.”, *Biochimica et Biophysica Acta*, Vol. 1123, no. 1, pp. 1–17, 1992.
145. Makowski, L., J. B. Boord, K. Maeda, V. R. Babaev, K. T. Uysal, M. A. Morgan, R. A. Parker, J. Suttles, S. Fazio, G. S. Hotamisligil, and M. F. Linton, “Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis.”, *Nature Medicine*, Vol. 7, no. 6, pp. 699–705, 2001.
146. Lefebvre, V., B. Dumitriu, A. Penzo-Méndez, Y. Han, and B. Pallavi, “Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors.”, *The international Journal of Biochemistry & Cell Biology*, Vol. 39, no. 12, pp. 2195–214, 2007.
147. Mwale, F., C. Billinghurst, W. Wu, M. Alini, C. Webber, A. Reiner, M. Ionescu, J. Poole, and A. R. Poole, “Selective assembly and remodelling of collagens II and IX associated with expression of the chondrocyte hypertrophic phenotype.”, *Developmental Dynamics*, Vol. 218, no. 4, pp. 648–62, 2000.
148. Nelea, V., L. Luo, C. N. Demers, J. Antoniou, A. Petit, S. Lerouge, M. R Wertheimer, and F. Mwale, “Selective inhibition of type X collagen expression in human mesenchymal stem cell differentiation on polymer substrates surface-modified by glow discharge plasma.”, *Journal of Biomedical Materials Research. Part A*, Vol. 75, no. 1, pp. 216–23, 2005.
149. Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T. K. Blackwell, D. Turner, R. Rupp, and S. Hollenberg, “The myoD gene family: nodal

point during specification of the muscle cell lineage.", *Science*, Vol. 251, no. 4995, pp. 761–6, 1991.

150. Li, Z., M. Mericskay, O. Agbulut, G. Butler-Browne, L. Carlsson, L. E. Thornell, C. Babinet, and D. Paulin, "Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle.", *The Journal of Cell Biology*, Vol. 139, no. 1, pp. 129–44, 1997.
151. Nagamoto, T., G. Eguchi, and D. C. Beebe, "Alpha-smooth muscle actin expression in cultured lens epithelial cells.", *Investigative Ophthalmology & Visual Science*, Vol. 41, no. 5, pp. 1122–9, 2000.
152. Reddi, A. H., "Bone and cartilage differentiation.", *Current Opinion in Genetics & Development*, Vol. 4, no. 5, pp. 737–44, 1994.
153. Narayanan, K., R. Srinivas, A. Ramachandran, J. Hao, B. Quinn, and A. George, "Differentiation of embryonic mesenchymal cells to odontoblast-like cells by overexpression of dentin matrix protein 1.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 98, no. 8, pp. 4516–21, 2001.
154. Fahey, G. M., "Dr. William Haseltine on Regenerative Medicine, Aging and Human Immortality", *Life Extension*, Vol. 8, no. 7, p. 58, 2002.
155. Price, J., C. Faucheux, and S. Allen, "Deer antlers as a model of Mammalian regeneration.", *Current Topics in Developmental Biology*, Vol. 67, pp. 1–48, 2005.
156. Bianco, P. and P. G. Robey, "Stem cells in tissue engineering.", *Nature*, Vol. 414, no. 6859, pp. 118–21, 2001.
157. Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W. G. Tong, J. Ross, J. Haug, T. Johnson, J. Q. Feng, S. Harris, L. M. Wiedemann, Y. Mishina, and L. Li, "Identification of the haematopoietic stem cell niche and control of the niche size.", *Nature*, Vol. 425, no. 6960, pp. 836–41, 2003.
158. Friedenstein, A. J., J. F. Gorskaja, and N. N. Kulagina, "Fibroblast precursors in normal and irradiated mouse hematopoietic organs.", *Experimental Hematology*, Vol. 4, no. 5, pp. 267–74, 1976.
159. Caplan, A. I., "Mesenchymal stem cells.", *Journal of Orthopaedic Research*, Vol. 9, no. 5, pp. 641–50, 1991.
160. Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak, "Multilineage

potential of adult human mesenchymal stem cells.", *Science*, Vol. 284, no. 5411, pp. 143–7, 1999.

161. Gronthos, S., A. C. W. Zannettino, S. J. Hay, S. Shi, S. E. Graves, A. Kortesidis, and P. J. Simmons, "Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow.", *Journal of Cell Science*, Vol. 116, no. Pt 9, pp. 1827–35, 2003.
162. Gerson, S. L., "Mesenchymal stem cells: no longer second class marrow citizens.", *Nature Medicine*, Vol. 5, no. 3, pp. 262–4, 1999.
163. Campagnoli, C., I. A. Roberts, S. Kumar, P. R. Bennett, I. Bellantuono, and N. M. Fisk, "Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow.", *Blood*, Vol. 98, no. 8, pp. 2396–402, 2001.
164. Tuan, R. S., G. Boland, and R. Tuli, "Adult mesenchymal stem cells and cell-based tissue engineering.", *Arthritis Research & Therapy*, Vol. 5, no. 1, pp. 32–45, 2003.
165. Yalvac, M. E., A. A. Rizvanov, E. Kilic, F. Sahin, M. A. Mukhamedyarov, R. R. Islamov, and A. Palotás, "Potential role of dental stem cells in the cellular therapy of cerebral ischemia.", *Current Pharmaceutical Design*, Vol. 15, no. 33, pp. 3908–16, 2009.
166. Miura, M., S. Gronthos, M. Zhao, B. Lu, L. W. Fisher, P. G. Robey, and S. Shi, "SHED: stem cells from human exfoliated deciduous teeth.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 100, no. 10, pp. 5807–12, 2003.
167. Liu, Y., Y. Zheng, G. Ding, D. Fang, C. Zhang, P. M. Bartold, S. Gronthos, S. Shi, and S. Wang, "Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine.", *Stem Cells*, Vol. 26, no. 4, pp. 1065–73, 2008.
168. Radio, N. M., J. S. Doctor, and P. A. Witt-Enderby, "Melatonin enhances alkaline phosphatase activity in differentiating human adult mesenchymal stem cells grown in osteogenic medium via MT2 melatonin receptors and the MEK/ERK (1/2) signaling cascade.", *Journal of Pineal Research*, Vol. 40, no. 4, pp. 332–42, 2006.
169. Fustin, J. M., H. Dardente, G. C. Wagner, D. A. Carter, J. D. Johnston, G. A. Lincoln, and D. G. Hazlerigg, "Egr1 involvement in evening gene regulation by melatonin.", *FASEB Journal*, Vol. 23, no. 3, pp. 764–73, 2009.
170. Svanberg, B., B. Arvidsson, A. Norrby, G. Rybo, and L. Sölvell, "Absorption of supplemental iron during pregnancy - a longitudinal study with repeated bone-

marrow studies and absorption measurements.", *Acta Obstetricia et Gynecologica Scandinavica. Supplement*, Vol. 48, pp. 87–108, 1975.

171. Karsenty, G., "Minireview: transcriptional control of osteoblast differentiation.", *Endocrinology*, Vol. 142, no. 7, pp. 2731–3, 2001.
172. Muruganandan, S., A. A. Roman, and C. J. Sinal, "Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program.", *Cellular and Molecular Life Sciences*, Vol. 66, no. 2, pp. 236–53, 2009.
173. Kawaguchi, H., T. Akune, M. Yamaguchi, S. Ohba, N. Ogata, U. Chung, N. Kubota, Y. Terauchi, T. Kadowaki, and K. Nakamura, "Distinct effects of PPARgamma insufficiency on bone marrow cells, osteoblasts, and osteoclastic cells.", *Journal of Bone and Mineral Metabolism*, Vol. 23, no. 4, pp. 275–9, 2005.
174. Shockley, K. R., O. P. Lazarenko, P. J. Czernik, C. J. Rosen, G. A. Churchill, and B. Lecka-Czernik, "PPARgamma2 nuclear receptor controls multiple regulatory pathways of osteoblast differentiation from marrow mesenchymal stem cells.", *Journal of Cellular Biochemistry*, Vol. 106, no. 2, pp. 232–46, 2009.
175. Jeon, M. J., J. A. Kim, S. H. Kwon, S. W. Kim, K. S. Park, S. W. Park, S. Y. Kim, and C. S. Shin, "Activation of peroxisome proliferator-activated receptor-gamma inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts.", *The Journal of Biological Chemistry*, Vol. 278, no. 26, pp. 23270–7, 2003.